Towards the design of tumour vaccines: induction of immune responses to self antigens

By Ayako Wakatsuki
The Edward Jenner Institute for Vaccine Research

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Immunobiology Unit
Institute of Child Health
University College London

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天国にいるおじいちゃんとおばあちゃんへ
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Abstract

Despite the increasing incidence of cancer worldwide, tumour immunotherapy available in clinics to date is very limited with variable results. As the number of cancer patients is expected to continue to rise, there is an increasing demand for good vaccines.

Evidence suggests that tumour cells arising spontaneously present self molecule-derived antigens. Due to the process of thymic selection, our immune system has been designed for self recognition but not self activation. This is achieved by T cell receptors bearing different affinities to different antigens. Briefly, T cells in the periphery can only respond to “high-affinity” antigens, and are incapable of eliciting a response to self antigens, or “low-affinity” antigens. Thus, in order to elicit an effective antitumour response we need to understand how our immune system responds to “low-affinity” self antigens.

To investigate the mechanisms of low-affinity recognition by T cells, the ability of differentially matured bone marrow-derived dendritic cells (DCs) to induce low-affinity T cell responses was assessed. In the syngeneic mixed lymphocyte reaction, pathogen-derived products (LPS, cholera toxin and pertussis toxin), but not cAMP inducers (dbcAMP, forskolin and PGE2) drive maturation and differentiation of DCs and subsequent Th1 responses. Pertussis toxin-treated DCs were found to be potent stimulators of naive syngeneic CD4+ and CD8+ T cells, and they do so independently of the signalling through MHC-TcR interaction or B7-mediated costimulation. In the F5 transgenic system, “low-affinity” antigen presented on mature DCs induced proliferation and IFNγ production of naive T cells, but not cytotoxic function. Remarkably, cytotoxicity of low-affinity targets was improved by priming T cells with “high-affinity” antigen. Thus, data suggest that there are limitations to the ability of DCs to induce low-affinity T cell responses, and that manipulation of antigens recognised by the T cells may be the key to inducing effective antitumour immune responses.
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<tr>
<td>ADP</td>
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<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human histocompatibility leukocyte antigens</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-Isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>IMDM</td>
<td>Isocove’s Modified Dulbecco’s medium</td>
</tr>
<tr>
<td>ko</td>
<td>Knock out</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NP</td>
<td>Influenza A virus nucleoprotein</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PTx</td>
<td>Pertussis toxin (holotoxin)</td>
</tr>
<tr>
<td>PTxB</td>
<td>Pertussis toxin B-oligomer</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
</tbody>
</table>
1.1 Overview of the immune system

1.1.1 Immune systems have evolved to combat infections

Multicellular organisms face debilitating and potentially life-threatening infections throughout their lives. Under such selective pressure for survival, host organisms have evolved the ability to recognise invading microbes and to eliminate them efficiently without causing damage to self.

Two types of defence mechanisms are used by host organisms against pathogens: constitutive and inducible. The constitutive defence mechanisms include the barrier functions of the body surfaces and mucosal epithelia of the respiratory, gastrointestinal and reproductive tracts. Certain antimicrobial peptides are constantly produced by the surface epithelial cells, and the antibacterial enzyme, lysozyme, which is constitutively present in excreted body fluids (e.g., tears). Commonly the constitutive defence mechanisms are present at sites of continuous interaction with microbes, and their destructive capacity is directed strictly at the microbes but not at the host cells and tissues. This is achieved by compartmentalisation of these mechanisms (body surfaces and cavities) or by the nature of the activities (e.g., lysozyme and defensins are destructive only for bacterial cell walls and not host cells).
Most of the host defence mechanisms are inducible upon infection. Therefore, their activation requires specific recognition of the infectious microorganisms. This type of defence mechanism consists of complex interactions between cells and products of the immune system, which will be discussed below.

1.1.2 The innate and adaptive immune systems

Two general systems of inducible immunity to infectious agents have been selected during evolution: innate and adaptive immunity. The innate immune system is a phylogenetically ancient defence mechanism, with some forms thought to be present in all multicellular organisms, whereas the adaptive immune system evolved about 400 million years ago and is found only in vertebrates (Thompson, 1995).

Key features of the mammalian innate immune system include: (1) the ability to rapidly recognise pathogen and/or tissue injury, and (2) the ability to signal the presence of danger to cells of the adaptive immune system. The principal components of innate immunity are phagocytic cells, mediators of inflammation as well as cells that mediate cytotoxicity. Thus, innate immunity provides the first lines of defence against microbes.

Adaptive immunity, on the other hand, requires 4-7 days to develop in response to infection. However, once initiated it exhibits exquisite specificity for distinct molecules, together with memory, the ability to remember and respond more vigorously and quickly to repeated exposure to the same microbe.
The essential difference between the two systems is the means by which they recognise microorganisms. The innate immune system has germline-encoded receptors for the recognition of microbial pathogens. This is in direct contrast to adaptive immunity, which is based on receptors with random specificities that are generated by somatic mechanisms during the ontogeny of each individual organism. In other words, cells in the adaptive immune system have not evolved to recognise pathogens directly. Thus, the generation of a specific immune response ultimately depends on the recognition of pathogens by the innate immune system.

1.1.3 The components of the immune system

All the cellular components of blood originate in the bone marrow. The cells of the immune system derive ultimately from the same precursor cells, hematopoietic stem cells in the bone marrow. Two types of progenitor cells, myeloid and lymphoid, are derived from these stem cells, and these progenitor cells give rise to the cells of the immune system. A summary of cell types derived from these two progenitor cells is shown in figure 1.1.
Figure 1.1 Cellular components of the immune system are derived from the hematopoietic stem cells in the bone marrow.

(CFU, colony-forming unit)
The innate immune system includes phagocytic cells (neutrophils, macrophages), natural killer (NK) cells, complement (plasma proteins that attack extracellular pathogens) and interferons (IFNs). Cells of the innate immune system use a variety of pattern recognition receptors to recognise patterns shared between pathogens, for example bacterial lipopolysaccharide (LPS), carbohydrates, and double-stranded viral RNA (dsRNA) (section 1.2).

The adaptive immune system consists of two distinct types of lymphocytes, B cells that are the precursors of antibody-producing cells, and T cells that mediate cell-mediated immunity. Precursors of these cell types are generated in the bone marrow, and further development of B and T cells takes place in the bone marrow or thymus, respectively. For this reason these organs are often referred to as primary or central lymphoid organs. Once functional lymphocytes are generated, they initiate adaptive immune responses in the secondary or peripheral lymphoid organs, sites that are specialised to trap antigens, and allow the initiation of adaptive immune responses. The peripheral lymphoid organs include the spleen, lymph nodes and mucosal immune systems such as the GALT (gut-associated lymphoid tissue) and BALT (bronchial-associated lymphoid tissue).
1.2 Innate immune recognition

The innate immune system is found in all multicellular organisms. It distinguishes self from non-self using germline-encoded receptors, many of which are believed to be evolutionarily conserved.

The recognition of pathogens is complex due to their enormous variability, molecular heterogeneity and high mutation rate. Thus, relatively few germline-encoded molecules must be able to recognise a vast variety of molecular structures associated with pathogens. Theoretically, a number of conditions need to be met by such molecular structures in order to be recognised by the receptors of the innate immune system: (1) the molecular structure recognised by the immune system must be shared by large groups of pathogens, thus representing molecular patterns rather than particular structures; (2) these molecular patterns must be conserved products of microbial metabolism that are not subject to mutation; and (3) the recognised structures need to be absolutely distinct from self antigens.

Over the last decade a large number of molecular structures and receptors that recognise them have been identified, many of which still require further investigation.

1.2.1 Recognition of pathogen-associated molecular patterns (PAMPs)

Germline-encoded receptors that mediate recognition of pathogens were termed pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997b). The ligands for these receptors were subsequently termed pathogen-associated molecular patterns, or PAMPs. To date, several types of protein domains involved in pattern recognition have been identified, the most prominent being the C-type lectin domain, the
scavenger receptor cysteine-rich domain and the leucine-rich repeat domain. So far, six protein families have been identified that are believed to play a central role in forming PRRs (Medzhitov and Janeway, 1997a). These are summarised in table 1.1.

Table 1.1 Families of protein domains that function as pattern recognition receptors.
[data taken from (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 1997a)]

<table>
<thead>
<tr>
<th>Protein family</th>
<th>Site of expression</th>
<th>Example</th>
<th>Ligands</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-type lectins</td>
<td>Plasma protein</td>
<td>Collectins (mannose-binding lectin)</td>
<td>Bacterial and viral</td>
<td>(Drickamer and Taylor, 1993; Sastry and Ezekowitz, 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Macrophages, DCs*</td>
<td>N-acetylgalactosamine</td>
<td>(Suzuki et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Macrophages, DCs</td>
<td>Macrophage C-type lectin receptor</td>
<td>GalNAc* receptor</td>
<td>(Chang et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Macrophage mannose receptor</td>
<td>Terminal mannose</td>
<td></td>
</tr>
<tr>
<td>Leucine-rich proteins</td>
<td>Macrophages, DCs*</td>
<td>CD14</td>
<td>LPS*</td>
<td>(Wright et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Monocytes, DCs, etc</td>
<td>Toll-like receptors</td>
<td>Various pathogen molecules</td>
<td>(Akira et al., 2001; Imler and Hoffmann, 2001)</td>
</tr>
<tr>
<td></td>
<td>B cells</td>
<td>RP105</td>
<td>LPS</td>
<td>(Ogata et al., 2000)</td>
</tr>
<tr>
<td>Scavenger receptors</td>
<td>Macrophages</td>
<td>Macrophage scavenger receptor</td>
<td>Bacterial cell wall</td>
<td>(Krieger and Herz, 1994)</td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>MARCO*</td>
<td>Bacterial cell wall</td>
<td>(Elomaa et al., 1995)</td>
</tr>
<tr>
<td>Pentraxins</td>
<td>Plasma protein</td>
<td>C-reactive protein</td>
<td>Phosphatidyl choline</td>
<td>(Gewurz et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Serum amyloid P</td>
<td></td>
<td>Bacterial cell wall</td>
<td>(Emsley et al., 1994)</td>
</tr>
<tr>
<td>Lipid transferases</td>
<td>Plasma protein</td>
<td>LPS binding protein</td>
<td>LPS, lipopolysaccharides</td>
<td>(Schumann et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>BPIP*</td>
<td></td>
<td>LPS, lipopolysaccharides</td>
<td>(Elsbach and Weiss, 1993)</td>
</tr>
<tr>
<td>Integrins</td>
<td>Macrophages, DCs,</td>
<td>CD11b/c:CD18</td>
<td>LPS</td>
<td>(Ingalls and Golenbock, 1995)</td>
</tr>
<tr>
<td></td>
<td>NK*, T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: DCs, dendritic cells; GalNAc, N-acetylgalactosamine; LPS, lipopolysaccharide; MARCO, macrophage receptor with collagenous structure; BPIP, bactericidal permeability increasing protein; NK, natural killer cells.

PRRs can be secreted proteins present in the body fluids. Alternatively, they can be expressed on the cell surface or in the cytoplasm. Functionally, PRRs can be involved in: (1) opsonisation of bacteria and viruses for phagocytosis or activation of complement by the lectin pathway (Fraser et al., 1998), (2) uptake of pathogens by phagocytes and DCs (Stahl and Ezekowitz, 1998), or (3) triggering of the signalling
pathways that result in the induction of transcription of a variety of immune effector genes, such as the antimicrobial peptides and inflammatory cytokines.

**Mammalian Toll-like receptors (TLRs)**

Recently, a group of primitive receptors, called Toll-like receptors (TLRs) have been identified in humans. Toll receptors are type I transmembrane proteins that are evolutionarily conserved between insects and humans (Anderson, 2000). In *Drosophila*, Toll is a key functional molecule in antifungal immunity (Lemaitre et al., 1996). A homologous family of Toll receptors, TLRs, was found to exist in mammals (Medzhitov et al., 1997c). Structurally, the extracellular portion of TLRs contains leucine-rich repeats, while the cytoplasmic domains are related to IL-1Rs. TLR family members are expressed differentially among immune cells (Muzio et al., 2000) and appear to respond to different stimuli. TLR expression is also observed in a variety of other cells, including vascular endothelial cells, adipocytes, cardiac myocytes and intestinal epithelial cells (Akira et al., 2001). To date, ten TLRs (TLR 1-10) have been reported. Those TLRs with known ligands and functions are summarised in table 1.2.
Ligands that are recognised by TLRs appear to be specific but diverse. However, the mechanisms by which the TLRs recognise their ligands are still unknown. It is possible that unknown coreceptors may be required for specific recognition of each ligand (e.g., CD14 is a coreceptor for recognition of LPS by TLR4 (Aderem and Ulevitch, 2000)).

### 1.2.2 Recognition of molecules other than PAMPs

Recognition of PAMPs is not the only system of innate immune recognition. A classic example is the way by which natural killer (NK) cells recognise altered self, as well as absence of self. Recognition of altered self is poorly understood, but may be directed, for example, at changes in glycosylation patterns of cell surface glycoproteins, or changes in the phospholipid composition of cell membranes. The best understood example is the mechanism by which surface MHC class I molecules
are detected by a NK cell receptor, Ly49, thereby allowing recognition of self and nonself cells (Lanier, 1998).

In addition to the recognition of self by NK cells, there is also evidence that other endogenous self molecules can be recognised by receptors of the innate immune system during an inflammatory response. Heat shock proteins (hsp60) are highly conserved molecules, whose expression dramatically increases in cells under stress conditions. Hsps, such as hsp60 (60kDa hsp) are released by necrotic but not apoptotic cells, and under these circumstances have potent immunostimulatory properties. It was recently demonstrated that mice bearing mutant TLR4 genes were resistant to hsp60-induced macrophage activation, suggesting that hsp60 activates TLR4 (Ohashi et al., 2000). Thus, it is possible that cells in the innate immune system are capable of detecting stressed or damaged cells by recognising the changes in the lipid and/or carbohydrate moieties expressed on the cell surface or by the expression of proteins not normally found in the outer cell membrane.
1.3 The adaptive immune system: generation and functions of lymphocytes

1.3.1 Clonal selection theory

Unlike the cells in the innate immune system, where recognition of infectious microbes is achieved by germline-encoded receptors, the specificity of T and B cells in adaptive immunity is determined by clonal receptor systems, which are generated by random somatic mutations. The concept that each T and B cell expresses receptors with a single specificity and maintains the expression as a clone, was first put forward by Jerne and Talmage, and later formulated as the “clonal selection theory” by McFarlane Burnet in the 1950s.

There are four basic principles of the clonal selection hypothesis. First, each lymphocyte bears a single type of receptor with a unique specificity. Second, high affinity interaction between such a receptor and a foreign molecule (antigen) leads to lymphocyte activation. Third, the differentiated effector cells derived from an activated lymphocyte will bear receptors of identical specificity to those of the parental cell from which that lymphocyte was derived. Finally, lymphocytes that express receptors specific for ubiquitous self molecules are deleted at an early stage in lymphoid cell development and are therefore absent from the repertoire of mature lymphocytes. It is now known that the process of selection for non-autoreactive lymphocytes takes place in the bone marrow for B cells and the thymus for T cells. Fifty years after it was first proposed, the theory of clonal selection of lymphocytes is still the most important principle in adaptive immunity. However, it is now known that B cells do not carry receptors of identical specificity once they become differentiated effector cells due to the process of somatic mutation.
1.3.2 Antigen recognition by lymphocytes

The adaptive immune system operates by recognition of an enormous variety of antigens. This is achieved by generation of a diverse repertoire of antigen receptors, T cell receptors (TcRs) and B cell receptors (BcRs).

TcRs and BcRs are related to each other, both in structure (figure 1.2) and the genetic mechanisms used to generate diversity during their development. One major difference between TcRs and BcRs is the way in which they recognise antigens. Whilst B cells are able to recognise antigen in a native form, T cells are only capable of recognising a peptide presented in the groove of major histocompatibility complex (MHC) molecules together with associated co-receptor molecules, CD4 and CD8. Two types of TcRs are known, αβ TcR and γδ TcR. γδ T cells are a distinct lineage from αβ T cells, and in humans less than 5% of T cells express γδTcR. For simplicity only αβ TcR will be considered in this chapter.

The αβ TcR is a heterodimer consisting of two transmembrane polypeptide chains covalently linked to each other by disulphide bonds (figure 1.2A). The extracellular portions contain N-terminal variable (V) domains and membrane-proximal constant (C) regions. The BcR, or immunoglobulin (Ig), is an Y-shaped molecule composed of light and heavy chains linked together by disulphide bonds (figure 1.2B). An Ig molecule can be further divided into a Fab fragment that binds antigen, and Fc fragment, which interacts with effector molecules and cells. Like the TcR, the Fab fragment has an N-terminal V region and a membrane-proximal C region (figure 1.2C). Thus, the Fab fragment of Ig resembles the TcR molecule structurally. For both T and B cells, recognition of antigen relies on the V domain of the receptors,
Figure 1.2 Comparison of structure of T cell receptor and B cell receptor (Ig).

(A) Schematic diagram of αβ TcR. (B) Schematic diagram of an immunoglobulin IgG molecule. (C) Fab portion of Ig molecule. V and C in the loop-structures refer to Ig-like variable and constant domains, respectively. Dotted line indicates intrachain and interchain disulphide bonds. N and C at the end of peptide chains refer to amino and carboxy termini of the polypeptide chains, respectively.
which are highly variable from one clone to another. This domain contains highly diverse regions, called complementarity determining regions (CDRs). These constitute the antigen binding site, and thus determine the antigen-binding specificity of TcRs and BcRs.

The diversity of BcRs and TcRs is generated by somatic recombination of gene segments that constitute the V region genes, V, D and J. Each of these genes consists of multiple segments that exists as arrays of tandem repeat. These gene segments are randomly selected and joined together by the lymphocyte-specific recombination activating gene (RAG)-1/2 proteins. The combinatorial associations that result from this process contribute to generation of receptor diversity. In addition, further diversity is created by varying the length of the nucleotide sequences during joining of VDJ gene segments. Finally, the pairing of the two polypeptide chains (α and β chains for TcR, heavy and light chain for BcR) serves to further multiply the diversity generated for each chain.

1.3.3 Overview of lymphocyte life cycles
Progenitors of both B and T cells arise in the bone marrow. While B cells complete the major part of their development in the bone marrow, T cells migrate to the thymus to complete their development. When lymphocytes are generated, these cells express antigen receptors that are formed by the random somatic gene rearrangement events as described above. This generates a great diversity, but the vast majority of lymphocytes produced in this way are not fit to function as a part of the adaptive immune system. Those lymphocytes that are either non-functional, or autoreactive are eliminated in selection processes that take place in the thymus for T cells
(discussed further in section 1.5.8), or in the bone marrow and spleen for B cells (Pulendran et al., 1997; Townsend et al., 1999). Lymphocytes that newly emerge into the periphery are termed naïve T cells or naïve B cells, as they have yet to encounter antigens. When these naïve lymphocytes encounter antigens that they recognise, they become activated in the presence of appropriate signals (discussed below). This process takes place in lymphoid organs such as lymph nodes and spleen. Activated lymphocytes first go through extensive proliferation (expansion of the clone), followed by differentiation into effector cells that immediately act at sites from which the antigens originated (e.g., site of infection). During the immune response memory cells are also generated. These remain at an increased frequency and can therefore generate large number of effector cells more rapidly upon secondary challenge with the same antigen.

1.3.4 T cell activation and functions

T cells recognise a processed peptide antigen bound to major histocompatibility complex (MHC) molecules expressed on antigen presenting cells (APCs). However, recognition of specific antigen by itself does not trigger activation of T cells, and requires a costimulatory, or secondary signal provided by professional APCs (details in section 1.5.7). Activated T cells proliferate rapidly in response to IL-2, which is produced in an autocrine or paracrine fashion. Proliferating T cells then develop into armed effector T cells, which act on target cells that display antigens on the cell surface. Effector T cells can mediate a variety of functions. Depending on the functional phenotypes and differential coreceptor expression (CD4 and CD8), T cells are classified into different subtypes. These are summarised in table 1.3.
Table 1.3 Functions of different subtypes of T cells.

<table>
<thead>
<tr>
<th>T cell subtypes</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4^+ T helper type 1 cells</strong></td>
<td>Activation of B cells by cell-cell mediated interaction</td>
</tr>
<tr>
<td>(CD4^+ Th1)</td>
<td>Cytokine secretion, which promotes:</td>
</tr>
<tr>
<td></td>
<td>B cell activation / differentiation</td>
</tr>
<tr>
<td></td>
<td>NK cell activation</td>
</tr>
<tr>
<td></td>
<td>Macrophage activation</td>
</tr>
<tr>
<td></td>
<td>T cell differentiation</td>
</tr>
<tr>
<td><strong>CD4^+ T helper type 2 cells</strong></td>
<td>Activation of B cells by cell-cell mediated interaction</td>
</tr>
<tr>
<td>(CD4^+ Th2)</td>
<td>Cytokine secretion, which promotes:</td>
</tr>
<tr>
<td></td>
<td>B cell activation / differentiation</td>
</tr>
<tr>
<td></td>
<td>T cell differentiation</td>
</tr>
<tr>
<td><strong>CD4^+ T regulatory cells</strong></td>
<td>Prevents IL-2 production from T cells</td>
</tr>
<tr>
<td>(CD4^+ T_{reg})</td>
<td>Cytokine secretion, which inhibits:</td>
</tr>
<tr>
<td></td>
<td>T cell activation</td>
</tr>
<tr>
<td></td>
<td>Activation of APCs</td>
</tr>
<tr>
<td><strong>CD8^+ cytotoxic T cells</strong></td>
<td>Induction of cytotoxicity in target cells</td>
</tr>
<tr>
<td>(CD8^+ Tc)</td>
<td>Cytokine secretion</td>
</tr>
</tbody>
</table>

CD4^+ T cells are generally termed T helper cells (Th), for B cell activation and antibody production requires interaction with CD4^+ T cells. CD4^+ T cells also assist in activation of other cell types, such as macrophages and NK cells. In addition, activation of CD8^+ cytotoxic T cells often requires CD4^+ T cells that recognise cognate antigen (Stohlman et al., 1998; von Herrath et al., 1996; Wild et al., 1999).

CD4^+ T cells are classified into two subtypes, Th1 and Th2, based on the profile of cytokines they produce (Constant and Bottomly, 1997). Cytokines produced by Th1 cells, for example, IL-2, IFNγ, and TNFβ, are generally associated with cell-mediated inflammatory immune responses. In contrast, Th2 cells produce cytokines such as IL-4, IL-5 and IL-10, that are mainly associated with humoral responses, and are essential in activation of B cells and induction of different types of antibody responses.

CD8^+ T cells are cytotoxic in nature. They directly lyse target cells, either by interaction with cell surface molecules (e.g., ligation of Fas molecules on target cells).
or via soluble molecules (perforin and granzyme). As mentioned above, their activation often requires “help” from CD4⁺ T cells that recognise cognate antigen. This is achieved either by paracrine production of IL-2, or alternatively, by priming professional APCs to activate CD8⁺ T cells directly. CD8⁺ T cells produce Th1-type cytokines (e.g., IL-2 and IFNγ). However, there is also evidence that some CD8⁺ T cells produce Th2-type cytokines (e.g., IL-4). Based on this, the terminology Tc1 and Tc2 were described to distinguish two different phenotypes of CD8⁺ T cells (Croft et al., 1994; Sad et al., 1995).

In addition to these “activating” cell types, so-called T-regulatory cells were characterised recently [reviewed in (Maloy and Powrie, 2001)]. These are a subset of CD4⁺ T cells and have been shown to inhibit immunopathology in a number of experimental settings. Although the mechanism of immune suppression is still unclear, production of anti-inflammatory cytokines, IL-10 and TGFβ, is known to contribute.

1.3.5 B cell development and functions

B cells are the only cell type that can produce antibodies. Antibodies are the antigen-specific products of B cells and play a major role in the humoral adaptive immune response, by neutralising and eliminating the antigens that induced their formation.

Like initiation of T cell responses, activation of B cells occurs in lymphoid organs. Recognition of antigen (that binds to the membrane Ig molecules) by naïve B cells does not always trigger activation of B cells. Recognition of non-protein antigens such as polysaccharides and lipids is capable of inducing activation of B cells on its
own. However, recognition of protein antigens alone does not activate B cells, and, like naïve T cells, B cells require second signals in order to become activated. These are provided by CD4\(^+\) T helper (Th) cells. These Th cells recognise peptide fragments derived from the antigen internalised and processed by the B cell and activate B cells by ligation of CD40 on the B cell surface. In addition, directed release of cytokines by Th cells further promotes activation and differentiation of B cells.

Following activation, B cells, like T cells, first undergo vigorous proliferation, followed by differentiation into either effector cells (antibody-secreting plasma cells) or memory cells. During this process, activated B cells form regions called germinal centres in the lymphoid organs, which are packed full of proliferating and differentiating B cells. Somatic hypermutation of V-region genes takes place here, by which the antigen-binding properties of the antibody may change. At this stage, selection for B cells that mediate high-affinity binding occurs in response to antigen displayed on the surface of follicular dendritic cells (DCs), whereas B cells that do not recognise the antigen go through programmed cell death. As the antibody response develops, the amount of antigen available for display on follicular DCs decreases progressively. This leads to selection for B cells that recognise the antigen with higher affinity. As a result, the average affinity of the secreted antibody and of membrane Ig on antigen-specific B cells increases after antigenic stimulation and is substantially higher in secondary than in primary responses. This is called affinity maturation and is a unique property of humoral immune responses to protein antigens.

Differences in effector functions of antibody responses depend on the isotype of the antibodies (which are defined by their heavy-chain C regions). There are five main
different isotypes, IgG, M, D, A and E. Some of these isotypes are also further divided into subclasses, such as IgG₁ and IgG₂a. When B cells are first generated, they produce antibodies of IgM isotype. In the germinal centre, the cytokine environment created by Th cells can alter the isotype of antibody. For example, IL-4 induces production of IgG₁ and IgE antibodies, while inhibiting the production of IgG₂a, IgG₃ and IgM antibodies. These differentially induced antibodies then contribute to the adaptive immune responses in the periphery.

1.3.6 Instructive role of the innate immune system in adaptive immune responses

As mentioned above, the adaptive immune system functions as a clonal system, whose immune responses depend on a large diversity of antigen recognition receptors that are generated by the process of somatic gene rearrangement. As a result, the specificity of the response that can be induced upon ligation of the receptor with antigen is not pre-determined. Thus, the adaptive immune system faces two problems upon encountering antigen. First, it needs to decide whether to respond or not (activation versus tolerance). Induction of an immune response is only appropriate if the antigen recognised is derived from a pathogen. Inappropriate activation of lymphocytes to self antigen or innocuous persistent environmental antigen may cause autoimmune responses and hypersensitivity, respectively. Second, the adaptive immune response needs to decide how to respond to the antigen. Protection from different groups of pathogen may require induction of different types of effector responses. Thus, in order to solve these problems the adaptive immune system needs information about the origin of the antigen.
Increasing evidence suggests that information about antigens is provided indirectly by the cells of the innate immune system (Fearon and Locksley, 1996; Janeway, 1992; Medzhitov and Janeway, 1997a). As described in section 1.2 of this chapter, molecules and factors that are associated with infection or tissue damage can be recognised by the innate immune system. By being able to discriminate between self and nonself molecules, the innate immune system transfers information about the antigen to the adaptive immune system, a process now thought to be achieved by a specialised type of APC, the dendritic cell (DC) (Banchereau and Steinman, 1998). While DCs are at an immature stage they sample the local microenvironment of the tissue. DCs then carry the information acquired in the tissue to secondary lymphoid organs, where adaptive immunity is initiated. Thus, DCs function as the sentinels of the immune system (Steinman, 1991) and are the key regulators of the adaptive immune system. The biology of DCs is further discussed in the next chapter.
1.4 Dendritic Cells

1.4.1 Overview of DC life cycle

Dendritic cells (DCs) are derived from hematopoietic progenitor cells in the bone marrow. DCs are found in almost all tissues, and depending on the location they have been given different names (Steinman, 1991). Examples are skin epidermal Langerhans cells, dermal/interstitial DCs, and T zone interdigitating cells. Studies of murine DC function have been largely based either on tissue-isolated DCs from skin (Langerhans cells), spleen, and thymus, or DCs generated from bone marrow in vitro. In humans, the sources of DCs are more restricted, and information on human DCs comes largely from DCs isolated from peripheral blood, epidermal Langerhans cells and peripheral blood monocyte (PBMC)-derived DCs generated in vitro.

DCs in culture exist in two functionally and phenotypically distinct states, immature and mature. Immature DCs are well equipped for antigen capture, but express relatively low levels of surface MHC class I and II products and costimulatory molecules. DCs mature upon exposure to activation- or maturation-driving signals, such as microbial products and/or inflammatory mediators such as proinflammatory cytokines. These mature DCs have a reduced capacity for antigen uptake but have an exceptional capacity for T cell stimulation. This transition is accompanied by a dramatic increase in surface expression of MHC class II, together with surface costimulatory molecules (e.g., CD80, CD86), MHC class I, and T cell adhesion molecules (e.g., CD48 and CD58).

Immature DCs reside in peripheral tissues and function as efficient phagocytes in vivo. Maturation triggering stimuli such as factors associated with infections and
tissue damage cause both cell differentiation and migration of DCs from peripheral tissue sites to secondary lymphoid organs. During the period of migration, DCs become potent T cell stimulating antigen presenting cells (APCs). Once in the T cell area of lymphoid organs, DCs stimulate antigen-specific T cells and initiate immune responses. After encountering and stimulating T cells, DCs are thought to die in the lymphoid organs, as they are not found in efferent lymphatics (Banchereau and Steinman, 1998; Steinman, 1991). By carrying information from the periphery to induce potent adaptive immune responses in lymphoid organs, DCs form a bridge between the innate and adaptive immune systems, and act as sentinels of the immune system (Steinman, 1991).

1.4.2 DC maturation

Transition from immature to mature DCs

Maturing DCs go through a number of dramatic changes, which are associated with a change in their function: from actively phagocytic cells to potent T cell stimulating APCs. The major changes in function and associated changes in expression of surface molecules are summarised in figure 1.3.

The best-studied cellular change in maturing DCs is the regulation of MHC class II expression. In immature DCs, abundant MHC class II molecules are synthesised, but they are mainly sequestered intracellularly in late endocytic compartments and lysosomes (Pierre et al., 1997). Upon antigen uptake, and receipt of maturation signals, antigens are first targeted to MHC class II-positive lysosomes. However, at this stage they are not efficiently utilised for the formation of MHC class II-peptide complexes. The DC maturation process is accompanied by the movement of MHC
class II-peptide complexes to peripheral non-lysosomal vesicles (Pierre et al., 1997). Approximately 48 hours after antigen uptake (Pierre et al., 1997), when DCs have migrated to T cell areas in vivo, MHC class II-peptide complexes reach the cell surface, presenting the antigens captured in peripheral tissue sites (Inaba et al., 2000; Turley et al., 2000). In addition, there is evidence that mature DCs can also generate functional peptide-MHC II complexes from newly internalised antigens (Inaba et al., 2000; Turley et al., 2000).

As summarised in figure 1.3, in synchrony with the upregulation of cell surface MHC class II expression, maturing DCs also accumulate MHC class I, various adhesion molecules and costimulatory molecules that account for the potent T cell stimulatory function of mature DCs. Morphologically, mature DCs extend long “dendritic” processes thereby increasing opportunities for interaction with T cells.
Figure 1.3 Maturation of dendritic cells (DCs) and associated functions and surface molecule expression.
Abbreviation: (h), restricted to human DCs; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; DC-LAMP, dendritic cell lysosome-associated membrane protein; DC-SIGN, dendritic cell-specific ICAM-3-grabbing nonintegrin.
Migration of DCs

The process of DC maturation is accompanied by acquisition of the ability to migrate from peripheral tissue to draining lymphoid organs. Maturing DCs lose adhesions to the peripheral tissue sites, reorganise the cytoskeleton, and acquire high cellular motility (Winzler *et al*., 1997). The migration event is driven by regulated expression of cell surface receptors. For example, DC-SIGN (CD209) binds ICAM-2, thereby mediating DC rolling and transendothelial migration (Geijtenbeek *et al*., 2000).

Accumulating evidence suggests that important roles are played by several chemokines in directing the migration of DCs. This is achieved by regulated expression of certain chemokine receptors in maturing DCs. The differential expression of chemokine receptors (whose ligands are summarised in table 1.4) on maturing DCs are described in figure 1.3. Immature DCs are first recruited to the site of inflammation by chemotactic factors such as a chemokine MIP-3α (which is exclusively expressed by epithelial cells). After antigen uptake, maturing DCs lose the expression of CCR6, the receptor for MIP-3α, thus losing responsiveness to this chemokine (Dieu *et al*., 1998; Sallusto *et al*., 1998; Sozzani *et al*., 1999). Upon maturation, DCs upregulate the chemokine receptor CCR7 (Yoshida *et al*., 1997) and accordingly acquire responsiveness to ligands of CCR7, MIP-3β and 6Ckine (Chan *et al*., 1999; Dieu *et al*., 1998; Yanagihara *et al*., 1998). As a result, maturing DCs leave the inflamed tissues and enter afferent lymphatics, possibly directed by 6Ckine expressed on lymphatic vessels (Gunn *et al*., 1998; Sacki *et al*., 1999). Mature DCs entering the draining lymph nodes are driven into the paracortical area in response to the production of MIP-3β and/or 6Ckine by cells spread throughout the T zone (Dieu *et al*., 1998; Ngo *et al*., 1998). Because these two chemokines attract both mature
DCs and naïve T cells (Gunn et al., 1998; Ngo et al., 1998), they are likely to play an important role in increasing the chance of antigen bearing DCs to encounter specific T cells.

Table 1.4 Chemokine receptors expressed on DCs and their ligands.

<table>
<thead>
<tr>
<th>Chemokine receptors</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>MIP-1α, MIP-1β, RANTES, MCP-3, MIP-5</td>
</tr>
<tr>
<td>CCR2</td>
<td>MCP-1, MCP-2, MCP-3, MCP-4</td>
</tr>
<tr>
<td>CCR4</td>
<td>TARC, MDC</td>
</tr>
<tr>
<td>CCR5</td>
<td>MIP-1α, MIP-1β, RANTES</td>
</tr>
<tr>
<td>CCR6</td>
<td>MIP-3α</td>
</tr>
<tr>
<td>CCR7</td>
<td>MIP-3β (ELC), SLC (6Ckine)</td>
</tr>
<tr>
<td>CXCR1</td>
<td>IL-8</td>
</tr>
<tr>
<td>CXCR4</td>
<td>SDF-1</td>
</tr>
</tbody>
</table>

*Abbreviations: CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T cell expressed and secreted; MCP, monocyte chemoattractant protein; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; ELC, EBi1 ligand chemokine; SLC, secondary lymphoid-tissue chemokine; IL, interleukin; SDF, stromal derived factor.

Factors that trigger maturation of DCs

DC maturation is typically triggered by products of microbial or viral pathogens, such as LPS, CpG DNA, or dsRNA (Cella et al., 1999; De Smedt et al., 1996; Hartmann et al., 1999; Henderson et al., 1997; Sparwasser et al., 1998). The response of DCs to these molecules is mediated via non-clonal recognition receptors expressed on the DC surface. An example is the family of Toll-like receptors (TLRs) [(Kaisho and Akira, 2001); and section 1.2.1]. Proinflammatory cytokines, such as TNFα and IL-1β, also trigger maturation (Cumberbatch and Kimber, 1992; Heufler et al., 1988). In addition, ligation of CD40, which mimics the interaction with activated T cells, can
mediate maturation of DCs (Caux et al., 1994a). Ligation of TLRs, IL-1 receptor and TNF-receptor each lead to NF-κB activation, a hallmark of mature DCs.

