Particle-mediated DNA immunisation: CD4+ T cell priming and cooperation

A thesis submitted by

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Revised abstract

In order to better understand how CD4+ T cells interact in vivo, a mouse model was established, involving the combined adoptive transfer of two populations of T cell receptor (TCR)-transgenic T cells and particle-mediated DNA delivery (PMDD) for the control of antigen presentation by dendritic cells. Since PMDD is a relatively recent method of immunisation, its effect on CD4+ T cells needed to be documented in the context of adoptive transfer models. These preliminary studies allowed the quantification of the CD4+ T cell responses to PMDD in vivo, and in particular, the appearance of specialised activated CD4+ T cells, namely Th1 and Th2, and demonstrated the crucial role of cytokines in the early phase of the immune response. Such models should prove useful for testing the concomitant inoculation of DNA antigen and immunomodulators, in the scope of rectifying the balance between these T cell subsets as a strategy for the treatment of many pathological conditions. These models were also used to examine the influence of the genetic background and the dose of DNA administered. A comparative study on migration and activation of naïve versus polarised CD4+ T cells was also carried out. To examine the cooperation between CD4+ T cells of different specificity, two populations of TCR-transgenic T cells recognising different antigens were co-transferred into the same adoptive host. PMDD was used as a tool for in vivo co-expression of two antigens, either on the same dendritic cell (linked) or not (unlinked). It was found that polarised T cells may considerably influence the fate of naïve T cells according to their cytokine profile. Cytokines confined to the dendritic cell cluster microenvironment appeared to play a more important role in the instruction of responding T cells. Moreover, linked presentation of two antigens to clones with a high and a low clonal frequency resulted in a strongly enhanced activation and effector function in the latter. These results demonstrated that cooperation between CD4+ T cells dominates over competition, is more efficient upon linkage with the same antigen-presenting cell and leads to better T cell instruction / deviation.
To my dear parents and brothers,
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<th>Definition</th>
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<tbody>
<tr>
<td>ACAD</td>
<td>activated T cell autonomous death</td>
</tr>
<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>combined adoptive transfer</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
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<tr>
<td>CFSE</td>
<td>5- (and 6-)carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>CTLA</td>
<td>cytotoxic T lymphocyte antigen</td>
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<td>CXCL</td>
<td>C-X-C chemokine</td>
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<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
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<tr>
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<td>dendritic cell</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide phosphate</td>
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<tr>
<td>DT</td>
<td>diphteria toxoid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ELISPOT</td>
<td>enzyme-linked immunospot</td>
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<tr>
<td>EpCAM</td>
<td>epithelial cell adhesion molecule</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>granulocyte/macrophage colony-stimulating factor</td>
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<td>Hank's balanced salt solution</td>
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<td>HBV</td>
<td>hepatitis B virus</td>
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<td>HEV</td>
<td>high endothelial venule</td>
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Hib  
*Haemophilus influenzae* type b

ICAM  
intercellular adhesion molecule

ICOS  
inducible costimulator

i.d.  
intradermal

IFN  
interferon

i.m.  
intramuscular

IL  
interleukin

IRES  
internal ribosome entry site

LAG  
lymphocyte activation gene

LC  
Langerhans cell

LCMV  
lymphocytic choriomeningitis virus

LFA  
lymphocyte function associated molecule

LPS  
ilopolysaccharide

MHC  
major histocompatibility complex

NK  
natural killer

OVA  
ovalbumin

PAMP  
pathogen-associated molecular pattern

PBS  
phosphate-buffered saline

PCC  
pigeon cytochrome C

PCR  
polymerase chain reaction

PE  
phycoerythrin

PMDD  
particle-mediated DNA delivery

PRR  
pattern recognition receptor

RAG  
recombination activating gene

RANK  
receptor activator of NF-κB

RNA  
ribonucleic acid

SLAM  
signalling lymphocytic activation molecule

SMAC  
supramolecular activation cluster

STAT  
signal transducer and activator of transcription

TCR  
T cell receptor

TCR-Tg  
T cell receptor transgenic

TGF  
tumour growth factor

Th  
helper T cell
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>Tim</td>
<td>T-cell immunoglobulin- and mucin-domain-containing molecule</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRANCE</td>
<td>TNF-related activation-induced cytokine</td>
</tr>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
</tr>
<tr>
<td>UCL</td>
<td>University College London</td>
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Chapter 1

General introduction
1.1. Introduction

DNA vaccines constitute a new and promising way to elicit protective immune responses to infectious organisms, or cancer, and also offer exciting possibilities for modulating or down-regulating on-going pathological responses to auto-antigens and allergens. However, the response of CD4+ T cells and their possible immunomodulatory roles have not been fully investigated in this context. CD4+ T cells play an important role in the adaptive immunity by regulating its different arms via specialised subsets, such as T helper (Th)1, Th2 and regulatory T cells. Dendritic cells (DCs) provide most of the information required for CD4+ T cells to be activated and develop into Th1 or Th2 cells (Section 1.2). They also provide them with a suitable environment where they may interact with each other more efficiently. Cooperation between T cells is usually required for optimum immune responses; however, the mechanisms underlying these interactions are only partially elucidated (Section 1.3). DNA vaccines offer several advantages over conventional vaccines (Section 1.4), but in the case of particle-mediated DNA delivery (PMDD), they also provide a valuable tool that can be used to dissect some fundamental aspects of CD4+ T cell cooperation.

1.2. T cell activation

The different steps leading to T cell activation are briefly examined in Sections 1.2.1-1.2.4. The activation of T cells, their subsequent, subtly regulated, differentiation and fate are more extensively reviewed in Sections 1.2.5-1.2.7, focusing mainly on the characteristics, effector functions and regulatory properties of Th1 and Th2 cells.

1.2.1. Lymphocyte and dendritic cell localisation and traffic

Lymphocytes are mainly present in primary (i.e. bone marrow, thymus) and secondary (i.e. spleen, lymph nodes) lymphoid organs as well as the blood stream. In secondary lymphoid organs, they are organised in compartments (e.g. B cells in follicles and T cells in paracortical zone) to which they are directed by the means of specific adhesion molecules and chemokines. For instance, naïve T cells and mature DCs are directed to lymph nodes if they express both CD62L (L-selectin) and CCR7,
a chemokine receptor (Bradley et al., 1994; Saeki et al., 1999; Baekkevold et al., 2001). They can circulate via the lymph (i.e. afferent and efferent lymphatics) or via the blood from which they are recruited in high endothelial venules (HEVs). DCs act as sentinels in the different tissues where they reside while T cells continuously traffic through the lymphoid organs in search of antigens presented by mature DCs in the context of danger signals (Gallucci and Matzinger, 2001).

DCs constitute a large and heterogeneous family of antigen-presenting cells (APCs) that exert a key role in initiating immune responses (Banchereau and Steinman, 1998; Bell et al., 1999). Different types of DCs, with their own localisation and phenotype, arise from precursors of myeloid and lymphoid lineages, under specific conditions such as the cytokine milieu in which they develop. They may perform different functions, such as thymic selection, B and T cell activation and modulation of immune responses (see also Section 1.2.5.2.5). For example, the research presented in this thesis has focused on immune responses that are predominantly mediated by Langerhans cells (LCs), which are resident in the skin (see also Section 1.4.3). LCs develop from CD34+ myeloid progenitors in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) and tumour growth factor (TGF)-β and in the absence of interleukin (IL)-4; they characteristically contain Birbeck granules and express markers such as CD1a, Langerhin, Lag and E-cadherin (Caux et al., 1999).

1.2.2. Antigen processing and presentation

DCs have been shown to be much more potent than macrophages or B cells in priming CD4+ T cells in vivo (Levin et al., 1993). DCs process both endocytosed and intracellular antigens onto class-II and class-I major histocompatibility complex (MHC) respectively, and present these antigens to CD4+ and CD8+ T cells respectively (Mellman et al., 1998; Théry and Amigorena, 2001; Unanue, 2002). Because of their particular ability to express class II MHC and up-regulate costimulatory molecules, DCs (as well as macrophages and B cells) are qualified as professional APCs. Some other cell types, referred to as amateur APCs, do not initiate immune responses, but can nonetheless be recognised by CD8+ T cells and natural killer (NK) cells if they present antigen on class I MHC or CD1 molecules.
respectively (Mellman et al., 1998). When non-haematopoietic cells are infected intracellularly (e.g. by viruses), antigen transfer from these cells towards APC (also known as cross-priming) is required (Sigal et al., 1999). To this end, DCs possess an alternative pathway by which exogenous antigen can be processed and presented on class I MHC (Huang et al., 1996; Brossart and Bevan, 1997).

1.2.3. Activation, maturation and migration of antigen-presenting cells

DCs can sense the presence of specific bacterial, viral and other microbial components, all referred to as pathogen-associated molecular patterns (PAMPs) by the means of pattern recognition receptors (PRRs; Reis e Sousa, 2001; for review). Toll-like receptors (TLR) constitute a family of PRRs (at least 10 members); they are capable of recognising a wide panel of molecules as different as lipoproteins, peptidoglycans (TLR-1/TLR-2, TLR-2/TLR-6), viral double-strand RNA (TLR-3), lipopolysaccharides (LPS) (TLR-4/CD14), flagellin (TLR-5), CpG motifs on bacterial DNA (TLR-9) (Medzhitov, 2001; for review). DCs up-regulate appropriate genes in response to PAMPs (Huang et al., 2001): first, genes that are important for innate immune responses such as inflammatory cytokines (tumour necrosis factor (TNF)-α, IL-1, IL-6) and second, genes required for migration to lymph nodes (CCR7) and induction of adaptive immune responses (e.g. MHC molecules, B7.1/B7.2 (CD80/CD86), CD40). They can also secrete important cytokines such as IL-2 (Granucci et al., 2001) and IL-12 (Reis e Sousa et al., 1997). Thus, the changes in gene expression during maturation of DCs reflect a dramatic shift from an activity of antigen processing to that of antigen presentation and immune cell stimulation. Up-regulation of CCR7 precedes the homing of DCs towards the paracortical region of draining lymph nodes (Saeki et al., 1999). The migration of LCs from the skin to draining lymph nodes can be induced by PAMPs such as CpG motifs (Ban et al., 2000) and is also impaired in CD40L-deficient mice, in which levels of TNF-α are abnormally low (Moodycliffe et al., 2000).

1.2.4. Immunological synapse and dendritic cell cluster

After entry into lymph nodes via the HEVs, T cells migrate through the paracortical area of lymph nodes. At this stage, they exhibit a relatively high motility (10.8-12.2
\[ \mu m/min \text{ in average; Miller et al., 2002a} \] and scrutinise the surface of DCs in the search for specific antigen. They can be selectively attracted by mature DCs that release DC-CK1 (Adema et al., 1997). If DCs present no antigen, T cells make only brief contacts (Steinman and Inaba, 1988; Gunzer et al., 2000; Stoll et al., 2002), lasting between 10 min and a few hours, which may however be of functional importance for the survival of naïve T cells (Revy et al., 2001). In contrast, T cells that encounter antigen-bearing DCs establish long-lasting contacts (>15 hours) and become static (Stoll et al., 2002, Miller et al., 2002a). The interactions between T cell receptor (TCR) and MHC/peptide complexes trigger the expression of other surface molecules that reinforce contact between T cells in DCs (Lanzavecchia and Sallusto, 2001; Krummel and Davis, 2002; for review). This adhesion is mainly mediated by LFA-1 (lymphocyte function associated molecule 1) – ICAM-1 (intercellular adhesion molecule 1, CD54) interactions, expressed on both DCs and T cells, and by LFA-3 (CD58) – CD2 interactions, expressed on DCs and T cells respectively (Inaba and Steinman, 1987; King and Katz, 1989). LFA-1 – ICAM-1 pairs, together with CD43 and CD45, constitute the peripheral supramolecular activation cluster (pSMAC), mainly a structural and adhesive component of the immunological synapse. The central supramolecular activation cluster (cSMAC) contains smaller interacting molecules involved in signalling (TCR – peptide/MHC and CD28 – B7.1/B7.2 pairs) or adhesion (LFA-3 – CD2 pair). Other molecules recruited in the cSMAC include CD4 or CD8, and later, CD40L (CD154) and CTLA-4 (cytotoxic T lymphocyte antigen 4, CD152). The organisation of these molecules within the synapse is arranged and maintained by a lipid raft, a rigid structure resulting from a modification in the composition of the cell membrane. The recruitment of CD4 along with its associated kinase Lck is important for the initiation of T cell activation (Inaba and Steinman, 1987; Viola et al., 1997, Bachmann et al., 1999; Holdorf et al., 2002).

DCs form clusters containing several T cells, but can also contain B cells from the same MHC haplotype as the DC (Inaba et al., 1984). Clustering and antigen presentation are required for 24-48 hours before T cells can independently proliferate (Austyn et al., 1983; Austyn et al., 1988; Jelley-Gibbs et al., 2000). After 48 hours, antigen-bearing DCs start to disappear (Ingulli et al., 1997), and activated T cells then regain high motility (5-12 \[ \mu m/min \]) and progressively leave the lymph nodes.
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(Stoll et al., 2002; Miller et al., 2002a). The longevity of DC clusters is prolonged by CD40L – CD40 interactions (Miga et al., 2001). Their dissociation may result from DC apoptosis induced by activated T cells via Fas ligation (Matsue et al., 1999), leaving T cells free to migrate out of the lymph node. Alternatively, DC apoptosis may occur after the detachment of T cells, resulting in the loss of survival signals. Indeed, activated T cells were also shown to maintain DC survival via TNF-related activation-induced cytokine (TRANCE) expression (Wong et al., 1997; Josien et al., 1999) and to stimulate them to express IL-1, IL-6, IL-12 and IL-15 (Josien et al., 1999). Finally, it is possible that DCs may be prematurely killed by activated cytotoxic T cells (CTLs).

1.2.5. T lymphocyte activation

T lymphocytes integrate a large quantity of information delivered by DCs, differentiate accordingly and may counter-regulate DC activity (Lanzavecchia and Sallusto, 2000; Lanzavecchia and Sallusto, 2001; for reviews). The use of antigen-specific TCR transgenic CD4+ and CD8+ T lymphocytes has led the way to much progress in the study of T cell selection (Murphy et al., 1990; Clarke et al., 2000) and in vivo activation (Pape et al., 1997a; Rogers et al., 1997; Kim et al., 1997; Jenkins et al., 2001).

1.1.5.1. Early signals in T cell activation

TCR ligation is the first signal inducing a cascade of events within the T cell. A minimum threshold of TCR ligation and a second signal are necessary to achieve full activation (Lanzavecchia and Sallusto, 2001; for review). The second signal is provided by the ligation between CD28 on T cells and B7.1 / B7.2 on DCs (Lenschow et al. 1996; for review). TCR signalling was reported to start even before the completion of the synapse formation (Lee et al., 2002a). The minimal time of TCR stimulation required for T cell proliferation is still subject to debate, but may be as short as 2 hours (van Stipdonk et al., 2001; Lee et al., 2002a) and as long as 20 hours (Iezzi et al., 1998), presumably depending on the strength of the two signals. TCR stimulation extending beyond two days leads to increased cell death and reduced cytokine production by surviving T cells (Jelley-Gibbs et al., 2000).
Costimulation through CD28 enables efficient priming with the need for less TCR engagement or lower antigen dose (Viola et al., 1996; Rogers and Croft, 2000). In addition to enhancing TCR signalling, it promotes cell division, survival and transcription of important cytokine genes (Lenschow et al. 1996; Sharpe and Freeman, 2002; for review). The differential role of B7.1 and B7.2 has been highlighted by several studies. First, B7.2 is up-regulated more rapidly than B7.1 and thus plays a more prominent role in the early stage of priming (Hathcock et al., 1994). B7.2 has been found in several studies to favour Th2 responses (see Section 1.2.5.2.3). B7.1 and B7.2 also support CD8+ T cell cytolytic function and expansion, respectively (King et al., 2001).

CD40L is rapidly and transiently up-regulated after T cell activation, peaking at 6-8 hours and back to normal at 24-48 hours (Roy et al., 1993). The ligation of CD40L with CD40 on DC provides a positive feedback that induces further up-regulation of B7 and adhesion molecules (ICAM-1) on DCs (Yang and Wilson, 1996; Cella et al., 1996; Vogel and Noelle, 1998). ICAM-1 was reported to also act as a costimulatory molecule for CD8+ but not CD4+ T cells (Deeths and Mescher, 1999). Activated T cells also express OX40, 4-1BB (CD137) and the T cell inducible costimulator (ICOS). OX40 signalling amplifies the expansion, migration and survival of CD4+ T cells (Maxwell et al., 2000; Gramaglia et al., 2000; Lane, 2000; Rogers et al., 2001), the cytokine expression by CD4+ T cells (for both Th1 and Th2 cells) and the expansion and cytotoxic activity of CD8+ T cells (De Smedt et al., 2002). 4-1BB and ICOS intervene more importantly (but not exclusively) in the maintenance of the CD8+ and CD4+ T cell responses, respectively (see Section 1.2.6.2). In addition to CD40 ligation, DC function may be further enhanced by pro-inflammatory cytokines, such as IL-1 and TNF-α, which can also directly act on T cells to boost their expansion in the presence of IL-2 for CD4+ or IL-2 + IL-12 for CD8+ T cells (Pape et al., 1997b; Curtsinger et al., 1999).
1.2.5.2. Differentiation signals

1.2.5.2.1. Relation between transcription and proliferation

The transcription of cytokine genes following activation is controlled by several cascades of signalling events (Rothenberg and Ward, 1996; Murphy et al., 2000). The IL-2 gene is one of the first to be transcribed in response to TCR/CD28 signalling. IL-2 supports T cell growth, and in the long term, promotes apoptosis (Van Parijs et al., 1999). Although most antigen-specific T cells end up activated, only a fraction of the cells efficiently divide, each of them generating approximately 10 daughter cells (Gudmundsdottir et al., 1999). T cells that are recruited and activated early and those with high affinity TCR are preferentially clonally expanded (Bousso et al., 1999; Kedl et al., 2002). Undifferentiated IL-2-producing T cells constitute an intermediate population that proliferate but require further signals to achieve commitment to a particular Th phenotype (Iezzi et al., 2001; Wang and Mosmann, 2001).

Activated T cells can later differentiate into two distinct subsets, Th1 and Th2, defined by the production of their own panel of cytokines and their migration patterns (O’Garra, 1998; Sallusto et al., 1998; Swain, 1999; for review). Th1 are important for helping CTLs and supporting cell-mediated immunity, such as delayed-type hypersensitivity, anti-viral and anti-tumoral responses. Th2 are more readily involved in humoral immunity, by influencing the function of B cells. Main features related to Th1 and Th2 cells are summarised in Table 1.1. Characteristic genes of Th1 and Th2 cells, such as interferon (IFN)-γ or IL-4 respectively, are induced later under the control of specific signal transducers and activators of transcription (STAT molecules) and other specific transcription factors (Kaplan and Grusby, 1998). They may be enhanced/modulated by environmental cytokines and other mediators (Murphy et al., 2000). The transcription of these genes is effective following chromatin rearrangement (epigenetic modification), which in the short term allows expression when cells start to divide and in the long term stabilises the phenotype adopted (Richter et al., 1999; Grogan et al., 2001). Interestingly, the transcription of IFN-γ and IL-4 appears differentially regulated by cell division. IFN-γ may be expressed before or during the S phase of the first division (Laouar and Crispe, 2000;
<table>
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<th>Th2</th>
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<td>Surface markers</td>
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<td>FasL_{high}, ICOS_{low}, CTLA-4_{low}</td>
<td>FasL_{low}, ICOS_{high}, CTLA-4_{high}</td>
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<td>IgG1, IgG3 (human)</td>
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<td>Factors favouring</td>
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<td>Cytokines</td>
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<td>Co-stimulation</td>
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<td>APC</td>
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<td>DC*, B cells</td>
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<tr>
<td>*DC-conditioning factors</td>
<td>CD40L, LPS, poly (I:C), IFN-γ</td>
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<td>Antigen dose</td>
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<td>Varying the antigen dose may shift the</td>
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<td>response towards Th1 or Th2</td>
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**Table 1.1.** Specific features of Th1 and Th2 cells.
Bird et al., 1998), while IL-4 appears to be expressed later, between the S phase of the first division and the third division (Richter et al., 1999; Bird et al., 1998). This correlates with the differential induction of IFN-γ and IL-4 mRNA (at 6 and 48 hours respectively; Lederer et al., 1996) and suggests a differential control of the transcription of these cytokines by the cell cycle. The choice of the type of response can either be “instructed” at the cell level during activation or “selected” at the population level, by factors such as differential growth and death rates between populations (Reiner and Seder, 1999). Some activated CD4+ T cells, termed Th0, exhibit an indecisive differentiation by the co-expression of both IFN-γ and IL-4, the latter being however more transiently expressed than the former (Openshaw et al., 1995). This phenotype may represent a pre-differentiated state, a transition state between Th1 and Th2 cells or the result of conditioning with Th1 and Th2 stimuli of equal importance.