DC maturation can also be initiated by a variety of factors that are not associated with infection. For example, DCs become activated by exposure to cells undergoing stress or necrosis (Gallucci et al., 1999). It was also shown that gentle disruption of cell-cell contacts induces maturation of DCs in culture (Gallucci et al., 1999). In addition, maturation of human monocyte-derived DCs was also shown to be induced by extracellular matrix proteins such as fibronectin and collagen type 1 (Brand et al., 1998), suggesting that interaction with these molecules during migration may further enhance the maturation of DCs.

1.4.3 Heterogeneity of DCs

Accumulating evidence suggests that there are a number of DC “subsets” with different phenotypes. Because the information available for murine and human DCs differs in some aspects, subsets of DCs in mice and humans are discussed separately below.

Mice

In mice, an increasing number of reports suggest that DCs are derived from both myeloid and lymphoid precursors. Evidence for the myeloid origin of DCs comes mainly from in vitro studies in which myeloid-committed precursors give rise to granulocytes, monocytes and myeloid DCs under the influence of GM-CSF (Inaba et al., 1993). Support for the existence of so-called lymphoid related DCs (Shortman et al., 1998) comes from the observation that progenitor cells for lymphoid cells (T cells,
B cells and NK cells) that express low levels of CD4 can develop into DCs that express CD8 and lack myeloid antigens such as CD11b, CD13 and CD33 (Ardavin et al., 1993; Wu et al., 1996). Unlike myeloid DCs, these DCs develop in the absence of GM-CSF (Saunders et al., 1996). Two major populations have been distinguished in mouse spleen, CD4-CD8α+DEC-205highCD11b(low) and CD4-CD8α-DEC-205lowCD11b(high) (Kronin et al., 1997), representing putative lymphoid-related and myeloid-derived DCs, respectively. More recently, a third subclass of DC in the spleen, with a CD4+CD8α-DEC-205lowCD11bhigh phenotype has been identified (Vremec et al., 2000). Whilst CD8α has been used as a marker to distinguish the myeloid and lymphoid subsets of DCs in mice (Wu et al., 1996), the recent demonstration that both CD8α+ DCs and CD8α- DCs can arise from myeloid progenitors, suggests that the presence of CD8α is not a reliable marker for lymphoid-derived DCs (Traver et al., 2000). Although the genetic mechanisms that control the development and function of these DCs are still poorly understood, RelB (a transcription factor, which is a member of the NF-κB family) has been shown to selectively regulate development of myeloid DCs, but not lymphoid DCs (Burkly et al., 1995; Wu et al., 1998). Conversely, mice bearing a mutant Ikaros gene are deficient in lymphoid, but not myeloid DCs (Wu et al., 1997), suggesting a role for Ikaros in regulation of lymphoid DCs. Myeloid and lymphoid DCs also exhibit differences in their requirement for growth factors. Flt3 ligand (Flt3-L), which targets hematopoietic progenitors in the bone marrow (Lyman and Jacobsen, 1998), expands both lymphoid and myeloid DCs upon injection (Maraskovsky et al., 1996), while GM-CSF preferentially expands the myeloid DC subset in vivo (Pulendran et al., 1998). However, clonal data to demonstrate that one cell is capable of producing T,
B, NK cells and DCs is still lacking, therefore these DCs were termed "lymphoid related" rather than "lymphoid derived".

Lymphoid and myeloid DCs differ also in localisation and function. Lymphoid DCs are localised in the T cell areas of the periarteriolar lymphatic sheaths (PALS) in the spleen and lymph nodes (Steinman et al., 1997). In contrast, myeloid DCs are in the marginal zone of the spleen (Steinman et al., 1997) but can be induced to migrate to the PALS under the influence of proinflammatory signals such as LPS (De Smedt et al., 1996). Lymphoid DCs were found to be less efficient in priming allogeneic CD4\(^+\) and CD8\(^+\) T cells than myeloid DCs in vitro (Kronin et al., 1996; Suss and Shortman, 1996). This was explained by the ability of lymphoid DCs to differentially regulate T cell responses. CD8\(\alpha^+\), but not CD8\(\alpha^-\) DCs express a high level of FasL and induce apoptosis in responding CD4\(^+\) T cells (Suss and Shortman, 1996). CD8\(^+\) T cell responses are regulated by CD8\(\alpha^+\) but not CD8\(\alpha^-\) DCs, by limiting the production of IL-2 from T cells (Kronin et al., 1996). However, in vivo, both lymphoid and myeloid DCs appear to prime antigen-specific CD4\(^+\) T cells efficiently (Maldonado-Lopez et al., 1999; Pulendran et al., 1999). Lymphoid DCs were found to produce higher levels of IL-12 (Hochrein et al., 2001; Maldonado-Lopez et al., 1999; Ohteki et al., 1999; Reis e Sousa et al., 1999) and IFN\(\alpha\) (Hochrein et al., 2001), and are less phagocytic than myeloid DCs (Leenen et al., 1998). In addition, CD8\(\alpha^+\) splenic DCs were shown to induce Th1 responses in vivo while CD8\(\alpha^-\) DCs preferentially primed Th2 responses (Maldonado-Lopez et al., 1999).
Humans

In humans, three main subsets of DCs have been recognised: epidermal DCs (Langerhans cells), myeloid DCs and lymphoid or plasmacytoid DCs (Banchereau and Steinman, 1998; Steinman et al., 1997).

Evidence suggests that both Langerhans cells and myeloid DCs are derived from the same CD34^+ precursors. In the presence of GM-CSF and TNFα, CD34^+ precursors can give rise to both epidermal DC (Langerhans cells, CD1α^+) via CD1α^+CD14^- precursors, or myeloid DCs (CD1α^+, CD11b^+ CD13^+, CD33^+, CD36^+) via CD1α^-CD14^+ precursors (Caux et al., 1996). The myeloid DCs were also shown to develop from CD34^+ precursors in the presence of GM-CSF and IL-4 (Rieser et al., 1998).

In contrast, the existence of lymphoid related DCs is controversial. DCs that lack myeloid markers, but express CD4 have been found in blood (O. Doherty et al., 1994) and in T cell areas of lymphoid organs (Grouard et al., 1997). These DCs, termed "plasmacytoid DCs", have a characteristic phenotype (CD1α^-, CD123^+, CD45RA^+, CD4^+, CD11^+), and migrate to lymphoid organs without inflammation or the presence of foreign antigens (Olweus et al., 1997). These DCs are also known to produce high levels of IFNα (Bendriss-Vermare et al., 2001). Whilst there is no solid evidence to support the idea that these DCs have a lymphoid origin, the demonstration that cultured human stem cells transduced with genes that interfere with lymphocyte development inhibited the development of CD123^+ plasmacytoid DC precursors, indicate that these DCs may be derived from a lymphoid lineage (Spits et al., 2000).
1.4.4 Function of DCs as "nature's adjuvant"

DCs are known to be the most potent stimulator of naïve T cells, and are the only cell type that can elicit an immune response to a novel antigen (Banchereau and Steinman, 1998). This ability of DCs to initiate primary immune responses has been well documented in numerous studies, both in vitro and in vivo (Steinman, 1991). The unique features of DCs, their migratory capacity allowing them to 'survey' the periphery and consequently initiate a potent antigen-specific immune response in the secondary lymphoid organs, indicate that they may have therapeutic potential, and they are often regarded as 'nature's adjuvant' (Schuler and Steinman, 1997).

Many recent studies have examined the ability of peptide-antigen-pulsed DCs to initiate antigen-specific T cell responses in vivo. Soluble-antigen-pulsed DCs were found to elicit potent antigen-specific T helper responses when injected into mice (Inaba et al., 1990). It has been demonstrated that such antigen-pulsed DCs interact with CD4⁺ Th cells in the PALS (Ingulli et al., 1997). In addition, Th cells primed by DCs were shown to interact with B cells and stimulate antigen-specific antibody production (Sornasse et al., 1992).

Strong in vivo CTL responses can also be induced by injection of mice with antigen-bearing DCs (Takahashi et al., 1993). In addition, DCs loaded with antigens in a non-peptide form have been shown to induce potent CTL responses in vivo. These include allogeneic DCs (McKinney and Streilein, 1989), protein-loaded DCs (Brossart and Bevan, 1997), DCs transfected with DNA (Rouse et al., 1994), DCs expressing virally encoded antigens (Kaplan et al., 1999; Specht et al., 1997) and DCs pulsed with RNA (Ashley et al., 1997).
The potent immunogenicity of DCs can not only induce an immune response to novel antigens, but also abrogate peripheral T cell tolerance against several antigens examined, which include soluble antigens (Pulendran et al., 1998), viral antigens (Shimizu et al., 1998), self antigens (Dittel et al., 1999), tumour antigens (Gong et al., 1998) and transplantation antigens (Steptoe et al., 1997).

It remains to be determined whether the special ability of DCs to prime T cells results from the expression of molecules unique to DCs or from the high density of molecules involved in DC-T cell interactions. MHC products and MHC-peptide complexes are expressed at 10- to 100-fold higher levels on DCs than on other APCs like B cells and monocytes (Inaba et al., 1997). Several adhesion molecules that mediate clustering between DCs and T cells, such as β1 and β2 integrins and members of the Ig superfamily (CD2, CD50, CD54 and CD58) (Bell et al., 1999), are expressed at high levels on mature DCs. So far, CD86 on DCs is known to be the most critical molecule for amplification of T cell responses (Caux et al., 1994b; Inaba et al., 1994). Other important T cell costimulatory molecules expressed on DCs include CD40 (Cella et al., 1996), 4-1BB ligand (DeBenedette et al., 1997), and OX40 ligand (Stuber et al., 1995).

1.4.5 Induction of tolerance by DCs

For a long time, much attention has been focussed on the exceptional ability of DCs to elicit T and B cell responses, and their potential as therapeutic immunological adjuvants. However, there is accumulating evidence suggesting that antigen presentation by DCs also contributes to inducing peripheral tolerance of T cells.
So far, the conventional view of DC function has been that DCs in the periphery pick up antigens and migrate into the T cell areas upon maturation, thereby initiating an antigen-specific immune response. The function of resident lymphoid DCs was considered to be separate from that of migratory DCs, in that DCs that reside in the T cell areas of lymphoid organs function as inducers of tolerance to self antigens (Steinman et al., 1997). The latter idea is based on the observation that in mice, DCs within T cell areas express high levels of self-antigens and functional Fas-L capable of inducing CD4+ T cell death (Suss and Shortman, 1996). However, if migratory myeloid DCs function solely to bring in antigens from the periphery and induce immunity, and resident lymphoid DCs only to present self-antigens and maintain self-tolerance (Steinman et al., 1997), the question arises: how do the lymphoid DCs acquire self antigens from the periphery, if these cells are not capable of migration? In addition, a different, but related question is, given that immature DCs are disseminated throughout the peripheral tissues and capture antigen from dying cells during the turnover of normal tissue, how are DCs prevented from inducing an immune response against self antigen? This question may partly be answered by data showing that freshly isolated DCs from lymphoid organs are functionally immature (Mellman and Steinman, 2001). However, these DCs from lymphoid tissues present large amounts of MHC products and MHC-peptide complexes on their surface in vivo (Inaba et al., 1998), which contrasts with the features of immature DCs in vitro. These "immature" DCs found in lymphoid organs are endocytically active and express relatively low levels of key costimulatory molecules such as CD86 and CD40 (Mellman and Steinman, 2001).
In addition, an old observation suggests that, even in the absence of overt stimuli, DCs traffic from tissue parenchyma to lymph nodes (Kelly et al., 1978). This was further supported by recent studies that DCs capture antigens against which immunity is normally avoided. For example, DCs that line the airways transport soluble macromolecules from the airway to the lymph nodes in the chest (Vermaelen et al., 2001). DCs in intestinal lymphatics were also shown to capture constitutively proteins placed in the gut lumen and fragments of apoptotic epithelial cells and carry the antigens to the T cell area of mesenteric lymph nodes (Huang et al., 2000). In these cases, if DCs were to be immunogenic, chronic inflammation would ensue.

These observations suggest that even in the absence of infection, DCs are constantly sampling the periphery and are migrating to the secondary lymphoid organs in an "immature" form, thus preventing T cell activation to self antigens captured in the periphery. This contradicts the paradigm set out earlier that DCs only migrate to draining lymph nodes when they have captured nonself antigen in the context of inflammatory stimuli.

So how do DCs achieve two contrasting roles: induction of immunity and tolerance? Recently, it was shown that DCs respond differently to damaged cells, depending on how they die; necrotic cells promote the maturation of DCs and strong CD4+ and CD8+ T cell stimulatory activity, whereas apoptotic cells fail to activate DCs (Gallucci et al., 1999; Sauter et al., 2000). Thus, in the absence of ‘danger’ signals DCs may be constantly inducing peripheral tolerance to self antigens acquired during the capture of apoptotic cells. Conceivably, the capture of proteins in the steady state,
i.e., in the absence of microbial or other perturbations, allows DCs to control tolerance to self and normal environmental constituents.

One mechanism that might be used by DCs to maintain self tolerance is the presentation of exogenous (primarily cell-derived) antigens to CD8\(^+\) T cells (cross presentation) or CD4\(^+\) Th cells, which may be mediated by presentation of antigens derived from apoptotic cell fragments by DCs (Albert et al., 1998). In support of this, Kurts et al demonstrated that in OVA-expressing transgenic mice, OVA-specific CD8\(^+\) T cells were rendered tolerant upon encounter with bone marrow-derived APCs in the lymph nodes draining the sites of OVA expression (Kurts et al., 1996; Kurts et al., 1998; Kurts et al., 1997).

Taken together, migratory DCs could have either T cell stimulatory function induced by danger signals, or tolerising function if they have captured antigen in the absence of danger signals. A recent study by Hawiger et al (Hawiger et al., 2001) demonstrated that in the absence of danger signals DCs \textit{in vivo} are indeed capable of migrating and carrying antigen captured in the periphery, and subsequently inducing tolerance of responding T cells. Therefore, it is likely that even within one lineage (not necessarily the lymphoid lineage) DCs do not only function as T cell stimulators, but in the absence of infection or 'danger' they migrate to lymphoid organs in an immature state, presenting self-antigen captured in the periphery, thereby maintaining peripheral tolerance.
1.4.6 Induction of different qualities of response by DCs

Two distinct subsets of CD4⁺ T helper (Th) cells are found in both rodents and in humans (section 1.3.4). They are classified in terms of their functional abilities and capacity to release unique profiles of cytokines generally associated with Th1 (inflammatory responses) and Th2 (humoral responses). T cell differentiation is probably affected by several factors, including the cytokine environment and antigen dose (Constant and Bottomly, 1997; O. Garra and Murphy, 1994). Since CD4⁺ T cells capable of mediating either Th1/Th2 type responses can only be isolated following a period of antigenic stimulation, the role of co-stimulatory signals, or APCs as a whole, in particular DCs, in shaping the differentiation pathway of naïve Th cells must be important because they provide the precursor Th cell with initial activation signals.

Current evidence suggests that the ability of DCs to induce different types of T cell responses is affected by: (1) the lineages from which the DCs are derived (Arpinati et al., 2000; Rissoan et al., 1999; Smith and de St Groth, 1999), (2) factors that influence the maturation of DCs (Vieira et al., 2000), and/or (3) the kinetics of DC maturation (Langenkamp et al., 2000). Throughout this thesis, DCs that mediate a Th1 response are denoted as DC1, and those that drive Th2 responses are denoted as DC2. Studies that support these ideas are described briefly below.

(1) DC lineage determines the function of DC1/DC2 (lineage hypothesis)

The idea that the lineage of DCs determines their Th1/Th2-mediating function was suggested by studies that compared the functional phenotypes of plasmacytoid T cell derived- and monocyte derived-DCs in humans (Arpinati et al., 2000; Rissoan et al.,
1999). It was shown that plasmacytoid T cell precursor-derived DCs (termed pDC2) had an inherent ability to induce Th2 responses while monocyte-derived DCs (pDC1) drove a Th1 response. Similarly, in mice, induction of Th1 and Th2 responses were shown to be induced by CD8+ "lymphoid" DCs and CD8- "myeloid" DCs, respectively (Smith and de St Groth, 1999), which was associated with the level of IL-12 produced by these subtypes of DCs in vivo (Maldonado-Lopez et al., 1999).

(2) DC1/DC2 differentiation is influenced by DC maturation conditions

The lineage hypothesis above is in disagreement with an increasing number of studies that support the idea that DCs derived from a single lineage can acquire a DC1/DC2 phenotype. For example, human plasmacytoid DCs, termed pDC2 (Rissoan et al., 1999) were shown to induce Th1 responses in the presence of CD40L and influenza virus (Cella et al., 2000). This is in direct conflict with an earlier study that showed pDC2 mediated Th2 responses (Rissoan et al., 1999). In addition, differentiation of monocyte-derived DCs into DC1 or DC2 phenotypes has been documented in many studies, both in mice (Heufler et al., 1996; Macatonia et al., 1995; Sato et al., 1999; Whelan et al., 2000) and humans (Vieira et al., 2000).

DC1 differentiation has been shown to be induced following stimulation with bacteria or bacterial products in murine DCs derived from bone marrow (Heufler et al., 1996; Whelan et al., 2000), murine splenic DCs (Heufler et al., 1996), and human monocyte-derived DCs (Heufler et al., 1996; Hilkens et al., 1997). Other DC1 mediating factors include influenza virus, CD40 ligation on DCs (Cella et al., 1996; Koch et al., 1996), and various cytokines such as TNFα and IFNγ (Liu et al., 1998; Macatonia et al., 1995; Sato et al., 1999).
Development of a DC2 phenotype was observed following stimulation of bone marrow-derived DCs with a filarial nematode product (Whelan et al., 2000). The presence of Th2 cytokines such as IL-4 during DC maturation also drives the differentiation of DC2 phenotype (Liu et al., 1998; Macatonia et al., 1995; Sato et al., 1999). In addition, accumulating evidence suggests a role for PGE$_2$ and other molecules that elevate intracellular cAMP levels in generating a DC2 phenotype (Gagliardi et al., 2000; Kalinski et al., 1997b).

(3) **DC1/DC2 differentiation is influenced by the kinetics of DC maturation**

The concept that the kinetics of DC maturation may influence the differential priming of responding T cells came from the observation that LPS-activated DCs that induced a Th1 response at an early stage induced a Th2 response at a later stage of maturation (Langenkamp et al., 2000). This earlier induction of a Th1 response correlated with the ability of DCs to produce IL-12.

While it is unclear to what extent the three mechanisms described above reflect what is happening *in vivo*, it is likely that the differential maturation of DCs is regulated by complex mechanisms. It is possible that all of these factors (DC lineage, environmental factors and the maturation status of DCs) contribute to and influence the differentiation of DC and the subsequent type of T cell response.
1.5 Tumour biology

1.5.1 Cancer epidemiology

World Health Organisation (WHO) data indicates that the global incidence of cancer is now over 10 million, with mortality of approximately 6 million. These numbers are soaring due to rapidly ageing populations in most countries. By the year 2020, it is estimated that there will be 20 million new cancer patients each year. Cancer arises from a series of uncontrolled cell divisions caused by either spontaneous mutations or infections by certain viruses carrying oncogenes. Cancerous cells reproduce in defiance of normal control, and become a serious problem when they metastasise.

In the UK, cancer is a major cause of morbidity, with nearly 260,000 new cases (excluding non-melanoma skin cancer) registered in 1997; the lifetime risk of developing cancer is more than one in three. In fact, cancer is the cause of a quarter (24 per cent) of all deaths in the UK (in 1999 there were 152,500 deaths from cancer). The disease is more likely to develop later in life, with around 65 per cent of cancers diagnosed in people over the age of 65. In people under the age of 75, deaths from cancer outnumber deaths from diseases of the circulatory system, including ischaemic heart disease and stroke. There are more than 200 different types of cancer, but four of them, lung, breast, large bowel (colorectal) and prostate, account for half of all deaths caused by cancer (figure 1.4).
In addition, figure 1.5 shows that many more people are dying from cancer in the UK compared to 50 years ago, and the number is still increasing. The main reason for this increase, despite the availability of improved medical and clinical facilities, is the increased longevity of the population and that most cancers occur later in life.
1.5.2 Therapeutic approaches

Currently available therapies (namely, radiotherapy, chemotherapy and surgery) have achieved only limited success (most metastatic cancers are refractory to these conventional therapies), and these therapies are usually accompanied by serious side effects. Therefore, the need to develop alternative approaches is enormous.

Recently developed approaches that are still under investigation are gene therapy and immunotherapy. Gene therapy refers to approaches that involve manipulation of DNA: DNA is used as a therapeutic vehicle to carry an essential ‘code’ either to correct an existing gene, or to deliver a novel gene [reviewed in (Dachs et al., 1997)]. Examples are delivery of toxin genes, ‘suicidal genes’ for tumour cell eradication, and genes encoding prodrug-activating enzymes. In contrast, immunotherapy involves manipulation of the immune system to reject the tumour. The development of the immunotherapeutic approach is discussed in this thesis.

Immunotherapy

Vaccination operates by modulating the natural immune response elicited by a given antigen. It can be given as a prophylactic measure, as in the case of vaccination against infectious agents, with the aim of eliciting quicker and specific responses when the body re-encounters the same pathogen, promoting effective elimination of the infection. In contrast, vaccination in cancer is usually therapeutic (with the exception of the prophylactic effect of Hepatitis B virus vaccination, which results in reduced incidence of liver cancer (Lee et al., 1998)), and is based on the enhancement and activation of the host immune system, to mount a specific response against the tumour.
Immunotherapy can be divided into two broad categories, passive and active. As a general rule, passive immunotherapy utilises products of effector cells (such as cytokines and antibodies), or cells that are already “primed” to function upon administration. An example in cancer therapy is direct administration of recombinant TNFα (Lejeune et al., 1998). In contrast, active immunotherapy involves strategies to initiate the immune effector functions, such as immunisation with tumour antigen (with or without adjuvants). The induction of an antitumour immune response by active immunotherapy is the main focus of this thesis, and is discussed further later in this chapter (section 1.5.12).

1.5.3 Role of the immune system in immune surveillance of tumour cells

The role of the immune system in searching for and eliminating tumour cells was first proposed almost 50 years ago by Thomas (Thomas, 1959), and further described in “the concept of immunological surveillance” by Burnet (Burnet, 1970). The immune surveillance hypothesis suggests that the major function of the immune system is to seek out and destroy new tumour cells as they arise. Thus, cancer was explained by the ability of tumour cells to avoid detection by the immune system (Burnet, 1970).

Evidence for the presence of immune surveillance in cancer is the association between immunosuppression and increased tumour incidence in transplant patients. However, available data strongly suggest that the immune response is directed against oncogenic viruses (Lee et al., 1998; Sheil, 1998). Over the last 20 years our understanding of the immune system has greatly increased, and it is clear that the interaction between the immune system and tumour cells is much more complex than originally suggested by Burnet.
1.5.4 Recognition of tumour cells by the innate immune system

Exposure to environmental stresses such as carcinogens, pyrogens, inflammatory cytokines and oxidants is inevitable for any cell and may predispose to neoplastic transformation. Thus, cells have developed complex responses that result either in successful repair and survival, or alternatively, recognition of failed repair, which culminates in apoptosis. If cells fail to repair mutations but survive programmed cell death, a successful system of immunosurveillance must defend the organism from tumour initiation. Notably, the role played by natural killer (NK) cells in detection and control of tumour cells has been studied in detail.

**NK cells**

It has long been recognised that NK cells can spontaneously kill tumour cells (Barlozzari et al., 1983; Riccardi et al., 1980). They mediate cytotoxicity via perforin and produce a variety of cytokines (including IFNγ) after interaction of their cell surface receptors with cognate ligands. NK cells are highly responsive to many cytokines, including IL-2, IL-12, IL-15 and IFNs, and rapidly increase their cytolytic, secretory, proliferative and anti-tumour functions.

NK cells achieve the destruction of tumour targets by the recognition of cell-surface structures. The killing activity of NK cells is regulated by interacting with receptors that generate counter-activating signals that block the induction of cytotoxicity. The specific structures recognised by such inhibitory receptors have been defined as MHC class I molecules. Thus, cells exhibiting normal MHC class I levels are spared NK cell mediated cytotoxicity, whereas tumour cells, many of which have completely or
partially lost MHC class I, are not. Three inhibitory receptor families have been
discovered: the killer cell Ig-like receptors (KIRs) (primates); the Ly49 lectin-like
homodimers (rodents); and the CD94-NKG2A lectin-like receptors (primates and
rodents). Recently, several other ligands for these receptors have also been described.
Examples are non-classical MHC molecules, such as HLA-E molecules and CD1
[reviewed in (Long, 1999; Ravetch and Lanier, 2000)].

Although the "missing self" hypothesis, in which cytotoxicity is controlled by the
action of inhibitory receptors, is valid, the observation that there are many class I-
deficient targets that are not recognised efficiently by NK cells has indicated the
existence of NK cell-specific activation receptors. Indeed, there are activating NK
receptors, such as FcR and CD2 that could transmit intracellular signals to initiate
cytotoxicity. In addition, NK-specific activation receptors have recently been
identified. Examples are families of NK cell c-lectin or Ig-like receptors (Yokoyama,
1998). Members of these families either contain an immunoreceptor tyrosine kinase-
based inhibitory motif (ITIM) that mediates inhibitory signals, or have a shortened
cytoplasmic domain that associates with an immunoreceptor tyrosine-based activation
motif (ITAM) bearing molecule. This latter group of receptors may be responsible for
directing the cytotoxic action of NK cells (Soloski, 2001). Therefore, NK cell
recognition is mediated by the opposing effects of two sets of NK receptors defined
operationally as activating and inhibiting receptors (Soloski, 2001).

Other components of the innate immune system
A subset of T cells that express some NK cell markers [NK T cells (Godfrey et al.,
2000)] have also shown the capacity to regulate both CTL and NK cell anti-tumour
activity. These cells recognise CD1d in the context of glycolipid ligands and rapidly secrete large amounts of Th1 and Th2 cytokines, linking the innate and acquired immune systems. Like NK cells, the reactivities of NKT cells are controlled by both inhibitory and stimulatory receptors. In addition, various investigators have established that macrophages can serve as both positive and negative mediators of the immune system. As positive effector cells, macrophages mediate direct anti-tumour cytotoxicity or presentation of tumour-associated antigens, which are effective strategies for the eradication of tumours. In contrast, tumour-induced macrophages demonstrate tumour cell growth promoting abilities that aid in tumour growth (Elgert et al., 1998; Mantovani et al., 1992), and these macrophages suppress many T and NK cell anti-tumour responses (Elgert et al., 1998).

1.5.5 Recognition of tumour cells by the adaptive immune system (anti-tumour responses)

As already discussed in section 1.3, the function of the adaptive immune system depends on the recognition of antigens. In experimental animal models, tumour antigens have been shown to induce both humoral and cell-mediated responses resulting in destruction of the tumour cells [reviewed in (Boon et al., 1994)]. With increasing evidence for the role of cytotoxic T cells (CTLs) in mediating destruction of tumour cells (Boon et al., 1994; van der Bruggen et al., 1991), much effort has been focussed on developing techniques to identify antigens that are recognised by CTLs (Boon et al., 1994; Van den Eynde and van der Bruggen, 1997). These antigens are described below.
Tumour antigens recognised by the immune system

Tumour cells arise as a result of cellular transformation, so it is not surprising that the majority of antigens expressed in tumour cells are "self" antigens. The tumour-associated "self" antigens (TAAs), together with antigens that are exclusively specific to tumours (TSAs), can be categorised into five classes (Boon et al., 1994; Van den Eynde and van der Bruggen, 1997; van der Bruggen et al., 1991; Wang and Rosenberg, 1999).

1. Unique tumour antigens generated by mutation, that are expressed exclusively in the tumour cells. Examples are oncogene and tumour-suppresser gene products (the most commonly altered tumour suppressor gene in cancer is p53) (Nigro et al., 1989), as well as other gene products such as the product of the β-catenin gene, which alters binding to E-cadherins (Rubinfeld et al., 1997).

2. Unique tumour antigens generated by changes in glycosylation patterns. For example, MUC-1, is a mucin gene product that is underglycosylated in breast and pancreatic tumour cells [reviewed in (Finn et al., 1995)].

3. Tumour associated antigens shared by many tumour types. These are typically the products of genes that have been reactivated. Best characterised are the products of the MAGE (melanoma antigen gene) gene family, for example, MAGE-1 and MAGE-3, which are expressed most frequently in melanoma (Rosenberg et al., 1996; Van den Eynde and van der Bruggen, 1997).

4. Tissue-specific differentiation antigens expressed by the normal tissue from which the tumour arose. These are non-mutated proteins derived from tissue specific differentiation antigens, such as the proteins of melanosomes (e.g., Mart-1, gp100 and TRP 1) in melanoma (Cox et al., 1994; Wang and Rosenberg, 1999).
5. Viral antigens in virus-associated tumours. These are viral antigens that can be a target of the immune response to tumour cells. Examples are E6 and E7 in Human Papillomavirus associated cancer (Beaudenon et al., 1986; Rickinson and Moss, 1997).

Eliciting TAAs-specific immune responses

Originally, vaccines that target unique tumour-specific antigens (TSAs) were preferred because of two fundamental advantages. Firstly, immune responses targeted against unique antigens would be exquisitely tumour-specific and produce no collateral damage to normal cells. Secondly, immune tolerance to tumour specific neoantigens would not be stringent as these would have arisen subsequent to development of the mature adult immune system. Thus, it is likely that breaking of tolerance to neoantigens would be easier than tolerance to self-antigens.

One major drawback in developing a vaccine targeted against unique tumour-specific antigens is that this method would, by necessity, be individualised rather than generic, thereby dramatically increasing the cost and labour intensiveness of treating large numbers of patients. Increasing evidence from recent studies that TAAs can elicit specific delayed-type hypersensitivity (DTH) and CTL responses that lead to clinical tumour regression (Jager et al., 1999; Marchand et al., 1995; Rosenberg et al., 1998) raises the possibility that TAAs can be used as a target for cancer immunotherapy.

1.5.6 Failure of the immune system to eliminate tumour cells

Despite the presence of recognised tumour antigens, and numerous effector mechanisms both in the innate and adaptive immune systems, cancers still develop in
a fully immunocompetent individual. So why does the immune system fail to eradicate tumour cells in most cancer patients?

As the immune system evolved under selective pressure imposed by infectious microorganisms, perhaps the simplest answer to this question may be that the immune system has not evolved to eliminate tumour cells. This probably reflects the fact that most cancers arise after the individuals have reproduced, thus lowering the chance of selection at the genetic level. Thus, understanding how the immune system has been selected to work, and its interaction with tumours is critical for designing an effective cancer vaccine.

1.5.7 Factors determining the immunogenicity of an antigen

As described earlier (section 1.3), adaptive immune responses involve the activation of T cells by specific antigen (with the exception of some B cell responses induced by thymus independent antigens, which play an important role in responses against extracellular bacteria). Initiating an effective response to tumour antigens certainly requires activation of T cells that recognise these antigens. The mechanism by which T cells “see” antigen in the periphery is discussed below.

Two signal model of T cell activation

How do T cells “know” when to become activated upon encounter with an antigen in the periphery? A model to address this question was first described by Bretcher and Cohn, originally to explain maintenance of self-tolerance (Bretscher and Cohn, 1970), and later by Lafferty and Cunningham to explain the requirement of APCs in graft rejection (Lafferty and Cunningham, 1975). This model states that naïve T cells need
to receive two independent signals in order to become activated: the first resulting from the engagement of TcR with MHC:peptide complexes presented on APCs ("signal 1"), and the second derived from engagement of costimulatory molecules with counter-receptors on T cells ("signal 2"). The best-characterised examples of the latter are B7 molecules on APCs, and CD28 and CTLA-4 on the responding T cells. Evidence suggests that signal 1 on its own sends a negative signal to the responding T cells (Schwartz, 1989). As a result, immune responses at the T cell level are ultimately dependent on costimulatory signals (signal 2) present at the time of antigen recognition. However, T cell activation is not an all-or-nothing event, and the nature of both signal 1 and signal 2 can influence the outcome of T cell responses, as discussed below.

1.5.8 Determinants of signal 1: generation of the T cell repertoire

Signal 1 is triggered through the TcR, and is usually derived from a physical interaction between the TcR on T cells and MHC:peptide complexes on APCs. The nature of the signal and how T cells respond to it, depends on how the TcR interacts with the peptide presented, which is in turn determined in the process of thymic selection.

TcR agonists, partial agonists and antagonists

How can the T cell response be influenced by the peptide it recognises? The outcome of any biological ligand-receptor interaction is determined by a number of factors including the binding strength (affinity) and efficacy of the ligand to provoke a biological response. Thus, a ligand (in this case a peptide) may have identical affinity for the receptor (TcR) but could exhibit different degrees of efficacy (Jameson and
Bevan, 1995a). A peptide can be defined as a TcR antagonist if it inhibits, by competition, particular biological responses (e.g., IL-2 production, proliferation) induced by another peptide (an agonist) recognised by the same TcR. A partial agonist, in contrast, may inhibit only a part of the responses mediated by an agonistic peptide. Thus, an antagonistic peptide has a lower efficacy than an agonistic peptide to induce a response, but may have the same affinity for the TcR. It is thought that the efficacy of peptides for TcR depends on a number of factors. These include the abilities of a peptide to induce: (1) TcR conformational change; (2) alterations in the kinetics and level of TcR clustering (TcR cross-linking); (3) changes in TcR clustering with the participation of other receptors (e.g., co-receptors); (4) induction of signalling pathways that mediate negative signals. It is unclear at this stage which of these factors plays a key role in modulating T cell responses. It is possible that different factors apply to different peptide-TcR interactions (Jameson and Bevan, 1995a).

**Thymic selection**

The repertoire of antigen specificities displayed by T cells is shaped during thymic development. In the thymus, first the genes encoding the α and β chains of the TcR undergo random rearrangement in immature CD4<sup>-</sup>CD8<sup>-</sup> (double negative, DN) or CD4<sup>+</sup>CD8<sup>+</sup> (double positive, DP) thymocytes, generating TcRs with great structural diversity (von Boehmer et al., 1989). Through selection processes in the thymus the diversity of the TcR repertoire is subsequently reduced (von Boehmer et al., 1989), eliminating 95%-99% of developing T cells, so that only 1%-5% of the cells produced in the thymus ever leave as mature T cells (Janeway, 1994).
In a selection process known as positive selection, T cells that are capable of MHC-restricted recognition of antigen are selected (Bevan, 1977; Zinkernagel et al., 1978). Thus, only those immature DP thymocytes that express TcRs with sufficient binding affinity for peptide:self-MHC complexes expressed on thymic stromal cells are given the signals to differentiate any further (Kisielow et al., 1988; Lo and Sprent, 1986; Sha et al., 1988). This process takes place in the thymic cortex. The majority of DP thymocytes do not undergo positive selection and are eliminated by programmed cell death (PCD). Engagement of TcRs with MHC class I molecules results in differentiation into CD4^CD8^ cells, whereas engagement of TcRs with MHC class II molecules leads to differentiation into CD4^CD8^- cells (Teh et al., 1988).

In contrast, during the process of negative selection, T cells that are potentially autoreactive are eliminated through clonal deletion (Schwartz, 1989). This process takes place in the thymic cortex, and especially at the cortico-medullary junction. In negative selection T cells are triggered to die if they bear receptors that would allow the mature T cell to respond to self-peptides bound to self MHC molecules (Hugo et al., 1993; Schwartz, 1989). Together these selection events generate a repertoire of T cells that are able to recognise a large diversity of antigens without being able to respond to self MHC molecules.

**Thymic paradox**

Both positive and negative selection involves interactions between TcRs and peptide/self-MHC complexes in the thymus. So how does this same interaction trigger two completely different cell fates during positive and negative selection? Many models have been proposed to resolve this apparent paradox ("thymic
paradox”). These are largely based on two hypotheses: the first assumes that qualitatively different signals mediate these selection processes, while the second suggests that they are driven by quantitatively different signals (Sebzda et al., 1999).

**Models for thymic selection**

The qualitative model proposes that mutually exclusive peptides promote positive and negative selection (Jameson et al., 1995b). This model was supported primarily by studies demonstrating that nonstimulatory antagonist peptides could promote positive selection. Using the OT-1 TcR transgenic mouse model specific for ovalbumin and H-2Kb, a correlation was drawn between positively selecting ligands and antagonist peptides, whilst agonist peptides were shown to promote clonal deletion (Hogquist et al., 1994; Jameson et al., 1994). The model was also supported by studies showing that interactions with altered peptide ligands transmit distinct intracellular signals, leading to altered ζ-chain phosphorylation and a lack of ZAP-70 activity (Madrenas et al., 1995; Sloan-Lancaster et al., 1994). However, while in some systems unique signals were observed in positive and negative selection (Jameson et al., 1995b), others have shown that antagonist peptides can induce clonal deletion (Page et al., 1994), inhibit negative selection (Williams et al., 1996), or inhibit T cell development (Spain et al., 1994), raising a question about the direct association between antagonist peptides and positive selection. Furthermore, it was shown that a panel of altered peptide ligands ranging from agonist to antagonist ligands can transmit a gradient of intracellular signals (Smyth et al., 1998), suggesting that differences observed in response to altered peptide ligands may be due to quantitative rather than qualitative differences in signalling (Hemmer et al., 1998a; Preckel et al., 1997)
The idea that the T cells are selected on the basis of the quantity of the signals received by developing thymocytes was first proposed by Sprent (Sprent et al., 1988), who suggested that TcR engagement resulting in negative selection arises from high-affinity interactions with MHC:peptide complexes, while positive selection arises from low-affinity interactions. This was further extended and represented as an "avidity model", which proposed that it is the avidity, and not just the affinity of individual TcR that accounts for the selection process (Ashton-Rickardt and Tonegawa, 1994b). Molecules that affect thymocyte-stromal cell avidity, such as coreceptors and adhesion molecules, will influence the duration and/or strength of TcR signalling and therefore have an impact on thymocyte fate. Thus, when the avidity is lower than a minimal threshold, the thymocyte is not rescued from programmed cell death (PCD), whilst when the avidity is above the threshold, and within a certain range, the signal for positive selection is delivered. When the avidity is higher than this range, then the signal for negative selection is delivered and the thymocyte is deleted. Importantly, this model illustrates that the avidity necessary for positive selection is lower than that required for negative selection, which, in turn, is lower than that required for activation of mature T cells. This provides a safety mechanism to prevent activation of T cells in response to self in the periphery. Direct evidence indicating that thymocyte selection may be governed by a quantitative avidity model came from studies using TcR transgenic mouse lines, where low concentrations of a strong peptide agonist induced positive selection, whereas clonal deletion occurred with high concentrations of the same peptide (Ashton-Rickardt et al., 1994a; Sebzda et al., 1994). These studies indicate that different concentrations of the same peptide can mediate both positive and negative selection. In addition, direct affinity measurement of the ligands used in a TcR transgenic mouse model
showed that positively selecting peptides had a lower affinity than negatively selecting ligands (Alam et al., 1996).

Although these two models may differ in the mechanism of selection, they can be merged into one picture, incorporating the factors that are considered crucial for the selection processes in these models. The reactivity of the thymocyte to a range of MHC:peptide complexes (and thus the selection of the thymocytes) can be described in a bell-shaped curve, as shown schematically in figure 1.6. A given TcR would recognise any given MHC:peptide complex with varying levels of reactivity (where reactivity is measured by activation of thymocytes induced by recognition of MHC:peptide complexes). The reactivity is influenced by the affinity and avidity of the complex for the TcR, as well as the quality of signals mediated by the interaction. Recognition of MHC:peptide complexes that result in poor signal generation would lead to death of the thymocyte by neglect, whilst recognition of MHC:peptide complexes resulting in a large signal would also lead to death of the thymocyte by deletion (negative selection). As a result, thymocytes would be selected to survive only upon interaction with MHC:peptide complexes that generate a low-intermediate signal (figure 1.6, area between dotted lines). The same MHC:peptide complex that selected the thymocyte to survive (marked x in the figure 1.6) may induce death by neglect or deletion if the ligand density decreases (blue line) or increases (red line), respectively. Thus, as a result of the selection process TcRs have been selected to recognise self MHC:peptide complexes with an affinity, which is high enough to allow stable interaction with the MHC:peptide complexes, but low enough not to trigger activation.
For simplicity of the terminology, the “reactivity” of the TcR to a given MHC:peptide complex is termed “affinity” from here on in this thesis.

1.5.9 Determinants of signal 2: activation of the innate immune system

Once the T cell repertoire is generated, the role of these T cells is to respond appropriately to a given signal 1 in the periphery. However, as described in section 1.5.7, peripheral T cells cannot be activated even when they receive a strong signal 1 unless accompanied by signal 2. Unlike signal 1, the vast majority of APCs cannot provide signal 2 unless the APCs themselves are activated. As T cells that were selected in the thymus have not been selected against recognition of tissue-specific
self antigens with high affinity (high signal 1), this requirement for signal 2 is an essential mechanism to maintain the peripheral tolerance of T cells to self-antigens.

The idea that the generation of signal 2 is achieved via activation of the innate immune system was first put forward by Janeway, who proposed that signal 2 is induced by distinctive recognition events involving evolutionarily primitive receptors that bind conserved microbial constituents (Janeway, 1992). He coined the term ‘infectious non-self’ for those structures that can bind to the conserved, non-clonal receptors on APCs and trigger the expression of costimulatory molecules. Thus the immune system can discriminate these from ‘non-infectious self’ where triggering of signal 2 does not occur. He suggests that cells that have receptors which can recognise these conserved microbial structures (e.g., LPS, double-stranded RNA) are selected over the course of evolution.

Whilst this model was widely accepted by immunologists, it was soon realised that the evolutionarily selected receptors cannot be the only mechanism by which the immune system ‘decides’ to mount a response. Not all pathogens express a ‘conserved’ structure vital for their survival which can be detected by the immune system, and there is always a chance that pathogens may evade immune recognition by a series of mutations under strong selective pressure. In addition, this model cannot provide explanations for a number of immunological observations such as allergy and alloreactivity. An extension of this model was therefore proposed (Ibrahim et al., 1995), which states that, in addition to utilising conserved cell surface receptors, APCs of the innate immune system can detect other external stimuli such as inflammatory cytokines, reactive oxygen intermediates and necrotic tissues, which are
associated with infection and/or tissue damage, and can respond to these by expressing signal 2. Thus, the outcome of antigen recognition by T cells depends on both the nature of the antigen and the microenvironment in which the APCs encounter it.

Independently of the above work, Matzinger proposed the so-called “danger model” (Matzinger, 1994) which states that the immune system has evolved to detect “danger” (which include factors associated with tissue damage and distress caused by infection or injury), but not to discriminate self and non-self. Whilst this theory challenged the current concepts of self tolerance and induction of immune responses, the idea that the innate immune system responds to “dangerous” cues thereby inducing an immune response is in agreement with the models proposed by Janeway and Ibrahim.

1.5.10 Response threshold for peripheral T cell activation

While it is the presence of both signal 1 and signal 2 which is necessary for inducing T cell activation, an interaction between a single TCR molecule and a MHC:peptide complex, even with the presence of signal 2, would not lead to T cell activation (Viola and Lanzavecchia, 1996). This suggests that there is a threshold level for the signal that needs to be reached in order to achieve full T cell activation. As discussed above, the strengths of signal 1 and signal 2 are determined independently, and T cell activation is triggered only when the summation of the two signals reaches a response threshold.
Signal 1 is an interaction between the MHC:peptide complex on an APC and the TCR on responding T cells. Almost every cell in our body expresses MHC class I [but at low levels (Rowe et al., 1983)], while high levels of MHC class II expression tends to be more restricted to 'professional' APCs. Since virtually any protein (be it 'self' or not derived from 'self') can be processed and presented on an APC at any point in time, we could assume that signal 1 is always present where there is a T cell-APC interaction (as empty MHC molecules are very unstable and rarely expressed on the cell surface). The strength of signal 1 in the periphery is believed to be influenced by a number of factors, such as that described by the 'avidity model' (Ashton-Rickardt and Tonegawa, 1994b). The avidity between an APC and responding T cell in an antigen-specific response is determined by both the affinity of individual MHC:peptide/TCR interactions, and the number of such interaction on T cells (density). Thus, strong avidity may be provided by high affinity and/or high density of the interactions, while a weak signal may be the result of low-affinity and/or low density interactions. However, strong avidity does not always result in T cell activation because the quality of signal provided to a T cell by peptide:TCR interactions is not uniform. Thus, some interactions may result in T cell activation (agonist peptides) while others may result in T cell tolerance (antagonist peptides) (Janeway, 1998; Janeway and Bottomly, 1996).

In contrast to signal 1, expression of high levels of signal 2 is restricted to activated APCs. The level of expression and therefore the strength of signal 2 depends on the cell type and can change dramatically depending on the activation status of APCs. For example, expression of costimulatory molecules on DCs is 10-100 times higher
than on any other APCs. During microbial infections, the density of co-stimulatory molecules is significantly up-regulated on APCs.

1.5.11 Mechanisms of T cell unresponsiveness to tumour antigens

Having defined the requirements for triggering T cell activation, it is easier to speculate how the immune system "sees" tumour cells. As mentioned earlier, most tumour antigens are derived from self molecules (section 1.5.5). The immune system has two ways to avoid a response targeted to self: (1) by the mechanism of thymic selection in which peripheral T cells have been selected to recognise self antigen with a low affinity (section 1.5.8), and (2) by peripheral tolerance mechanisms where a response to tissue-specific antigens is controlled by imposing a requirement for signal 2 for T cell activation (section 1.5.9). In other words, provided that signal 1 is not high, any antigen could appear as "self" antigen in the absence of signal 2.

The early stages in tumour transformation are 'quiet' processes in that they do not cause inflammation or tissue destruction. This allows tumour cells to have enough time to proliferate, and some may accumulate surface neoantigens without being "detected" by the immune system. When tumour cells die, fragments of the transformed cells can be picked up by APCs, but again lack of cues to activate the innate immune system means that tumour antigens would be presented without appropriate T cell activating signals.

At later stages, however, it is possible that cells undergoing damage and necrotic death caused by tumour cells may provide signals such as cytokines or heat shock proteins (HSPs) which would activate the innate immune system, including DCs.
(Gallucci and Matzinger, 2001). This would provide sufficient signal 2 to T cells recognising antigens derived from tumour cells. Only in the presence of such "dangerous" cues could tumour antigens become a target for adaptive immunity. However, the observation that spontaneous regression and complete elimination of tumour cells in cancer patients is rare, suggests that simply providing signal 2 may not be sufficient to eliminate tumour cells. Thus, the main problem with induction of an effective anti-tumour immune response is that tumour antigens are 'seen' by the immune system as no different from any other 'self' antigens. Understanding how the immune system regulates its response towards self antigens may provide clues to inducing an effective immune response against self-derived tumour antigens.

In addition to the poorly induced T cell response to tumour antigens, immune responses against tumour cells may be made even more difficult by active immune evasion by tumour cells. Having evaded innate surveillance mechanisms and established a tolerant environment, tumours can progress by turning their genetic instability to advantage and evade the adaptive immune response in various ways. The known mechanisms include: (1) down-regulation of MHC or the tumour antigens themselves (Ferrone and Marincola, 1995); (2) secretion of immunosuppressive cytokines such as TGFβ and IL-10 (Elgert et al., 1998); (3) expression of molecules such as FasL to kill attacking lymphocytes (Walker et al., 1998).

1.5.12 Eliciting immune responses to tumours

Evidence that the immune system perceives self-derived tumour antigens as "self" comes from experimental animal models. For example, in animal models where mice were immunised against TRP-1, TRP-2, gp100 or tyrosinase, immunity against the
melanocyte antigens induced vitiligo (Bowne et al., 1999; Colella et al., 2000; Hara et al., 1995; Naftzger et al., 1996; Overwijk et al., 1999). In addition, clinical observations have suggested that the appearance of vitiligo during melanoma progression or after therapy is correlated with an improved prognosis (Houghton et al., 2001). Thus, antitumour immune responses and autoimmune responses could be viewed as opposite faces of the same coin (Nanda and Sercarz, 1995). If this is indeed the case, understanding the mechanism of how self tolerance is broken to cause autoimmune disease may be the clue for designing an effective anti-tumour vaccine.

From the model proposed above (section 1.5.10) describing the response threshold for signal 1 and signal 2 to trigger peripheral T cell activation, it is likely that a high signal 2 would be required in order to elicit a response to an antigen providing a low signal 1. Indeed, means of enhancing anti-tumour responses through the use of adjuvants have been investigated for more than a century.

The role of adjuvant: introducing signal 2

By definition, an adjuvant is a substance that non-specifically enhances the immune response to an antigen. Adjuvants are believed to work by providing an artificial 'danger' signal which drives APC activation, so that captured tumour antigen can be presented in an 'immunogenic' form, i.e., providing signal 2 to the T cell (Fuchs and Matzinger, 1996).

The first example of the use of adjuvants in tumour immunotherapy was Coley's demonstration in 1893 that administration of extracts of pyogenic bacteria caused
sporadic anti-tumour regression [reviewed in (Starnes, 1992)]. Sixty years later, Lindenmann and Klein showed that vaccination with influenza virus infected tumour cell lysates generated enhanced systemic immune responses against a challenge with the original tumour cells (Lindenmann and Klein, 1967). They termed this approach “xenogenisation”. It was thought that a burst lymphokine production in response to influenza antigens resulted in amplification of normally weak responses to the poorly immunogenic endogenous tumour antigens. The response generated was shown to be critically CD4+ T cell dependent. More recently, the concept of presenting “adjuvants” at the same site as tumour antigens has been tested with some success (Chiodoni et al., 1999; Davis, 2000; Melcher et al., 1998; Tamura et al., 1997). For example, BCG immunisation enhanced rejection of some tumours, particularly bladder carcinoma [reviewd in (Davis, 2000)]. Other approaches include immunisation with tumours transduced with various cytokines, of which GM-CSF has produced the greatest degree of T cell immunity relative to irradiated nontransduced tumour cells [reviewed in (Greten and Jaffee, 1999)]. Other cytokines that stimulate innate immunity, such as IL-15 and type I IFNs, must also be more thoroughly evaluated in this context. Other strategies include the genetic engineering of tumour cells to express MHC and costimulatory molecules that are critical for T or NK cell activation (Cayeux et al., 1995; Townsend and Allison, 1993). In animal studies it has already been shown that transfection of tumours with B7-1 and MHC class II or cytokines, or blockade of CTLA-4 results in immunity against wildtype tumour challenges [reviewed in (Greten and Jaffee, 1999)].
Removing the safeguards: suppressing the suppressing mechanisms

The immune system has many safety mechanisms for preventing self-tissue destruction in order to avoid autoimmune disease. A number of regulatory or suppressor T cell subsets maintain self-tolerance and represent potentially formidable barriers to successful anti-tumour immune responses. These include NKT cells (Godfrey et al., 2000), CD25⁺CD4⁺ T cells (Sakaguchi, 2000a; Sakaguchi, 2000b) and CD8αα⁺γδ T cells (Hanninen and Harrison, 2000). NKT cells can prevent autoimmune diabetes in NOD mice (Hammond et al., 1998) and CD25⁺CD4⁺ T cells mediate protection against autoimmune gastritis (Sakaguchi, 2000a; Sakaguchi, 2000b).

Accumulating evidence suggests that these safe-guarding regulatory cells have a role in inhibiting tumour immunity, and that depletion of these cells results in improved cancer diagnosis. For example, NKT cells were shown to prevent complete tumour regression in a mouse model by inhibiting CTL-mediated anti-tumour immunity in an IL-13 dependent manner (Terabe et al., 2000). Depletion of CD25⁺CD4⁺ T cells can abrogate immunological unresponsiveness to syngeneic tumours in vivo, resulting in spontaneous tumour-specific CTL and NK cell cytotoxicity (Onizuka et al., 1999; Shimizu et al., 1999). This CD25⁺CD4⁺ subset appears to suppress the activation and proliferation of other CD4⁺ and CD8⁺ T cells in an antigenic nonspecific manner through direct contact with APCs (Sakaguchi, 2000a; Sakaguchi, 2000b). In addition, depletion of γδ T cells has also been shown to facilitate CTL- and NK cell-mediated tumour rejection (Seo et al., 1999), although it is not clear whether these are the same as the CD8αα⁺γδ T cells that prevent autoimmune diabetes (Hanninen and Harrison, 2000). Taken together, these observations suggest that interfering with the action of
suppressor or regulatory cells may be beneficial for treatment of cancer. This may involve a simple approach such as transiently depleting these cells or inhibiting their effector molecules (e.g., IL-13 for NKT cells). Thus, a strategy that incorporates both the enhancement of tumour antigen-targeted responses, and the suppression of regulatory functions of the immune system may result in promoting tumour rejection (Smyth et al., 2001).
1.6 DC-based tumour immunotherapy

1.6.1 DCs as adjuvants for tumour immunotherapy

As described in chapter 1.4, DCs are the only cell type that can elicit an immune response to a novel antigen, and are also known to be the most potent stimulators of naïve T cells (section 1.4.4). The ‘adjuvant’ properties of DCs clearly suggest a therapeutic potential and considerable attention has been given recently to the use of DCs in both murine and human models of cancer vaccines. Given that the presence of DCs \textit{in vivo} is not sufficient to control tumour cells in untreated cancer patients, it could be argued that there is a lack of tumour antigen presentation by DCs \textit{in vivo} (Schuler and Steinman, 1997), or alternatively, that tumour antigen presentation by DCs does not lead to T cell mediated resistance to tumours. As discussed above (section 1.4.5), DCs that acquire antigens from the periphery, including antigens derived from tumour cells, do not necessarily induce immunity. In fact, in the absence of DC maturation signals DCs are likely to present tumour antigens without sufficient T cell activation signals, resulting in tolerance.

Thus, in order to induce an effective antitumour immune response using DCs as a tool, there is a need to combat the obstacles that (1) tumour antigens may not access DCs \textit{in vivo}, and (2) presentation of tumour antigens by DCs may be inducing tolerance \textit{in vivo}. The best-studied approach is to use bone marrow-derived DCs (BmDC), phenotypically distinct and extremely potent APCs, to present tumour associated antigens and generate tumour specific immunity. Support for this strategy comes from animal studies demonstrating that DCs, when loaded \textit{ex vivo} with tumour antigens and administered to tumour bearing hosts, can elicit T cell mediated tumour
destruction (Boczkowski et al., 1996; Celluzzi et al., 1996; Labeur et al., 1999; Mayordomo et al., 1995; Zitvogel et al., 1996).

**1.6.2 DC immunotherapy in murine tumour models**

Most of the experiments to demonstrate generation of antitumour responses by DCs in vivo have utilised DCs isolated ex vivo (e.g., splenic DCs) or in vitro generation of DCs (e.g., BmDC), followed by loading of the DCs with tumour antigen and injection of the antigen-bearing DCs into syngeneic animals as a cancer vaccine.

The ability of in vitro generated BmDC to induce tumour-specific immunity has been demonstrated in a number of studies (Ashley et al., 1997; Celluzzi et al., 1996; Fields et al., 1998; Kaplan et al., 1999; Labeur et al., 1999; Mayordomo et al., 1995; Paglia et al., 1996; Porgador et al., 1996; Specht et al., 1997; Zitvogel et al., 1996). Much of the earlier work concentrated on immunisation with BmDC loaded with defined peptide tumour antigens (Celluzzi et al., 1996; Labeur et al., 1999; Mayordomo et al., 1995; Paglia et al., 1996; Porgador et al., 1996), which resulted in protection from subsequent tumour challenge (Celluzzi et al., 1996; Labeur et al., 1999; Mayordomo et al., 1995; Paglia et al., 1996; Porgador et al., 1996), as well as partial or complete regression of established tumours (Labeur et al., 1999; Mayordomo et al., 1995). Importantly, generation of tumour immunity induced by BmDC was shown to correlate with the DC maturation stage (Labeur et al., 1999), supporting the notion that maturation of DCs at the time of antigen presentation is crucial in inducing tumour immunity.
Although the tumour antigen-specific immunity induced by peptide-loaded DCs has been very successful experimentally, this strategy would not immediately benefit cancer patients whose tumours express as yet uncharacterised T cell epitopes. Therefore, a number of alternative approaches to the use of defined peptide tumour antigen have also been investigated. For example, Zitvogel et al utilised unfractionated, acid-eluted tumour peptides (bound to MHC class I) (Storkus et al., 1993) loaded onto BmDC, which were effective in regression of both fibrosarcoma and adenocarcinoma (Zitvogel et al., 1996). In addition, BmDC loaded with tumour lysate were also shown to be successful both in protection from tumour challenge and therapy of pulmonary metastases (Fields et al., 1998).

Considering that tumour tissues available from cancer patients may be limited, gene-based vaccination strategies have also been examined by a number of investigators, so as to minimise the quantity of tumour tissues required for vaccine preparation. For example, Boczkowski et al demonstrated, in a murine model of lung metastasis, that splenic DCs loaded ex vivo with total RNA of tumour cells were as effective as peptide-pulsed DCs in inducing tumour antigen-specific CTLs in vivo as well as in induction of tumour regression (Boczkowski et al., 1996). A further improved approach is the use of virally transduced DCs expressing tumour antigens (Kaplan et al., 1999; Song et al., 1997; Specht et al., 1997). This allows prolonged antigen presentation in vivo, as transduced DCs synthesise tumour antigens themselves and present them on the cell surface. BmDC that were transduced with a model tumour antigen (Song et al., 1997; Specht et al., 1997) and melanoma-associated antigens (Kaplan et al., 1999) were shown to be effective in regression of established tumours.
Other approaches include the use of DCs fused with tumour cells (Gong et al., 1997; Gong et al., 1998), and transduction of tumour cells in such a way that DCs would be targeted indirectly (Chiodoni et al., 1999). The former strategy was proven to be successful in a study where carcinoma cells fused with DCs induced tumour rejection (Gong et al., 1998). Indirect targeting of DCs was achieved by, for example, transducing tumour cells with GM-CSF and CD40L. This approach was shown to reduce the tumorigenicity of murine carcinoma in vivo (Chiodoni et al., 1999), although its therapeutic potential is yet to be determined.

One of the major problems with the use of DC-based vaccines is the generation of sufficient number of DCs to use in immunisation. Expansion of DCs using hematopoietic growth factors has been suggested. In particular, the effect of Flt3L in inducing expansion of DC number (Maraskovsky et al., 1996) has been incorporated into a number of DC-based or DC-targeted vaccine trials with some success (Pawlowska et al., 2001).

Taken together, DC based immunotherapy in murine models has been very successful, and these studies have established the rationale for evaluating tumour antigen-bearing DCs as therapeutic vaccines in humans.

1.6.3 Human clinical trials

In contrast to DC-based tumour therapy in animal models, a more limited number of successful results have been obtained in human clinical trials.
Based on the animal experiments, a number of different approaches to induce antitumour immunity have been taken in DC-based tumour therapy in humans. Most studies to date have utilised in vitro generated autologous DCs (typically peripheral blood monocyte-derived DCs that are generated in the presence of GM-CSF and IL-4) (Bakker et al., 1995; Boczkowski et al., 2000; Brossart et al., 2000; Eibl et al., 1997; Heiser et al., 2000; Nair et al., 2000; Nestle et al., 1998; Schuler-Thumer et al., 2000), whilst a few have used ex vivo isolated DCs (Fong et al., 2001; Hsu et al., 1996). Approaches that have been examined to date include in vitro/ex vivo manipulation of DCs such as loading of DCs with defined tumour peptide antigen (Bakker et al., 1995; Brossart et al., 2000; Fong et al., 2001; Nestle et al., 1998; Schuler-Thumer et al., 2000), DCs incubated with idiotypic protein (Hsu et al., 1996), DCs pulsed with tumour RNA (Boczkowski et al., 2000) or tumour lysate (Nestle et al., 1998), RNA-transfected DCs (Heiser et al., 2000; Nair et al., 2000) and DCs physically fused with tumour cells (Kugler et al., 2000).

Whilst most of these in vitro/ex vivo manipulated DCs were shown to be successful in induction of tumour antigen-specific responses as measured by in vitro T cell assays such as generation of tumour antigen-specific CTls and proliferation, only a few studies demonstrated some degree of correlation between these in vitro T cell functions and therapeutic effect on tumours in vivo (Fong et al., 2001; Hsu et al., 1996; Kugler et al., 2000; Nestle et al., 1998).

To date, results are still variable where the therapeutic potential of DC-based vaccines has been tested in real cancer patients, however, some successes are also being reported. For example, autologous DCs pulsed with a cocktail of known tumour-
associated melanocyte-differentiation antigens were used to treat advanced melanoma patients (Nestle et al., 1998). Out of 32 patients who were treated in this study, three complete and five partial responses were noted. More recently, the effect of autologous DCs pulsed with an altered peptide ligand of carcinoembryonic antigen (CEA) was examined in twelve patients with colon or non-small cell lung cancer (Fong et al., 2001). Flt3L was also included in the treatment, which expanded the number of DCs 20-fold in vivo. This resulted in induction of CD8\(^+\) T cell-mediated antitumour responses, and two out of twelve patients showed dramatic tumour regression, one had a partial regression and two showed stable disease. One of the most successful vaccine trials to date is one in which DCs loaded with idiotype protein were used to treat patients with follicular B-cell lymphoma (Hsu et al., 1996). The DCs were isolated by leukapheresis and co-cultured with idiotypic immunoglobulin from autologous lymphoma. All four patients treated developed measurable antitumour cellular immune responses, three patients had a complete or partial tumour regression, and none developed significant side effects. In addition to the use of tumour antigen-loaded DCs, Kugler et al developed an "electrofusion" technique to generate tumour-DC hybrid cells (Kugler et al., 2000). Seventeen patients with advanced renal cell cancer were vaccinated with hybrid cells. The DCs used were of a mature phenotype (CD83\(^+\)) because DCs were treated with TNF\(\alpha\) in culture before vaccination. Four patients demonstrated a prolonged complete response, two showed a partial response, two had stable disease and the remainder had progressive disease. CD8\(^+\) T cells were shown to migrate to the site of DC vaccination and 65% of patients developed positive DTH reactions.
Taken together, whilst the successes of DC-based immunotherapy in humans are still limited, attempts to generate effective antitumour immunity in cancer patients using this approach are ongoing. The differences observed in the efficacy of DC vaccines between murine models and human trials may be largely due to the fact that patients in such trials are restricted to those with late stage of malignancy, who often received extensive chemotherapy (some of which may be immunosuppressive) prior to the vaccine trial. In addition, findings made in the murine tumour model may not directly reflect what happens in human cancer. Thus, while studies in animal models provided valuable information, there is a substantial need for an improvement in vaccine design for human tumours, which would induce an antitumour immune response for a long enough period to eliminate tumour cells.

1.6.4 DC-targeted vaccines: from in vitro/ex vivo to in vivo methods

As discussed above, most of the DC-based vaccination protocols have utilised in vitro/ex vivo manipulated autologous DCs with some limited success. However, for a cancer vaccine to be used widely and accessible to a large number of cancer patients, not only should the design of the vaccine be generic but also inexpensive. All of the examples illustrated above involve extraction of patients’ DCs and/or tumour cells and modification of them in vitro, which means that the preparation of vaccine would need to be individualised and therefore, expensive. Thus, ideally a vaccine needs to be designed so that in vivo targeting of DCs is possible.

So far, attempts to develop in vivo targeting of DC-induced antitumour immunity have not been reported. However, there is evidence to suggest that such in vivo delivery of tumour antigen into DCs is possible. For example, Diebold et al have
developed a DNA delivery system where DCs are targeted on the basis of their surface mannose receptor expression (Diebold et al., 1999). More recently, a method for *in vivo* targeting of DCs to deliver DNA has been developed. This method relies on the ability of DCs to internalise antigen-IgG complexes resulting in effective priming of antigen specific CD4\(^+\) and CD8\(^+\) T cells in mice (You *et al*., 2001). A better understanding of DC-induced antitumour responses *in vivo*, together with development of effective vector systems that would allow selective targeting of DCs *in vivo*, may make it possible to design a generic cancer vaccine that is applicable to a large population of cancer patients.
1.7 Objectives

To date, most attempts to initiate an antitumour immune response in tumour-bearing hosts have involved the use of adjuvants and more recent studies have focussed on the use of DCs as an adjuvant that can initiate an immune response to tumour antigens. These approaches are based on the assumption that an immune response can be elicited against a weakly immunogenic tumour antigen (which gives low signal 1) by increasing the levels of signal 2. However, given that immunotherapy targeted at tumour antigens using adjuvants has had variable results and complete eradication of tumours in cancer patients is extremely rare, it is likely that the immunotherapy approaches currently used for treating cancer patients are far from optimal.

The original idea for the use of adjuvants came from successful vaccination protocols used for controlling infections. However, vaccination used for infections and cancer differ in two main aspects: first, vaccination for infections involves the use of foreign antigen, as opposed to self-derived antigen in cancer, and second, vaccination for infection is usually prophylactic, whereas immunisation for cancer is usually used for therapeutic purposes. If the model proposed in section 1.5.10 is correct, which suggests that a high level of signal 2 may compensate for low signal 1, the difference in efficacy of vaccination against infection and cancer may be explained by the difference in signal 1. This raises the question whether further optimisation of signal 2 can induce an effective immune response to low signal 1.

In order to design an efficacious antitumour vaccine, the mechanism of induction of immune responses to low-affinity antigen needs to be clear. This thesis attempts to examine the relative importance of signal 1 and signal 2 for triggering T cell
activation in response to low-affinity antigens. Bone marrow-derived DCs were used as a model APC. In addition, in order to establish the potential of DCs for manipulating the quality of immune responses, the mechanisms by which differentially-matured DCs trigger different types of T cell responses are also examined in this thesis.
Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Animals

BALB/c mice (H-2^d), C57BL/6 mice (H-2^b), DO11.10 αβ TCR transgenic mice (H-
2^d), IFNγ knock out mice (H-2^b), I-A knock out mice (H-2^b), β2m knock out mice (H-
2^b), and F5 TCR transgenic mice (F5 Rag^/-) were bred and maintained in the specific
pathogen free unit of the Institute for Animal Health, Compton, Berkshire. IL-12
deficient mice (IL-12p40^/-) were a kind gift from Dr. Adrian Mountford, Department
of Biology, University of York. Mice used were between 6 and 12 weeks of age.

2.1.2 Tissue culture media

RPMI-1640 (Life Technologies Ltd., Paisley) and Isocove's Modified Dulbecco's
medium (IMDM; Life Technologies) were used. These were supplemented with
reagents as detailed below:

1. RPMI-CM: used for culturing RMA-S cells and EL4 cells as well as for primary
cultures of mouse spleen and lymph node cells. RPMI-1640 medium was
supplemented with 10% v/v heat-inactivated FCS* (Harlan Sera-Lab),
penicillin/streptomycin (100U/ml, 100μg/ml, respectively), and 2mM L-glutamine
(Life Technologies).

2. BM-CM: used for culturing bone marrow-derived dendritic cells (BmDC), and in
all the functional assays using BmDC. IMDM with Glutamax-1, and 25mM
HEPES (Life Technologies) supplemented with 10% v/v heat-inactivated FCS*,
penicillin/streptomycin (100U/ml, 100μg/ml, respectively), 2mM L-glutamine and 50μM 2-mercaptoethanol (2-ME) (Sigma Chemical).

3. CTL-CM: used for generation of effector cells in CTL assay. IMDM with Glutamax-1 (Life Technologies), supplemented with 10% v/v heat-inactivated FCS**, penicillin/streptomycin (100U/ml, 100μg/ml, respectively), 2mM L-glutamine and 50μM 2-mercaptoethanol.

Table 2.1 General tissue culture reagents used in the studies

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>Sigma Chemical, Dorset</td>
<td>D-2650</td>
</tr>
<tr>
<td>FCS*</td>
<td>Harlan Sera-Lab</td>
<td>009501 (batch 91607)</td>
</tr>
<tr>
<td>FCS**</td>
<td>Life Technologies, Paisley</td>
<td>10106</td>
</tr>
<tr>
<td>IMDM</td>
<td>Life Technologies, Paisley</td>
<td>31980</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Life Technologies, Paisley</td>
<td>61870-010</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Life Technologies, Paisley</td>
<td>25030</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>Sigma Chemical, Dorset</td>
<td>M-7522</td>
</tr>
<tr>
<td>Penicillin</td>
<td>Life Technologies, Paisley</td>
<td>15140-114</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>Life Technologies, Paisley</td>
<td>15031-016</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Sigma Chemical, Dorset</td>
<td>T-8154</td>
</tr>
</tbody>
</table>

2.1.3 Cell lines

RMA-S cells and EL4 cells were maintained in RPMI-CM at 37°C, 5% CO₂. These cells were kept in liquid N₂ for long term storage as described below (section 2.2.2).
2.1.4 Plasticwares

Plastics used for tissue culture were radiation sterilised by the manufacturers.