1.2.5.2.2. Th1 cells

Th1 cells are mainly characterised by the production of type 1 cytokines (IFN-γ, TNF-α/β, IL-2). In addition, they specifically up-regulate the chemokine receptors CCR1, CCR5 and CXCR3 (Sallusto et al., 1998). Other markers include TRANCE, lymphocyte activation gene (LAG)-3, T-cell immunoglobulin- and mucin-domain-containing molecule (Tim)-3 and signalling lymphocytic activation molecule (SLAM). Their function is briefly described below.

Th1 development is induced by IL-12 (Hsieh et al., 1993; Scott, 1993; Robinson and O’Garra, 2002). DCs (Cella et al., 1996; Koch et al., 1996; Reis e Sousa et al., 1997) and macrophages (Scott, 1993, Shu et al., 1995) can generate great quantities of IL-12 after stimulation with CD40L (expressed on activated T cells) and/or TLR-binding microbial components (LPS, bacterial DNA, viral RNA, etc). IL-12 is a heterodimer (p70) made of p35 and p40 subunits. If expressed at high levels, IL-12p40 competes with bioactive IL-12p70 and inhibits its function (Gillessen et al., 1995). IL-12 binding on its receptor induces the transcription of Th1-specific genes such as IFN-γ via STAT4. The transcription of IFN-γ is also controlled by T-bet in CD4+ (Szabo et al., 2000; Mullen et al., 2001) but not in CD8+ T cells (Szabo et al.,
IFN-γ enhances the responsiveness to IL-12 (Hu Li \textit{et al.}, 1997; Smeltz \textit{et al.}, 2002) and its presence is essentially required within the first few hours of T cell activation (Hu Li \textit{et al.}, 1997; Schuhbauer \textit{et al.}, 2000). It also acts in synergy with CD40L for a more efficient secretion of IL-12 by DCs (Snijders \textit{et al.}, 1998; Mailliard \textit{et al.}, 2002). NK cells, NK T cells (Arase \textit{et al.}, 1996) and/or CD8+ T cells (Das \textit{et al.}, 2001; Mailliard \textit{et al.}, 2002) may constitute the early source of IFN-γ, which sensitises DCs and macrophages for IL-12 production. However, protective Th1 responses can be driven by a minimal elicitation of IL-12 on DC and endogenous IFN-γ production by CD4+ T cells, in the absence of exogenous IFN-γ from non-T cells (Wakil \textit{et al.}, 1998). IFN-γ also has an important role in the stability of the Th1 phenotype by maintaining repression of Th2 cytokine genes (Zhang \textit{et al.}, 2001).

IL-18 was identified as an IFN-γ-inducing factor, hence its former name IGIF (Okamura \textit{et al.}, 1995), and its receptor is specifically expressed on Th1 cells (Xu \textit{et al.}, 1998). IL-18 enhances the production of IFN-γ induced by IL-12, but via a STAT4 independent pathway (Kohno \textit{et al.}, 1997; Robinson \textit{et al.}, 1997). It also up-regulates IL-2 and IL-12 receptors on Th1 cells (Kohno \textit{et al.}, 1997; Xu \textit{et al.}, 1998; Chang \textit{et al.}, 2000) and increases CD8/CD4 ratio (Okamoto \textit{et al.}, 1999). More recently, IL-18 also was found to enhance Th2 responses in the absence of IL-12, or in BALB/c mice which have low responsiveness to IL-12 (Xu \textit{et al.}, 2000; Nakanishi \textit{et al.}, 2001). Finally, differentiation of naïve CD4+ T cells into Th1 can also be enhanced by IL-27, a heterodimer made of two subunits, which are related to the two IL-12 subunits (Pflanz \textit{et al.}, 2002; Robinson and O’Garra, 2002), or by type I interferons (IFN-α/β), produced in large amounts by virally-infected cells and CD40L-activated plasmacytoid DCs (Cella \textit{et al.}, 2000; Moser and Murphy, 2000). IL-27 binds the T cell cytokine receptor (TCCR), previously identified as required for Th1 responses (Chen \textit{et al.}, 2000) and synergises with IL-12, while IFN-α/β can drive Th1 responses independently and may also inhibit IL-12 (Byrnes \textit{et al.}, 2001).

2002). It is induced by IFN-γ via STAT-1 signalling, thus independently of IL-12 (Afkarian \textit{et al.}, 2002). T-bet expression can be inhibited by TGF-β, also in an IL-12R-independent manner (Gorelik \textit{et al.}, 2002).
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TRANCE is preferentially expressed on Th1 cells (Chen et al., 2001). Stimulation of TRANCE by immobilised receptor activator of NF-κB (RANK).Fc enhances IFN-γ secretion, which reflects positive bi-directional signals between DCs and T cells (Chen et al., 2001). TRANCE-expressing T cells were indeed previously reported to improve survival and stimulatory capacities of RANK-expressing DCs (Wong et al., 1997; Anderson, 1997; Josien et al., 1999). LAG-3 is induced upon activation and by IFN-γ, inhibited by IL-4 and its possible role in T cell differentiation is not known (Romagnani, 1997). SLAM is expressed on both committed Th1 and Th2 populations, but is absent in Th2 clones and appears functional only in Th1 cells. In fact, stimulation of SLAM results in the enhancement of IFN-γ production by Th1 cells but has no effect on Th2 cells (Castro et al., 1999). Tim-3, another marker of full Th1 commitment, appears after at least 3 rounds of stimulation in vitro. Cross-linking of Tim-3 in vivo results in the activation and expansion of macrophages (Monney et al., 2002).

1.2.5.2.3. Th2 cells

Th2 cells preferentially secrete type 2 cytokines (IL-4, IL-5, IL-13) as well as the inhibitory cytokine IL-10. After repeated stimulation (most effectively in presence of IL-6), they also express the T1/ST2 marker for which there is no characterised ligand (Meisel et al., 2001). Cross-linking of T1/ST2 stimulates the production of type 2 cytokines and expansion of Th2 cells (Meisel et al., 2001). CD30 was also reported to be a Th2 marker (Del Prete et al., 1995a) linked with the expression of type 2 cytokines in humans and mice (Del Prete et al., 1995b; Nakamura et al., 1997a). However, other data have contested these findings in humans (Hamann et al., 1996; Spinozzi et al., 1997).

The main cytokine driving Th2 differentiation is IL-4, but its initial source remains obscure. Activation-induced autocrine IL-4 production may constitute the most common source (Noben-Trauth et al., 2000; Noben-Trauth et al., 2002), but this production may not always be sufficient. Differentiation of naive into Th2 cells may rely on an exogenous source of IL-4 (Ben-Sasson et al., 2000), such as that produced by pre-activated T cells (Gollob and Coffman, 1994; Croft and Swain, 1995). Other
sources of IL-4 include NK T cells (Yoshimoto et al., 1995; Chen and Paul, 1997) and Rauscher leukaemia virus-infected DCs (Kelleher et al., 1999). If initially primed in the absence of IL-4, T cells fail to subsequently produce IL-4 even when restimulated in Th2 conditions (Hu Li et al., 1997). Signals that induce the production of autocrine IL-4 are not yet fully understood. There is growing evidence that CD4 engagement can trigger a small amount of endogenous IL-4 in an IL-4-independent manner, which is required for subsequent amplification in a positive feedback loop (Campbell et al., 2000; Campbell et al., 2001). In the absence of CD4 engagement, CD4+ T cells are unable to mount Th2 responses (Brown et al., 1997; Fowell et al., 1997). Cross-linking of CD4 could be lifting an inhibition of IL-4 production since inhibitors of Src-family tyrosine kinases such as the CD4-associated Lck favour Th2 polarisation (Gimsa et al., 1999).

Main components of the Th2 signalling pathway are STAT6, GATA-3 and c-Maf. GATA-3, which got its name from the consensus sequence of its binding site, regulates the expression of IL-4, IL-5 and IL-13 and is involved in the chromatin remodelling of these genes (Lee et al., 2000; Kishikawa et al., 2001). It is normally induced by the IL-4 receptor via STAT6, however it may still be activated in IL-4- and STAT6-deficient mice, suggesting an IL-4-independent alternative pathway (Finkelman et al., 2000), presumably downstream of CD4. GATA-3 can activate its own transcription and thus contribute to the stability of the phenotype (Ouyang et al., 2000). Its expression as a transgene evokes the emergence of committed Th2 cells that express T1/ST2 (Nawijn et al., 2001). It is repressed in naïve T cells by FOG (Friend of GATA) (Kurata et al., 2002). The c-Maf transcription factor stimulates specifically the transcription of the IL-4 gene (Kim et al., 1999) and is involved in both IL-4-dependent and independent Th2 differentiation (Ho et al., 1998). Interestingly, if CD28 signalling occurs prior to rather than simultaneously to CD3 (TCR) signalling, IL-4 expression is also favoured, via the activation of STAT6 (Oki et al., 2000).