Table 2.2. Plasticwares used in the studies

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal tubes, 30ml</td>
<td>Sterlin</td>
<td>128C</td>
</tr>
<tr>
<td>Bijou, 7ml</td>
<td>Sterlin</td>
<td>129A</td>
</tr>
<tr>
<td>15ml polypropylene tubes</td>
<td>Falcon</td>
<td>2096</td>
</tr>
<tr>
<td>50ml polypropylene tubes</td>
<td>Falcon</td>
<td>2070</td>
</tr>
<tr>
<td>23G x 1” needle</td>
<td>Terumo Europe, Belgium</td>
<td>NN-2325R</td>
</tr>
<tr>
<td>25cm² tissue culture flasks</td>
<td>TRP, Switzerland</td>
<td>9026</td>
</tr>
<tr>
<td>75cm² tissue culture flasks</td>
<td>TRP, Switzerland</td>
<td>9076</td>
</tr>
<tr>
<td>24 well tissue culture plate</td>
<td>NUNC™, Nalge Nunc International, Denmark</td>
<td>146485</td>
</tr>
<tr>
<td>96 well U-bottomed plate</td>
<td>NUNC, Nalge Nunc International</td>
<td>163320</td>
</tr>
<tr>
<td>Immulon 4HBX plate</td>
<td>Dynex Technologies</td>
<td>3855</td>
</tr>
<tr>
<td>F96 MaxiSorp plate</td>
<td>NUNC, Nalge Nunc International</td>
<td>439454A</td>
</tr>
<tr>
<td>Freezing container (Cryo 1°C)</td>
<td>NALGENE, Nalge Nunc International</td>
<td>5100-0001</td>
</tr>
<tr>
<td>70µm cell strainer</td>
<td>Falcon</td>
<td>2350</td>
</tr>
</tbody>
</table>

2.1.5 Reagents used to stimulate BmDC (modulins)

Table 2.3 Reagents used to stimulate BmDC

<table>
<thead>
<tr>
<th>Modulins</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccaride <em>Escherichia coli</em></td>
<td>Sigma Chemical</td>
<td>L-4524</td>
</tr>
<tr>
<td>serotype 055:B5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-CD40 antibody (clone 1C10)</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>MAB440</td>
</tr>
<tr>
<td>dibutyryl cyclic AMP (dbcAMP)</td>
<td>Sigma Chemical</td>
<td>D 0260</td>
</tr>
<tr>
<td>forskolin</td>
<td>Sigma Chemical</td>
<td>F 0922</td>
</tr>
<tr>
<td>Prostaglandin E₂ (PGE₂)</td>
<td>Sigma Chemical</td>
<td>P 6532</td>
</tr>
<tr>
<td>Pertussis holotoxin from <em>Bordetella pertussis</em> (PTx)</td>
<td>Calbiochem</td>
<td>516560</td>
</tr>
<tr>
<td>Pertussis toxin B-oligomer from <em>Bordetella pertussis</em> (PTxB)</td>
<td>Calbiochem</td>
<td>516852</td>
</tr>
<tr>
<td>Cholera toxin (CTx)</td>
<td>Sigma Chemical</td>
<td>C 8052</td>
</tr>
</tbody>
</table>
2.1.6 **Cytokines**

Recombinant cytokines were used to promote growth and/or differentiation of DCs or T cells in *in vitro* experiments.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IL-2</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>402-ML</td>
</tr>
<tr>
<td>Human IL-2</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>202-IL</td>
</tr>
<tr>
<td>Mouse GM-CSF</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>415-ML</td>
</tr>
<tr>
<td>Mouse IL-4</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>404-ML</td>
</tr>
<tr>
<td>Mouse IL-10</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>417-ML</td>
</tr>
<tr>
<td>Mouse IL-12</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>419-ML</td>
</tr>
<tr>
<td>Mouse IL-18</td>
<td>R&amp;D Systems, Abingdon, Oxon</td>
<td>422-ML</td>
</tr>
</tbody>
</table>

2.1.7 **Materials used to isolate cells by magnetic sorting**

Magnetic cell sorting developed by Miltenyl Biotec (Germany) was used to isolate cells by magnetic beads bound to monoclonal antibodies specific for cell surface markers of interest.

<table>
<thead>
<tr>
<th>Product</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse CD4 (L3T4) Microbeads</td>
<td>492-01</td>
</tr>
<tr>
<td>Mouse CD8a (Ly-2) Microbeads</td>
<td>494-01</td>
</tr>
<tr>
<td>Mouse CD11c (N418) Microbeads</td>
<td>520-01</td>
</tr>
<tr>
<td>Mouse CD62L (L-selectin) Microbeads</td>
<td>497-01</td>
</tr>
<tr>
<td>MS cell separation columns</td>
<td>130-042-201</td>
</tr>
<tr>
<td>LS cell separation columns</td>
<td>130-042-401</td>
</tr>
<tr>
<td>MiniMACS separation unit</td>
<td>130-042-102</td>
</tr>
<tr>
<td>MidiMACS separation unit</td>
<td>130-042-302</td>
</tr>
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### 2.1.8 Antibodies

#### Table 2.6A. Antibodies against murine cytokines

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>FITC</td>
<td>JES6-5H4</td>
<td>Rat IgG_2b</td>
<td>Pharmingen</td>
<td>18174A</td>
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<tr>
<td>IL-4</td>
<td>PE</td>
<td>BVD4-1D11</td>
<td>Rat IgG_2b</td>
<td>Pharmingen</td>
<td>18035A</td>
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<tr>
<td>IFN_γ</td>
<td>FITC</td>
<td>XMG1.2</td>
<td>Rat IgG_1</td>
<td>Pharmingen</td>
<td>18114A</td>
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<tr>
<td>IL-10</td>
<td>None</td>
<td>JES5-2A5</td>
<td>Rat IgG_1</td>
<td>Pharmingen</td>
<td>18140D</td>
</tr>
<tr>
<td>IL-12p40/70</td>
<td>Biotin</td>
<td>C17.8</td>
<td>Rat IgG_2a</td>
<td>Pharmingen</td>
<td>18482D</td>
</tr>
<tr>
<td>IL-12p40/70</td>
<td>None</td>
<td>C17.8</td>
<td>Rat IgG_2a</td>
<td>Pharmingen</td>
<td>18480D</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>None</td>
<td>C15.6</td>
<td>Rat IgG_1</td>
<td>Pharmingen</td>
<td>18491D</td>
</tr>
<tr>
<td>IL-18</td>
<td>None</td>
<td>51817.111</td>
<td>Rat IgG_2a</td>
<td>Pharmingen</td>
<td>20171D</td>
</tr>
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</table>

#### Table 2.6B. Antibodies directed to murine BmDC surface antigens

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
<td>FITC</td>
<td>2D7</td>
<td>Rat IgG_2a, \kappa</td>
<td>Pharmingen</td>
<td>01204D</td>
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<tr>
<td>CD11c</td>
<td>PE</td>
<td>HL3</td>
<td>Hamster IgG, group 1, \lambda</td>
<td>Pharmingen</td>
<td>09705B</td>
</tr>
<tr>
<td>CD40</td>
<td>FITC</td>
<td>HM40-3</td>
<td>Hamster IgM</td>
<td>Pharmingen</td>
<td>09404D</td>
</tr>
<tr>
<td>CD49d</td>
<td>FITC</td>
<td>R1-2</td>
<td>Rat IgG_2b, \kappa</td>
<td>Pharmingen</td>
<td>01274D</td>
</tr>
<tr>
<td>CD54</td>
<td>FITC</td>
<td>3E2</td>
<td>Hamster IgG, group 1, \lambda</td>
<td>Pharmingen</td>
<td>01544D</td>
</tr>
<tr>
<td>CD80/B7-1</td>
<td>FITC</td>
<td>GL1</td>
<td>Rat IgG_2a</td>
<td>Pharmingen</td>
<td>09604D</td>
</tr>
<tr>
<td>CD80/B7-1</td>
<td>None</td>
<td>1G10</td>
<td>Rat IgG_2a, \kappa</td>
<td>Pharmingen</td>
<td>01940D</td>
</tr>
<tr>
<td>CD86/B7-2</td>
<td>FITC</td>
<td>GL1</td>
<td>Rat IgG_2a</td>
<td>Pharmingen</td>
<td>09274D</td>
</tr>
<tr>
<td>CD86/B7-2</td>
<td>None</td>
<td>PO3</td>
<td>Rat IgG_2b, \kappa</td>
<td>Pharmingen</td>
<td>09880D</td>
</tr>
<tr>
<td>H-2K_b</td>
<td>PE</td>
<td>AF6-88.5</td>
<td>Mouse IgG_2a, \kappa</td>
<td>Pharmingen</td>
<td>06105A</td>
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<tr>
<td>H-2K_d</td>
<td>PE</td>
<td>SF1-1.1</td>
<td>Mouse IgG_2a, \kappa</td>
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<td>06095A</td>
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<tr>
<td>H-2D_d</td>
<td>FITC</td>
<td>KH95</td>
<td>Mouse IgG_2b, \kappa</td>
<td>Pharmingen</td>
<td>06114D</td>
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<tr>
<td>I-A_b</td>
<td>PE</td>
<td>M5/114.15.2</td>
<td>Rat IgG_2b, \kappa</td>
<td>Pharmingen</td>
<td>06355A</td>
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<tr>
<td>I-A_d/I-E_d</td>
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<td>2G9</td>
<td>Rat IgG_2a, \kappa</td>
<td>Pharmingen</td>
<td>06344D</td>
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### Table 2.6C. Antibodies directed to murine T cell surface antigens

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Catalogue Number</th>
</tr>
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<tbody>
<tr>
<td>CD2</td>
<td>FITC</td>
<td>RM2-5</td>
<td>Rat IgG₂κ, κ</td>
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<td>01174D</td>
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<tr>
<td>CD3</td>
<td>None</td>
<td>145-2C11</td>
<td>Hamster IgG, group 1, κ</td>
<td>Pharmingen</td>
<td>01080D</td>
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<tr>
<td>CD4</td>
<td>PE</td>
<td>H129.19</td>
<td>Rat IgG₂κ, κ</td>
<td>Pharmingen</td>
<td>18035A</td>
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<tr>
<td>CD4</td>
<td>Biotin</td>
<td>H129.19</td>
<td>Rat IgG₂κ, κ</td>
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<td>09002A/D</td>
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<tr>
<td>CD8a</td>
<td>PE</td>
<td>53-6.7</td>
<td>Rat IgG₂κ, κ</td>
<td>Pharmingen</td>
<td>01045B</td>
</tr>
<tr>
<td>CD8a</td>
<td>APC</td>
<td>53-6.7</td>
<td>Rat IgG₂κ, κ</td>
<td>Pharmingen</td>
<td>01049A</td>
</tr>
<tr>
<td>CD8a</td>
<td>Biotin</td>
<td>53-6.7</td>
<td>Rat IgG₂κ, κ</td>
<td>Pharmingen</td>
<td>01042A/D</td>
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<td>CD25</td>
<td>PE</td>
<td>PC61</td>
<td>Rat IgG₁</td>
<td>Pharmingen</td>
<td>09985B</td>
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<tr>
<td>CD28</td>
<td>None</td>
<td>37.51</td>
<td>Hamster IgG, group 2, λ</td>
<td>Pharmingen</td>
<td>01675D</td>
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<tr>
<td>CD28</td>
<td>PE</td>
<td>37.51</td>
<td>Hamster IgG, group 2, λ</td>
<td>Pharmingen</td>
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<tr>
<td>CD44</td>
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<td>1M7</td>
<td>Rat IgG₂κ, κ</td>
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<td>CD69</td>
<td>PE</td>
<td>H1.2F3</td>
<td>Hamster IgG, group 1, λ</td>
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<td>CDw137</td>
<td>PE</td>
<td>1AH2</td>
<td>Rat IgG₁, κ</td>
<td>Pharmingen</td>
<td>09845A</td>
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<tr>
<td>CD152</td>
<td>PE</td>
<td>UC10-4F10-11</td>
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<td>CD154</td>
<td>PE</td>
<td>MR1</td>
<td>Hamster IgG, group 3, κ</td>
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### Table 2.6D. Antibodies directed to other murine cell surface antigens

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Catalogue Number</th>
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<tr>
<td>CD11b</td>
<td>PE</td>
<td>M1/70.15</td>
<td>Rat IgG₂κ, κ</td>
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<tr>
<td>CD16/32</td>
<td>None</td>
<td>2.4G2</td>
<td>Rat IgG₂κ, κ</td>
<td>Pharmingen</td>
<td>01241A</td>
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<tr>
<td>Gr-1/Ly-6G</td>
<td>FITC</td>
<td>RB6-8C5</td>
<td>Rat IgG₂κ, κ</td>
<td>Pharmingen</td>
<td>01214D</td>
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<tr>
<td>F4/80</td>
<td>PE</td>
<td>F4/80</td>
<td>Rat IgG₂κ, κ</td>
<td>Serotec</td>
<td>MCA497PE</td>
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**Antibodies against pertussis toxin (PTx)**

Purified mouse anti-PTx monoclonal antibodies (anti-PT subunit S1, S2, S3, S4 and S5) were a kind gift from Dr. Xing, National Institute for Biological Standards and Control, UK.
### Table 2.6E. Isotype control antibodies

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>Rat IgG₁, κ</td>
<td>None</td>
<td>R3-34</td>
<td>Pharmingen</td>
<td>20610D</td>
</tr>
<tr>
<td>Rat IgG₂a, κ</td>
<td>PE</td>
<td>R35-95</td>
<td>Pharmingen</td>
<td>11025A</td>
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<tr>
<td>Rat IgG₂a</td>
<td>FITC</td>
<td>R35-95</td>
<td>Pharmingen</td>
<td>11024C</td>
</tr>
<tr>
<td>Rat IgG₂a</td>
<td>APC</td>
<td>R35-95</td>
<td>Pharmingen</td>
<td>11029A</td>
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<tr>
<td>Rat IgG₂a, κ</td>
<td>None</td>
<td>R35-95</td>
<td>Pharmingen</td>
<td>20620D</td>
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<tr>
<td>Rat IgG₂b, κ</td>
<td>PE</td>
<td>A95-1</td>
<td>Pharmingen</td>
<td>11185A</td>
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<tr>
<td>Rat IgG₂b, κ</td>
<td>FITC</td>
<td>A95-1</td>
<td>Pharmingen</td>
<td>11184C</td>
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<tr>
<td>Rat IgG₂b, κ</td>
<td>None</td>
<td>A95-1</td>
<td>Pharmingen</td>
<td>11180D</td>
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<tr>
<td>Hamster IgG, group 1, λ</td>
<td>PE</td>
<td>G235-2536</td>
<td>Pharmingen</td>
<td>11125A</td>
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<tr>
<td>Hamster IgG, group 1, λ</td>
<td>Biotin</td>
<td>G235-2536</td>
<td>Pharmingen</td>
<td>11122C</td>
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<tr>
<td>Hamster IgG, group 2, λ</td>
<td>None</td>
<td>B81-3</td>
<td>Pharmingen</td>
<td>11220D</td>
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<td>Hamster IgG, group 2, λ</td>
<td>PE</td>
<td>Ha4/8</td>
<td>Pharmingen</td>
<td>11145A</td>
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<tr>
<td>Hamster IgG, group 3, λ</td>
<td>PE</td>
<td>A19-4</td>
<td>Pharmingen</td>
<td>11165A</td>
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### Table 2.6F. Secondary antibodies

<table>
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<th>Specificity</th>
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<tr>
<td>Streptavidin</td>
<td>APC</td>
<td>Pharmingen</td>
<td>13049A</td>
</tr>
<tr>
<td>Goat F(ab')₂, anti-mouse IgM/IgG/IgA</td>
<td>FITC</td>
<td>Southern Biotechnology</td>
<td>1012-02</td>
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</table>
2.1.9 Peptides

Peptides recognised by the transgenic T cells of DO11.10 and F5 transgenic mice were synthesised by Genosys Biotechnologies, Cambridge.

Table 2.7 Peptides used for studying transgenic models

<table>
<thead>
<tr>
<th>Name</th>
<th>Derivation of peptide</th>
<th>Sequence</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA^{323-339}</td>
<td>Chicken ovalbumin OVA</td>
<td>ISQAVHAHAHEINEAGR</td>
<td>H-2A&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>NP68</td>
<td>Influenza nucleoprotein (A/NT/60/68)</td>
<td>ASNENMDAM</td>
<td>H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NP4Q</td>
<td>Influenza nucleoprotein</td>
<td>ASNQNMDAM</td>
<td>H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NP34</td>
<td>Influenza nucleoprotein (A/PR/8/34)</td>
<td>ASNENMETM</td>
<td>H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

2.1.10 Miscellaneous reagents

Table 2.8 Miscellaneous reagents used in the study

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<thead>
<tr>
<th>Reagents</th>
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<tr>
<td>New born calf serum</td>
<td>Life Technologies</td>
<td>26010</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma Chemical</td>
<td>A-4503</td>
</tr>
<tr>
<td>NaN&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Sigma Chemical</td>
<td>S-8032</td>
</tr>
<tr>
<td>PMA</td>
<td>Sigma Chemical</td>
<td>P-8139</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Sigma Chemical</td>
<td>L-0634</td>
</tr>
<tr>
<td>PHA</td>
<td>Sigma Chemical</td>
<td>L-9132</td>
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<td>Normal mouse serum</td>
<td>Sigma Chemical</td>
<td>S-7273</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma Chemical</td>
<td>P-1379</td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;Cl</td>
<td>Sigma Chemical</td>
<td>A-0171</td>
</tr>
<tr>
<td>TMB</td>
<td>Pharmingen</td>
<td>2642KK</td>
</tr>
<tr>
<td>PermeaFix™</td>
<td>Ortho Diagnostic Systems</td>
<td>775999</td>
</tr>
</tbody>
</table>

2.1.11 Radioactive Isotopes

Tritiated <sup>3</sup>H-thymidine and Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> were both purchased from Amersham Pharmacia Biotech.
2.2 Methods

2.2.1 Cell Viability Test by Trypan Blue Exclusion Method
A small volume of cell suspension to be counted (e.g., 10µl) was mixed with the same volume of trypan blue solution (Sigma Chemical). This was then layered on a cell counting chamber (Weber Scientific International) and viewed under a light microscope (Leica) at a magnification of ×100. Viable cells which had excluded trypan blue were counted and the number of the cells in the suspension was calculated as follows:
Number of viable cells in 25 squares × 2 (dilution factor) × 10⁴ = number of cells/ml

2.2.2 Cryopreservation and Retrieval of Cells
Cells to be cryopreserved were counted, and between 5 × 10⁶ and 2 × 10⁷ cells were then resuspended in 1ml FCS with 10% v/v DMSO per vial of cryotube (Greiner). These were placed in a freezing container (Cryo 1°C, NALGENE) which allows gradual decrease in temperature (1°C per minute) and stored at −80°C overnight before being transferred to the liquid N₂ storage. For retrieval of frozen cells, cryotubes were thawed in 37°C water bath. Cells were then washed twice in cold supplemented medium before counting and cultured at 37°C.

2.2.3 Bone Marrow DC (BmDC) Preparation from Mice
Femurs and tibias were removed under sterile conditions. Bone marrow was isolated by removing the ends of the bone with a pair of scissors and forceps and flushing out marrow with media using a 23G x 1” needle (Terumo Europe, Belgium). Bone
marrow cells were cultured in BM-CM and 10ng/ml recombinant mouse GM-CSF (R&D Systems) at approximately $10^6$ cells/ml in 75cm$^2$ tissue culture flasks (TPP). At day 3 (72 hours), non-adherent cells were removed by replacing the medium completely with fresh BM-CM with GM-CSF. At day 5 (120 hours), semi-adherent cells were removed from the culture flask, resuspended in fresh BM-CM at $10^6$ cells/ml and cultured in 24 well tissue culture plates (NUNCTM, Nalge Nunc International, Denmark) for 24 hours (unless otherwise stated), with or without addition of modulins (table 2.3).

2.2.4 Immunofluorescent Labelling of Cell Surface Markers

Cells to be examined were preincubated at $10^7$ cells/ml in blocking buffer (described below) for 15 minutes at 4°C before labelling with antibodies to minimise non-specific binding. PBS containing 0.1% w/v bovine-serum albumin (BSA) (Sigma) and 0.01% NaN$_3$ (Sigma) with 5% v/v normal mouse serum (Sigma) was used as a blocking buffer for labelling with rat- or hamster-derived antibodies, while 5% v/v FCS replaced the mouse serum for labelling with mouse-derived antibodies. Antibodies directed to cell surface markers (section 2.1.8) were diluted in the blocking buffer. $10^6$ cells were labelled with 25μl of the diluted antibodies for 15 minutes at 4°C, followed by two washes with cold PBS containing 0.01% NaN$_3$. Labelled cells were examined by flow cytometry using a FACScalibur (Becton Dickinson, Oxford, Oxon).

2.2.5 Immunofluorescent Labelling of Intracellular Cytokines

Following appropriate treatment, cells were further stimulated at $10^6$ cells/ml in RPMI-CM with 50ng/ml PMA (Sigma) and 500ng/ml ionomycin (Sigma) for 6 hours
at 37°C 5% CO₂ in order to amplify the cytokine production. After 2 hours of the 6 hour incubation Brefeldin A (Sigma) was added at a final concentration of 10μg/ml. These cells were harvested and incubated with the blocking buffer (section 2.2.4) at 10⁷ cells/ml for 15 minutes at 4°C. 10⁶ cells were first labelled with the antibodies directed to cell surface markers and washed as described earlier. 100μl PermeaFix™ (Ortho Diagnostic Systems) was added to the cells (which permealises and fixes the cells) and incubated at room temperature for 45 minutes in the dark. This was followed by two washes with cold PBS containing 0.01% NaN₃. Cells were then labelled with 25μl of diluted antibodies for intracellular cytokines for 30 minutes at 4°C, followed by two washes with cold PBS containing 0.01% NaN₃. Cells were examined by flow cytometry using a FACScalibur.

### 2.2.6 Labelling Cells with CFSE

Cells to be labelled were resuspended at 5 x 10⁷ cells/ml in serum-free PBS. A 10mM stock solution of carboxyfluorescein diacetate succinimidyl ester (CFSE, Cambridge bioscience, Cambridge, UK) in DMSO was added to a final concentration of 5μM and incubated at 37°C for 10 minutes. At the end of the incubation period, the cells were immediately washed 3 times in cold BM-CM.

### 2.2.7 Confocal Microscopy and Image Acquisition

Cells to be examined by confocal microscopy were prepared as described in section 2.2.4. Labelled cells (2x10⁵) were mounted on microscope slides using cytospin (Shandon) and allowed to dry. Samples were then examined by laser confocal microscopy (Leica TCS NT, Leica). Areas of interest were selected by conventional fluorescence microscopy and scanned using the ×40, ×60 or ×100 oil immersion...
objectives. Samples were scanned in a raster fashion to produce an electronic image of 1024×1024 pixels. The filter settings were altered manually to match the excitation and emission spectra of the fluorochromes. Images in X-Y sections were obtained from sequential scans in a Z plane.

2.2.8 Peptide Binding Assay

Efficiency of a peptide to bind MHC class I molecule was assessed using RMA-S cells (Ljunggren et al., 1990; Schumacher et al., 1990). RMA-S is a murine lymphoma cell line and expresses H-2D^b/K^b stably at 26°C but not at higher temperatures in the absence of added exogenous peptide (Schumacher et al., 1990). However, the expression can be stabilised at 37°C upon binding to a peptide (Ljunggren et al., 1990; Schumacher et al., 1990). RMA-S cells were cultured at 10^6 cells/ml in RPMI-CM. 100μl of the cell suspension was plated in each well of 96 well U-bottom plates, and incubated for 5 hours to overnight at 26°C. 1-2 hours before the end of the incubation period, titrated concentrations of peptides were added to the cells. The plates were then transferred to 37°C and incubated for 2-8 hours. Efficiency of the peptide binding was determined by measuring the expression of H-2K molecules on the cell surface by flow cytometry.

2.2.9 Purification of CD4^+ and CD8^+ T cells

CD4^+ T cells and CD8^+ T cells from BALB/c or C57BL/6 mice were isolated directly by using anti-mouse CD4/8 magnetic columns (Miltenyl Biotec). Briefly, spleen and peripheral lymph nodes were removed from mice and made into single cell suspensions in RPMI-CM by filtering through 70μm cell strainers. RBC from the cell suspension were lysed by treatment with 168mM NH₄Cl in PBS at room temperature
for 10 minutes. RBC were then removed by brief centrifugation to sediment the lysed RBC. The RBC-depleted cell suspension was washed in RPMI-CM, counted, and preincubated in PBS containing 0.5% w/v BSA and 5% v/v normal mouse serum for 15 minutes at 10^8 cells/ml to minimise non-specific binding of the antibodies. Cells were then labelled with anti-mouse CD4/8 magnetic beads and isolated according to the manufacture’s recommendation. For cytokine studies in the DO11.10 transgenic system, single cell suspensions of splenocytes made from DO11.10 transgenic mice were first positively selected for cells expressing CD62L using the MACS columns as described above. CD62L positive cells were then stained with PE-conjugated CD4 antibody (H129.19, Pharmingen) and were sorted using the MoFlo sorter (Fort Collins, Colorado, USA). Purity of the sorted cells was examined by flow cytometry.

2.2.10 Mixed Lymphocyte Reaction (MLR) and Proliferation Assay
Splenocytes or sorted CD4^+/CD8^+ T cells were cultured at 10^6 cells/ml. γ-irradiated (2,500rads) BmDC were cultured at varying concentrations (from 10^4 cells/ml to 10^6 cells/ml). 100μl of each culture was mixed in 96 well plates (NUNC™, Nalge Nunc International) and incubated at 37°C, 5% CO_2. Unless otherwise stated, these were left for 3 days, after which 0.5 μCi/well of ^3H-thymidine was added, and the culture was harvested 8-16 hours later for measurement of thymidine incorporation using a MicroBeta scintillation counter (Wallac, Turku, Finland).

2.2.11 Peptide-Specific Responses Induced by BmDC
Day 5 BmDC were cultured overnight in the presence or absence of modulins, together with titrated concentrations of ovalbumin peptide (OVA^{323-339}) for DO11.10 studies, or NP peptides for F5 studies. Purified CD4^+/CD8^+ T cells (10^4-10^5 cells)
were cultured together with γ-irradiated (2,500 rads) BmDC (10^3-10^5 cells) in 96 well plates and incubated at 37°C, 5% CO₂ for 3 days (unless otherwise stated). For assessing the proliferation of T cells, 0.5 μCi/well of ^3H-thymidine was added, and the culture was harvested 8-16 hours later for measurement of thymidine incorporation using a MicroBeta scintillation counter. For cytokine analysis, ionomycin (Sigma) and PMA (Sigma) were added at final concentrations of 500 ng/ml and 50 ng/ml respectively, and the supernatant was removed after 8-16 hours. Cytokines were analysed by ELISA (section 2.2.11) and/or intracytoplasmic staining (section 2.2.5).

2.2.12 Cytokine Assay (ELISA)

Capture antibody (monoclonal antibody) for the cytokine to be measured was made up to 4 μg/ml in PBS. 100 μl of this was used to coat each well of 96 well plates (Immulon plates, Dynex Technologies) at room temperature overnight. Plates were washed three times with wash buffer (PBS with 0.05% tween-20), then blocked for 1 hour at room temperature with 10% v/v new born calf serum in PBS (300 μl/well). Plates were washed as before and standards for the assay and supernatants to be tested were added to the wells and incubated at room temperature for 2 hours. Standards used were recombinant cytokines made into 8 serial dilutions in 1% w/v BSA in PBS. Plates were washed, and 100 μl/well of detection antibody (biotinylated antibody) was added to the wells and incubated for 2 hours at room temperature. Plates were washed three times, and 100 μl per well of HRP-conjugated streptavidin was added and incubated for 30 minutes at room temperature. Plates were washed again, and 100 μl of substrate solution (1:1 mixture of tetramethylbenzidine (TMB) and H₂O₂) was added to the wells. The reaction was stopped after 15-30 minutes by addition of
50μl 2N H₂SO₄. Absorbance was read at 450nm within 30 minutes of stopping the reaction.

2.2.13 Mouse IL-12 Bioassay (all steps were carried out in sterile conditions)

The protocol from Current Protocols in Immunology (Gately et al., 2001) was followed.

A. Preparation of lymphoblasts

Human PBMC were made into a single cell suspension in RPMI-CM with 50μM 2-ME at 5 x 10⁵/ml, and 20ml of this was cultured in T75 tissue culture flask in the presence of 10μg/ml PHA (Sigma) for 3 days at 37°C 5% CO₂. The culture was split into two by addition of 20ml RPMI-CM with 50μM 2-ME, mixing and transferring 20ml into a new T75 flask. Human recombinant IL-2 was added at 1ng/ml, and incubated overnight at 37°C 5% CO₂. Cells were washed 3 times in RPMI-CM and counted before incorporation into the bioassay.

B. Preparation of IL-12p70 plates for bioassay

Capture antibody for IL-12p70 (R&D and Pharmingen) was made up to 5μg/ml in PBS. 100μl of this was used to coat each well of 96 well immunoplates (MaxiSorp, Nunc) at room temperature overnight.

C. Assay set up

IL-12p70 antibody-coated plates were washed with sterile water three times. Plates were then blocked with 200μl per well of 5% v/v NCS and 0.5% w/v BSA in PBS, for 1 hour at 37°C. Plates were washed again as before, and 100μl of recombinant IL-12 (as standard) and samples to be tested were added to the wells and incubated for 2½ - 3 hours at room temperature. Plates were washed again as before, and 2 x 10⁴ lymphoblasts were added to each well of the plate. After 2 days incubation at 37°C,
proliferation of the lymphoblasts were assessed by measuring the $^3$H-thymidine incorporation (described in section 2.2.9).

2.2.14 Quantification of Intracellular cAMP

Day 5 BmDC ($2-5 \times 10^6$) were cultured in 96 well U-bottom plates for 2 hours. Titrated concentrations of cAMP inducers (dbcAMP, PGE$_2$, forskolin, PTx and CTx see table 2.3) were added to the cells, with or without 100µM IBMX (Sigma), for varying time points of between 30 seconds and 1 hour. Cells were quickly pelleted, lysed and intracellular cAMP was quantified using the cAMP enzyme immunoassay kit (BIOTRA, Amersham Pharmacia Biotech).

2.2.15 Cytotoxicity Assay (Chromium Release Assay)

Day 5 BmDC were stimulated overnight with modulins and NP peptides. BmDC were then washed 3 times in CTL-CM and $2 \times 10^6$ cells were mixed with $2 \times 10^7$ RBC-depleted splenocytes/lymph node cells, or purified CD$^8^+$ T cells from F5 transgenic mice in a total of 10ml CTL-CM in T25 tissue culture flasks. This was left at 37°C, 5% CO$_2$ for 3 days. $1-2 \times 10^7$ EL4 cells were labelled with 3.7MBq Na$_2^{51}$CrO$_4$ for 1½ hours and loaded with titrated concentrations of NP peptides. EL4 target cells were then washed, and plated at $10^4$ cells per well of 96 well U-bottom plates. Titrated numbers of F5 effector cells were added, and the plates were incubated at 37°C, 5% CO$_2$ for 4-6 hours. Chromium release in the supernatant was quantified using a MicroBeta scintillation counter.
Chapter Three

The Syngeneic MLR, a Model for Low-Affinity Interactions

3.1 Introduction

Naïve T cells encounter antigens in the periphery in the form of MHC:peptide complexes. The outcome of this interaction depends on a number of factors. These include: (1) affinity of the TcR for the MHC:peptide complexes, often termed signal 1, (2) avidity of the TcR for the MHC:peptide complexes (which amplifies signal 1), and (3) 'cues' to respond to signal 1, such as the presence of co-stimulatory cell surface molecules (such as B7-CD28 interactions) and secretion of cytokines, which are collectively termed signal 2 (section 1.5.7). As discussed earlier in section 1.5.8, it is likely that as a result of thymic selection, tumour antigens derived from self molecules (presented as MHC:peptide complexes) are seen with low-affinity by the TcR (or low signal 1). Thus, one potential way of inducing an anti-tumour response may be to amplify signal 2. Presently there is a great deal of research being carried out in designing adjuvants to “help” tumour vaccines (Schuler and Steinman, 1997; Young and Inaba, 1996).

A model has been schematically presented in figure 3.1, in order to understand the relative contribution of signal 1 and signal 2 in triggering a T cell response. This predicts that signal 1 and signal 2 need to reach a threshold in order to trigger T cell effector function (dotted line). For example, in infections, “foreign” peptides presented on MHC are likely to be “seen” with high affinity by TcR. In addition, inflammation and tissue injury associated with infection may trigger activation of the
innate immune system and APC, which leads to elevation of signal 2 (section 1.5.9). Thus, it is likely that T cell responses in infections result from relatively high levels of both signal 1 and signal 2. This model predicts that where signal 1 is high, the threshold required for signal 2 to trigger a T cell response may be minimal (e.g., alloreactivity). In contrast, a relatively high level of signal 2 is required to achieve T cell activation in response to low signal 1 (e.g., autoimmunity). If these assumptions are correct, then it should be possible to induce a T cell response to a self-derived tumour antigen (low signal 1) by amplifying signal 2.

![Figure 3.1 Model to describe the relative importance of signal 1 and signal 2 in triggering T cell activation.](image)

The sum of signal 1 and signal 2 needs to reach a threshold (dotted line) in order to trigger a T cell effector function. In cancer, where signal 1 is low (box arrow) we predict that T cells can be triggered by amplifying the level of signal 2.

It is thought that immunological adjuvants generally work by activation of innate immunity (section 1.5.12). A number of mechanisms have been suggested to explain how adjuvants work. For example, it has been argued that adjuvants such as immunostimulating complexes (ISCOMs) and liposomes work by facilitating antigen transport and presentation by APC (Zinkernagel et al., 1997). However, recent studies reveal that some adjuvants act through molecules that are involved in delivering signal 2 (Schijns, 2000). Thus, it is likely that factors that cause elevation of signal 2 are important in the mechanism of adjuvanticity.
In order to examine the importance of amplified signal 2 in triggering a T cell response to low-affinity antigens, the syngeneic mixed lymphocyte reaction (MLR) system was chosen as a model. This system represents an interaction between TcR and self MHC:peptide complexes \textit{in vitro}, and provides a model for dissecting the regulatory mechanism underlying T cell responses to "self" (or low-affinity antigens).

Bone marrow-derived DCs (BmDC) can be activated \textit{in vitro} to acquire a mature phenotype with elevated expression of co-stimulatory molecules, and thus can be used as a model APC with varying levels of signal 2. Specifically, the question was asked: would differential maturation of DCs by products of pathogens or endogenous factors result in activation of syngeneic T cells? To do this BmDC were stimulated with LPS and anti-CD40 antibody (aCD40), both of which are factors known to promote activation of DCs. For convenience, unstimulated DCs are termed DC0, LPS-matured DCs are denoted as DC^{LPS} and anti-CD40-treated DCs as DC^{aCD40}.

### 3.2 Objectives

The objective of this chapter is to test the hypothesis: amplification of signal 2 leads to induction of syngeneic CD4\(^+\) and CD8\(^+\) T cell responses.
3.3 Experimental Results

3.3.1 Biology of BmDC

Generation of DCs from bone marrow (BmDC) was first described by Inaba et al (Inaba et al., 1992). Whole bone marrow cell cultures were set up in the presence of GM-CSF. On day 3, non-adherent cells were removed, leaving only the adherent/semi-adherent cells in culture. On day 5, DCs were enriched by selecting the semi-adherent cells and replating. This two step-selection of the semi-/non-adherent cells typically resulted in a culture with 85-90% CD11c^+ BmDC (figure 3.2). These cells consistently exhibited high levels of MHC class II expression, with low to intermediate expression of MHC class I, CD80, CD86 and CD40 molecules. There were contaminating macrophages (F4/80^+) and granulocytes (Gr-1^+) in the culture that typically consisted of: <9% F4/80^+ CD11c^+; <0.5% F4/80^+ CD11c^-; and <5% Gr-1^+ CD11c^- (data not shown). The CD11c^+ BmDC cultured in this way were defined functionally as potent APC with exceptionally high T cell stimulatory capability in MLRs (Inaba et al., 1992).
Figure 3.2 Typical immunophenotype of day 5 BmDC.