Th2 responses were also found to be impaired in mice deficient in monocyte chemoattractant protein (MCP)-1 (Gu et al., 2000), a chemokine that binds to CCR2 and CCR4. Interestingly, the latter is preferentially expressed on Th2 cells, while the former, expressed on both Th1 and Th2 (Sallusto et al., 1998), was found to be more
important for Th1 responses (Boring et al., 1997). In synergy with IL-4, macrophage-derived IL-6 favours Th2 differentiation (Rincón et al., 1997), and also inhibits Th1 differentiation, by interfering with the IFN-γ signalling pathway (Diehl et al., 2000). However, a contradictory report found that, in IL-6-deficient mice, the production of IL-4 is increased while IFN-γ is inhibited after polyclonal activation of spleen T cells (Tanaka et al., 2001). In the same report, exogenous IL-6 inhibited Th2 differentiation without affecting Th1 development. Finally, in contrast with IFN-α/β, which induces IFN-γ and IL-10, IFN-τ stimulates the production of IL-4, IL-5 and IL-10 (Soos et al., 2002).

In terms of costimulatory molecules, B7.2 appears more important than B7.1 for the development of Th2 responses (Freeman et al., 1995; Kuchroo et al., 1995; Ranger et al., 1996; Corr et al., 1997). Furthermore, ICOS, which binds to B7h (or B7-H2, a B7 homologue), is expressed at higher levels on Th2 than Th1 cells (Coyle et al., 2000; McAdam et al., 2000) and is required for IL-4 production (Dong et al., 2001; Tafuri et al., 2001). ICOS engagement stimulates type 2 cytokines upon activation by APC/peptide but both type 1 and type 2 cytokines upon CD3 triggering (McAdam et al., 2000). It also enhances cytokine expression by memory Th1 and Th2 cells (Khayyamian et al., 2002). Similarly, OX40 signalling was shown to enhance IL-4 production and Th2 responses in several reports (Ohshima et al., 1998; Flynn et al., 1998; Tanaka et al., 2000), but it can also favour Th1 responses initially and amplify any ongoing responses regardless of the type of response (De Smedt et al., 2002). Surprisingly, CD40 and its ligand CD154 were found to be crucial for the generation of a Th2 response against soluble egg antigen from Schistosoma mansoni, but not for the Th1 response directed against Propionibacterium acnes (MacDonald et al., 2002), an observation contrasting with the well established role of CD40 for the release of IL-12 (Section 1.2.5.2.2).

1.2.5.2.4. Cross-regulation between Th1 and Th2 cells

Th1 and Th2 cells usually antagonise each other by the cytokines they secrete, but can also support each other in some aspects (Morel and Oriss, 1998; Paludan, 1998; for reviews). Th1 and Th2 cytokines are mutually inhibitory. IFN-γ selectively
reduces the proliferation of Th2 cells (Gajewski and Fitch, 1988), and one possible
mechanism is by interfering with the proliferative effect that IL-1 has on Th2 cells
(Oriss et al., 1997). In contrast, Th1 are influenced neither by IL-1 nor by IFN-γ,
because they lack expression of both IL-1R (Solari et al., 1990) and IFN-γRβ (Pernis
et al., 1995), the latter being required for the transmission of the anti-proliferative
signal of IFN-γ. Inversely, IL-4 secreted by Th2 cells can decrease the expression of
IL-12 receptor in Th1 cells (Szabo et al., 1997), inhibit IL-12 expression (Koch et
al., 1996; Ohshima and Delespesse, 1997) and switch on type 2 cytokine genes,
resulting in some cases in the reversion from a Th1 to a Th2 phenotype (Perez et al.,
1995; Mocci and Coffman, 1995). In contrast, the commitment of Th2 cells may be
stabilised by specific loss of IL-12Rβ2 (Rogge et al., 1997; Szabo et al., 1997),
preventing reversion to Th1 phenotype (Szabo et al., 1995). Nakamura et al. (1997b)
suggested that in the absence of IL-4, Th2 cells may revert to a Th1 phenotype, but
since the distinction between the two subsets was based on CD30 expression, the
accuracy of such observation may be questionable. The duration of the polarising
stimuli is important for an irreversible commitment of helper T cells to a specific
phenotype (Murphy et al., 1996). At the transcriptional level, the stabilisation of
the phenotype is achieved by the fact that GATA-3 and T-bet are mutually repressive
(Asnagli and Murphy, 2001). T cells stimulated with both IL-4 and IFN-γ do not
differentiate and predominantly produce IFN-γ if rechallenged with IL-12 (Hu Li et
al., 1997).

T cell growth factors (IL-2, IL-4) act as positive signals by which Th1 and Th2 cells
can enhance one another’s proliferation. IL-4 may increase the expansion of Th1
cells without affecting their phenotype if IL-12 is also present (Oriss et al., 1999). In
fact, IL-4 was found to be important for Th1 responses, as IL-4-/- mice have impaired
anti-tumour immunity (Schüler et al., 1999).

1.2.5.2.5. Modulation of Th1/Th2 responses

The origin of DC and the cytokine microenvironment in which they ultimately
develop may affect the outcome of the immune response. In humans, monocyte-
derived myeloid DC favour Th1 differentiation while plasmacytoid DC (CD4+ CD3-
CD11c- preferentially induce Th2 responses (Rissoan et al., 1999). However, the latter may also stimulate strong antiviral Th1 responses in appropriate conditions by the expression of high levels of IFN-α (Cella et al., 2000). In mice, lymphoid/myeloid DC from Flt3 ligand-treated mice (CD11c+ CD11b<sup>low</sup>) and myeloid DC from GM-CSF treated mice (CD11c+ CD11b<sup>high</sup>) predominantly stimulate Th1 and Th2 responses respectively (Pulendran et al., 1999). This subdivision appears to correlate with the mouse CD8α+ and CD8α- subsets respectively (Maldonado-López et al., 1999; Moser and Murphy, 2000). Additionally, IL-10-treated splenic DCs were also shown to favour Th2 responses (Liu et al., 1998). The heterogeneity of DCs makes it difficult to link the DC lineage with a predefined helper T cell response; however, exogenous factors (PAMPs, cytokines) consistently condition DCs into favouring one or another response, in particular by the stimulation or the inhibition of IL-12 production (Kalinski et al., 1999). Factors stimulating IL-12 production by DC1 include CD40L, LPS, poly (I:C) and IFN-γ, whereas prostaglandin E2 (PGE2), vitamin D, IL-4, IL-10, TNF-α and TGF-β were reported to inhibit IL-12 expression and/or enhance DC2 function (Müller et al., 1998; Kalinski et al., 1999; Lanzavecchia and Sallusto, 2000; Maldonado-López and Moser, 2001; for reviews). CD8+ DCs that have been sensitised through CD40 induce high levels of CD40L and low levels of CTLA-4 on Th1 cells. Conversely, in the absence of CD40 ligation, these DCs generate Th1 cells that are CD40L<sup>low</sup> and CTLA-4<sup>high</sup> (Fallarino et al., 2002). APCs other than DCs may also differentially regulate helper T cell differentiation. Macrophages are able to secrete IL-12 (Scott, 1993) and express a specific ligand, M150, which costimulate the production of IFN-γ and IL-2 on CD4+ T cells (Agrewala et al., 1994), thus stimulating more efficiently Th1 responses. In contrast, B cells do not secrete IL-12 (Guery et al., 1997), express higher levels of B7.2 (Hathcock et al., 1994), which may be more important for Th2 responses (see Section 1.2.5.2.3) and preferentially stimulate IL-4 production (Mason, 1996; Macaulay et al., 1997). A more recent study showed that Th2 responses, but not Th1, are profoundly impaired in B cell-deficient mice (Bradley et al., 2002).

The dose and/or affinity of antigen may determine the choice between a preferential Th1 or Th2 response (Constant et al., 1995; Constant and Bottomly, 1997). High
doses of protein or peptide antigens favoured Th1 responses (Hosken et al., 1995; Rogers and Croft, 1999) while high doses of foreign cells/pathogens (Bretscher et al., 1992; Bancroft et al., 1994; Ismail and Bretscher, 1999) appeared to have the opposite effect. The effect of the affinity of TCR/peptide and/or MHC/peptide interactions on the development of Th1 and Th2 responses was extrapolated as a general effect of TCR signal strength (Leitenberg and Bottomly, 1999; for review). Thus, the variability of peptide / MHC affinity between different MHC haplotypes may explain differential types of immunity between individuals (Murray, 1998), hence the link between particular MHC haplotypes and some pathological conditions. LFA-1 – ICAM interactions also support Th1 responses, possibly by enabling tighter and longer contacts between T cells and DCs (Salomon and Bluestone, 1998; Luksch et al., 1999). Cross-linking of OX40 and CD28 also lower the dose threshold required for IFN-γ and IL-5/IL-13 production at high and low peptide doses respectively (Rogers and Croft, 2000). Moreover, the preponderant Th1 response obtained with high peptide dose may be skewed to a Th2 response by blockade of LFA-1 (Rogers and Croft, 2000).

The duration of TCR signalling is another parameter that can modulate the differentiation of CD4+ T cells. Th2 development requires a more prolonged TCR signalling than Th1 (Iezzi et al., 1999). Differential TCR signalling has an evident influence on the levels of mitogen-activated protein (MAP)-kinase, protein kinase C and cytosolic calcium, which can then determine an initial bias towards Th1 or Th2, in absence of IL-12 or IL-4 (Noble et al., 2001). No such initial bias was observed for CD8+ T cells to differentiate into Tc1 or Tc2 in the absence of polarising cytokines (Noble et al., 2001).

Finally, the fate of T cells may be strongly influenced by the timing of their encounter with DCs. DCs that have been stimulated with LPS for less than 24 hours express IL-12 (for which mRNA levels peak at 8 hours) and stimulate the differentiation of CD4+ T cells into Th1 cells (Langenkamp et al., 2000). In contrast, DCs that have been stimulated for more than 24 hours can no longer produce IL-12 and may produce some IL-10 instead (mRNA levels peak at 18 hours); thus T cells encountering such DCs differentiate into Th2 cells (or fail to polarise with lower antigen dose) (Langenkamp et al., 2000).
1.2.5.3. Inhibitory signals

In the absence of costimulation, most importantly of CD28 stimulation, TCR signalling leads to a state of non-responsiveness termed anergy (Schwartz, 1997; Van Gool et al., 1999). Anergic cells survive but are refractory to proliferation and IL-2 production, even if restimulated with costimulation. They can only proliferate in the presence of exogenous IL-2. Anergy is thought to play a role in peripheral tolerance and anergic cells may participate in the regulation of immune responses rather than just being non-functional (Taams and Wauben, 2000).