Day 5 BmDC (BALB/c) were harvested and labelled with antibodies specific for a number of DC maturation markers and appropriate controls as described in materials and methods (chapter 2). Numbers indicated are percentage positive cells in the quadrant. These data are representative of 12 separate experiments.
3.3.2 Primed BmDC can trigger syngeneic T cell responses

While unstimulated BmDC (DC0) were potent stimulatory cells in the allogeneic MLR, they were poor stimulators of syngeneic CD4\(^+\) and CD8\(^+\) T cells (figure 3.3). However, BmDC stimulated in the presence of LPS and/or aCD40 (DC\(^{LPS}\), DC\(^{aCD40}\) or DC\(^{LPS/aCD40}\)) provoked proliferation of syngeneic CD4\(^+\) T cells, but remained poor stimulators of syngeneic CD8\(^+\) T cells (figure 3.4). It is possible that syngeneic CD4\(^+\) T cell responses induced by the stimulated DCs were in part due to elevated levels of signal 2. To test this, we measured the surface expression of some of the co-stimulatory molecules, CD80, CD86 and CD40, which are known to be upregulated upon activation of DCs. Both DC\(^{LPS}\) and DC\(^{LPS/aCD40}\) exhibited elevated expression of CD80, CD86 and CD40 (figure 3.5). In contrast, DC\(^{aCD40}\) expressed a relatively low level of CD86 and CD40. This may be partly due to the masking effect of CD40 molecules with the stimulating aCD40. Interestingly, while expression of MHC class I is clearly increased on DC\(^{LPS}\) and DC\(^{aCD40}\), these cells showed no enhancement in the expression of MHC class II. Only when DCs were stimulated with LPS together with aCD40 (DC\(^{LPS/aCD40}\)) was there a modest increase in the expression of MHC class II.
Figure 3.3 Unstimulated BmDC, DC0, trigger a poor syngeneic MLR.

CD4+ (A) or CD8+ (B) T cells (5x10^4) from BALB/c mice were cultured in 96 well plates with syngeneic (BALB/c, open circles) or allogeneic (C57BL/6, closed circles) BmDC (1x10^3). Proliferation was analysed at the various time indicated above by adding 3H-thymidine (0.5μCi) to the culture for 8 hours prior to measuring 3H-thymidine incorporation. These data are means of quadruplicate wells (± SD) from one representative experiment out of five performed.
Figure 3.4 Primed DCs induce syngeneic CD4+ T cell response.

CD4+ (A) or CD8+ (B) T cells (5x10^4) from BALB/c mice were cultured in 96 well plates with syngeneic (BALB/c) BmDC (1x10^4) for 72 hours. The DCs had previously been stimulated for 24 hours with medium alone (DC0), LPS and/or anti-CD40 antibody (aCD40). Proliferation was measured by ^H-thymidine incorporation. The results are means of quadruplicate points (±SD) from one representative experiment out of five.
Figure 3.5 Immunophenotype of BmDC stimulated with LPS and/or anti-CD40 antibody (aCD40).

Day 5 BmDC were cultured overnight with medium alone (DC0), LPS (DC^{LPS}; 10μg/ml) and/or aCD40 (DC^{aCD40}; 2μg/ml or DC^{LPS+aCD40}). These cells were then labelled with fluorochrome-conjugated antibodies (line histograms) or appropriate isotype-matched controls (shaded histograms). The numbers shown indicate the mean fluorescence intensity. These data are representative of 10 separate experiments.
3.3.3 “CD4 help” does not trigger syngeneic CD8^ T cell responses

There may be a number of reasons to explain the relative unresponsiveness of CD8^ T cells to syngeneic DCs compared to CD4^ T cells. It has been suggested that there is a requirement for “help” provided by CD4^ T cells in order to fully activate CD8^ T cells in response to exogenous antigens (Bennett et al., 1997; Keene and Forman, 1982). Indeed, it has been shown that CD40-mediated signalling “conditions” DCs to acquire the ability to activate CD8^ T cells to exogenous antigens (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). However, DC^CD40 failed to induce proliferation of syngeneic CD8^ T cells (figure 3.4). Thus it is possible that CD8^ T cells require signals from CD4^ T cells other than that provided by CD40-mediated signalling.

To investigate this, proliferation of CD8^ T cells in the presence of both syngeneic DCs and CD4^ T cells was examined by immunofluorescence (figure 3.6). Carboxyfluorescin diacetate succinimidyl ester (CFSE) is a fluorescent dye that can be incorporated and retained in living cells (Lyons and Parish, 1994). Cell divisions of CFSE labelled cells result in decreased fluorescence as measured by FACS analysis. The results show that while a clear reduction in the fluorescence intensity was evident in CD8^ T cells cultured with allogeneic DCs (figure 3.6E), such a decrease was not observed in CD8^ T cells cultured with syngeneic DC0 or DC^{LPS} (figure 3.6A and B). Importantly, addition of syngeneic CD4^ T cells to these cultures did not induce proliferation of CD8^ T cells in response to syngeneic DC0 or DC^{LPS} (figure 3.6C and D).
Figure 3.6 CFSE analysis of CD8⁺ T cell proliferation in response to syngeneic DCs in the presence of CD4⁺ T cells.

CFSE labelled CD8⁺ T cells were cultured with syngeneic DC0 (A), syngeneic DCTLPS (B), CD4⁺ T cells and syngeneic DC0 (C), CD4⁺ T cells and syngeneic DCTLPS (D), or with allogeneic DCs (E) to assess proliferation of CD8⁺ T cells. CFSE labelled CD8⁺ T cells (F) or CD4⁺ T cells (G) were cultured alone as negative controls. Cell divisions result in reduction of the CFSE fluorescence intensity. The experiment was repeated three times, and one representative data set is shown.
To further examine whether co-culture with syngeneic CD4\(^+\) T cells would trigger DNA synthesis in CD8\(^+\) T cells in response to syngeneic DC0 or DC\(^{\text{LPS}}\), incorporation of \(^3\text{H}\)-thymidine by CD8\(^+\) T cells in response to syngeneic DC0 or DC\(^{\text{LPS}}\) in the presence of irradiated syngeneic CD4\(^+\) T cells was analysed. As shown in figure 3.7, DNA synthesis of CD8\(^+\) T cells was not triggered by stimulation with syngeneic DC0 or DC\(^{\text{LPS}}\) in the presence of irradiated CD4\(^+\) T cells (figure 3.7A and B, open bars). However in the presence of IL-2, high levels of \(^3\text{H}\)-thymidine uptake were observed in CD8\(^+\) T cells in response to syngeneic DCs (figure 3.7A and B, shaded bars). The presence of DCs was necessary for this since CD8\(^+\) T cells cultured with IL-2 alone did not incorporate \(^3\text{H}\)-thymidine (figure 3.7 legend). Thus, whilst it is clear that syngeneic DCs could not induce CD8\(^+\) T cells to progress from the G\(_0\) to S phase of the cell cycle, this experiment indicated that DCs can make CD8\(^+\) T cells responsive to IL-2, which may activate cell cycle progression from the G\(_0\) to the G\(_1\) phase.

Syngeneic CD4\(^+\) T cells do produce low levels of IL-2 in response to DCs (figure 3.8A). In addition, syngeneic CD8\(^+\) T cells express a low level of IL-2 receptor, CD25 (figure 3.8B). However, CD8\(^+\) T cells do not respond to syngeneic CD4\(^+\) T cells (figure 3.6 and 3.7). Thus, it is likely that IL-2 produced by the syngeneic CD4\(^+\) T cells was not sufficient to trigger proliferation of syngeneic CD8\(^+\) T cells in the mixed culture.
Figure 3.7 $^3$H-thymidine incorporation in CD8$^+$ T cells in response to syngeneic DCs in the presence of CD4$^+$ T cells.

T cells ($5 \times 10^4$) were cultured alone with syngeneic DC0 (A) or DC$^{^{\text{LPS}}}$ (B) with (shaded bars) or without (open bars) the presence of IL-2 (1ng/ml) for 72 hours. Proliferation was measured by $^3$H-thymidine incorporation. CD4$^+$ T cells capable of proliferation in response to BmDC (I) were irradiated (CD4$^*$) and their ability to help the proliferation of CD8$^+$ T cells was assessed (IV). In the absence of DCs, neither CD4$^+$ nor CD8$^+$ T cells proliferated even in the presence of IL-2 (CD4$^+$ T cells alone: 36 cpm; CD4$^+$ T cells plus IL-2: 48 cpm; CD8$^+$ T cells alone: 118 cpm; CD8$^+$ T cells plus IL-2: 86 cpm). The data are means of quadruplicate points ($\pm$SD) from one representative experiments out of 2 performed.
Figure 3.8 IL-2 production from syngeneic CD4+ T cells.

Purified CD4+ T cells (1×10^6) were cultured with syngeneic DC0, DC_LPS, DC_wCD40 or DC_LPS+wCD40 (1×10^5) and supernatants were collected after 24 hours for quantifying IL-2 by ELISA (A). The data shown are means of quadruplicate points (±SD) from one representative experiment out of three. Purified CD8+ T cells were stimulated with syngeneic DCs as in A, and expression of CD25 was examined 24 hours later by FACS (B). The data are single points from one representative experiment out of two performed.
3.3.4 Differential requirements for triggering CD4\(^+\) and CD8\(^+\) T cells

There are number of factors which may account for the difference between CD4\(^+\) and CD8\(^+\) T cells in the proliferative response to syngeneic DCs: (1) the availability of immunogenic peptides presented on MHC molecules; (2) the number of such MHC:peptide complexes; and (3) the differential requirement for levels of co-stimulation, or signal 2 (soluble and/or cell-cell contact dependent factors) by the different cell types.

It is difficult to examine what is occurring in the syngeneic system in terms of what comprises signal 1, as the peptides being presented by MHC remain undefined. It is possible that antigenic materials from the foetal calf serum (FCS) in the culture medium have been taken up, processed and presented on self MHC molecules. However it is unlikely that the presentation of FCS-derived antigens is important in mediating the syngeneic MLR, since DCs cultured in mouse serum were equally stimulatory (data not shown).

The apparent differences in CD4\(^+\) and CD8\(^+\) T cell responses can also be explained, if CD4\(^+\) and CD8\(^+\) T cells possess a different threshold of signal 1 and/or signal 2 for them to be able to proliferate. In order to quantify the threshold of the signals required to trigger these T cells, purified CD4\(^+\) and CD8\(^+\) T cells were stimulated with varying concentrations of immobilised anti-CD3 antibody (aCD3), a substitute for signal 1. As shown in figure 3.9A, CD8\(^+\) T cells required a 10 times higher amount of CD3 triggering ("signal 1") than CD4\(^+\) T cells to proliferate. Addition of "signal 2" by means of stimulation with soluble anti-CD28 antibody lowered this threshold by a log for both CD4\(^+\) and CD8\(^+\) T cells (figure 3.9B). Interestingly, further addition of
IL-2 did not alter the threshold nor significantly enhance the proliferative response of these cells (figure 3.9C).
Figure 3.9 CD8+ T cells require higher level of CD3-mediated signals for proliferation than CD4+ T cells.

CD4+ or CD8+ T cells (1×10^5) were cultured in 96 well plates for 72 hours with titrated amounts of plate-bound anti-CD3 antibody (aCD3, filled symbols) or isotype-matched control antibody (open symbols) (A), together with anti-CD28 antibody (10µg/ml) (B) or with anti-CD28 antibody and IL-2 (100ng/ml) (C). Proliferation was measured by 3H-thymidine incorporation. These data are means of quadruplicate wells (+SD) from one representative experiment out of three performed.
3.4 Discussion

In this chapter T cell responses to a low affinity interaction ('self') were used as a model for induction of a T cell response to tumour antigens. The T cell proliferative responses that results from the syngeneic MLR are thought to be due to a subpopulation of T cells recognizing peptides presented by self MHC molecules. It is possible that the antigenic materials have been derived from FCS used in the study. However, DCs cultured in isologous serum are equally stimulatory to DCs cultured in FCS [(Nussenzweig and Steinman, 1980) and (Whelan and Rigley, unpublished results)], thus excluding the possibility that the T cell responses were mediated against high-affinity, "foreign" antigens. Despite the poorly defined signal 1 (nature and concentration of peptide-MHC complexes) in this system, it is our contention that it represents a low-affinity interaction between TcR and self MHC molecules: BmDC that are powerful stimulators of allogeneic MLR and antigen-specific T cell responses (Steinman, 1991) failed to trigger syngeneic T cells, thus providing a model for low signal 1.

In contrast to the above findings, murine lymphoid DCs (Nussenzweig and Steinman, 1980) and BmDC (Inaba et al., 1992) have both been reported to trigger a strong syngeneic MLR. A possible explanation for this is that lymphoid DCs represent a different subtype of DCs, thus accounting for the different results obtained with these cells. Another, possibly more important factor that may differ between our system and earlier work, is the cleanliness of the culture systems: mice utilised in this study are likely to be exposed to lower levels of pathogens, and as a result DCs derived from these mice are relatively immature, or unprimed. In addition, the FCS used in
this study was screened for low endotoxin level (3.3pg/ml) which may account for the
differences in the functional phenotypes of the DCs.

In this study BmDC cultured in GM-CSF for 5 days were further matured in the
presence of either LPS (DC\textsuperscript{LPS}) and/or aCD40 (DC\textsuperscript{aCD40}, DC\textsuperscript{LPS/aCD40}). Unstimulated
DCs (DC\textsubscript{0}) were potent stimulatory cells in allogeneic MLR, but failed to induce
proliferation of syngeneic CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells. In contrast, DC\textsuperscript{LPS}, DC\textsuperscript{aCD40} and
DC\textsuperscript{LPS/aCD40} acquired an ability to trigger proliferation of syngeneic CD4\textsuperscript{+} T cells, but
not CD8\textsuperscript{+} T cells. Furthermore, these CD8\textsuperscript{+} T cells did not proliferate in response to
syngeneic DCs even when stimulated in the presence of CD4\textsuperscript{+} T cells, whilst retaining
the ability to proliferate in response to exogenous IL-2. As syngeneic CD4\textsuperscript{+} T cells
were capable of producing low amounts of IL-2, this suggests that the amounts
produced by syngeneic CD4\textsuperscript{+} T cells were not sufficient to trigger proliferation of
syngeneic CD8\textsuperscript{+} T cells.

Given that there was an elevation in the expression of co-stimulatory molecules (B7
molecules and CD40) on DC\textsuperscript{LPS} and DC\textsuperscript{aCD40} but not of MHC class II molecules, it is
likely that an increase in signal 2 was sufficient for triggering syngeneic CD4\textsuperscript{+} T cells,
but not CD8\textsuperscript{+} T cells. Three possible explanations were considered to explain the
difference between CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the proliferative responses to syngeneic
DCs: (1) the availability of immunogenic peptides presented on MHC molecules; (2)
the density of such MHC:peptide complexes (signal 1); and (3) the differential
requirement for signal 2 (soluble and/or cell-cell contact dependent factors).
Although we could not test the availability of immunogenic MHC-peptides in this
system, the difference between CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses is not likely to have
reflected antigen presentation on MHC molecules for two reasons: (1) DCs cultured in the presence of isologous serum are as stimulatory as DCs cultured in foetal calf serum; and (2) maturation of DCs in this system resulted in pronounced upregulation of MHC class I molecules but not MHC class II molecules. Therefore it may be more reasonable to consider that CD4⁺ and CD8⁺ T cells may possess different thresholds of signal 1 and/or signal 2 for initiation of proliferative responses. Indeed, CD8⁺ T cells required 10 times higher TCR triggering than CD4⁺ T cells as measured by the amount of immobilised CD3 antibody required to trigger T cell proliferation. This indicates that the threshold of signal 1 required to trigger CD8⁺ T cells is higher than that of CD4⁺ T cells. It is interesting to note that addition of "signal 2" to this system, by means of adding soluble anti-CD28 antibody, lowered the threshold of CD3 triggering ("signal 1") 10 fold for both CD4⁺ and CD8⁺ T cells. This suggests that "signal 2" in the form of CD28 ligation acts equally on both cell types provided that "signal 1" is high enough. Thus, these data offer a plausible explanation for the earlier observation that syngeneic CD4⁺ and CD8⁺ T cells did not respond equally to the same amount of signal 2 provided by DCs, because the signal threshold of the signal 1 for CD8⁺ T cells is higher than that of CD4⁺ T cells.

A possible explanation for the above results is that control of CD8⁺ T cell response to "self" is more stringently regulated than for CD4⁺ T cells. Given that activation of CD8⁺ T cells may lead to destructive pathology in an immune response and can be extremely dangerous if generated against self, it is likely that these cells are under a strict regime of immunological control. The observation that DCs induced syngeneic CD8⁺ T cells to become responsive to stimulation with IL-2 suggests that DCs had partially activated these T cells, probably from the G₀ to the G₁ phase of the cell cycle.
(Roska and Lipsky, 1985). This supports the notion that the threshold of signal 1 for CD8^+ T cells is higher than that of CD4^+ T cells, and that the required threshold of signal 1 must be reached before signal 2 can act on T cells.

In many examples of immune responses, development of cytotoxic T cells requires CD4^+ T cell help (Bennett et al., 1997; Keene and Forman, 1982). There is accumulating evidence suggesting that the interaction between CD40L on CD4^+ T cells and CD40 on APC is critical in activating the APC to present antigens productively to CD8^+ T cells (Matzinger, 1999; van Kooten and Banchereau, 1997). In addition, recent in vivo studies suggest that this 'help' can be replaced by ligation of CD40 on DCs (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

In the present study proliferation of CD8^+ T cells in response to self was not observed, even in the presence of CD4^+ T cells. In addition, CD40 ligated DCs that had an enhanced capacity to stimulate syngeneic CD4^+ T cells failed to trigger activation of syngeneic CD8^+ T cells. Whilst these data may simply reflect the difference between in vivo and in vitro regulation of CD8^+ T cell responses, a more likely explanation is the difference in the nature of signal 1. Studies implicating CD40 ligation and conditioning of DCs have utilised exogenous 'high affinity' peptides: Ridge et al examined the killing response to H-Y antigens whilst OVA and E1B antigens were used as a target in the studies by Bennett and Schoenberger, respectively. In addition, mouse BmDC stimulated with aCD40 which fail to generate cytotoxicity of syngeneic T cells were able to do so in the presence of a foreign peptide derived from OVA protein (Kelleher and Beverley, 2001).
It is interesting to note that DCs in the lymph nodes and the spleen present self antigens on MHC molecules (Inaba et al., 1997; Inaba et al., 1998), suggesting that T cells are encountering self antigens continually, but evidently this process does not lead to autoimmune diseases. In fact, numerous studies indicate that such interactions between T cells and self MHC molecules on APC are essential for survival of T cells (Goldrath and Bevan, 1999). If the model described in figure 3.1 is correct, T cells that encounter self antigens presented by professional APC may become activated in certain conditions, such that self antigens are presented with high signal 2 (e.g., during the course of an infection). However, most individuals do not develop any symptomatic autoimmunity following an infection, which suggests that T cell interactions with low-affinity self antigens may not lead to full activation of T cells, and the subsequent generation of effector cells. This is supported by the findings in this chapter: syngeneic stimulation with a strong signal 2 was not sufficient to trigger proliferation of CD8+ T cells even in the presence of CD4+ T cells. Thus, the proposed model may illustrate an efficient mechanism for maintenance of peripheral non-responsiveness whilst avoiding harmful autoreactivity. However, at the same time these data suggest that increasing signal 2 may not be an effective means of triggering low affinity anti-tumour responses. 

Taken together, the earlier model described in figure 3.1 can be extended to include the difference in the response threshold between CD4+ and CD8+ T cells. The CD3 triggering experiments demonstrated that the threshold of signal 1 for T cell triggering was higher for CD8+ T cells than for CD4+ T cells, while CD28 triggering-mediated signal 2 exerted similar effects on both cell types. In addition, it was shown in the syngeneic MLR system that providing stronger signal 2, by means of stimulating with
"mature DCs", was sufficient to trigger CD4⁺ but not CD8⁺ T cells. Thus, it is likely that there are two levels of signal thresholds that need to be reached for triggering T cells in the periphery: (1) threshold for signal 1; and (2) threshold for the sum of signal 1 and signal 2. The proposed model is illustrated schematically in figure 3.10. This model suggests, unlike the model described in figure 3.1, that the amount of signal 1 must reach a threshold level before co-stimulatory signals (signal 2) can act on T cells. It also illustrates the observation that syngeneic CD4⁺ and CD8⁺ T cells did not respond equally to the same amount of signal 2 provided by DCs, because the threshold of signal 1 required by CD8⁺ T cells for activation is higher than that required by CD4⁺ T cells.

A major limitation of the syngeneic MLR system in studying the relative importance of signal 1 and signal 2 in triggering a T cell response is the poorly defined signal 1. Although syngeneic MHC-TCR interaction has been shown to mediate a "low-affinity" interaction (Sandberg et al., 1999), there is a need for a system which provides a well-defined low signal 1.
The F5 TcR transgenic system provides partial agonist, antagonist, and full agonist peptides in the context of MHC class I H-2D\(^b\) (Smyth et al., 1998). Thus, the antagonistic peptide was utilised as a model for a low-affinity antigen, and assessed the pattern of responsiveness of transgenic CD8\(^+\) T cells to the peptide presented on a variety of conditioned DCs. These data will be discussed in chapter 6 of this thesis.
Chapter Four

Effect of cAMP elevation on DC differentiation

4.1 Introduction

Chapter 3 dealt with quantitative aspects of T cell activation in response to a model low-affinity antigen. Another important consideration for vaccine design is the type or quality of immune responses elicited as a result of T cell activation.

4.1.1 Functional differentiation of T cells

As reviewed in the introduction chapter (section 1.3.4), immune responses can be broadly categorised into two types: cell-mediated inflammatory responses and humoral responses. Activated T cells play a considerable role in mediating these different responses, as they become differentiated into functionally distinct phenotypes that are associated with distinct patterns of cytokine production. For example, T cells are classified as type 1 when they secrete IL-2, IFNγ and TNFβ, and are mainly associated with cell-mediated responses. In contrast, T cells that produce IL-4, IL-5, IL-6, IL-9 and IL-10, which are mainly important for humoral responses, are categorised as type 2 (Mosmann and Sad, 1996). CD4⁺ T helper cells that produce a type 1 cytokine profile are termed Th1, and those T helper cells that secrete type 2 cytokines are termed Th2. More recently, similar terminology was used to describe functionally different CD8⁺ T cells (termed Tc1 and Tc2, respectively) (Mosmann and Sad, 1996). In infections, a particular type of response is often beneficial whilst induction of an inappropriate response may lead to pathology. For example, infections with many intracellular pathogens correlate with protective type 1
responses, while type 2 responses are required to induce protective responses to most extracellular helminth infections. In the case of cancer where direct killing of tumour cells by cytotoxic T cells (CTL) is believed to be important, a vaccine that would induce type 1 responses may be beneficial.

The functional differentiation of T cells is thought to be influenced by a number of factors. The cytokine environment, antigen dose, nature of antigens, type of APCs and genetic background all contribute to this differentiation process (Constant and Bottomly, 1997; O. Garra and Murphy, 1994). Given that T cell differentiation occurs early after activation (Seder and Paul, 1994; Toellner et al., 1998), it is likely that a major role is played by the APCs, in particular DCs, in shaping the differentiation pathway of naïve T cells, because they provide the precursor T cell with its initial activation signal (Banchereau and Steinman, 1998). Indeed, the concept that DCs play a role in shaping the resulting T cell response has been demonstrated in a number of studies, both in mice and in humans [section 1.4.6; for reviews see (Kalinski et al., 1999; Moser and Murphy, 2000; Reid et al., 2000)].

4.1.2 Working hypothesis

It is clear from numerous studies that DCs can differentiate into DC1/DC2 phenotypes in response to micro-environmental factors during the maturation process (section 1.4.6). For instance, LPS (from Escherichia coli) promotes differentiation of bone marrow-derived DCs into a DC1 phenotype, while a filarial nematode product ES-62 (from Acanthocheilonema viteae) induced DC2 differentiation (Whelan et al., 2000). These results led to the hypothesis that products of pathogens ("modulins") can induce differential maturation of DCs (section 1.4.6). Janeway and co-workers
[Medzhitov and Janeway, 1997b; section 1.2.1] proposed that the innate immune system recognises pathogens using a set of pattern recognition receptors (PRRs). Crosslinking of PRRs by interaction with "modulins" leads to the activation of APCs that are then able to activate T cell function. Thus, it is plausible that the interactions mediated by the PRRs shape the type of immune responses elicited. However, this is not the only mechanism by which DCs differentiate into functionally distinct phenotypes, as factors other than pathogen products also mediate this process, such as cytokines, CD40 ligation, inflammatory mediators such as PGE$_2$ and various cAMP inducers such as dbcAMP and forskolin (section 1.4.6).

4.1.3 Mechanism of DC1/DC2 differentiation

Whilst evidence for the ability of DCs to differentiate into functionally distinct DC1 or DC2 phenotypes is accumulating, the mechanisms of such functional differentiation is poorly understood. One possible mechanism is the role of IL-12 in differentiation of DC1 phenotype. Several studies indicate that a DC1 phenotype correlates with the production of IL-12 by DCs (Heufler et al., 1996; Hilkens et al., 1997; Langenkamp et al., 2000; Rieser et al., 1997; Steinbrink et al., 1997), while a DC2 phenotype correlates with the reduced production of IL-12 by DCs (Gagliardi et al., 2000; Kalinski et al., 1997a; Kalinski et al., 1997b; Langenkamp et al., 2000; Panina-Bordignon et al., 1997). Thus, differentiation of DC1 phenotype appears to be associated with the ability of DCs to produce IL-12, while failure to do so may result in DC2 phenotype. However, there is inconsistent findings that suggest the role of some of the factors that were shown to promote IL-12 production from DCs (such as elevation in cAMP levels and CD40 ligation on DCs), suggesting that the process of DC1/DC2 differentiation process may be influenced by a number of other factors,
such as methods used for DC preparation and DC culture conditions. This is discussed below.

(1) **Role of cAMP elevation in differentiation of DCs**

PGE$_2$ is an inflammatory mediator produced by a variety of cell types, and is well known for its cAMP elevating function. A role for PGE$_2$ in DC2 differentiation was first observed by Kalinski and co-workers (Kalinski et al., 1997b). Upon stimulation with PGE$_2$, DCs failed to produce IL-12 and a concomitant increase in IL-10 production was observed. Similarly, other cAMP elevating agents such as cholera toxin (CTx) and forskolin were also able to induce a DC2 phenotype (Gagliardi et al., 2000). In contrast to these studies, other workers have shown that PGE$_2$ promotes IL-12 production by DCs, and importantly other cAMP inducers, such as dbcAMP and forskolin, were able to mimic the effect mediated by PGE$_2$ (Rieser et al., 1997; Steinbrink et al., 2000). However, it should be noted that in these studies high levels of IL-12 production was only observed when DCs were stimulated together with inflammatory cytokines such as TNFα (Rieser et al., 1997; Steinbrink et al., 2000), IL-1β or IL-6 (Steinbrink et al., 2000). Thus, although the results suggesting the role of a range of cAMP inducers in directing the differentiation of DC1 or DC2 are inconsistent, it is likely that intracellular cAMP levels are involved in the mechanism of DC1/DC2 differentiation.

(2) **Role of CD40 ligation in differentiation of DCs**

CD40 ligand (CD40L) is expressed on activated T cells, and ligation of CD40 on the DC surface has been shown to induce maturation of DCs (Caux et al., 1994a; van Kooten and Banchereau, 1997). Some investigators have demonstrated that CD40
ligation on DCs (by soluble CD40L, anti-CD40 antibody or interaction with CD40L expressing cells) promote differentiation towards a DC1 phenotype, which was accompanied by a dramatic upregulation of IL-12 production (Cella et al., 1996; Koch et al., 1996), and subsequent stimulation of Th1 responses (Cella et al., 1996). In contrast, others have shown that CD40 engagement alone was insufficient to induce IL-12 production by DCs, and that at least one additional exogenous factor (IFNγ) (Hilkens et al., 1997; Snijders et al., 1998) was required. Thus, the role of CD40 ligation in mediating DC1/DC2 differentiation remains controversial.

If the working hypothesis proposed above (section 4.1.2) is correct, DCs would acquire DC1/DC2 phenotypes upon activation, mediated by recognition of pathogen products or infection/tissue injury-related factors. Thus, DCs would not differentiate into DC1/DC2 in the absence of cues for activation. In order to test this hypothesis, the effect of cAMP elevating agents from various sources and CD40 ligation on BmDC were examined.

4.2 Objectives

The primary objective of this chapter was to determine the role of the elevation of intracellular cAMP levels in DC differentiation. A secondary objective was to mimic the presence of T cells by ligation of CD40 on the surface of DCs to see whether this influences DC1/DC2 differentiation.
4.3 Experimental results

4.3.1 Effect of intracellular cAMP elevation on DC maturation

In order to assess the role of intracellular cAMP levels in DC activation and differentiation, five reagents known to elevate cAMP were used: PGE₂, forskolin, dbcAMP, cholera toxin (CTx) and pertussis toxin (PTx). As expected, stimulation of BmDC with these reagents resulted in elevation of intracellular cAMP (figure 4.1). However, not all of these cAMP-inducing reagents induced maturation of BmDC. CTx and PTx induced upregulation of some of the DC maturation markers, but changes were not as large as compared to LPS-stimulated DCs (figure 4.2). PGE₂ induced upregulation of CD80 and CD86 albeit at low levels. In contrast, dbcAMP or forskolin failed to upregulate any of the cell surface markers that were studied.
Day 5 BmDC (BALB/c; 5×10^5 cells/well) were cultured in 96 well plates and stimulated with a range of cAMP elevating agents for up to 1 hour. IBMX (100μM) was added to the cells at the same time the cAMP inducers were added. After the stimulation, cells were lysed and intracellular cAMP levels were measured by ELISA (Pharmacia). The data are means of duplicate wells (SD < 5% in all cases) from one representative experiment out of three performed.
Figure 4.2 Comparison of DC maturation markers upon stimulation with cAMP inducers.

Day 5 BmDC (BALB/c) were cultured overnight with the following cAMP inducers: dbcAMP, forskolin, PGE$_2$, cholera toxin (CTx) and pertussis toxin (PTx). LPS was used as a positive control. DCs were then washed and labelled with fluorochrome-conjugated antibodies to I-A$^b$/I-E$^d$, CD80, CD86 and CD40. The relative change in expression of these molecules was calculated by the median of DCx/median of DC0. The dotted line indicates the expression of the markers on DC0. These data are from one representative experiment out of five.
The changes observed in the expression of maturation markers on the surface of DCs were partially reflected in their ability to stimulate allogeneic MLRs (figure 4.3). While stimulation of the DCs with PGE₂, forskolin, or dbcAMP did not enhance the ability of DCs to induce proliferation of allogeneic splenocytes (figure 4.3, A, B and C), DCs treated with CTx and PTx showed a marked increase in their ability to stimulate allogeneic T cells (figure 4.3 D and E). In particular, the response generated by DCPT increased dramatically with increasing PTx concentration. In comparison, the response generated by DCCT peaked between CTx concentrations of 1-10 ng/ml, and the response decreased thereafter. This decline in proliferation correlated with reduced viability of DCs upon stimulation with higher concentrations of CTx, as assessed by the trypan blue exclusion method (data not shown). In contrast, with the exception of DCPT, none of these DCs were able to trigger proliferation of syngeneic responder cells. At a PTx concentration of 1 μg/ml, DCPT induced dramatic proliferation of syngeneic splenocytes comparable to that observed in the allogeneic responses (figure 4.3E).
Figure 4.3 Allogeneic and syngeneic MLRs generated by DCs treated with cAMP inducers.

Day 5 BALB/c BmDC were cultured overnight with varying concentrations of PGE₂ (A), forskolin (B), dbcAMP (C), cholera toxin (CTx; D) or pertussis toxin (PTx; E). These DCs (1×10⁶) were cultured with BALB/c (open bars) or C57BL/6 (shaded bars) splenocytes (1×10⁵). Proliferation was measured by thymidine uptake after 72 hours. The data in each graph are means of triplicate wells (±SD) from one representative experiment out of three (A-D) or four (E) performed.
4.3.2 Role of intracellular cAMP elevation in DC1/DC2 differentiation

The above data indicated that not all cAMP elevating reagents induced maturation of DCs in terms of surface markers and induction of MLRs. However, given that unstimulated DCs (DC0) are fully capable of inducing cytokine production from DO11.10 Tg CD4^ T cells (Macatonia et al., 1993), it was possible that DCs treated with cAMP elevating agents may be capable of mediating DC1/DC2 differentiation. DO11.10 Tg CD4^ T cells recognise a peptide derived from chicken ovalbumin (OVA\textsuperscript{323-339}). These CD4^ T cells were used as a readout system to examine the ability of cAMP elevating reagents to induce DC1/DC2 differentiation. To investigate this, IL-2 produced from DO11.10 Tg CD4^ T cells in response to differentially stimulated DCs was quantified. A dramatic enhancement in IL-2 production was observed in response to DCs stimulated with low concentrations of PTx (figure 4.4). However, such enhancement of IL-2 was not observed when DCs were stimulated with the other cAMP inducers. In addition, considerable enhancement of IFN\gamma production by T cells was observed in response to DC\textsuperscript{PTx} (figure 4.5). There was also a modest increase in IFN\gamma production in response to DC\textsuperscript{CTX}. No changes in the level of IL-4 were detected in response to DC\textsuperscript{PTx} or DC\textsuperscript{CTX}, indicating that both PTx and CTx acted as DC1 inducers. No clear induction of IFN\gamma or IL-4 was observed in response to DC\textsuperscript{PGE\textsubscript{2}}, DC\textsuperscript{dbcAMP} or DC\textsuperscript{forskolin}. 
Figure 4.4 IL-2 production by DO11.10 Tg CD4+ T cells in response to DCs stimulated with cAMP inducers.

Day 5 BALB/c BmDC were stimulated overnight with varying concentrations of PGE₂, forskolin, dbcAMP, cholera toxin (CTx) or pertussis toxin (PTx). These DCs (1x10⁵) were then cultured with DO11.10 Tg CD4⁺ CD62Lhigh T cells (1x10⁵) in the presence of OVA323-339 (100nM). Supernatants were removed after 24 hours and IL-2 was quantified by ELISA. The data are means of duplicate points (SD < 10% in all cases) from one representative experiment for each cAMP inducer. Each of these experiments was performed at least three times.
Day 5 BALB/c BmDC were stimulated overnight with varying concentrations of PGE₂, forskolin, dbcAMP, cholera toxin (CTx) or pertussis toxin (PTx). These DCs (1×10⁵) were then cultured with DO11.10 Tg CD4⁺ CD62L⁺ T cells (1×10⁵) in the presence of OVA323-339 (100nM) for 72 hours. Cells were then stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 8 hours before supernatants were removed for cytokine analysis by ELISA. Data are presented as percentage change from the amount of cytokines produced by DC0. The actual quantities of the cytokines are shown (pg/ml) in the graphs. The data are means of duplicate points (SD < 10%) from one representative experiment out of three similar experiments performed.
IL-12 secreted by DCs has been shown to be an important factor in Th1 inducing function (Heufler et al., 1996; Macatonia et al., 1995). Thus, it was likely that both PTx and CTx induced IL-12 production by DCs. As expected, the supernatant of DC^{LPS} culture contained high levels of IL-12p70 (figure 4.6). However, in contrast, DC^{CTx} and DC^{PTx} culture supernatants both contained relatively small levels of this cytokine. In addition, insignificant levels of IL-12 was detected in the cultures of DC0, DC^{PGE2}, DC^{dbcAMP} and DC^{forskolin}.

**Figure 4.6 IL-12 production by DCs upon stimulation with cAMP inducers.**
Day 5 BmDC (BALB/c) were stimulated with medium alone (DC0), PGE2 (10^{-6} M), dbcAMP (10^{-6} M), forskolin (10^{-6} M), CTx (1\mu g/ml), PTx (1\mu g/ml), LPS (1\mu g/ml) or aCD40 (1\mu g/ml) for 24 hours and supernatants were removed for quantification of IL-12p70 by ELISA.
4.3.3 CD40 ligation amplifies the function of DCs in T cell activation

CD40L is expressed on activated T cells as well as on memory T cells. DCs from various sources become activated upon CD40 ligation *in vitro* [(van Kooten and Banchereau, 1997); see Chapter 3], suggesting that maturation of DCs may be enhanced by encountering activated T cells *in vivo*. In order to test whether CD40 engagement leads to DC activation and enhancement of subsequent T cell activation, the ability of aCD40-treated DCs (DC\textsuperscript{aCD40}) to stimulate allogeneic MLRs was assessed (figure 4.7). As expected, LPS stimulation (DC\textsuperscript{LPS}) resulted in an increased capacity to stimulate allogeneic T cells, while DC\textsuperscript{aCD40} only marginally upregulated this function. However, when aCD40 was used in combination with LPS to drive maturation, it further increased the proliferative response. Thus aCD40 further enhanced the stimulatory function of mature DCs in an additive fashion.

IL-2 is one of the earliest cytokines produced by T cells upon activation. As a measure of antigen specific T cell responses, IL-2 produced by DO11.10 Tg CD4\textsuperscript{+} CD62L\textsuperscript{high} T cells in response to exogenously added OVA\textsuperscript{323-339} peptide was analysed. Enhanced production of IL-2 by CD4\textsuperscript{+} T cells stimulated by DC\textsuperscript{LPS} was observed (figure 4.8). CD40 ligation alone (DC\textsuperscript{aCD40}) provoked only a marginal increase in IL-2 production. However, when DCs were co-cultured with LPS and aCD40 (DC\textsuperscript{LPS,aCD40}) a dramatic enhancement in IL-2 production was observed in an additive manner (figure 4.8).
Figure 4.7 CD40 ligation on DCs elevates proliferation of allogeneic splenocytes.

Day 5 BALB/c BmDC were cultured overnight in medium (DC0), or with LPS (100ng/ml; DC LPS), aCD40 (50ng/ml; DC aCD40) or LPS and aCD40 together (DC LPS aCD40). BmDC (1×10⁴) were cultured with allogeneic splenocytes (1×10⁶) in 96 well plates, and proliferation was measured after 4 days. The data are means of triplicate wells (±SD) from one representative experiment out of three.

Figure 4.8 CD40 ligation on DCs amplifies IL-2 production from DO11.10 Tg CD4+ T cells.

Purified DO11.10 Tg CD4+ T cells (1×10⁵) were stimulated with BALB/c BmDC (1×10⁴) stimulated with LPS (100ng/ml; DC LPS), aCD40 (50ng/ml; DC aCD40) or both together (DC LPS aCD40), in the presence of OVA^{323-339} (100nM). Supernatant was removed after 24 hours and IL-2 production was measured by ELISA. The data are means of duplicate points (±SD) from one representative experiment out of seven performed.
4.3.4 CD40 ligation does not induce DC1 differentiation

The literature suggests contrasting results for the role of CD40 ligation in generating an IL-12 producing, DC1 phenotype (Cella et al., 1996; Hilkens et al., 1997; Koch et al., 1996; Snijders et al., 1998). This may reflect differences in sources of DCs, culture conditions and/or the extent of CD40 engagement in these studies. In order to assess whether CD40 engagement on DCs leads to differentiation into a DC1 phenotype, BmDC were stimulated with either aCD40 alone, or together with LPS (positive control for DC1 inducer). The function of these DCs in skewing the subsequent Th1/Th2 responses was assessed by analysing the cytokines produced by DO11.10 Tg CD4+ CD62L high T cells. Stimulation with DCs treated with varying concentrations of aCD40 did not change the cytokine profile of these CD4+ T cells, although there was a marginal decline in IL-4 production with increasing concentration of aCD40 (figure 4.9A and C). Furthermore, aCD40 triggering of LPS-stimulated DCs had no effect on the DC1 phenotype mediated by LPS (figure 4.9B and D).

In addition, CD40 ligation of DCs stimulated with PGE2 or dbcAMP did not result in an enhancement of IL-4 secretion from DO11.10 Tg CD4+ T cells, while there was a slight enhancement of IFNγ levels (figure 4.10). Thus, it appears that CD40 ligation does not result in DC1/DC2 differentiation of DCs in this system, but rather acts as an amplifier of a committed response.
Figure 4.9 CD40 ligation does not induce DC1 differentiation.

Day 5 BmDC (Balb/c) were stimulated overnight with titrated concentrations of anti-CD40 antibody (aCD40) with or without LPS (1 μg/ml). CD62L$^{\text{high}}$ CD4$^+$ T cells ($1 \times 10^5$) from DO11.10Tg mouse spleens were stimulated with these DCs ($1 \times 10^6$) in the presence (grey circles 100pM, black circles 10nM) or absence (open circles) of OVA$^{323-339}$ for 72 hours. Cells were then stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 8 hours before supernatants were removed for cytokine analysis by ELISA. IFNγ production by CD4$^+$ T cells in response to DCs stimulated with aCD40 alone (A), or DCs stimulated with aCD40 and LPS (B), and IL-4 production from CD4$^+$ T cells in response to DCs stimulated with aCD40 alone (C), or DCs stimulated with aCD40 and LPS (D), are shown. These data are means of duplicate wells from one representative experiment out of three performed. SD < 5% in all cases.
Figure 4.10 CD40 ligation does not enhance production of IFNγ or IL-4 by DC\textsuperscript{PGE\_2} or DC\textsuperscript{dbcAMP}.

Day 5 BmDC (Balb/c) were stimulated overnight with medium alone, PGE\textsubscript{2} (10\textsuperscript{-6} M), or dbcAMP (10\textsuperscript{-6} M) with or without aCD40 (2\mu g/ml). CD62L\textsuperscript{high} CD4\textsuperscript{+} T cells (1×10\textsuperscript{5}) from DO11.10 Tg mouse spleens were stimulated with these DCs (1×10\textsuperscript{6}) in the presence of OVA\textsuperscript{323-339} (100nM) for 72 hours. Cells were then stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 8 hours before supernatants were removed for cytokine analysis by ELISA. These data are means of six experiments (±SE).
4.4 Discussion

Increasing evidence suggests that DCs can differentiate into distinct phenotypes which can trigger Th1 (DC1) or Th2 (DC2) responses (Kalinski et al., 1999; Moser and Murphy, 2000; Reid et al., 2000). This offers a potentially attractive method of targeting appropriate types of immune responses to combat various types of diseases. However, the mechanisms of this functional differentiation of DCs are poorly understood. Based on the hypothesis that DC1/DC2 differentiation occurs upon recognition of pathogen products and infection/injury-mediated factors, the effects of elevation of intracellular cAMP and CD40 ligation on DC function were studied in this chapter.

It was found that an elevation of intracellular cAMP induced by PGE$_2$, dbcAMP, forskolin, CTx and PTx did not correlate with DC maturation (figure 4.2 and 4.3) or Th1/Th2 cytokine patterns produced by responding CD4$^+$ T cells (figure 4.4 and 4.5). In fact, CTx and PTx were the only cAMP elevating reagents found to induce both maturation of DCs, as measured by surface molecule expression and MLRs, and induction of T cell cytokine responses. Both DC$^{PTx}$ and DC$^{CTx}$ induced increased IFN$\gamma$ production from responding CD4$^+$ T cells but not IL-4 production, suggesting that these DCs exhibited a DC1 phenotype. CD40 ligation on DCs resulted in enhanced proliferation (allogeneic MLR, figure 4.7) and IL-2 production (DO11.10 Tg, figure 4.8) of responding T cells, but had no effect on Th1/Th2 differentiation as measured by cytokine secretion (figure 4.9 and 4.10).

Based on early studies that indicated a role of cAMP elevating reagents in DC1/DC2 differentiation (Gagliardi et al., 2000; Kalinski et al., 1997a; Kalinski et al., 1997b;
Rieser et al., 1997; Steinbrink et al., 1997), five reagents known to elevate cAMP were tested in this study: PGE$_2$, dbcAMP, forskolin, CTx and PTx. The molecular mechanisms regulating the enzyme responsible for cAMP synthesis, adenylate cyclase, involve a complex coupling system with different stimulatory or inhibitory receptors and corresponding GTP binding proteins, or G-proteins (G$_s$ and G$_i$). A G-protein is composed of three different polypeptide chains, $\alpha$, $\beta$, and $\gamma$. The $\alpha$ chain of G$_s$ proteins binds GTP and activates adenylate cyclase, while binding of the $\alpha$ chain of G$_i$ proteins inhibits the activation of this enzyme. This process is tightly regulated by hydrolysis of GTP to GDP, which inactivates the ability of G-proteins to bind to adenylate cyclase. This activation process of adenylate cyclase is schematically illustrated in figure 4.11.

![Diagram of the process of adenylate cyclase activation](image)

**Figure 4.11 Process of adenylate cyclase activation.**

Ligand (e.g., PGE$_2$) binding alters the conformation of receptors (토키), causing the receptor to bind to a G protein (토키). This reduces the affinity of G protein for GDP. GDP then dissociates, allowing GTP to bind (토키). This causes G$_\alpha$ to dissociate from the G protein complex, allowing binding to adenylate cyclase (토키). This leads to activation of the enzyme and cAMP synthesis results (토키). Meanwhile, dissociation of the ligand returns the receptor to its original conformation (토키). GTP is hydrolysed (토키) and causes the G protein to dissociate from the adenylate cyclase (which becomes inactive) (토키).
While forskolin acts on adenylate cyclase directly to activate the enzyme, CTx, PTx and PGE\(_2\) act upon the complex of molecules that activate the enzyme (dbcAMP is an cAMP analogue). Cholera toxin mediates ADP ribosylation of \(\alpha\_s\), which prevents hydrolysis of GTP, resulting in continuous activation of adenylate cyclase. In contrast, pertussis toxin catalyses the ADP ribosylation of \(\alpha\_i\). This prevents the G\(_i\) complex from interacting with receptors, and as a result the complex remains bound to GDP and is unable to inhibit adenylate cyclase activity. PGE\(_2\) interacts with specific membrane receptors coupled to G\(_s\) protein, which in turn activates adenylate cyclase.

The effects of a range of cAMP inducers and other modulins studied in this thesis on DC function are summarised in table 4.1.

**Table 4.1 Changes in function of DCs upon differential stimulation (relative to DC0).**

<table>
<thead>
<tr>
<th></th>
<th>DC(_{\text{PGE2}})</th>
<th>DC(_{\text{dbcAMP}})</th>
<th>DC(_{\text{forskolin}})</th>
<th>DC(_{\text{CTx}})</th>
<th>DC(_{\text{PTx}})</th>
<th>DC(_{\text{PS}}^*)</th>
<th>DC(_{\text{ES2**}})</th>
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<tr>
<td>DC maturation markers</td>
<td>++</td>
<td>+/−</td>
<td>+/−</td>
<td>+++</td>
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<td>Allogeneic MLR</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>Syngeneic MLR</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>−</td>
<td>−</td>
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<tr>
<td>IL-12 production</td>
<td>−</td>
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<td>−</td>
<td>+/−</td>
<td>+</td>
<td>++</td>
<td>+/−</td>
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<tr>
<td>DO11.10 Tg CD4+ T cell cytokine responses</td>
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<tr>
<td>IL-2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++</td>
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<td>++</td>
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<tr>
<td>IFN(\gamma)</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>−</td>
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<tr>
<td>IL-4</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td></td>
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</tbody>
</table>

* DC\(_{\text{PS}}^*\) is included as stimulator of DC1, while ** DC\(_{\text{ES2}}^*\) is typical of DC2 phenotype [Whelan et al., 2000]; data not shown. Scale: − denotes no difference from DC0 phenotype; ++++ denotes maximum expression/response obtained within the systems tested; each + accounts for \(\%\) of the maximum expression/response observed.
The data shows clearly that whilst CTx and PTx induced both maturation and differentiation of DCs, other cAMP inducers (PGE$_2$, forskolin and dbcAMP) did not. Thus, this suggests that an increase in intracellular cAMP level is not likely to be responsible for the resulting maturation of DCs in response to CTx and PTx, as not all cAMP inducers caused maturation of DCs. This also implies that a component of CTx and PTx other than that induces cAMP elevation is responsible for maturation stimulus of DCs, necessary to achieve functional differentiation of DCs. In contrast to the work presented in this chapter, earlier studies implicated the role of elevating cAMP in DCs differentiation by examining the effects of cAMP inducers on human monocyte-derived DCs (Gagliardi et al., 2000; Kalinski et al., 1997a; Kalinski et al., 1997b; Rieser et al., 1997; Steinbrink et al., 1997). Thus the differences in the type of DCs may explain the different results obtained between this study and earlier studies.

The importance of the interaction between CD40L on activated T cells and CD40 expressed on B cells, monocytes and DCs in enhancement of APC function has been well documented (van Kooten and Banchereau, 1997). CD40 ligation on BmDC resulted in a small, but significant enhancement of the allogeneic MLR (figure 4.7) and IL-2 production from DO11.10 Tg CD4$^+$ T cells (figure 4.8). However, when aCD40 was used in conjunction with LPS to stimulate DCs (DC$^{LPS/aCD40}$), both of these responses were amplified in an additive manner. Interestingly, the response correlated with the level of costimulatory molecules expressed on DCs (Chapter 3, figure 3.5). DC$^{aCD40}$ failed to induce Th1 or Th2 responses, as indicated by the levels of IFN$\gamma$ and IL-4 produced by responding CD4$^+$ T cells (figure 4.9). Furthermore, in contrast to the amplifying role on allogeneic MLR and IL-2 production induced by DC$^{aCD40}$, further enhancement of the Th1 cytokine production induced by DC$^{LPS}$ was
not observed, suggesting that ligation of CD40 on DCs has an insignificant influence on Th1/Th2 differentiation (figure 4.9). Similarly, enhancement of Th1/Th2 responses by \( \text{DC}^{\text{PGE}_2} \) and \( \text{DC}^{\text{dbcAMP}} \) was not observed upon ligating CD40 on these DCs (figure 4.10).

An enhanced production of IL-12p70 by \( \text{DC}^{a\text{CD}40} \) was not observed in this study (figure 4.6). This agrees with earlier studies that demonstrated that mere ligation of CD40 on DCs is not sufficient to trigger production of this cytokine (Hilkens et al., 1997; Snijders et al., 1998). This is not surprising, given that CD40L is expressed on both Th1 and Th2 cell types. It would be potentially dangerous if, as a result of any DC-T cell interactions, DCs only acquired an ability to produce high levels of IL-12 and induced Th1 responses. Thus, Th1 responses may require strict regulation, and subsequently IL-12 production by DCs may be under stringent control. The differences in the literature relating to the role of CD40 ligation on IL-12 production by DCs may reflect the differences in methods used for preparation of DCs, lineages of DCs, methods of CD40 ligation used and/or time period at which the cytokine was measured.

The two studies that agree with the observations presented in this chapter examined the response of human monocyte-derived DCs to soluble CD40L. In contrast, studies indicating a direct role of CD40 ligation in the induction of IL-12 used a CD40L-transfected cell line to stimulate human monocyte-derived DCs (Cella et al., 1996), or an antibody against CD40 (aCD40) to stimulate mouse splenic DCs (Koch et al., 1996). In addition, it is likely that the extent of DC maturation in these two studies may explain the differences obtained in this chapter. For example, murine DCs were
shown to take a longer period than human DCs for maturation in response to aCD40 (Kelleher and Beverley, 2001; Koch et al., 1996). Thus, it is possible that DCs stimulated with aCD40 in the work presented in this chapter may have an enhanced secretion of IL-12 at a later time point. In addition, since low levels of contamination with endotoxin may be sufficient to trigger maturation of DCs, high levels of IL-12 production in response to ligation of CD40 in some of the previous studies may be due to the presence of endotoxin.

One of the most surprising findings in this chapter is that DC\textsuperscript{PTx} triggered a potent syngeneic MLR at a level almost comparable to that of the allogeneic MLR. The generation of such a syngeneic response has not been described before by DCs stimulated with other modulins tested. Both PTx and CTx have been used as mucosal adjuvants in vaccine trials (Hormozi et al., 1999; Lycke, 1997; Rappuoli et al., 1999). However, the mechanism of their adjuvanticity remains largely unknown. It is plausible that these toxins activate the immune system by activating DCs. This is further investigated in the following chapter in this thesis.

In summary, the DC\textsuperscript{1} phenotype resulted from stimulation with bacterial toxins including LPS, CTx and PTx, all of which induced maturation of DCs. In contrast, other cAMP inducers that failed to activate DCs had no effect on DC\textsuperscript{1}/DC\textsuperscript{2} differentiation. In addition, CD40 ligation resulted in amplification of some of T cell responses (proliferation and IL-2 production), but not differential cytokine production. As CD40 does not function as a pattern recognition receptor (PRR), these results are consistent with the hypothesis that modulins that are recognised by the PRR on DCs would activate and differentiate DCs. Thus, the conclusion from this
chapter is that, at least for murine BmDC, a maturation-driving signal is a prerequisite of the modulins for functional differentiation of DCs. It is likely that these signals are mediated via recognition of the modulins by the PRRs on DCs.
Chapter Five

Mechanisms of DC^{PTx}-mediated T cell responses

5.1 Introduction

Pertussis toxin (PTx) is an exotoxin from *Bordetella pertussis*, and is a key virulence factor in infection with this organism. It consists of six polypeptides (S1 to S5) held together by noncovalent interactions that are arranged in an A-B structure similar to cholera toxin (CTx) and *Escherichia coli* heat-labile toxin (LTx) (Tamura *et al.*, 1982). The active A protomer of the toxin is composed of a single S1 subunit with catalytic ADP-ribosyltransferase activity. The B-oligomer component of the toxin (PTxB) has a pentameric structure, which consists of five subunits; S2, S3, S4 and S5 in a 1:1:2:1 ratio. Exposing PTx to 5M urea dissociates the S1 subunit from PTxB and disrupts PTxB into three moieties: an S2-S4 dimer, an S3-S4 dimer and an S5 monomer (Tamura *et al.*, 1982). These results suggest that PTxB may consist of the two dimers, each bound to the S5 subunit (figure 5.1A).

Considerable evidence suggests that PTxB binds PTx to cells and dramatically increases the efficiency with which the S1 subunit gains entry into cells. It has been demonstrated in a number of studies that receptors for PTx contain carbohydrates (Kaslow and Burns, 1992). However, the identity of the protein(s) to which these carbohydrates are linked remains unknown. PTx binds to a 165KDa protein found in detergent extracts of CHO cells (Brennan *et al.*, 1988), while a 43KDa protein was found to be the receptor(s) on human Jurkat T cells (Rogers *et al.*, 1990). Detergent extracts of T lymphocytes contain a 70KDa protein that either directly or as part of a
complex binds to PTx (Clark and Armstrong, 1990). Thus, proteins of various sizes on different cell types associate with PTx. However, it remains to be demonstrated that binding of PTx to these proteins is a first or exclusive step in the entry of the toxin into cells. There is also evidence for PTx binding to glycolipids (Tuomanen et al., 1988), and thus glycolipids may also participate in the binding and/or entry of the toxin into cells.

Upon binding to glycoconjugate receptors, PTx is thought to enter the cells by directly penetrating the plasma membrane without the need for an endocytic process (Kaslow and Burns, 1992). PTx then requires activation in order to gain ADP ribosyltransferase activity. First, the S1 subunit needs to be dissociated from PTxB, the process that takes place in the presence of ATP (Mattera et al., 1986). Once dissociated a single disulphide bond within the S1 subunit is reduced, which promotes the formation of a NAD binding site that allows the catalysis of the ADP-ribosylation of G-proteins (Kaslow and Burns, 1992). The current model of PTx activation steps is summarised in figure 5.1 (Kaslow and Burns, 1992).
PTx exerts a variety of biological effects, and therefore many names have been applied to PTx, such as lymphocytosis promoting factor (LPF), histamine-sensitising factor (HSF) and islet activating protein (IAP). Importantly, purified PTxB alone has many effects on immune function: aggregation and phospholipase C activation of human platelets (Banga et al., 1987); adhesion of the human monocyte cell line U937 (Wong et al., 1996); increase in inositol phosphate production and intracellular calcium levels of Jurkat T cells (Rosoff et al., 1987); IL-2 production and increase in intracellular calcium of human peripheral T cells (Rogers et al., 1990); proliferation of mouse splenocytes (Tamura et al., 1983); elevation of co-stimulatory molecules on APCs (Ryan et al., 1998); and induction of Th1 and/or Th2 (Ryan et al., 1998; Ryan et al., 1997) responses in vitro and in vivo.
In the previous chapter, PTx was found to be the only reagent, among all the cAMP inducers tested, to induce potent proliferative response from syngeneic splenocytes at a level almost equivalent to an allogeneic MLR. One interpretation is that residual toxin in the culture acted as a T cell mitogen, as suggested by a number of studies (Rosoff and Mohan, 1992; Rosoff et al., 1987; Tamura et al., 1983). However, a role of accessory cells was not excluded in the study that demonstrated the effect of PTx on splenic T cells (Tamura et al., 1983). Furthermore, studies that claimed PTx as a T cell mitogen in accessory cell-free conditions utilised systems such as the Jurkat T cell line and human T cell IL-2 blasts (Rosoff and Mohan, 1992; Rosoff et al., 1987), both of which are activated and may not necessarily reflect how the toxin induces its effect on naïve T cells. Thus, in this chapter mechanisms that enable PTx-treated DCs (DC^{PTx}) to induce a strong “low-affinity” response were investigated. In addition, the effect of DC^{PTx} on differentiation of T cells was examined.

5.2 Objectives

The objectives of this chapter are to investigate the mechanisms by which DC^{PTx} enhance T cell proliferation induced by low-affinity interactions and subsequent activation of type 1 or type 2 T cell responses.
5.3 Experimental Results

5.3.1 DC\textsuperscript{PTx} and DC\textsuperscript{PTxB} are potent stimulators of syngeneic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells

Chapter 3 demonstrated a differential requirement to trigger effector function of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in syngeneic interactions. Whereas DC\textsuperscript{LPS} or DC\textsuperscript{CD4} were capable of inducing proliferation of syngeneic CD4\textsuperscript{+} T cells, presumably by amplifying signal 2, these DCs failed to induce syngeneic CD8\textsuperscript{+} T cell responses. A plausible explanation for this was that different levels of signal 1 were required to trigger activation of these T cells.

In Chapter 4, BmDC stimulated with PTx (DC\textsuperscript{PTx}) were shown to induce strong proliferative responses of syngeneic splenocytes (figure 4.3E). In order to establish which populations of splenocytes were proliferating in response to syngeneic DC\textsuperscript{PTx}, purified syngeneic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (CD62L\textsuperscript{high}) were tested for their responsiveness to DC\textsuperscript{PTx}. As shown in figure 5.2, DC\textsuperscript{PTx} induced strong proliferation of both syngeneic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. In addition, DCs stimulated with the binding subunit (PTxB) of PTx (DC\textsuperscript{PTxB}) provoked significant responses of both syngeneic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, although these were not as large as the responses induced by DC\textsuperscript{PTx}. Importantly, T cells cultured alone with the same concentration of the toxins used to stimulate DCs failed to proliferate. Thus, in the absence of DCs, toxin alone at this concentration had no mitogenic effect on these T cells. T cell stimulation by DC\textsuperscript{PTx} and DC\textsuperscript{PTxB} involved both antigen specific and antigen non-specific interactions, since proliferation of DO11.10 Tg CD4\textsuperscript{+} T cells was triggered by DC\textsuperscript{PTx} or DC\textsuperscript{PTxB} in the absence of the peptide antigen, and addition of the antigen further enhanced the proliferative response (figure 5.3).
Figure 5.2  DC_{PTx} and DC_{PTxB} are potent stimulators of syngeneic CD4+ and CD8+ T cells.

BALB/c CD4+ (A) or CD8+ (B) T cells (1×10^5) were stimulated with syngeneic (BALB/c) DC0, DC stimulated with PTx (5μg/ml; DC_{PTx}) or PTx B-oligomer (5μg/ml; DC_{PTxB}) at a ratio of T cells:DCs at 10:1. T cells were stimulated with PTx or PTx B-oligomer alone as controls (T_{PTx} and T_{PTxB}). Proliferation of T cells was measured with tritiated thymidine after 72 hours. The data are means of quadruplicate wells (±SD) from one representative experiment out of five separate experiments conducted.
Figure 5.3 Proliferation of DO11.10 Tg CD4+ T cells in response to syngeneic DC^{PTx} and DC^{PTxB}.

DO11.10 Tg CD4+ T cells (1x10^5) were cultured with syngeneic BmDC (1x10^6) treated with titrated concentrations of PTx (A) or PTxB (B) in the presence of OVA peptide as indicated above. Proliferation of CD4+ T cells was assessed by measuring tritiated thymidine incorporation after 72 hours. The data are means of triplicate points (±SD) from one representative experiment out of three performed.
5.3.2 Elevation of cAMP levels does not compensate for the absence of A-protomer in DC$^{PTxB}$-mediated T cell proliferative responses

The difference in the potency of the T cell responses induced by DC$^{PTx}$ and DC$^{PTxB}$ suggests that the presence of the A-protomer of the toxin enhances the effect triggered by the B-oligomer of the toxin. Since the A-protomer exerts its effect by ADP-ribosyltransferase activity, thereby inducing an elevation of intracellular cAMP levels, we hypothesised that high levels of cAMP might compensate for the difference between DC$^{PTxB}$ and DC$^{PTx}$. To test this, BmDC were stimulated with PTxB in the presence of a cAMP analogue, dbcAMP, and their ability to stimulate syngeneic T cells was examined (figure 5.4). Consistent with the earlier observations (figure 4.3C), stimulation with dbcAMP alone had no effect on the T cell stimulatory function of DCs. Importantly, addition of dbcAMP to DC$^{PTxB}$ did not result in enhancement of the T cell stimulatory ability of DC$^{PTxB}$, suggesting that elevation of cAMP levels is not sufficient to mimic the responses mediated by DC$^{PTx}$. 
Figure 5.4 Effect of cAMP elevating agent (dbcAMP) on syngeneic T cell stimulatory capacity of DC<sup>PTxB</sup>.

BALB/c CD<sup>4+</sup> (A) or CD8<sup>+</sup> (B) T cells (1×10<sup>5</sup>) were stimulated with syngeneic (BALB/c) DC (5×10<sup>4</sup>) that were treated with medium alone (DCO), PTx B-oligomer (5μg/ml; PTxB), three concentrations of dbcAMP (see legends) or dbcAMP together with PTx B-oligomer. Proliferation of T cells were measured after 72 hours by thymidine incorporation. The data are means of quadruplicate wells (±SD) from one experiment.
5.3.3 Maturation of BmDC is enhanced by PTx, but not PTxB

It is possible that PTx and PTxB may “super activate” DCs, inducing expression of high levels of signal 2, which may explain the potent T cell stimulatory ability of DC\textsuperscript{PTx} and DC\textsuperscript{PTxB}. Indeed, DC\textsuperscript{PTx} exhibited a phenotype of matured DCs, with elevated expression of co-stimulatory molecules (CD80, CD86 and CD40), MHC molecules and adhesion molecules, in patterns similar to DC\textsuperscript{LPS}(figure 5.5). This was not likely to be due to the contamination of PTx with endotoxin, as PTx contained very low amount of LPS (43.5 EU/ml; 4.35ng/ml), which is several orders lower than the concentration required to trigger maturation of DCs (data not shown). Interestingly, such changes were not observed in DC\textsuperscript{PTxB}. Apart from some elevation in the levels of MHC class I, CD40 and CD11a expression, DC\textsuperscript{PTxB} showed no sign of further maturation. Thus, T cell responses mediated by DC\textsuperscript{PTxB} are not due to elevated levels of costimulatory molecules such as CD80 and CD86. The differences observed between PTx and PTxB in the effects on DC maturation may reflect their ability to activate DCs to stimulate T cells.
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Figure 5.5 Maturation of BmDC is enhanced by stimulation with PTx, but not PTxB.

Day 5 BmDC (C57BL/6) were cultured overnight with medium alone (DC0), LPS (10μg/ml; DC_{LPS}), PTx (5μg/ml; DC_{PTx}) or PTxB (5μg/ml; DC_{PTxB}). These cells were then labelled with fluorochrome-conjugated antibodies (line histograms) or appropriate isotype-matched controls (shaded histograms). Values indicate the mean fluorescence intensity. The data are representative of three separate experiments.
5.3.4 DC^{PTx} and DC^{PTxB} are capable of inducing proliferation of syngeneic T cells in the absence of MHC molecules

There are a number of possible mechanisms by which DC^{PTx} and DC^{PTxB} induce potent proliferative response of syngeneic T cells. First, since PTx is a glycoprotein it is possible that the toxins may be processed and presented by DCs, although the probability of naïve T cells or DO11.10 Tg CD4^+ T cells recognising such PTx-derived antigens is quite low. Second, stimulation with PTx might alter the functional phenotype of DCs, which might have resulted in expression of co-stimulatory molecules other than those measured above (figure 5.5), and/or secretion of soluble factors that may be stimulatory for T cells. Third, residual toxin in the culture or on the surface of DC^{PTx} and DC^{PTxB} might have a direct effect on T cells.

To test whether presentation of antigen(s) derived from the toxins is crucial in T cell responses to DC^{PTx} and DC^{PTxB}, syngeneic T cell responses to BmDC derived from mice deficient in MHC class I (β2m ko) or MHC class II (I-A ko) were examined (figure 5.6). Surprisingly, in the absence of MHC molecules DC^{PTx} and DC^{PTxB} were almost equally capable of inducing proliferation of both syngeneic CD4^+ and CD8^+ T cells (figure 5.6, A and B). In fact, the maximum suppression of T cell responses caused by the lack of MHC molecules was about 30%. Thus, these results demonstrated not only that MHC presentation of toxin-derived antigen(s) by DCs is not important, but also that signalling mediated via MHC molecules is not crucial in T cell responses mediated by DC^{PTx} and DC^{PTxB}. Thus, conventional “signal 1” mediated by the interaction between MHC:peptide complexes and TcR does not play a role in this system.
Figure 5.6 DC<sup>PTx</sup> and DC<sup>PTxB</sup> induce proliferation of syngeneic T cells in the absence of MHC molecules.

C57BL/6 CD<sup>4+</sup> or CD<sup>8+</sup> T cells (1x10<sup>5</sup>) were stimulated with DC0, DC<sup>PTx</sup> or DC<sup>PTxB</sup> (at varying ratios) derived from C57BL/6 mice or mice deficient of MHC class I (A) or class II (B) molecules. Proliferation of T cells were measured after 72 hours by thymidine incorporation. DCs derived from C57BL/6, β<sub>2</sub>m ko and I-A ko mice were labelled with antibodies against H-2K<sup>b</sup> and I-A<sup>b</sup>/I-E<sup>b</sup> (C, shaded histograms), or with appropriate control antibodies (C, line histograms). The data are means of quadruplicate wells (±SD) from one representative experiment out of two (β<sub>2</sub>m ko) or three (I-A ko) experiments performed.
5.3.5 B7-mediated signalling is partially responsible for the syngeneic T cell proliferative responses mediated by DC^{PTx} and DC^{PTxB}

Our earlier observation that DC^{PTx} exhibited a mature DC phenotype with high levels of CD80 (B7-1), CD86 (B7-2) and CD40 comparable to DC^{LPS} (figure 5.5) suggests that signals mediated by these co-stimulatory molecules would, at least in part, contribute to the T cell responses induced by DC^{PTx}. To test this, syngeneic T cells were stimulated with DC^{PTx} and DC^{PTxB} in the presence of blocking antibodies to B7-1 and/or B7-2, and the proliferative response was examined (figure 5.7). While blocking antibody to B7-1 alone had no effect on subsequent proliferation of T cells, blocking B7-2 mediated signalling resulted in up to 40% inhibition of CD4^+ T cell response induced by DC^{PTx}. In addition, although PTxB did not increase levels of B7-1 or B7-2 expression on DCs (figure 5.5), a reduction in T cell response was obtained by blocking B7-2 in both CD4^+ and CD8^+ T cells in response to DC^{PTxB}. When both B7-1 and B7-2 were blocked, the inhibition was further enhanced in all cases except the CD4^+ response to DC^{PTx}. These results suggest that B7-mediated signalling by DC^{PTx} and DC^{PTxB} is indeed an important part of, but not crucial in, triggering the proliferation of syngeneic T cells.
Figure 5.7 Blocking B7-mediated signalling partially inhibits proliferation of syngeneic T cells in response to DC^{PTx} and DC^{PTxB}.

BALB/c CD4^{+} (A) or CD8^{+} (B) T cells (1×10^{5}) were stimulated with DC0, DC^{PTx} or DC^{PTxB} (1×10^{4}) for 72 hours in the presence of rat antibodies to CD80 (B7-1) alone, CD86 (B7-2) alone, B7-1 and B7-2 together or isotype-matched controls. Proliferation of the responding T cells was measured by thymidine incorporation. The data are means of triplicate wells (±SD) from one representative experiment out of two performed.
5.3.6 Effect of residual PTx on syngeneic T cell responses

In all experiments thus far described, DCs stimulated with the toxin were washed extensively to avoid carry over of any residual toxin. Thus, it is unlikely that any unbound toxin was able to interact with T cells in the co-culture between DC\textsuperscript{PTxB} and syngeneic T cells. However, it was possible that the toxin that remained bound to the surface of DCs might be available to interact directly with T cells.

Upon treatment with PTx or PTxB, BmDC became increasingly adherent, which was evident from the clustering of DCs observed in cultures treated with PTx or PTxB (figure 5.8). This has also been observed when CHO cells were treated with PTx (Fujiwara and Iwasa, 1989). It is thought that the cell adhesion is mediated mainly via the bound toxin on the cell surface. In order to examine whether the toxin directly interacts with T cells in DC\textsuperscript{PTx}-T cell co-culture, syngeneic CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells were co-cultured with DC\textsuperscript{PTx} or DC\textsuperscript{PTxB}, and distribution of the toxin was examined by immunolabelling and flow cytometry. As shown in figure 5.9A (left column), toxin remained bound on the surface of DC\textsuperscript{PTx}. In contrast, only a low level of toxin was detected on the surface of DC\textsuperscript{PTxB} (figure 5.9B left column). Interestingly, the level of the toxin detected on the cell surface was indifferent in DCs that were incubated with the toxin for a period between 3 minutes and 24 hours. Cell surface-bound PTx was also detected on T cells (figure 5.9A, right column), indicating that residual toxin in the co-culture do bind directly to T cells. Again, relatively low level of PTxB was found to be bound on the surface of T cells (figure 5.9B, right column).
Figure 5.8 Morphology of BmDC stimulated with different modulins.

Day 5 BmDC (C57BL/6) were treated overnight with medium alone (DC0), LPS (DC\textsuperscript{LPS}; 10µg/ml), aCD40 (DC\textsuperscript{aCD40}; 2µg/ml), LPS and aCD40 together (DC\textsuperscript{LPSaCD40}), PTx (DC\textsuperscript{PTx}; 5µg/ml) or PTxB (DC\textsuperscript{PTxB}; 5µg/ml), and analysed under a phase contrast microscope (×400).
### Figure 5.9 Distribution of PTx and PTxB in DC<sup>PTx</sup>/DC<sup>PTxB</sup>-T cell culture.