Fully activated T cells require inhibitory signals to limit and regulate the immune response. Following activation, T cells up-regulate CTLA-4, which is homologous to CD28. While CD28 is constitutively expressed on naïve T cells and stimulates proliferation upon ligation with B7.1/B7.2, CTLA-4 is induced by activation, binds to B7.1/B7.2 molecules with much greater affinity than CD28 and its signalling inhibits T cell proliferation (Chambers et al., 2001). Furthermore, CTLA-4 is more strongly expressed on Th2 than on Th1 cells (Alegre et al., 1998). It is possible that high levels of CTLA-4 are less necessary in Th1 cells, because strong TCR stimulation, which favours the development of these cells (Leitenberg and Bottomly, 1999; for review), also induces a more efficient recruitment of CTLA-4 in the immunological synapse (Egen and Allison, 2002). CTLA-4 signalling represses IL-4 and up-regulates TGF-β1 production (Kato and Nariuchi, 2000). TGF-β prevents CD4+ T cell differentiation into Th2 or Th1 cells by inhibition of GATA-3 or T-bet, respectively (Gorelik et al., 2000; Gorelik et al., 2002), but has no effect on memory Th2 cells (Lúdvíksson et al., 2000). TGF-β1 also inhibits memory Th1 functions via the down-regulation of IL-12Rβ2 (Lúdvíksson et al., 2000). Another marker, PD-1 (programmed death gene 1), was recently identified on activated T and B cells and binds to two other members of the B7 family, B7-H1 (PD-L1) and B7-DC (PD-L2). It mediates the inhibition of proliferation and cytokine production (Greenwald et al., 2002; Sharpe and Freeman, 2002; for reviews).

In addition, T cells engage into a slow progression towards apoptosis, a process called activation-induced cell death (AICD). As part of this process, they up-regulate
FasL, a ligand that triggers apoptosis upon binding to Fas. FasL expression is promoted by IL-2 and STAT5 signalling (Van Parijs et al., 1999). One group (Ramsdell et al., 1994) showed that Th1 cells express higher levels of FasL and are more susceptible to apoptosis than Th2 cells, while another group gave evidence that both Th1 and Th2 cells express Fas and FasL, but Th2 cells may be better protected from apoptosis by expressing high levels of Fas-associated phosphatase (FAP)-1 (Zhang et al., 1997). The greater propensity of Th1 cells to undergo cell death may represent a more stringent down-regulation of these potentially more harmful cells. A third group also reported similar levels of Fas and FasL expression between Th1 and Th2 cells, but also an equal susceptibility to AICD (Watanabe et al., 1997). FasL can be induced on Langerhans cells upon CD40 ligation and mediate the apoptosis of activated T cells (Shibaki and Katz, 2001).

Fas-independent AICD may also be induced by another type of death receptor, the TNF receptors. AICD mediated by Fas and TNF receptors is caspase-dependent (Hildeman et al., 2002). In contrast, activated T cell autonomous death (ACAD) occurs upon the loss of protection by anti-apoptotic factors in favour of pro-apoptotic elements of the Bcl-2 family. ACAD is independent of death receptors and caspases (Hildeman et al., 2002).

1.2.6. Fate of activated T cells

1.2.6.1. Relocation and recirculation of effector and memory cells

T cells that have received a short TCR stimulation are able to proliferate but not to differentiate (Iezzi et al., 2001). They retain expression of CD62L and CCR7 so that they remain localised in lymph nodes with naïve cells but can respond faster than them upon re-encounter with antigen. In contrast, fully activated T cells acquired specialisation in terms of cytokine production and chemokine receptor expression (Sallusto et al., 1998). These effector cells then migrate to a different site where they perform appropriate functions. Th1 cells preferentially express CCR1, CCR5 and CXCR3, normally migrate to inflamed tissues (such as the mucosa for CCR5+ cells; Sallusto et al., 1998) where they activate macrophages and help the function of CD8+ T cells and NK cells. However, retention of Th1 cells in lymph nodes may be
induced if CXCL10, a ligand for CXCR3, is secreted by DCs (Yoneyama et al., 2002). In contrast, tissue infiltration by Th1 cells is increased if CXCL10 is blocked. Th2 cells express higher levels of CCR3 and CCR4 and migrate to the edge of B cell-rich areas to support proliferation and class-switching of B cells (Section 1.3.1), as well as to peripheral sites (such as the skin for CCR4+ cells; Campbell et al., 1999) to mediate recruitment of eosinophils by the secretion of IL-5. Furthermore, it was reported that murine Th1 cells also express considerably higher levels of CCR7 (Randolph et al., 1999) and CD62L (Van Wely et al., 1999) compared with Th2 cells, enabling them to relocate to T cell-rich zone while Th2 cells are virtually excluded from those areas. In contrast, human memory Th2 cells express higher levels of CD62L than Th1 memory cells (Kanegane et al., 1996).

Memory T cells (CD45RO+ in human or CD45RB\textsuperscript{low} in mice) are distinguishable from naïve T cells (CD45RA+ in human or CD45RB\textsuperscript{high} in mice) and evolve into two subsets with different homing features (Sallusto et al., 1999). While CD4+ CD45RA+ T cells are all CD62L\textsuperscript{high} CCR7+, CD45RA- T cells are either CD62L\textsuperscript{high} CCR7+ (central memory) or CD62L\textsuperscript{low} CCR7- (effector memory). Central memory cells appear non-polarised and only produce IL-2 upon activation, whereas effector memory T cells express lower levels of IL-2 and higher levels of IFN-\(\gamma\), IL-4 or IL-5. Moreover, effector memory T cells weakly respond to IL-15 compared with central memory T cells, but some of them may develop into the latter (Geginat et al., 2001). CD8+ T cells share the same kind of memory subdivision as CD4+ T cells, but in addition, some naïve CD8+ T cells are also CD62L\textsuperscript{low} CCR7-, which may possibly allow them to be activated in the periphery. Effector (cultured with IL-2) and memory (cultured with IL-15) CD8+ T cells exhibit different migration properties (Weninger et al., 2001). The idea that activated T cells become CD62\textsuperscript{low} CCR7- and thus cannot recirculate to lymph nodes has been challenged by several reports. T cells conditioned with phytohemagglutinin and/or IL-2 showed an increased chemotactic responsiveness to CCR7 ligands (i.e. CCL19 and CCL21) (Willimann et al., 1998). Likewise, CD62L\textsuperscript{low} CD4+ populations, previously activated and differentiated \textit{in vivo}, may re-express CCR7 and retain their ability to respond to CCR7 ligands (Debes et al., 2002).
1.2.6.2. Memory and effector functions

The development into long-lived memory cells from the pool of activated T cells is characterised by an acquired responsiveness to IL-15 by CD4+ (Geginat et al., 2001) and CD8+ T cells (Sprent and Surh, 2001). IL-15 may rescue T cells from programmed cell death (Marrack et al., 2000; Sprent and Surh, 2001; for reviews), which correlates with the inversion of anti- vs. pro-apoptotic factors in favour of the anti-apoptotic ones during memory formation (Garcia et al., 1999). IL-15 can also enhance IFN-γ and IL-4 mRNA levels in activated CD4+ and CD8+ T cells (IFN-γ only in CD8+ T cells) (Borger et al., 1999). IL-15R and IL-2R share the same β and γ chain, but unlike IL-15, IL-2 induces the down-regulation of the common γ chain, leading to progressive death by apoptosis (Li et al., 2001). Whether memory T cells derive from effector T cells or from activated T cells that did not develop into effectors is still a controversy (Sprent and Sirh, 2001; Kaech et al., 2002).

The effector function of activated/memory CD4+ and CD8+ T cells is maintained in the periphery via costimulatory molecules that are induced on T cells upon activation, such as ICOS, 4-1BB and OX40. B7h (B7-H2) is expressed on lymphoid and non-lymphoid tissues and stimulates cytokine production by both Th1 and Th2 cells via ICOS (McAdam et al., 2000; Khayyamian et al., 2002), but appears essential for the maintenance of Th2 responses (McAdam et al., 2000; Dong et al., 2001; Tafuri et al., 2001; Liang and Sha, 2002). Conversely, the expression of B7-H3 in the periphery appears to be involved in the maintenance of Th1 responses, but its ligand on T cells has not yet been identified (Liang and Sha, 2002). 4-1BB is induced in higher levels on CD8+ than in CD4+ T cells (Wen et al., 2002) and was reported to promote survival of both CD8+ and CD4+ T cells (Cannons et al., 2001) and to amplify and maintain CTL activity in the effector/memory response (Shuford et al., 1997; Cannons et al., 2001; Bertram et al., 2002; Wen et al., 2002). Its ligand 4-1BBL can be induced on APC by CD40L (Diehl et al., 2002). Stimulation of OX40 during priming also contributes significantly to the generation of a greater frequency of memory cells (Gramaglia et al., 2000). OX40 and 4-1BB both appear to enhance survival by preventing AICD (Weinberg et al., 1998; Vinay and Kwon,
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1998; for reviews) and 4-1BB-mediated memory functions are still maintained in IL-15<sup>-/-</sup> mice (Miller et al., 2002b).

1.2.7. Regulatory T cells

Two apparently distinct subsets of CD4+ T cells have been shown to down-regulate immune responses. On the one hand, CD4+ CD25+ T cells are immuno-suppressive in a contact-dependent (that may involve CTLA-4), cytokine-independent, antigen-nonspecific manner (Thornton and Shevach, 2000; Shevach, 2001). They require TCR stimulation, CD28 costimulation and IL-2 for survival and function (Papiernik et al., 1998; Thornton and Shevach, 2000; Salomon et al., 2000). On the other hand, Tr1 and Th3 secrete high levels of IL-10 and TGF-β respectively, which are implicated in the suppressive effect of these cells (Groux et al., 1997; Fukaura et al., 1996). Such regulatory T cells may acquire their function in contact with immature DC (Roncarolo et al., 2001) or may, in the case of CD4+ CD25+ cells, constitute a proper lineage that is generated in the thymus (Shevach, 2001). Interestingly, it was demonstrated that anergised T cells expressing the gene related to anergy in lymphocytes (GRAIL) or Tr1-like cells secreting IL-10 can be generated from CD4+ CD25- precursors upon induction by CD4+ CD25+ regulatory T cells (Ermann et al., 2001; Dieckmann et al., 2002). Since CTLA-4 stimulates the production of TGF-β (Kato and Nariuchi, 2000), it is possible that regulatory T cells can also induce Th3 cells expressing TGF-β.