Day 6 BmDC stimulated with medium alone (DC0), PTx (5μg/ml; DC<sup>PTx</sup>) or PTxB (5μg/ml; DC<sup>PTxB</sup>) for a varying period were cultured overnight with purified T cells. These cells were then labelled with fluorochrome-conjugated antibodies against PTx (A) or PtxB (B). Data shown have been gated on CD11c positive cells (DCs) or CD3 positive cells (T cells). The shaded histograms indicate cultures containing DC0 (median of DCs: 2.74; median of T cells: 1.04), and line histograms show cultures containing DC<sup>PTx</sup> (A) or DC<sup>PTxB</sup> (B). The values indicated are median fluorescence intensity. The data are representative of two separate experiments.

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In order to study where the toxin binds on DCs, DC\textsuperscript{PTx} labelled with anti-PTx antibody were examined by confocal microscopy. While CD11c was distributed in small patches over the surface (figure 5.10, red fluorescence), PTx staining was more or less evenly distributed over the whole cell surface (figure 5.10, green fluorescence). This suggests an abundant surface receptor for PTx, possibly carbohydrates or glycolipids.

Having observed that incubation of DCs with the toxin for a period between 3 minutes and 24 hours did not result in different amounts of the toxin being detected on the DC surface, it was possible that syngeneic T cells were stimulated directly by the toxin bound on the surface of DCs. To test this possibility, DCs stimulated with the toxin for a period between 3 minutes and 24 hours were cultured with purified syngeneic CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells, and the ability of these DCs to stimulate T cells were assessed by T cell proliferation. As shown in figure 5.11, even after 3 minute incubation with PTx, DC\textsuperscript{PTx} triggered strong proliferation of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. These responses increased with an increasing period of DC stimulation with the toxin. In contrast, the effect of PTxB on T cell stimulatory ability of DCs was only evident when DCs were incubated with PTxB for a period longer than 6 hours. These data suggest that the presence of the PTx, but not PTxB, on the DC surface alone was sufficient to trigger proliferation of syngeneic T cells.
Figure 5.10 Distribution of PTx bound to the surface of BmDC.

Day 5 BmDC (C57BL/6) cultured overnight with PTx (5μg/ml) were labelled with anti-PTx antibodies (FITC, shown in green) and/or anti-CD11c antibody (Texas red, shown in red), and analysed using a confocal microscope. The areas of overlapping staining between CD11c and PTx appear orange.
Figure 5.11 Prolonged incubation with toxin is required for stimulation of syngeneic T cells by DC\textsuperscript{ptx}, but not by DC\textsuperscript{ptx}.

BALB/c CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells (1×10\textsuperscript{5}) were stimulated with BmDC (1×10\textsuperscript{5}) stimulated with PTx or PTxB for varying periods as shown above. Proliferation of the responding T cells were measured by thymidine incorporation. The data are means of quadruplicate wells (±SD) from one representative experiment out of two conducted.
5.3.7 Induction of antigen specific CD4⁺ T cell responses by DC⁹Tx and DC⁹TxB

In the previous chapter, DC⁹Tx stimulated with low doses (100ng/ml) of the toxin induced high levels of IFNγ production from DO11.10 Tg CD4⁺ T cells (figure 4.5). In addition, PTx was found to be the only cAMP inducer that enhanced the ability of DCs to trigger IL-2 production from DO11.10 Tg CD4⁺ T cells. To examine whether DC⁹TxB would trigger Th1 differentiation of CD4⁺ T cells in a similar manner, naïve DO11.10 Tg CD4⁺ T cells were stimulated with DC⁹TxB and cytokines produced from CD4⁺ T cells were analysed as before (figure 5.12). In contrast to DC⁹Tx which induced Th1 cytokines, DC⁹TxB were found to activate both Th1 and Th2 cytokine responses, as shown by an increase in IL-2, IFNγ, and significantly IL-4.

5.3.8 Effect of DC⁹Tx and DC⁹TxB on differentiation of syngeneic CD4⁺ and CD8⁺ T cells

In contrast to the DO11.10 Tg system, the effect of DC⁹Tx and DC⁹TxB on CD4⁺ T cell differentiation was different in the syngeneic system. Whilst DC⁹Tx still induced IL-2 and IFNγ production, a Th2 cytokine IL-4 was also enhanced (figure 5.13A). In contrast, stimulation of CD4⁺ T cells with DC⁹TxB resulted in IFNγ production, but not IL-2 or IL-4 production. In syngeneic CD8⁺ T cells, both DC⁹Tx and DC⁹TxB provoked high levels of IFNγ production (figure 5.13B), but not IL-4. In addition, IL-2 was induced by DC⁹Tx, but not by DC⁹TxB.
Figure 5.12  Cytokine produced from DO11.10 Tg CD4⁺ T cells in response to DCPtx and DCPTxB.

Day 5 BmDC (BLAB/c) were stimulated overnight with medium alone (DC0), or varying concentrations of PTx or PTxB. CD62L⁺'CD4⁺ T cells (1x10⁵) from DO11.10 Tg mouse spleens were stimulated with these DC (1x10⁴) in the presence (coloured bars) or absence (open bars) of OVA peptide (10nM) for 72 hours. Cells were then stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 8 hours before supernatants were removed for cytokine analysis by ELISA. The data are single points from one representative experiment out of six similar experiments performed.
Figure 5.13 Type 1 and type 2 T cell cytokine responses induced by syngeneic $DC_P^{PT}$ and $DC_P^{PTB}$.

Day 5 BmDC (C57BL/6) were stimulated overnight with medium alone (DC0), PTx (5µg/ml) or PTxB (5µg/ml). CD62L$^{hi}$ syngeneic CD4$^{+}$ T cells (A) or CD8$^{+}$ T cells (B) ($5\times10^5$) were stimulated with BmDC ($5\times10^5$) for 72 hours. Supernatants were then removed for cytokine analysis by ELISA. The data are single points from one representative experiment out of seven similar experiments conducted.
5.3.9 High IFNγ production by T cells is not mediated by IFNγ produced by DC<sup>PTx</sup> or DC<sup>PTxB</sup>

Splenic DCs are known to produce IFNγ under certain conditions (Fukao et al., 2000). BmDCs were also shown to produce this cytokine in response to IL-12 and IL-18 (Stober et al., 2001). Thus, it was possible that IFNγ produced by DC<sup>PTx</sup> or DC<sup>PTxB</sup> contributed to the IFNγ measured in the supernatant. To test this possibility, supernatants from day 6 DC<sup>PTx</sup> and DC<sup>PTxB</sup> cell cultures were collected before they were co-cultured with T cells and analysed for cytokine production. Stimulation with PTx, PTxB or LPS and aCD40 did not increase the amounts of IFNγ, IL-4 or IL-2 produced from DCs (figure 5.14A and data not shown). To rule out the possibility that these DCs may produce IFNγ after co-culture with T cells, DCs were generated from mice deficient in this cytokine and IFNγ production from T cells in response to these DCs was analysed. As shown in figure 5.14B, DC<sup>PTx</sup> and DC<sup>PTxB</sup>, incapable of producing IFNγ, induced production of IFNγ from responding CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which was comparable to that induced by wildtype DCs (upper panel). In contrast, culture of wildtype DCs with T cells deficient in IFNγ resulted in a complete absence of this cytokine (figure 5.14B, lower panel), confirming that this cytokine was exclusively produced by the responding T cells and not by DCs.
Figure 5.14 IFNγ produced by DCs is not involved in the production of IFNγ by T cells in response to syngeneic DCPTxB or DCPTxB.

A: Day 5 BmDC (C57BL/6) were stimulated with medium alone (DC0), PTx (5μg/ml; DC-PTx), PTxB (5μg/ml; DC-PTxB), or LPS (10μg/ml) and aCD40 [2μg/ml; (DC-LPS/aCD40)] and supernatants were tested for IFNγ and IL-4 by ELISA after 1 hour (1h), 4 hours (4h), 8 hours (8h), or overnight (o/n). B: After the overnight stimulation, these DCs (1×10³) (C57BL/6 or IFNγ ko) were used to stimulate syngeneic CD4⁺ or CD8⁺ T cells (1×10⁴) for 72 hours. Supernatants were then removed and IFNγ was quantified by ELISA. These data are single points (A) or means of quadruplicate points (±SD) (B) from one representative experiment out of four (A) or two (B) separate experiments.
5.3.10 DC$^{PTx}$, but not DC$^{PTxB}$, produce high levels of proinflammatory cytokines

Differentiation of T cells into type 1 (Th1/Tc1) or type 2 (Th2/Tc2) phenotypes is induced early after activation, and is largely influenced by the cytokine environment. To test whether DC$^{PTx}$ and DC$^{PTxB}$ achieve differentiation of T cells by secretion of either quantitatively or qualitatively different cytokines as compared to those produced by DC$^{LPS}$, some of the cytokines that are known to be produced upon DC activation were quantified (figure 5.15). DC$^{PTx}$ produced high levels of IL-1β and IL-6 comparable to DC$^{LPS}$. In addition, relatively high amounts of IL-1α and IL-12p70 were produced, although at lower levels than DC$^{LPS}$ or DC$^{LPS/αCD40}$. On the other hand, an elevation of these cytokines was not observed in DC$^{PTxB}$, with the exception of IL-12p70, which was produced at a higher level than by DC0. Only low levels of IL-10 and IL-18 were produced by both DC$^{PTx}$ and DC$^{PTxB}$, which were similar to that of DC0. These results are consistent with the expression of DC maturation markers in response to LPS, PTx or PTxB (figure 5.5): DC$^{PTx}$ had a similar maturation pattern to DC$^{LPS}$, while such a change was not observed in DC$^{PTxB}$. Thus, none of these cytokines tested seemed to be unique to DC$^{PTx}$ or DC$^{PTxB}$, and therefore are unlikely to be responsible for the induction of T cell responses by these DCs.
Figure 5.15 Cytokine profiles of DCs stimulated with PTx and PTxB.

Day 5 BmDC (C57BL/6) were stimulated overnight with medium alone (DC0), PTx (5µg/ml), PTxB (5µg/ml), LPS (10µg/ml), aCD40 (2µg/ml) or LPS and aCD40, and supernatants were removed for cytokine analysis by ELISA. These data are single points from one representative experiment out of five separate experiments.
5.3.11 IFNγ production mediated by DC^{PTx} and DC^{PTxB} is independent of IL-12

IL-12 is known to be an important factor in mediating Th1 responses in many biological systems (Gately et al., 1998; Trinchieri, 1995). Since production of this cytokine was induced in both DC^{PTx} and DC^{PTxB} (figure 5.15), it seemed likely that IL-12 produced from these DCs played a role in differentiation of T cells to become an IFNγ-producing phenotype. To test whether IL-12 produced by DCs is important in mediating IFNγ production by T cells in response to DC^{PTx} and DC^{PTxB}, a neutralising antibody was used to deplete IL-12, and IFNγ production by T cells was analysed. As shown in figure 5.16, IFNγ production by CD4⁺ or CD8⁺ T cells in response to DC^{PTx} and DC^{PTxB} was not reduced in the presence of the neutralising antibody (figure 5.16A). In addition, IL-4 production by CD4⁺ or CD8⁺ T cells was not affected by the presence of the neutralising antibody to IL-12 (figure 5.16B).

Although the above data suggest that IL-12 is not important for inducing IFNγ production by T cells, it is difficult to analyse the extent of the neutralising effect of the antibody. Thus, mice that are deficient in synthesising IL-12 (IL-12 ko) were studied. Functional IL-12 is made up of two polypeptides, IL-12p35 and IL-12p40. The IL-12 ko mice lack the expression of IL-12p40, thus preventing the formation of functionally bioactive IL-12p70 molecules (Magram et al., 1996). Surprisingly, upon stimulation with PTx, PTxB or LPS and aCD40, BmDC derived from the IL-12 ko mice (DC^{IL-12ko}) produced both IL-12p70 and IL-12p40 detectable by ELISA, although at levels lower than the wildtype (figure 5.17A). Since these mice were homozygous for the IL-12p40 ko alleles (A. Mountford, personal communication), a plausible explanation for this observation is that a molecule that resembles the conformationally active form of p40 was synthesised in these mice. This is possible,
since these IL-12 ko mice have a deletion of exon 3 of the p40 gene, while the rest of the gene remains unaffected (Magram et al., 1996).

To test whether the immunoactive IL-12p70 produced by the DC\textsuperscript{IL-12ko} was biologically functional, a bioassay for IL-12p70 was carried out as described previously (Gately et al., 2001). As shown in figure 5.17B, whereas DCs derived from wildtype (C57BL/6) mice produced bioactive IL-12p70 at levels that correlate with immunodetectable IL-12p70 (figure 5.17A and B), DCs derived from the IL-12 ko mice produced no detectable amount of bioactive IL-12p70, thus confirming that the BmDC derived from IL-12p40 ko mice do not produce biologically functional IL-12.

These DCs were then used to stimulate syngeneic T cells and the cytokines produced were quantified. As expected from previous studies (Cella et al., 1996; Snijders et al., 1998), IFN\textgamma induced by DC\textsuperscript{LPS/\alphaCD40 \textsuperscript{p}cLPs/\alphaCD40} \textsuperscript{DC} was indeed dependent on IL-12, as a significant reduction (40–50\%) in IFN\textgamma production by both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells was observed in response to DC\textsuperscript{IL-12ko} (figure 5.17C). Similarly, a lack of IL-12 reduced the amount of IFN\textgamma produced by CD8\textsuperscript{+} T cells in response to DC\textsuperscript{PTxB} (–50\%), suggesting a partial role of IL-12 in induction of IFN\textgamma. However, the absence of IL-12 had no effect on the amount of IFN\textgamma produced by CD4\textsuperscript{+} T cells in response to DC\textsuperscript{PTxB} and by CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in response to DC\textsuperscript{PTx}. Importantly, under none of these conditions was IL-12 from DCs required for IFN\textgamma production by T cells. This suggests a role for factor(s) other than IL-12 in the induction of IFN\textgamma responses. Interestingly, IL-4 produced by CD4\textsuperscript{+} T cells in response to DC\textsuperscript{PTxB} was also reduced in the absence of IL-12, suggesting that IL-12 does not only function as an inducer of Th1 responses.
Figure 5.16 Neutralising antibody to IL-12 has no effect on T cell IFNγ and IL-4 production in response to syngeneic DCPTx or DCPTxB.

Day 5 BmDC (C57BL/6) were stimulated overnight with medium alone (DC0), PTx (5µg/ml; DCPTx), PTxB (5µg/ml; DCPTxB) or LPS (10µg/ml) with aCD40 (2µg/ml; DCCLPS/aCD40). These DC (1×10⁶) were cultured with syngeneic CD4+ or CD8+ T cells (1×10⁶) in the absence (open bars) or presence of rat antibody (5µg/ml) against mouse IL-12 antibody (black bars) or isotype control (grey bars) for 72 hours. Supernatants from the culture were then removed and analysed for IFNγ and IL-4 by ELISA. The data are means of quadruplicate wells (±SD) from four similar experiments.
Figure 5.17 DC\textsuperscript{PTx} and DC\textsuperscript{PTxB} induce IFN\textgreek{y} production by syngeneic T cells in the absence of IL-12.

BmDC derived from C57BL/6 (IL-12p40\textsuperscript{++}) or IL-12p40 deficient mice (IL-12p40\textsuperscript{--}) were stimulated with medium alone (DC0), PTx (5\mu g/ml; DC\textsuperscript{PTx}), PTxB (5\mu g/ml; DC\textsuperscript{PTxB}) or LPS (10\mu g/ml) with aCD40 [2\mu g/ml; (DC\textsuperscript{LPS/aCD40})]. IL-12p40 and IL-12p70 from these cultures were tested by ELISA (A), and functionality of IL-12p70 was measured by a bioassay (B, see section 2.2.12). These DC were used to stimulate syngeneic CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells for 72 hours, and cytokines produced by T cells were measured by ELISA (C). The data are means of duplicate wells (±SD) from one representative experiment out of four performed.

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5.3.12 IFNγ production mediated by DC^{PTx} and DC^{PTxB} requires cell-cell contact between T cells and DCs

It is possible that soluble factors other than the cytokines measured above may play a crucial role in induction of syngeneic T cell responses. Two preliminary experiments were set up to test whether the soluble factors alone were sufficient to trigger T cell responses mediated by DC^{PTx} or DC^{PTxB}. In the first experiment, DC^{PTx} or DC^{PTxB} were cultured with syngeneic CD4^+ or CD8^+ T cells in transwells, such that T cells were exposed to any secreted molecules produced by DCs (and vice versa) without cell-cell contact. As shown in figure 5.18, secretion of IFNγ was not observed from CD4^+ T cells or CD8^+ T cells cultured with DC^{PTxB} without physical contact with the DCs.

In the second experiment, the role of secreted factors produced upon DC^{PTx}-T cell contact in mediating IFNγ production by T cells was examined. Cell culture supernatants of DC0 cultured with syngeneic CD4^+ T cells were replaced partially or wholly with supernatant of DC^{PTx}-T cell co-culture after 24 hours or 48 hours, and IFNγ production from these T cells was assessed. As shown in figure 5.19, transfer of DC^{PTx}-T cell supernatant was not sufficient to induce IFNγ production by T cells stimulated with DC0. In addition, proliferation of these T cells was not detected (data not shown). Thus, these preliminary experiments suggest that DC^{PTxB} need to be in physical contact with T cells in order to mediate proliferation of, and IFNγ production by these T cells.
Figure 5.18 IFNγ production by syngeneic T cells requires contact with DCPTx or DCPτx

Day 6 BmDC (C57BL/6; 5x10^5) stimulated overnight with medium alone (DC0), LPS (10μg/ml; DC^{LPS}), PTx (5μg/ml; DCP^{PTx}), or PTxB (5μg/ml; DCP^{PTxB}) were cultured with syngeneic CD4^+ or CD8^+ T cells (5x10^5) either in the same wells (controls), or in transwells to avoid cell-cell contact between DCs and T cells (transwell experiments). After 72 hours, cytokines produced by T cells were measured by ELISA. The data are means of duplicate wells (±SD) from one experiment.

Figure 5.19 Soluble factors produced by DCPτx-T cell interaction are not sufficient to trigger IFNγ production by syngeneic T cells.

Day 6 BmDC (C57BL/6; 1x10^5) stimulated overnight with medium alone (DC0), or PTx (5μg/ml; DCP^{PTx}) were cultured with syngeneic CD4^+ T cells (1x10^5). After 24 or 48 hours the indicated proportion of supernatant in DC0-T cell cultures was replaced with supernatant of DCP^{PTx}-T cell cultures. At 72 hours these cells were washed and were further incubated for 8 hours in the presence of PMA (50ng/ml) and ionomycin (500ng/ml). Cytokines produced by T cells were then measured by ELISA. The data are means of duplicate wells (±SD) from one experiment.
5.4 Discussion

Pertussis toxin (PTx) is an exotoxin produced by *Bordetella pertussis*, the causative agent of whooping cough. PTx has long been used as a mucosal adjuvant, alongside other bacterial toxins such as cholera toxin (CTx) and *Escherichia coli* heat-labile toxin (LTx) that share similar A-B structure. However, little is understood about the mechanisms of its adjuvanticity due to its complex and diverse effects on the immune system.

In this chapter, PTx was found to enhance maturation of BmDC, as shown by upregulation of MHC molecules and some of the co-stimulatory and adhesion molecules, induction of IL-12 production and significantly, stimulation of syngeneic CD4^+^ and CD8^+^ T cells to proliferate at levels almost comparable to allogeneic MLRs (chapter 4). These T cell responses were accompanied by high levels of IFNγ and/or IL-4 production. These effects of PTx were largely, but not completely, mimicked by the binding subunit of the toxin, B-oligomer (PTxB). Findings in this chapter, together with relevant information on DC0 and DC^{LPS} (or DC^{LPS/αCD40}) are summarised in table 5.1.
Table 5.1 Summary of DC phenotypes studied in Chapter 3, 4 and 5.

<table>
<thead>
<tr>
<th>DC phenotypes</th>
<th>DC0</th>
<th>DC_{LPS}</th>
<th>DC_{PTx}</th>
<th>DC_{PTxB}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expression of DC maturation markers</td>
<td>−</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>Production of proinflammatory cytokines</td>
<td>−</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Production of IL-12</td>
<td>−</td>
<td>++++</td>
<td>++</td>
<td>+</td>
</tr>
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</table>

DO11.10 Tg CD4+ T cell responses

<table>
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<th>DC_{LPS}</th>
<th>DC_{PTx}</th>
<th>DC_{PTxB}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>IL-2 production</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>IFNγ production</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>IL-4 production</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

Syngeneic T cell responses (CD4+ T cells in red, CD8+ T cells in blue)

<table>
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<th>DC_{LPS}</th>
<th>DC_{PTx}</th>
<th>DC_{PTxB}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation (MHC class I ko)</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Proliferation (MHC class ko)</td>
<td>−</td>
<td>N.D.</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Proliferation (anti-B7-1 antibody)</td>
<td>−</td>
<td>N.D.</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Proliferation (anti-B7-2 antibody)</td>
<td>−</td>
<td>N.D.</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Proliferation (anti-B7-1/B7-2 antibodies)</td>
<td>−</td>
<td>N.D.</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>IL-2 production</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IL-4 production</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>IFNγ production</td>
<td>+</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Dependence on IL-12 for IFNγ production

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>DC0</th>
<th>DC_{LPS}</th>
<th>DC_{PTx}</th>
<th>DC_{PTxB}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dependence on IL-12 for IFNγ production</td>
<td>−</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Scale: − represents absence of a response (or expression); ++++ represents maximum response (expression); +/- represents < 20% of maximum response (expression) with variation between experiments; each + indicates 20% of maximum response (expression) obtained in experiments; N.D: not determined.
The two-signal model of T cell activation suggests that activation of effector T cell function of naïve T cells is brought about by two signals: (1) signals mediated by MHC:peptide–TcR interaction (signal 1), and (2) co-stimulatory signals (signal 2). Based on this, a model was presented to explain the relative importance of these signals for T cell triggering, which suggests that the level of signal 1 must reach a threshold before signal 2 can act upon it (chapter 3, figure 3.10).

This chapter was based on a surprising finding that in the syngeneic interaction, where signal 1 is low, both CD4^+ and CD8^+ T cells proliferated extensively in response to DC^{PTx} and DC^{PTxB} (figure 5.2). Given that such responses were not observed with DCs stimulated with any other reagents tested (chapter 3 and 4), this raised the question: how do DC^{PTxB} differ from DCs stimulated with other modulins (e.g., LPS and aCD40)?

Three possible mechanisms were initially considered to explain how DC^{PTxB} may stimulate potent proliferative responses of syngeneic T cells. First, as PTx/B is a glycoprotein there was a possibility that the toxin may have been processed and presented by DCs, thus contributing to an enhanced signal 1. Second, PTx/B may have induced activation and differentiation of DCs in such a way that these DCs were equipped with an increased T cell stimulatory capacity (e.g., an increased signal 2). Finally, residual PTx/B in the culture may directly induce T cells to proliferate. The likelihood of these mechanisms is discussed below.
Role of signal 1 (via MHC-TcR interaction) in DC\textsuperscript{PTx/B}-mediated T cell responses

The observations that both DC\textsuperscript{PTx} and DC\textsuperscript{PTxB} were able to mount a strong proliferative response of syngeneic CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in the complete absence of MHC class I or MHC class II (albeit at reduced levels; figure 5.6), suggest that conventional signal 1 mediated via MHC-TcR interaction is not crucial in the T cell responses triggered by DC\textsuperscript{PTxB}. It is possible that in the absence of MHC class I or II, DCs may be able to utilise the available MHC to compensate and stimulate CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells. However, this is unlikely since DC0 lacking MHC class II failed to stimulate proliferation of allogeneic CD4\textsuperscript{+} T cells (data not shown). Thus, the potent T cell stimulatory function of DC\textsuperscript{PTx/B} is not a result of an increased signal 1 mediated via PTx/B-derived peptide presentation by DC\textsuperscript{PTx/B}. An earlier study that examined the mitogenic effect of PTx on IL-2 blasts indicated that pre-stimulation of cells with CD3/TcR leads to loss of response to a subsequent stimulation with PTx (Rosoff and Mohan, 1992). Thus, it is possible that PTx stimulates downstream TcR signalling in PTx-treated T cells without triggering TcR engagement.

Role of signal 2 in DC\textsuperscript{PTx/B}-mediated syngeneic T cell responses

PTx induced elevated levels of co-stimulatory molecules, B7-1 (CD80), B7-2 (CD86) and CD40, and adhesion molecules, CD11a, CD49d and CD54, on the DC surface (figure 5.5). In contrast, such changes were not induced by stimulation with PTxB (except for H-2K, CD40 and CD11a). It is possible that DC\textsuperscript{PTxB} may have expressed an elevated level of co-stimulatory molecules other than those measured in this study. Thus, an elevation in signal 2 may explain the different potency of T cell responses mediated by DC\textsuperscript{PTx} and DC\textsuperscript{PTxB} (compared to DC\textsuperscript{LPS}), but it does not explain how these DCs induce a strong proliferation of syngeneic T cells.
B7-mediated signalling was, however, important in stimulation of the syngeneic T cells, as shown by the inhibition in T cell proliferative response in the presence of blocking antibodies against B7 molecules (figure 5.7). Finding that the inhibitory effect of these antibodies was more pronounced on DC^{PTxB}-mediated responses than in responses triggered by DC^{PTx}, indicate that dependence on B7 signalling in DC^{PTxB}-mediated T cell responses is greater than in DC^{PTx}-induced responses. Interestingly, while antibody against B7-1 failed to induce inhibition on its own, it acted in an additive manner when used together with anti-B7-2 antibody. Thus, signalling through B7-2 is important for triggering the T cell proliferative response by these DCs, and its effect is further enhanced by signalling through B7-1.

Preliminary data demonstrate that soluble factors produced by DC^{PTxB} or DC^{PTxB}-T cell co-cultures are not sufficient to trigger proliferation or cytokine production by syngeneic T cells (figure 5.18/19 and data not shown). This strongly suggests that signal 2 in the form of soluble molecules secreted by DC^{PTxB} is either not important, or is insufficient on its own to trigger these T cell responses.

**Role of residual toxin in triggering proliferation of syngeneic T cells**

Whilst the receptor(s) for PTx remains largely unknown, it was evident that the toxin binds to the surface of DCs with high affinity, since cell surface-bound PTx was detected even after a few minutes incubation of DCs with the toxin, and the toxin remained bound after several cycles of vigorous washing (figure 5.9). Thus, it was surprising to find that “residual” toxin in the culture was found to be bound directly to T cells in the DC^{PTxB}-T cell co-cultures (figure 5.9). The observation that DC^{PTx}
exhibited a potent T cell stimulatory function regardless of whether the DCs were incubated with the toxin for 3 minutes or 24 hours (figure 5.11), is consistent with the amount of the toxin detected on the DC surface, but not on the T cells (figure 5.9). Interestingly, a much longer incubation period (6 hours) was required for PTxB to have its effect on DCs (figure 5.11). This may reflect the efficiency of the PTx and PTxB in binding to receptor(s) on DCs. Whilst a high level of the toxin was detected on DC^{PTx} (figure 5.9A), only a low level was observed on DC^{PTxB} (figure 5.9B). Similarly, a relatively high level of PTx was found on T cells in DC^{PTx}–T cell co-culture compared to T cells in DC^{PTxB}–T cell co-culture (figure 5.9). However, it is difficult to interpret these data as the differences between these molecules in binding to receptor(s), or to become internalised, are largely unknown.

**Type 1 and type 2 T cell responses induced by DC^{PTxB}**

DC^{PTx} induced a high level of IFNγ production by responding syngeneic CD4^+ and CD8^+ T cells as well as DO11.10 Tg CD4^+ T cells. Such cytokine production was also evident in T cells stimulated with DC^{PTxB}, but the quantity of IFNγ produced was lower than that induced by DC^{PTx}. This may reflect the difference in the levels of T cell proliferation induced by DC^{PTx} and DC^{PTxB}. In addition, production of IL-4 was also observed in syngeneic CD4^+ T cells and DO11.10 Tg CD4^+ T cells. In the case of syngeneic CD4^+ T cells, DC^{PTx}, but not DC^{PTxB}, mediated IL-4 production (figure 5.13). However, the reverse is true for DO11.10 Tg CD4^+ T cells (figure 5.12). These contrasting results may reflect the differences in signal 1 between the two systems.
IL-12-independent IFNγ production mediated by DC^{PTxB}

The mechanisms of DC^{PTxB}-induced IFNγ production were studied in some detail in this chapter. Contrary to the literature that suggests a major role of IL-12 in induction of IFNγ production by T cells (Trinchieri, 1995), a requirement for IL-12 in DC^{PTxB}-induced IFNγ production was not observed (figure 5.16/17). While DC^{PTxB}-mediated IFNγ production was partially dependent on IL-12, IFNγ induced by DC^{PTx} was completely independent of IL-12.

Recently, a role for IL-18 in amplifying IFNγ production independent of IL-12 was described (Muller et al., 2001; Xing et al., 2000). However, IL-18 is not likely to be responsible for the induction of IFNγ, as there was no pronounced IL-18 production by DC^{PTx} or DC^{PTxB} either from wildtype DCs (figure 5.15) or IL-12 ko DCs (data not shown).

To rule out that the possibility that other soluble factors may mediate the IFNγ response by DC^{PTxB}, the effect of DC^{PTxB}-T cell culture supernatant in inducing IFNγ production was analysed (figure 5.19). The supernatant of DC^{PTx}-T cell culture at 24 and 48 hours did not appear to contain soluble factors responsible for induction of IFNγ production. It is possible that secreted molecules that were crucial in mediating the IFNγ response had been taken up by the T cells, or that the half-life of such molecules was insufficient for them to be effective. However, a more likely explanation is that the IFNγ response is mediated by direct physical interaction with DC^{PTxB}. The functional differentiation of T cells is thought to occur early in their activation process (Toellner et al., 1998), which suggests that crucial steps in
determining the differentiation of T cells in response to DC\textsuperscript{PTx/B} may have occurred within the first 24 hours of DC-T cell contact.

A model: mechanisms of DC\textsuperscript{PTx/B}-mediated T cell responses

Two major questions that have been raised in this chapter are: (1) how do DC\textsuperscript{PTx/B} induce syngeneic T cell activation and differentiation?; and (2) what accounts for the differences between T cell responses mediated by DC\textsuperscript{PTx} and DC\textsuperscript{PTxB}? Whilst the answers for neither of the questions are clear, a number of clues have arisen to explain what may be happening between DC\textsuperscript{PTx/B} and the T cells.

The absence of "conventional" signal 1 mediated via MHC-TcR interactions indicates that MHC-TcR interaction is bypassed by both PTx and PTxB. Given that cell-cell contact is required to achieve DC\textsuperscript{PTx/B}-mediated T cell responses, signal 1 in this system is likely to be driven by, and T cell responses induced by, at least one of the following: (1) cross-linking of CD3 molecules (and other molecules); (2) direct mitogenic effect by the toxin [other than by the mechanism in (1)]; and/or (3) an increase in the overall avidity of DC\textsuperscript{PTx/B}-T cell interaction.

Whether the cell surface-bound toxin was responsible for mediating the subsequent T cell responses remains unclear. This could be established by further studies using blocking antibodies against toxin bound to DCs. However, the finding that only a few minutes of incubation of DCs with PTx was sufficient to induce a potent T cell response indicate that, at least for DC\textsuperscript{PTx}, the toxin bound on the DC surface may be responsible for triggering the T cell responses.
PTx has been shown to act as a T cell mitogen in a number of studies (Rosoff and Mohan, 1992; Rosoff et al., 1987; Tamura et al., 1983). For example, activation of splenocytes (Tamura et al., 1983) and Jurkat T cells (Rosoff et al., 1987) was observed following treatment with the toxin. In this chapter, purified CD4^+ or CD8^+ T cells failed to proliferate (figure 5.2) or to exhibit upregulated expression of early activation markers such as CD25 and CD69 (data not shown) in response to stimulation with PTx/B. However, it is possible that, the toxin bound on the DC surface may be able to activate T cells, either by cross-linking of CD3 (and other molecules), or directly stimulating T cells together with the co-stimulatory molecules expressed on DCs. Alternatively, given that both DC^{PTx} and DC^{PTxB} exhibited strong adherence to each other, it is possible that these DCs may have interacted with T cells with a higher than normal avidity, resulting in a stronger, and prolonged signalling from these DCs. This adherence of DCs mediated by the toxin may be due to (1) cross-linking by the toxin on the cell surface (Kaslow and Burns, 1992), and/or (2) upregulation of adhesion molecules other than those measured in this study (CD11a, CD49d, and CD54).

What accounts for the differences between the effect of PTx and PTxB on DCs? Biochemically, the only difference between these molecules is the presence or the absence of a catalytic subunit, S1 (or A-subunit). The finding that co-administration of PTxB and a cAMP elevating agent (dbcAMP) did not compensate for the difference between the two molecules, together with the observation that PTxB takes a longer period to exert its effect on DCs than PTx, suggests that the difference may be explained by the efficiency of these molecules in binding to receptor(s) on DCs. This may also explain why PTx, but not PTxB, induced activation of DCs.
The current hypothesis that has emerged from the findings in this chapter is that T cell activation by DC^{PTx} is likely to be mediated, at least in part, by toxin bound to the surface of DCs. However, how the bound toxin induces its effect is currently unknown. Despite the long history of use of PTx as a mucosal adjuvant, surprisingly little is known about its mechanisms of action. DCs, as well as other APCs, have evolved to recognise “danger” associated with a toxin and initiate potent T cell responses. However, it is debatable whether the polyclonal T cell responses induced by the toxin help the immune system to clear infection. It is possible that the bacteria may use such a toxin as a mechanism to evade a specific immune response. Further understanding of the biochemistry of the toxin and the interaction with its receptor(s) is necessary to understand the effects mediated by PTx and PTxB.
Chapter Six

F5 transgenic system

(Regulation of "anti-self" T cell responses)

6.1 Introduction

In Chapter 3, the effect of an elevation of signal 2 in activation of syngeneic T cells was examined based on the hypothesis that an amplification of signal 2 would result in induction of low-affinity T cell responses. Although the syngeneic MLR provided some valuable information on how naïve T cells may behave in response to low-affinity antigens, the major limitation of this model was an undefined signal 1. Thus, in this chapter, to further investigate how to induce a T cell response to a defined low signal 1, the F5 transgenic (Tg) system was chosen.

The F5 Tg mouse was generated from a cytotoxic T cell clone F5 (Mamalaki et al., 1992), which recognises a peptide of the influenza virus A/NT/60/68 nucleoprotein [NP-(366-374)] in the context of H-2D^b (Townsend et al., 1984; Townsend et al., 1986). The F5 Tg TcR specifically recognises a nonamer peptide NP68, ASNENMDAM. Injection of NP68 peptide into the F5 Tg mice induces clonal depletion of double-positive immature thymocytes in the thymus as well as activation of mature T cells in the periphery (Mamalaki et al., 1992). In addition, in F5/NP68/RAG-1^−/− Tg mice, which express the NP68 peptide epitope endogenously (Mamalaki et al., 1993), thymocytes are deleted in the transition stage between the CD4^+CD8^+TcR^int and CD4^−CD8^−TcR^hi subsets (Mamalaki et al., 1996). Thus, NP68 is recognised with high-affinity by the F5 TcR.
A related peptide, NP34 (ASNENMETM) is a naturally occurring peptide of the influenza virus A/PR/8/34 nucleoprotein. It differs from NP68 by two amino acid residues at 372 (D→E) and 373 (A→T), positions that are thought to be involved in TcR contact (Young et al., 1994). In contrast to NP68, NP34 is incapable of stimulating mature T cells from F5 Tg mice (Mamalaki et al., 1992; Tanaka et al., 1993) or a T cell line derived from these mice. Injection of NP34 had no effect on the size or the expression of activation markers on mature CD8+ T cells in the thymus or the periphery (Mamalaki et al., 1992). Furthermore, NP34 was shown to be able to antagonise negative selection of F5 thymocytes induced by NP68 in an in vitro model (Williams et al., 1996). In support of these studies, in the F5/NP68/RAG-1−/− Tg mice that express NP68 endogenously, deletion of thymocytes by negative selection in foetal thymic organ culture (FTOC) was prevented by addition of antagonist NP34 (Williams et al., 1998). Therefore, NP34 acts as a functional antagonist of NP68, implying that it is a low affinity ligand for the F5 TcR.