T-T presentation between CD4+ and/or CD8+ T cells represents another contact-dependent mechanism of regulation in both human and mice. This mechanism results in anergy and apoptosis, appears to be CTLA-4-independent and not attributed to a lack of costimulation (Chai et al., 1998). The unresponsive T cells generated become refractory to further activation by normal APCs (Taams et al., 1999). Cytokine production by both Th1 and Th2 can be down-regulated upon T-T presentation (De Vita et al., 1998). CD8+ T cells may exert immuno-suppressive functions on CD4+ T cells as well as on APC (Vukmanovic-Stejic et al., 2001; for review).
Th2 cells, which are not conventionally considered as a professional regulatory population, show however some important regulatory properties. They can secrete IL-10, share with CD4+ CD25+ cells some selectively up-regulated genes (Zelenika et al., 2002) and help rectifying autoimmune conditions (Müller et al., 1998; for review). Other evidence supporting the role of Th2 cells in the down-modulation of T cell responses is the stimulation of IL-10 transcription on Th2 cells by IL-4 (Schmidt-Weber et al., 1999). If the induction of anergy is prevented by early treatment with anti-CTLA-4, activated CD4+ T cells preferentially differentiate into Th2 cells, presumably more suitable for down-regulating the immune response (Nakata et al., 2000; Kato and Nariuchi, 2000). Interestingly, while Th2 cells secrete both IL-4 and IL-10 and support IgE production, Tr1 cells suppress IgE responses in vivo, an inhibition largely mediated by IL-10 (Cottrez et al., 2000). Finally, IL-4 was shown to restrain CTL responses (King et al., 2001) and is used in several models of autoimmunity to counteract detrimental Th1 and CTL responses by immune deviation (Röcken et al., 1996; Kim et al., 2001; Garren et al., 2001).

1.2.8. Concluding remarks on T cell activation

CD4+ T cells activation and differentiation are very complex and highly regulated processes. Some of the factors involved in this regulation are either poorly understood or were found to have contradictory roles in separate studies. Thus, it is important to comprehend the behaviour of these cells in all the different contexts of an immune response. In particular, not much is known about the responses of these cells in the context of DNA vaccination or of the DC-T cell cluster.
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1.3. T cell cooperation and competition

1.3.1. B cell-T cell cooperation

B cell activation also requires two signals, one through its B cell receptor (BCR) and the other from T cells. In the absence of T cell help, B cells become anergic, except with T cell-independent antigens, in which case they may secrete IgM but fail to undergo class switching. It was demonstrated a long time ago that B cells require the collaboration from T cells that are also responding to related parts of the same antigen (Mitchison, 1971), except in a transplantation model, where T cells may help B cells in an antigen-independent manner via the co-recognition of allogeneic DCs (Kelly et al., 1996). Activated T cells can drive activation of B cells by signalling through CD40L – CD40, in the same way as DCs (Vogel and Noelle, 1998; for review). Helper T cells that express the CCR4 and CXCR5 chemokine receptor migrate to the edge of the B cell follicles in the lymph node where they perform help (Garside et al., 1998; Sallusto et al., 1998). It has been thought for a long time that Th2 were the main subset involved in B cell help, because Th2 responses are generally associated with humoral responses. Th2 cells secrete IL-4, which induces the proliferation of B cells and the production of IgG1 and IgE isotypes. However, Th1 cells can induce class switching towards IgG2a and recently, Th1 and Th2 cells were found to help B cells with the same efficiency (Smith et al., 2000a).

1.3.2. Cooperation between T cells

1.3.2.1. The DC cluster and its microenvironment

A dendritic cell is able to attract and activate several T cells at a time. This results in a series of immunological synapses, which lead us beyond the concept of mutual communication between two cells (Creusot et al., 2002). Thus, the DC cluster contributes to the formation of a network of responding T cells. These cells may recognise different epitopes (likely to be derived from a common pathogen) and need to collaborate in order to tackle more efficiently the pathogen in question. In particular, CD8+ T cells are not usually efficiently activated in the absence of helper T cells (Keene and Forman, 1982). In fact, DCs require prior sensitisation by CD4+
T cells (Bennett et al., 1997), unless they are activated by some potent viral stimuli (Buller et al., 1987; Liu and Mullbacher, 1989). In this context, the DC acts like a bridge that ensures proximity between responding cells, enabling them to communicate using cytokines (paracrine activity), and also relay synapse-dependent information from T cell to T cell via the DC (epicrine activity). An illustrated summary of possible mechanisms of cooperation between T cells is shown in Figure 1.1.

After the demonstration of B-T cell cooperation, T-T cell cooperation was envisaged. Since T cells are not able to activate each other (though they may “deactivate” each other, as mentioned in Section 1.2.7), an APC acting as a bond between them was a plausible hypothesis. The concept of linkage between two or more T cells via the corecognition of the same dendritic expressing structurally related antigens was then envisaged. Some antigens cannot generate a detectable delayed-type hypersensitivity (DTH) response if they are not physically coupled with another one (Tucker and Bretscher, 1982; Bretscher, 1986). The expression of coupled or structurally related antigens by a DC thus leads to the recruitment of two clones or more, giving rise to the model of the three cell cluster (Mitchison and O’Malley, 1987).

Outside the context of the DC cluster, direct interactions between T cells (or between T and B cells) can occur by CD27 – CD70 interactions (Lens et al., 1998; for review). CD27 is expressed on mature T, B and NK cells, rapidly up-regulated then progressively lost after activation, and recovered in memory cells. CD70 is up-regulated in some T and B cells following antigenic stimulation. CD70 is more strongly expressed on CD8+ than on CD4+ T cells. Th1-polarising (IL-12) and pro-inflammatory conditions (IL-1α, TNF-α) enhance CD70 expression on CD4+ T cells while Th2-polarising conditions (IL-4) inhibit it. CD27-CD70 interactions occur during the expansion phase and signalling through CD27 supports proliferation.

1.2.2.2. Cooperation between CD4+ and CD8+ T cells

As stated above, efficient cytotoxic responses normally require the collaboration between helper and cytotoxic T cells on the surface of DCs (i.e. in a three cell cluster), a situation achieved only by antigen linkage (Mitchison and O’Malley,
Figure 1.1. Possible mechanisms for cooperation between T cells in the DC cluster microenvironment.
1987; Stuhler and Schlossman, 1997; Bennett et al., 1997). The major costimulatory molecules involved in CD4-CD8 cooperation are CD40L and CD40 (Vogel and Noelle, 1998; Toes et al., 1998; for reviews), which are expressed on T cells and DC respectively. Following CD40-CD40L ligation, T cells induce the release of large amounts of IL-12 by DC (Shu et al., 1995; Celia et al., 1996; Koch et al., 1996). IL-12 promotes the development of Th1 cells, which in turn help the proliferation and function of CD8+ T cells by secreting IL-2 and IFN-γ. IL-12 production by DC occurs within the first day after CD40 ligation or LPS stimulation, after which the secretion is irreversibly shut down (Langenkamp et al., 2000). Furthermore, CD40 ligation increases expression of MHC molecules and important costimulatory molecules such as CD80, CD86 and ICAM-1 (Yang and Wilson, 1996; Celia et al., 1996). Deficiency in class II MHC, CD40 and CD40L leads to impaired CTL responses while activation of CD40 by the means of anti-CD40 antibodies restores normal CTL responses in CD4-depleted, class II MHC knock-out or CD40L-deficient mice (Ridge et al., 1998; Bennett et al., 1998; Schoenberger et al., 1998). In the case of DNA immunisation, CD40 stimulation by antibodies or B7.1 co-expression with antigen (but not B7.2) only partially compensated for a class II MHC deficiency (Chan et al., 2001). On the basis of these data, a widely accepted model proposes the following sequence of events occurring during a brief period of time and in a three-cell cluster (i.e. CD4+ T cell – DC – CD8+ T cell): TCR ligation inducing rapid CD40L up-regulation in T cells, CD40-CD40L ligation then leads to up-regulation of costimulatory molecules by DC and the release of IL-12; the additional CD80/CD86-CD28 ligation complete the full activation of T cells, which can then proliferate and respond to environmental cytokines such as IL-12.

It was later found that optimal CD8+ T cell responses require more than CD40L-induced DC sensitisation because CD4+ T cell help still occurs to some extent in CD40−/− mice (Lu et al., 2000). If DCs are initially sensitised by CD4+ T cells and then cultured with CD8+ T cells only, they are able to activate the latter considerably better if the supernatant from the first culture is present. These authors thus demonstrated both epicrine (other than via CD40) and paracrine help from CD4+ to CD8+ T cells. Help from CD8+ to CD4+ T cells was also reported in the form of IFN-γ, which is produced in high quantities by CD8+ T cells (Mailliard et al., 2002).
IFN-γ synergises with CD40 signals to boost the release of IL-12 by DC (Snijders et al., 1998) and to enhance the expression of IL-12 receptor on T cells (Smeltz et al., 2002), thus supporting the generation of Th1 cells. In addition, activated CD8+ T cells, which up-regulate CD70, can cross-link with CD27 on CD45RA+ (naïve) helper T cells and promote up-regulation of CD40L expression (Stuhler et al., 1999). In return, these helper T cells can enhance DC function via CD40-CD40L ligation, and hence participate in the activation of further CD8+ T cells. Consequently, secretion of IFN-γ and expression of CD70 constitute two positive feedback mechanisms by which CD8+ T cells indirectly amplify their own response.

The precursor frequency of cells responding to antigen appears to be an important factor in the requirement for help since the adoptive transfer of CD8+ T cells in sufficient numbers is able to circumvent their dependence on CD4+ T cell help in MHC-II deficient mice (Wang et al., 2001; Mintem et al., 2002). Surprisingly, the mechanism does not involve CD40-induced DC sensitisation, normally achieved by CD4+ T cells (Mintem et al., 2002).

1.3.2.3. Cooperation between CD4+ T cells

Considerably less data are available about how helper T cells cooperate between each other. It is likely that some if not all the mechanisms previously described also apply in this context. Using somatic transgene immunisation to achieve linkage expression of antigenic determinants on B cells (used as APC), it was demonstrated that the co-expression of a dominant epitope can raise the response to a subdominant/cryptic epitope to detectable levels. In absence of the dominant epitope, the response to the subdominant/cryptic epitope could be partially retrieved using activating antibodies for CD40 and OX40 (Gerloni et al., 2000). These results suggested that paracrine (cytokines such as IL-2; Malek, 2002) and/or other epicrine factors are also involved in the linked cooperation between CD4+ T cells, reflecting the findings by Lu et al. (2000) on the cooperation between CD4+ and CD8+ T cells.

A role of cytokines was demonstrated in vitro by the reconstitution of three-cell clusters using DC, naïve and polarised T helper cells (Schuhbauer et al., 2000). Polarised Th1 or Th2 cells were able to drive the differentiation of naïve T cells,
mediated by paracrine IFN-$\gamma$ or IL-4 respectively. Indeed, this effect was significantly enhanced upon linkage of both the polarised and naïve cells around the same DC and was abrogated using neutralising antibodies to these cytokines. This effect may also have an epicrine component, since Th1 can enhance IL-12 production by DC in an IFN-$\gamma$-independent contact-dependent manner (Ria et al., 1998), while Th2 are able to inhibit this IL-12 expression via IL-4 and IL-10 (Koch et al., 1996; Ohshima and Delespesse, 1997; Ria et al., 1998). In later experiments where irradiated mice were reconstituted with splenocytes and immunised with sheep red blood cells, it was shown that a greater number of cells injected lead to a mixed Th1/Th2 response while lower numbers preferentially induce a Th1 response (Ismail and Bretscher, 2001). This shift towards Th2 was attributed to a differential frequency of CD4+ T cells present in the injected splenocytes, suggesting that the Th2 responses required further cooperation between these cells.

1.3.3. Competition between T cells

Should the number of dendritic cells or the availability of antigen become limiting, T cells may compete with each other. Adoptively transferred OVA-specific CD8+ T cells were shown to compete with endogenous OVA responders (Kedl et al., 2000). A high number of transferred CD8+ T cells resulted in abrogation of previously detectable endogenous response, as assessed by tetramer staining; and the injection of extra APCs in mice rendered this response detectable again. Competition was observed between CD8+ T cells specific for the same or different antigens present on the same MHC by the same APC (Kedl et al., 2000). Some degree of competition was also observed between CD8+ T cells specific for different peptide/MHC complexes (Grufman et al., 1999; Kedl et al., 2002) but another group found no evidence for such competition (Probst et al., 2002). Intraclonal competition was also described in the context of CD4+ T cells, resulting in relatively reduced clonal expansion when CD4+ T cells were present at high precursor frequency (Smith et al., 2000b; Laouar and Crispe, 2000). However, no evidence was found for interclonal competition between CD4+ T cells.

Competition between clones that are specific for a given antigenic peptide is likely to be determined by the affinity of the TCR for the MHC-peptide complex. On the one
hand, high affinity CD8+ T cells gain advantage over low affinity ones, because they can interact longer with APCs and deprive them of peptide/MHC complexes, through a process of transfer from APC to T cell (Kedl et al., 2002). As a consequence, APCs that have first interacted with high affinity CD8+ T cells become subsequently less capable of stimulating other CD8+ T cells, a mechanism believed to favour affinity maturation within the pool of responding T cells. On the other hand, however, studies on the regulation of CTLA-4 recruitment suggest that the stronger the TCR stimulation, the more potent the CTLA-4-mediated inhibition (Egen and Allison, 2002). This type of counter-regulation may represent a mechanism by which the immune system ensure the polyclonality of the response. Overall, for optimal protective responses, the immune system may have to ensure that T cells with the highest TCR affinity are selected from each pool specific for a given epitope, so that efficient responses are directed against several related epitopes from a given antigen/pathogen.

1.3.4. Concluding remarks on T cell cooperation and competition

Cooperation between T cells is an important, sometimes indispensable, process that ensures the efficacy of immune responses. Although T cell activation has been extensively documented, much less is known about how this activation is influenced by the concomitant activation of other T cells. Studies reviewed in this section have shown that the presentation of related antigens can lead to cooperation and/or competition depending on the T cell populations involved (i.e. CD4+ or CD8+), their respective TCR affinity and the immunogenicity of the different epitopes presented. These mechanisms have been poorly examined in the in vivo context, in particular regarding CD4+ T cells, and may be further dissected by the introduction of well-characterised antigens into DCs, related (i.e. linked) or not, using PMDD.
1.4. DNA vaccines

DNA vaccines have been shown to efficiently elicit responses in both CD4+ and CD8+ T cells. In addition, PMDD may constitute a useful tool for investigating the in vivo interactions between T cells, because it directly transfects cells, allows no or little antigen transfer and can flexibly incorporate regulatory molecules.

1.4.1. Origins and concepts in DNA vaccines

Intramuscular (i.m.) saline injection of plasmid DNA containing reporter genes can lead to durable expression (up to 2 months) of these genes in mice (Wolff et al., 1990). Similarly, gene gun was found to be efficient in transfecting mammalian cells with plasmid DNA in vivo (Yang et al., 1990) and subsequently in inducing immune responses (Tang et al., 1992). Initially used to transfect cells with hard cell walls such as plant cells, the gene gun as a method of vaccination is also known as particle-mediated DNA delivery (PMDD), or biolistic immunisation. It involves the bombardment into tissues (usually the skin) of DNA-coated gold micro-particles propelled in a jet of high-pressure helium.

An important feature of DNA vaccination is that it involves the uptake of plasmid DNA by APC. This DNA is then transported to the nucleus for transcription. Antigenic proteins are then expressed in the cytoplasm upon translation and processed onto class-I MHC. In addition, proteins secreted by transfected APCs and non-APCs can be endocytosed by APCs and loaded onto class-II MHC. Therefore, in contrast with conventional protein-based vaccines, DNA vaccines can potentially induce strong class I MHC-mediated cytotoxic responses while still generating effective humoral responses. Among various possible combinations of methods and routes for administering DNA, only few of them have been extensively investigated during the past decade, including injection of plasmid DNA in saline (i.m. and intradermal (i.d.) routes), PMDD and liposome encapsulation. The efficacy of these methods has been documented in numerous models (Robinson and Torres, 1997; Donnelly et al., 1997; Gurunathan et al., 2000; for reviews).
1.4.2. Doses and immunostimulatory properties of DNA

In a comparative study, PMDD was shown to be more effective than other methods of genetic vaccination, with a requirement for 250-2500 times less DNA required than the other routes (Fynan et al., 1993). In another study, significant immune responses were elicited with as little as 16 ng DNA per mouse while 5000-fold more DNA was required with i.m. or i.d. injections to achieve comparable responses (Pertmer et al., 1995). Finally, responses induced by PMDD in mice were much more reproducible than with i.m. injection (Yoshida et al., 2000).

However the nature of the immune response stimulated by the two methods is strikingly different. PMDD consistently favours Th2 responses irrespective of the target tissue (skin, muscle) while injection of DNA in either muscle or skin preferentially induces Th1 responses (Feltquate et al., 1997). It was proposed that this could be attributed to the different amounts of DNA delivered by the two methods. Plasmid DNA (of bacterial origin) has intrinsic immunostimulatory properties due to its content of unmethylated CpG motifs. These motifs act as PAMPs to activate APC (Tighe et al., 1998), by binding on TLR-9 (Hemmi et al., 2000; Bauer et al., 2001). Therefore, CpG-rich plasmids or high doses of DNA more efficiently induce the expression of IL-12, IL-18 and type I interferons, and promote Th1 immune responses (Roman et al., 1997; Leclerc et al., 1997). As a result, the inability of PMDD to induce strong Th1 responses may be due to insufficient amounts of CpG DNA administered. However, a recent finding suggested that some other effect inherent to the gene gun, such as the release of a mediator that suppress IFN-γ production in the skin, results in Th2 responses (Weiss et al., 2002).

1.4.3. Antigen localisation and transfer

Following injection, DNA has been found to be taken up by a variety of cell types present in the muscle tissue (Wolff et al., 1990) or in the dermis and epidermis (Raz et al., 1994). However, the direct inoculation of DNA into lymph nodes may lead to stronger CTL responses compared with the conventional routes mentioned before (Maloy et al., 2001). It remains controversial as to whether direct transfection or cross-priming is the major role for DC in DNA immunisation (Corr and Tighe, 1997;
Takashima and Morita, 1999). Cross-priming represents the transfer of antigen from non-APC to APC populations. In the case of i.m. DNA immunisation, it was found that CTL responses induced by DNA injection predominantly rely on cross-priming (Corr et al., 1999). This was consistent with the previous report that plasmid DNA which produces a cytoplasmic form of antigen induces poor CTL responses compared with a secreted or a membrane bound form when injected i.m. (Boyle et al., 1997). In contrast, others demonstrated that the uptake of DNA by APC after i.m. injection makes a greater contribution to the induction of CTL responses than the uptake of secreted protein (Whitton et al., 1999; Sbai et al., 2002). However, the best antibody production is only achieved using plasmids encoding secreted forms of antigen (Boyle et al., 1997; Whitton et al., 1999). Intramuscular immunisation may be less effective than PMDD in directly transfecting APCs, which may explain why much higher doses of DNA are required with the former. In addition, the muscle contains relatively few DCs and the DNA may be carried rapidly, presumably via the blood stream, to other tissues, where it can be picked up by DCs. This idea is supported by the observation that an antibody response is not abrogated if muscle tissue at the injection site is ablated after 10 min following injection of plasmid DNA (Torres et al., 1997).