Taken together, these studies demonstrate that the peripheral TcR in the F5 Tg mice interact with NP68 as if it was a high affinity ligand, and with NP34 as if it was a low affinity ligand. Thus, the F5 system provides a model to study the regulation of T cell responses to a low-affinity “self” (NP34) antigen as well as a high-affinity “non-self” (NP68) antigen.
6.2 Objectives

The objective of this chapter is to test the hypothesis that amplification of signal 2 would lead to induction of T cell effector functions to the model low-affinity antigen, NP34. Specifically, would an elevated signal 2 enhance F5 T cell responses to low-affinity NP34 such that these T cells would respond as if to high-affinity NP68?

6.3 Experimental Results

6.3.1 F5 CD8^ sup T cell responses to NP68 and NP34 presented by BmDC

In order to establish that NP68 and NP34 would act as high- and low-affinity antigens respectively, F5 Tg CD8^ sup T cell responses to these peptides presented by BmDC were first examined.

As expected, F5 Tg CD8^ sup T cells proliferated extensively in response to NP68 presented by BmDC (figure 6.1A). This response was further enhanced when DCs stimulated with LPS (DC\textsuperscript{LPS}) were used. DC\textsuperscript{LPS} lowered the threshold for peptide to trigger T cell responses by 1-2 log of peptide concentration. In the same system, F5 Tg CD8^ sup T cells almost completely failed to respond to NP34 presented by DCs (figure 6.1B). However, these CD8^ sup T cells began to proliferate when DCs were pulsed with very high concentration of peptide (>1\mu M). Similar to the effect seen with DC\textsuperscript{LPS} pulsed with NP68, stimulation with DC\textsuperscript{LPS} improved the response to NP34. The proliferation of F5 Tg CD8^ sup T cells was accompanied by IL-2 production (figure 6.2). Relative to the difference in cell proliferation induced by peptide-loaded DC0 and peptide-loaded DC\textsuperscript{LPS}, the elevation in IL-2 production induced by peptide-loaded DC\textsuperscript{LPS} compared to DC0 was more pronounced.
Figure 6.1 Proliferative responses of F5 CD8+ T cells to NP68 and NP34.

Purified F5 Tg CD8+ T cells (1x10^5) were stimulated with DC0 or DC^{LPS} (C57BL/6) at ratios of DCs to T cells of 1/10 or 1/100. DCs were pre-loaded with titrated concentrations of the agonistic NP68 peptide (A) or the antagonistic NP34 peptide (B). The proliferation was measured after 72 hours by ^3H-thymidine incorporation. The data are means of quadruplicate wells (±SD) from one representative experiment out of four performed.
Figure 6.2  IL-2 production by F5 CD8\(^+\) T cells in response to stimulation with NP68 and NP34.

Purified F5 Tg CD8\(^+\) T cells (1×10\(^3\)) were stimulated with DC0 or DC\(^{LPS}\) (1×10\(^4\); C57BL/6) that were pre-loaded with titrated concentrations of the agonistic NP68 peptide (A) or the antagonistic NP34 peptide (B). IL-2 in the culture supernatant was quantified by ELISA 24 hours after the experiment was set up. The data are means of triplicate wells from one representative experiment out of two performed. The SD of these data were less than 5%.  

\[\text{IL-2 (pg/ml)}\]

\(\text{[NP68] nM}\)

A

\(\text{[NP34] nM}\)

B
6.3.2 NP68 and NP34 bind to H-2D\textsuperscript{b} with the same efficiency

NP68 and NP34 were previously shown to bind with similar efficiency to H-2D\textsuperscript{b} (Williams et al., 1996). To ensure that this is the case with the peptides used in this study, peptide-binding assays using RMA-S cells were performed. RMA-S is a murine lymphoma cell line and expresses H-2D\textsuperscript{b}/K\textsuperscript{b} stably at 26°C but not at higher temperatures in the absence of added exogenous peptide (Schumacher et al., 1990). However, the expression can be stabilised at 37°C upon binding to a peptide (Ljunggren et al., 1990; Schumacher et al., 1990). Thus, efficiency of binding of a peptide to H-2D\textsuperscript{b} can be measured using this cell line. Various amounts of NP68 and NP34 were cultured with RMA-S cells and the relative levels of H-2D\textsuperscript{b} molecules stabilised in each case was analysed. As shown in figure 6.3, H-2D\textsuperscript{b} binding curves were similar for both of these peptides, indicating that NP68 and NP34 bind to H-2D\textsuperscript{b} with the same efficiency.

Thus, BmDC present “high-affinity” NP68 peptide to F5 Tg T cells, resulting in strong proliferation and IL-2 production, while BmDC presenting “low-affinity” NP34 induced weak proliferation and low IL-2 production. These results confirmed that NP68 acts as a model for high signal 1 and NP34 for low signal 1 in this system.
Figure 6.3 NP68 and NP34 binds to H-2D^b with the same efficiency.

RMA-S cells (1×10^6) incubated overnight in 96 well plates at 26°C were loaded with a titrated concentrations of NP peptides as indicated above. These were then further incubated at 37°C for 2 hours before the surface expression of H-2D^b molecules was examined by flow cytometry (see chapter 2 Materials and Methods). The dotted line indicates the level of H-2D^b expression when no peptide was added. Means of triplicate wells (±SD) are shown from one representative experiment out of three conducted.
6.3.3 DC<sup>LPS</sup> enhances F5 CD8<sup>+</sup> T cell activation signals

The elevation of proliferative responses and IL-2 production by peptide-loaded DC<sup>LPS</sup> seen in figure 6.1 and 6.2 may have reflected the differences in kinetics of F5 Tg T cell activation. To test this, expression of several T cell activation markers were examined at a various time points after stimulation with DC loaded with NP68 or NP34 (figure 6.4).

F5 Tg CD8<sup>+</sup> T cells responded equally to NP68 presented by DC0 and DC<sup>LPS</sup>, both in terms of kinetics and levels of expression of activation markers, with the exception of CD152 (CTLA-4). Expression of CD152 was induced at a later point in CD8<sup>+</sup> T cells responding to DC0, and only reached half the level of that induced on CD8<sup>+</sup> T cells by DC<sup>LPS</sup>. CD152, which binds to CD80 and CD86, is responsible for inhibitory signalling, and is induced upon activation of T cells, thus acting as a negative feedback mechanism for controlling T cell activation. DC<sup>LPS</sup> may have induced the expression of this molecule more efficiently by providing a stronger signal than DC0. For example, DC<sup>LPS</sup> express higher levels of CD80 and CD86, both of which bind to CD28 and CD152, than do DC0. However, expression of CD28 (another molecule that binds to both CD80 and CD86) induced by DC<sup>LPS</sup> was not higher than that induced by DC0.

In contrast, F5 Tg CD8<sup>+</sup> T cells stimulated by NP34-loaded DC0 and DC<sup>LPS</sup> expressed CD2, CD28 and CD152 equally and with similar kinetics, while CD25, CD44 and CD69 were expressed at higher levels by CD8<sup>+</sup> T cells stimulated by DC<sup>LPS</sup>, than CD8<sup>+</sup> T cells stimulated by DC0. It is difficult to assess the difference in
the expression of CDw137 and CD154 induced by DC0- and DC\textsuperscript{LPS}-stimulation, due to their low levels of expression.

NP34-induced CD8\textsuperscript{+} T cells were found to express comparable levels of some of the activation markers to that of NP68-stimulated CD8\textsuperscript{+} T cells. In particular, the levels of CD2, CD28, and CD152 induced by NP34-loaded DC0 were almost as high as the levels induced by NP68-loaded DC0. Expression of CD25, CD44, and CD69 was much lower in CD8\textsuperscript{+} T cells stimulated by NP34-loaded DC0 than that by NP68-loaded DC0. When presented by DC\textsuperscript{LPS}, however, NP34 induced a substantial increase in the levels of CD25, CD44 and CD69, but still lower than that induced by NP68. Importantly, the kinetics of expression of these markers was very similar on NP68 and NP34-stimulated T cells.
Figure 6.4 Expression of T cell activation markers upon stimulation of F5 CD8+ T cells with NP68 and NP34.

Purified F5 CD8+ T cells (1×10^5) were stimulated with DCs (1×10^4; C57BL/6) treated with medium (DC0; circles) or LPS (DCLPS; triangles) in the presence of 1μM NP68 or NP34. T cells were then harvested and labelled with antibodies against T cell activation markers (filled symbols) or isotype-matched controls (open symbols) as shown above. The data are single points from a representative experiment out of three performed.
### T cell activation markers

- **DC0, T cell activation markers**
- **DC^{LPS}, T cell activation markers**
- **DC0, isotype controls**
- **DC^{LPS}, isotype controls**
6.3.4 NP34 does not act as a functional antagonist of NP68

Previously NP34 was described as a functional antagonist of NP68. NP34 inhibited the killing of NP68-loaded targets in an in vitro CTL assay (Williams et al., 1996) and also inhibited deletion of CD8^+ F5 Tg thymocytes in an in vitro-model of negative selection (Williams et al., 1996). To establish whether NP34 behaves as a functional antagonist of NP68 in the system used in this chapter, CTLs were generated by stimulation of F5 Tg peripheral CD8^+ T cells with NP68-loaded BmDC in vitro, and their ability to kill NP68 loaded targets in the presence of NP34 was examined. Surprisingly, the presence of NP34 failed to lower the efficiency of NP68-specific killing (figure 6.5A). Even in the presence of a high concentration of NP34 (up to 10^7 times the concentration of NP68), inhibition of NP68-specific killing was not further enhanced. In the absence of pre-pulsing with NP68 peptide, NP68-primed effector cells were able to kill NP34-loaded target cells, albeit at lower levels than they killed NP68-loaded targets (figure 6.5B).
Figure 6.5 NP34 peptide does not antagonise the killing of NP68 loaded target cells.

CTLs were generated from F5 Tg CD8+ T cells by stimulation with NP68-loaded BmDC (C57BL/6). Target cells used were EL4 that were pre-pulsed with 10pM NP68 (A) or no peptide (B), prior to a second incubation with the indicated concentration of peptide NP68 or NP34. CTLs were added to a final E:T ratio of 50:1. Means of quadriplicate wells are shown from one representative experiment out of three conducted. SD of the means were less than 10% in each case.
6.3.5 Amplifying signal 2 does not enhance CTL activity

Having established that stimulation of F5 Tg CD8\(^+\) T cells with NP68 resulted in the generation of efficient killer cells, the effect of amplified signal 2 on their cytotoxic function was analysed.

In order to do this, F5 Tg splenocytes were stimulated with NP peptide-loaded DC0, DC\(^{LPS}\), DC\(^{CD40}\) and DC\(^{PtX}\), thus providing the T cells with higher levels of signal 2. As shown in figure 6.6, an enhancement in the CTL function of effector cells by priming with these matured DC was not observed for either NP68 or NP34. Thus, these and earlier results (at least in case of DC\(^{LPS}\)) suggest that an elevation of signal 2 at the time of priming amplifies both proliferation (figure 6.1), IL-2 production (figure 6.2) and expression of some of T cell activation markers (figure 6.4) on these T cells, but not CTL function.

CTL function is often associated with secretion of IFN\(\gamma\) (Trinchieri and Perussia, 1981). In order to test whether the amplified signal 2 results in increased secretion of this cytokine by the F5 Tg T cells, supernatants from the CTL cultures set up above were tested for IFN\(\gamma\). Similar levels of IFN\(\gamma\) were found in supernatants of NP68-specific T cells stimulated with matured DCs and those that were stimulated with DC0 (figure 6.7). In marked contrast, a significant enhancement in the production of IFN\(\gamma\) was observed in response to NP34 when T cells were stimulated with matured DC, albeit at much lower levels than NP68-induced IFN\(\gamma\) production. Thus, it is possible that when signal 1 is high (NP68), DC0 may induce a maximum CTL response from these T cells. On the other hand, when signal 1 is low (NP34), many
Effector functions may be enhanced by elevation of signal 2, namely, proliferation, IL-2 production and IFNγ production, but not cytotoxic capability.

Figure 6.6 Maturation of DCs does not improve the ability of F5 effector cells to kill target cells.

CTLs generated from F5 Tg CD8+ T cells by stimulation with NP68- (A) or NP34- (B) (1μM) loaded BmDC (C57BL/6). CTL activity was assessed by performing 51Cr-release assays. EL4 cells loaded with NP68 (A) or NP34 (B) 1μM were used as target cells. A range of effector:target ratios were included as shown above. Means of quadruplicate wells are shown from one representative experiment out of three conducted. SD of the means were less than 10% in each case.
Figure 6.7 IFNγ production in culture supernatant from F5 T cells stimulated with NP68- and NP34-loaded DCs.

Pooled splenocytes and lymph node cells (1×10^5) from the F5 mice were stimulated with BmDC (1×10^4; C57BL/6) loaded with NP68 or NP34 (1μM). Supernatants from these cultures were collected after 72 hours and IFNγ was quantified by ELISA. The data are means of quadruplicate wells (±SD) from one representative experiment out of two performed.
6.3.6 Low affinity targets are killed more efficiently by high-affinity primed effector cells

An earlier observation that NP68 effector cells were able to kill NP34-loaded target cells (figure 6.5B), albeit at a lower level than NP68-loaded targets, suggests that the cytotoxicity of the target cells was not completely peptide-specific. If this is the case, NP68 effector cells should also kill NP34 targets, while NP34 effector cells should be able to kill NP68 targets. To test this hypothesis, the ability of F5 effector cells to kill target cells presenting peptide that differs from the priming peptide was assessed. Consistent with earlier observations, when effector cells were generated in response to high-affinity NP68, CTLs were able to efficiently kill NP68-loaded target cells (figure 6.8). In comparison, effector cells generated from priming with low-affinity NP34, killed NP34 targets poorly. Importantly, NP68-primed effector cells were able to kill NP34-loaded targets more efficiently than NP34-primed T cells. Interestingly, NP34-primed effector T cells were able to lyse NP68-loaded targets better than NP34-loaded targets. Thus, these results suggest that killing of low-affinity target cells is achieved more efficiently by priming the effector T cells with a high-affinity peptide.
Figure 6.8 Priming of F5 effector cells with NP68 improves the ability to kill NP34-loaded targets.

CTLs were generated from F5 Tg splenocytes in response to DCs (C57BL/6) loaded with NP68 (A) or NP34 (B) at a concentration of 1μM. Target cells used were EL4 cells loaded with NP68 or NP34 (both at 1μM). Effector cells were added to a range of final E:T ratios as shown above. Means of triplicate wells are shown from one representative experiment out of three performed. SD of the means were less than 10% in each case.
6.4 Discussion

In this chapter, the ability of differentially matured DC to induce CD8\(^+\) T cell responses to a low-affinity antigen, NP34, using the F5 Tg system, was examined in order to understand the effect of elevation of signal 2 in induction of T cell effector function in a low-affinity (“anti-self”) interaction.

NP68 is a peptide that is recognised by the F5 Tg CD8\(^+\) T cells, and induces activation of peripheral T cells as well as deletion of thymic T cells in the F5 Tg mice (Mamalaki et al., 1992). Therefore NP68 can be used as a model peptide for a high-affinity, “non-self” antigen (strong signal 1). In contrast, NP34 is a natural variant of NP68, unable to activate peripheral T cells of F5 Tg mice, or to cause negative selection of thymocytes, and thus can be used as a model for a low-affinity, “self” antigen (weak signal 1). In addition, NP34 was shown to specifically inhibit effector functions of NP68 in vivo and in vitro, thus acting as a functional antagonist of NP68.

NP68 presented on BmDC induced potent activation of F5 Tg CD8\(^+\) T cells, resulting in proliferation, IL-2 production, and efficient killing of NP68-loaded target cells, which was associated with a high level of IFN\(\gamma\) production. Some of these responses (proliferation and IL-2 production) were further amplified when matured DCs (e.g., DC\(^{LPS}\)) were used to stimulate these CD8\(^+\) T cells. However, increases in CTL activity and IFN\(\gamma\) production were not observed when CD8\(^+\) T cells were primed by differentially matured DC. These results suggest either that (1) an elevation of signal 2 is not sufficient to enhance later stages of effector functions, and/or (2) that NP68-specific responses mediated by DC0 were already optimal for CTL function and IFN\(\gamma\) production.
NP34 was previously described as a peptide that is not “recognised” by the F5 TcR (Mamalaki et al., 1992). Thus, it was surprising to find that NP34-loaded DCs were able to induce activation of F5 Tg CD8⁺ T cells in this study. Whilst proliferation and IL-2 production from the F5 Tg CD8⁺ T cells in response to NP34-loaded DCs were poor compared to those induced by NP68-loaded DCs, interestingly, some of the T cell activation markers expressed by NP34-stimulated T cells were found to be comparable to that of NP68-stimulated T cells. This may be due to the partial activation of NP34-stimulated T cells, and that the induction of effector functions of these T cells may require stronger signals than do the expression of activation markers. None of the parameters used to measure T cell activation in this study (expression of T cell activation markers, proliferation, IL-2 production and cytotoxicity function) were in fact shown to be positive in the previous studies. This is likely to be due to a high level of signal 2 derived from BmDC at the time of interaction between MHC:peptide complex and TcR. Indeed, stimulation of these T cells with DC^{LPS}, which express higher levels of signal 2 such as CD80, CD86 and CD40, improved the NP peptide-specific T cell responses in this study (all but cytotoxic function).

The observation that NP34 did not behave as a functional antagonist of NP68 in this study (figure 6.5A) may be due to one or more of the following factors: (1) inefficient competition between the NP peptides due to unavailability of binding sites (i.e., H-2Dᵇ molecules saturated by pre-pulsing with NP68 peptide); (2) use of BmDC as priming APCs for the generation of CTL [previous work utilised splenocytes as APCs (Williams et al., 1996)]; and (3) primary CTLs were examined in this study, whereas
previous work utilised secondary CTLs (Williams et al., 1996). To test the first possibility the experiment should be repeated using a lower concentration of NP68 peptide to pre-pulse the target cells. In addition, target cells may be loaded with the two peptides simultaneously. An alternative approach is to perform a “cold” (^51Cr-unlabelled) competition experiment where killing of cold NP34 targets is competed with the killing of ^51Cr-labelled NP68 targets. The second explanation that APCs used for priming CTLs may be responsible for the differences, is possible, if the CTLs generated by BmDC are better effector cells, and are able to respond to very low levels of NP68 on target cells. In fact, CTLs generated by NP68-loaded DCs kill target cells loaded with as low as 10^-4 nM NP68 (figure 6.5B). The third possibility that the difference between this study and the previous work (Williams et al., 1996) is due to the type of CTLs used, is unlikely because one would expect the memory response to be more potent than the primary effector responses. However, it is possible if the secondary response is more easily inhibited than the primary response. Thus, whilst NP34 has been regarded as “antagonist” for the F5 Tg TcR in a number of studies (Mamalaki et al., 1992; Williams et al., 1996; Williams et al., 1998), this peptide failed to function as an antagonist in the system used in this study. Thus, the term “agonist/antagonist” may be misleading in describing the function of a peptide, as the definition of peptides may depend on experimental conditions (e.g., peptide concentration) and sensitivity of the readout assay used to measure T cell stimulation.

The hypothesis that an elevation of signal 2 leads to induction or enhancement of effector functions generated against a low signal 1 was based on an assumption that a high level of signal 2 would compensate for a low signal 1. This was shown to be the case in some of the effector functions studied, namely proliferation, IL-2 production
and expression of T cell activation markers. However, this did not extend to enhancement of other effector functions (CTL and IFNγ production). Further studies to compare CTL function and IFNγ responses at varying time points are required to confirm this observation.

It was evident from CTL studies that effector T cells generated against one peptide were able to kill target cells loaded with a different peptide. This was not due to non-specific killing of peptide-loaded cells, as target cells that were loaded with a third peptide, NP4Q (ASNQNMDAM), were not lysed by these effector cells (data not shown). NP4Q has the same binding affinity to H-2D^b as NP68 and NP34 (Williams et al., 1996), but did not stimulate proliferation of F5 Tg T cells nor induce expression of T cell activation markers (data not shown). The findings that killing of low-affinity NP34-loaded targets was achieved more efficiently by effector cells that were primed with the high-affinity NP68, and that NP34-primed effector cells induced greater specific lysis of NP68-loaded targets than NP34-loaded target cells, suggest that (1) the TcR is not completely peptide-specific, but promiscuous, and that (2) effector functions of T cells depend on TcR affinity.

The concept of TcR promiscuity is not new in the field of immunology. In fact, promiscuity is one of the main features of TcRs (Mason, 1998). As a result of thymic selection, only those cells bearing TcRs with a low-affinity for self antigens are able to mature, and enter the periphery (section 1.5.8). Once in the periphery, these T cells encounter a large diversity of antigens, and they become activated upon interaction with high affinity antigens (in the presence of signal 2). In other words, every TcR in the periphery was selected to recognise self antigens at a low affinity, and every TcR...
must therefore have its high affinity counterparts (figure 6.9). What has emerged from
the data in this chapter is that it is possible to induce a response to a low-affinity
antigen by triggering the same T cells with high-affinity antigen.

This has some important implications in tumour immunotherapy. To date, tumour
antigens have been used for vaccination of patients in order to induce an anti-tumour
response. However, this approach has obtained variable success. These results can be
explained by the data obtained in the F5 model, which suggests that vaccination
programs attempting to increase signal 2 may not be sufficient to induce an effective
anti-tumour responses. An effective immune response may need to be primed by an
antigen that has a high affinity for the TcR present in the periphery of cancer patients,
that only recognise tumour antigens with low affinity. Theoretically, this can be
achieved if non-self derived antigens are identified that have high-affinity for those
TcR that recognise low-affinity tumour antigens.
Clearly, further studies are required to understand the mechanisms of induction of low-affinity NP34 responses by high-affinity NP68 peptide. Previously, NP68 and NP34 were shown to stimulate qualitatively, but not quantitatively similar signals in F5 Tg thymocytes (Smyth et al., 1998). TcR ligation of Tg thymocytes with NP68 and NP34 resulted in phosphorylation of the same molecules, CD3ζ, CD3ε, ZAP-70, Syk, Vav, SLP-76 and pp36-38, but the extent of phosphorylation was higher in NP68 than in NP34-triggered thymocytes (Smyth et al., 1998). It is possible that similar differences may occur in peripheral T cells in response to these peptides. Thus, a strong signal mediated by high-affinity NP68 may be capable of priming the T cells to respond to a subsequent encounter with a weaker signal. Conversely, T cells that were originally activated with a weak signal may respond efficiently to a stronger signal mediated through the same TcR. Thus, it would be useful to examine the quantity/quality of the T cell signals mediated by NP68 and NP34 at the priming stage as well as at the effector stage of CD8⁺ T cells. In addition, information on in vivo priming of CTLs in the F5 system would provide a better insight into what is more likely to occur in response to a low-affinity tumour antigen in cancer patients.

A number of systems can be utilised as proof of principle experiments for the above hypothesis. For example, mdm2 is a self protein involved in cell-cycle control and is often overexpressed in transformed cells (Bueso-Ramos et al., 1993). A low-avidity self peptide derived from this protein (mdm100) was identified (Dahl et al., 1996) that can prime CTLs in a mouse tumour model only if CTLs were primed with mdm100 in the context of allogeneic MHC (Sadovnikova and Stauss, 1996). Importantly, these CTLs were able to discriminate healthy cells which express normal levels of mdm2 from tumour cells that express high levels of mdm2. Using this model, a high-affinity
antigen for TcR recognising the mdm2 peptide could be theoretically identified by screening a non-self peptide library: the identified peptide would then be used for priming the CTL, and the ability of the effector cells to selectively kill tumour cells could be assessed *in vivo* (figure 6.10).

**Figure 6.10 Use of mdm2 to test the working hypothesis: A high affinity antigen exists for a defined low affinity TcR.**

Mdm2 is a self protein overexpressed on many transformed cells. Priming the immune system with a peptide that has high affinity to the TcR that recognises mdm2 peptide could generate effective immune responses to high-avidity targets.
Chapter Seven

General Discussion

To meet the increasing demand for cancer vaccines, many studies have been carried out to examine the efficacy of various tumour vaccines in animal models. One of the most recent and successful approaches is the use of tumour antigen-loaded autologous DCs (Chapter 1.6). The rationale of this approach is based on the function of DCs as the most potent stimulators of T cells (Chapter 1.4 and 1.6). While these studies with animals have given promising results, vaccine trials in humans have been disappointing. This may be due partly to the fact that such trials in humans are restricted to patients with late stage malignancy, and often these patients have had extensive chemotherapy (some of which may be immunosuppressive) prior to the vaccine trial.

Importance of signal 2 in induction of low-affinity responses

The original idea for the use of adjuvants came from vaccination protocols used for controlling infections. These approaches are based on the assumption that an immune response can be elicited against a weakly immunogenic tumour antigen (which gives low signal 1) by increasing the level of signal 2. However, to date, immunotherapy that has targeted tumour antigens using adjuvants has had variable results. Fuchs et al pointed out that immunotherapy of established cancer often leads to only transient tumour regression, and that to achieve complete tumour regression a “danger” signal must be maintained in association with the tumour antigen (Fuchs and Matzinger, 1996). However, injecting inflammatory substances (such as BCG) repeatedly into
tumours rarely induces detectable systemic immunity (Fuchs and Matzinger, 1996). These observations suggest that simply administering the tumour antigen with a high signal 2 may not be sufficient to induce an appropriate immune response leading to eradication of tumour.

In order to improve the current approach, understanding the mechanisms of induction of immune responses to low-affinity tumour antigens is crucial. The experiments described in this thesis attempted to examine the relative importance of signal 1 and signal 2 in the induction of low-affinity (low-signal 1) responses.

In this thesis, murine bone marrow-derived DCs (BmDC) were used as a model APC. While these DCs exhibited immature DC phenotype when cultured in the presence of GM-CSF alone, they were potent stimulators of allogeneic MLRs (Chapter 3). Only when “matured” in the presence of stimuli (such as LPS) did these DCs acquire a phenotype capable of inducing proliferation of “low-affinity”, syngeneic CD4⁺, but not CD8⁺ T cells (Chapter 3). These results demonstrated that although BmDC are known to be potent stimulators of T cells, there are limitations to the ability of DCs to induce T cell responses. Considering that signal 2 mediated by mature DCs in this system is likely to be higher than under physiological conditions (because of the high DC:T cell ratio used in these in vitro experiments), the level of signal 1 in the system may be the limiting factor for the T cell stimulatory ability of DCs. Findings in the F5 Tg TcR system support this idea (Chapter 6). NP34 peptide, which is recognised by the F5 TcR with “low-affinity” (or low signal 1), induced low levels of T cell activation, compared to responses induced by “high-affinity” NP68 peptide. Importantly, increasing signal 2 in this system, by stimulating these Tg T cells with
mature DCs, resulted in only partial improvement of the responses. In fact, mature DCs failed to prime effective CTLs against low-affinity NP34 antigen. Thus, in systems where signal 1 is low, DCs may not be able to function as effective inducers of T cell responses.

**Induction of “anti-self” responses**

The idea that induction of antitumour immunity involves triggering of “anti-self” or autoimmune responses is based on the findings that most of the tumour antigens that are potential targets of the cancer vaccine are in fact unmodified self antigens [reviewed in (Pardoll, 1999)]. Thus, principles learned from the dissection of mechanisms by which tolerance is broken to cause autoimmune disease, could be applied to cancer immunotherapy targeted toward tissue-specific autoantigens.

For a long time, autoimmunity has been explained by an underlying hypothesis that initiating autoantigens represent “cryptic” epitopes, which, because they are not normally presented at high density, do not induce activation or active tolerance in the periphery (Kumar and Sercarz, 1995). Therefore, induction of autoimmune responses could occur in two different ways: when the cryptic epitopes become presented at higher density, and/or when these epitopes are presented in the context of active immune responses induced by infection with a foreign pathogen (i.e., high signal 2) (Karlsen and Dyrberg, 1998). During infection, inflammatory signals such as cytokines and other “signal 2” can activate T cells against foreign antigens derived from the pathogen. If such antigen mimics cryptic self epitopes, this can also lead to “anti-self” T cell responses. If these responses can become appropriately activated and sustained, they are capable of recognising lower concentrations of the self epitope.
on normal tissues and therefore can initiate an autoimmune reaction. One such example is the mimicry between an epitope from the spirochete causing Lyme disease and LFA-1, a candidate target for immune responses in Lyme-associated arthritis (Gross et al., 1998).

More recently, a number of studies have demonstrated that autoimmunity can be induced by an antigen that may not necessarily have sequence homology with an autoantigen (Hemmer et al., 1998b; Martin et al., 2001; Maverakis et al., 2001). For example, Hemmer et al demonstrated, by using human CD4+ autoreactive T cell clones, that these T cells can recognise antigens that show no sequence homology to the agonistic peptide, and that the amino acid sequence of an immunostimulatory peptide can be predicted by the relative influence of each amino acid (Hemmer et al., 1998b). This illustrates that TcR recognition is highly degenerate (Mason, 1998), and importantly, that an autoimmune response can be induced by a T cell response to a peptide completely unrelated to a self antigen. The flexibility in TcR antigen recognition has also been demonstrated in thymic selection where unrelated peptides can select the same TcR (Pawlowski et al., 1996) and the same MHC:peptide complex can select T cells with very different antigen specificities (Ignatowicz et al., 1997). These findings suggest a potential new approach in tumour immunotherapy, as antitumour responses could be triggered with peptides with no sequence relationship to tumour-derived MHC binding peptides.

**Importance of signal 1 in induction of low-affinity responses**

The demonstration that, in the F5 Tg TcR system (Chapter 6), T cell effector responses (CTL activity) against low-affinity antigen (NP34) can be induced by
priming the T cells with high-affinity antigen (NP68) is perhaps not surprising considering the promiscuous nature of the TcR. In fact, the use of tumour antigen-related peptide ("altered peptide ligand") for vaccination has been documented in a number of studies, which resulted in improved antitumour responses (Fong et al., 2001; Overwijk et al., 1998).

In an animal model of melanoma, CTLs against a melanocyte antigen, gp100, were elicited by immunisation of mice with human gp100, which fortuitously generates a response against the corresponding murine gp100 epitope (Overwijk et al., 1998). In addition, Fong et al demonstrated that immunisation of human patients with autologous DCs loaded with an altered peptide ligand of carcinoembryonic antigen (CEA) generated tumour regressions in some of the patients, which correlated with expansion of CEA-specific CD8⁺ T cells (Fong et al., 2001). Although these data were not interpreted in terms of "TcR affinity" of the antigens used to prime T cell responses, it is possible that T cells that can recognise the low affinity target tumour antigens were primed by an antigen that interacted with the same T cells with higher affinity.

Towards the design of improved DC-based tumour vaccines

Observations made in autoimmunity suggest that an effective antitumour vaccine needs enhancement of both signal 1 and signal 2. To date, DC-based immunotherapy of tumours has focussed very little on the former.

Theoretically, the strength of signal 1 could be improved by increasing the density of the MHC:tumour antigen complexes on DCs, thereby increasing the "avidity" rather
than the affinity of the interaction. In addition, data in this thesis (Chapter 6) as well as from other studies (Fong et al., 2001; Overwijk et al., 1998) suggest that enhancement of affinity may improve signal 1 significantly, resulting in enhanced anti-tumour (or low-affinity) responses. Where the TcR that recognises a given tumour antigen is known, a high affinity peptide recognised by that TcR may be predicted, as demonstrated by Hemmer (Hemmer et al., 1998b). Peptide antigens synthesised from the amino acid sequence predicted this way could then be tested for their immunogenicity in vitro and in vivo.

An alternative approach to increasing signal 1 was taken by Stauss and co-workers, where a tumour antigen (which is otherwise tolerised) was presented in the context of non-self MHC molecules (Sadovnikova and Stauss, 1996; Stauss, 1999). This approach took advantage of the fact that antigen recognition (and therefore tolerance) is self MHC-restricted.

Enhancement of signal 2 could be achieved by a number of reagents that induce maturation of DCs in vitro. In addition, if cell-mediated (CTL) antitumour responses are important in control of tumours, manipulation of DCs to induce a Th1 (Tc1) type of response may be essential. However, care must be taken with the choice of reagents used to manipulate DCs. Whilst it is clear that DCs respond differently to different types of stimuli (Chapter 4 and 5), it is likely that numerous other factors, such as the type of DCs, antigen dose and stage of DC maturation, would influence the outcome of T cell-DC interactions.
In summary, a DC-based tumour vaccine should (1) improve the signal 1 that primes the response, (2) enhance signal 2 by promoting the maturation of DCs, and (3) mediate Th1/Tc1 responses by manipulation of DC1 differentiation. Once such a vaccine protocol is optimised, accessibility of the vaccines needs to be improved (e.g., \textit{in vivo} targeting of DCs avoiding \textit{ex vivo} or \textit{in vitro} manipulations) in order to benefit a wider range of cancer patients.

\textbf{Future perspectives}

The findings in the F5 Tg model have exemplified TcR promiscuity, illustrating that priming with a "high-affinity" peptide could result in the efficient killing of otherwise ignored "low-affinity" targets. As discussed in Chapter 6, proof of principle experiments must be performed to show that such phenomena can be exploited in tumour immunotherapy. In order to do this, several steps need to be accomplished: (1) identification of defined tumour associated MHC class I epitopes; (2) identification of TcR that would recognise the defined epitopes; (3) screening for peptides (from a nonsel self peptide library) that would bind to the TcR; (4) identification of "high-affinity" peptides defined by functional assays; (5) incorporation into animal tumour models. Once the strategy have been shown to elicit effective antitumour responses in mice, it can then be applied to human cancers. With rapid progress in defining potential tumour associated antigens and HLA associated epitopes in cancer patients, it should be possible to identify sets of "high-affinity" altered peptides by combining bioinformatics technology and \textit{in vitro} functional assays (e.g., CTL assays). These could then be used to immunise most individuals against their tumours by DNA vaccination or by using synthetic peptide epitopes together with an appropriate adjuvant to enhance \textit{in vivo} CTL responses.
28 February, 2002

First Active Bank
PO BOX 458
St Peter Port
Guernsey
Channel Islands
GY1 5AE

Dear Sir

Opening up three new sterling (GBP) call accounts

I am writing to ask you to open two new sterling call accounts, in addition to my existing account (number 2458001) into which I intend to transfer money. I need to open these accounts before I transfer money into them (I shall be doing this in the next two to three weeks). I wish to have the interest earned on these two accounts, which will be of a capital nature, paid into a separate account in order to keep income and capital separate. For this purpose I would like to open up a third new sterling call account. Please open this third account by transferring £10 000 from account 2458001.

As soon as these accounts have been established please send me the account details as well as details of how to make electronic transfers into these accounts in different currencies (including GBP, Euro and USD).

Thank you in advance,

Yours sincerely.
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