Particle-mediated DNA delivery involves a completely different route of immunisation. Langerhans cells present in the epidermis (up to 5% of epidermal cells) are believed to play the major role as APC in the responses to PMDD. Gene expression lasts up to 12 days in keratinocytes but only two days in lymph node DCs (Akbari et al., 1999), so that the former can act as a reservoir of antigen for cross-priming. No primary antibody response is detectable if the target site is removed within 24 hours after immunisation, suggesting that this is the time needed for a sufficient number of transfected LCs to migrate out of the skin towards draining lymph nodes or for transfected keratinocytes to produce antigens for cross-priming (Torres et al., 1997; Klinman et al., 1998). Nevertheless, it has also been demonstrated that some cells, which have already migrated by 12 hours, are important for the memory response despite undetectable primary response (Klinman et al., 1998). As controversially as for DNA injection, PMDD was first reported to rely mostly on directly transfected DC for the initiation of good CTL responses (Condon et al., 1996; Porgador et al., 1998), however this was recently contradicted.
by a study demonstrating that genes under promoters specifically expressed in keratinocytes induce better CTL and antibody responses than genes under a promoter specifically expressed in DC and macrophages (Cho et al., 2001).

Numerous non-transfected DC also migrate to lymph nodes, because antigen-expressing cells constitute only 2-3% of migrating cells (Bot et al., 2000), resulting in only 50-100 transfected DCs per lymph node (Porgador et al., 1998). This suggests that the methods used, PMDD (Porgador et al., 1998), scarification (Akbari et al., 1999) or intradermal DNA injection (Bot et al., 2000), all lead to local damage and inflammatory signals that, in addition to CpG motifs present on the DNA (Ban et al., 2000), are sufficient to induce migration of many LCs. Transfected and non-transfected DCs may nonetheless exchange/acquire antigens later in the lymph node by direct transfer (Knight et al., 1998), by the mean of exosomes (Théry et al., 2002; for review), or by engulfment of apoptotic bodies from dying transfected DC (Albert et al., 1998).

1.4.4. Prospects for DNA vaccination

1.4.4.1. Control of the expression of antigens

Advances in vector design have brought new prospects in improving and controlling gene expression for vaccination purposes. Non-integrating non-replicating plasmids guarantee transient expression and meet safety and ethical issues. A choice can be made between strong promoters, such as cytomegalovirus (CMV), Rous sarcoma virus (RSV) or SV40 promoters, for high levels of transcription in all transfected tissues (Thompson et al., 1993), or more specific ones, such as a DC-specific CD11c promoter (Brocker et al., 1997).

The antigen can be expressed as a fusion with a ligand for targeting to a specific site (CD62L in lymph nodes) or to a specific type of cell (CTLA-4 for binding to B7 molecules on DC) (Boyle et al., 1998). The targeting of the antigen after expression is an effective way to elicit a desired type of immunity. For example, cytoplasmic localisation of OVA antigen is best for CTL-mediated immunity upon i.d. but not i.m. DNA injection (Boyle et al., 1997). In contrast, targeting antigen for secretion is
essential for eliciting antibody production (Boyle et al., 1997; Whitton et al., 1999). To this end, the production of antibodies against normally non-secreted viral and bacterial antigens may be strongly enhanced by the use of an IgG signal sequence to induce secretion (Svanholm et al., 1999).

1.4.4.2. Use of immunoregulatory molecules as adjuvants

Adjuvants are often used in combination with vaccines in order to improve their efficacy. They may be employed to differentially activate DCs and modulate the nature (Th1 vs. Th2) of a response (e.g. cytokines, CpG), to preserve the native conformation of an antigen for better neutralising antibodies, to help cytosolic localisation of antigen for more efficient cellular responses, to target antigens towards professional APC’s specific receptors using coupled ligands-antigens and/or to allow a slow and continuous release of antigen in the body (Cox and Coulter, 1997). One such adjuvant used in this research, complete Freund’s adjuvant (CFA), provides a potent stimulus for the induction of cell-mediated immunity (Ke et al., 1995).

Genetic adjuvants are used for concomitant expression with antigens in order to augment the efficacy of the response or influence its type. The most widely studied genetic adjuvants (Pasquini et al., 1997, Scheerlinck, 2001; for review) are immunoregulatory molecules such as cytokines (Maecker et al., 1997; Kim et al., 1997), chemokines (Youssef et al., 1998) and/or costimulatory molecules (Corr et al., 1997; Iwasaki et al., 1997). The cytokine, for instance, may be fused with the antigen (Maecker et al., 1997), co-expressed on a different plasmid (Kim et al., 1998), or co-expressed on the same plasmid under control of its own promoter or an internal ribosome entry site (IRES). The alteration in the immune response is generally consistent with the bias expected from the known mechanism of action of those soluble and cell-surface mediators. If PAMPS can be expressed from plasmid DNA, such as bacterial toxins (Arrington et al., 2002), they can also constitute potent genetic adjuvants. As mentioned above, plasmid DNA may be used as an adjuvant by itself, by varying its content in CpG motifs. Immunostimulatory CpG DNAs may constitute a natural adjuvant of choice, because they can stimulate strong Th1/Th2
responses with minimal toxicity compared with other adjuvants (Weeratna et al., 2000).

The delivery of immunoregulatory molecules by PMDD can also be used to further dissect the mechanisms involved in T cell activation and cooperation. For example, specific inhibitory proteins may be co-expressed with antigens to evaluate the relative contribution of some of the numerous signals mentioned in this chapter.

Being still relatively recent method, no successful DNA vaccines has emerged from human clinical studies. However, the combination of conventional and genetic immunisation in prime-boost strategies is offering promising prospects (Ramsay et al., 1999). Many mechanisms related to the induction of immune responses by DNA are yet to be fully understood.
1.5. Introduction to the PhD project

This research has two major objectives. First, it aims to document the activation of CD4+ T cells, and the subsequent development of the two subsets Th1 and Th2, in response to DNA immunisation by gene gun (PMDD). The early responses of these CD4+ T cell subsets to DNA have not been characterised in detail before, as many of the previous studies have been mostly focused on late antibody responses and/or on cytotoxic effector responses. The use of two strains of TCR transgenic mice in an adoptive transfer systems allows a more sensitive and robust measurement of the CD4+ T cell responses, both in term of magnitude and type (i.e. Th1/Th2 balance). Parameters examined include timing, genetic background and dose of DNA. Other aspects such as the migration of cells before and after immunisation, and a comparison with a non-DNA type of immunisation, are also studied in this research. The results from this first part are shown and discussed in Chapters 3 (see also Creusot et al., 2001), 4 and 5.

Second, PMDD is also employed as a tool to introduce different antigens in vivo under different conditions. The goal of these studies is to investigate the interactions between two populations of CD4+ T cells of different antigen/MHC specificity in vivo. The two TCR-transgenic populations independently described in the first part are then adoptively transferred together into a compatible host and the behaviour of one population in the absence or the presence of the other one is assessed following PMDD. Parameters examined include linkage versus non-linkage (when the two antigens are expressed by the same DC versus by different DCs), polarisation (to see if polarised Th1 or Th2 cells can influence naïve T cells of a different clone) and precursor frequency (to check if the relative frequency between the two populations influences their interaction). The data obtained from these studies are contained in Chapters 6 and 7.
Chapter 2

Materials and methods
Note: Experiments related to Chapter 3 were conducted at GlaxoSmithKline R&D laboratories in Stevenage (UK) whereas other chapters summarise work performed at the Windeyer Institute of Medical Sciences, UCL. Material & methods apply to the latter unless otherwise specified.

2.1. Mice

2.1.1. Transgenic mice

DO.11.10 mice (BALB/c background, H-2^d haplotype), donors of the ovalbumin (OVA_{323-339}) epitope-specific transgenic T cells (Murphy et al., 1990), were originally obtained from Dr Ken Murphy (Washington University School of Medicine, St. Louis, MO, USA) and a colony was maintained at Bury Green Farm, GlaxoSmithKline, UK.

OVA-B10D2 mice (DO.11.10 backcrossed into B10D2, H-2^d haplotype) were provided at the 9^{th} backcross stage by Dr Thomas Kamradt (Deutsches Rheumaforschungszentrum, Berlin, Germany) and bred with B10D2 mice for a further 3-5 backcrosses before breeding with B10A for experiments. Offspring from OVA-B10D2 x B10D2 and OVA-B10D2 x B10A breedings were screened by FACS staining of TCR clonotype on peripheral blood lymphocytes.

5CC7 rag2^{-/-} mice (B10A background, H-2^k haplotype), carrying the pigeon cytochrome C (PCC_{88-104}) epitope-specific TCR transgene (Berg et al., 1989), were given by Dr William Paul (NIH, Bethesda, Maryland, USA). As 5CC7 rag2^{-/-} mice are homozygous, offspring from 5CC7 rag2^{-/-} and 5CC7 rag2^{-/-} x B10D2 breedings do not required screening for TCR expression.

OVA-B10D2, OVA-B10D2 x B10A F1, 5CC7 rag2^{-/-} and 5CC7 rag2^{-/-} x B10D2 F1 were bred in UCL facilities (the last two in pathogen-free isolators).

DO.11.10, OVA-B10D2 x B10A F1 and 5CC7 rag2^{-/-} x B10D2 F1 mice were used as donors at 8-12 weeks of age.
2.1.2. Recipient mice

BALB/c mice were obtained from Charles River UK (Chapter 3) or Harlan UK (Chapter 4). B10A, B10D2 and B10D2 x B10A F1 mice were purchased from Harlan UK. All recipient mice were used at 6-10 weeks of age and experiments were carried out under UK ethical guidelines.

2.2. Plasmids

2.2.1. The pVAC1 family of vectors

pVAC1 (4627 bp, vector with no insert; see Figure 2.4) and pVAC1.OVA (6502 bp, expressing the whole ovalbumin protein; Figure 2.1) were kindly supplied by Dr Michelle Young and Dr Ian Catchpole (GlaxoSmithKline, UK). pVAC1 vectors are pCI-based and contain a CMV minimal promoter, IRES and SV40 poly(A) sequence.

2.2.2. Synthesis and cloning of the PCC gene

The amino acid sequence of PCC is available on PIR database (Accession ref. CCPY), however the DNA sequence has not been established. Since only the epitope region differs from the chicken cytochrome C (PIR, accession ref. CCCH; Limbach and Wu, 1983; Figure 2.2), we used the DNA sequence from the latter (Genebank accession number K02303; Limbach and Wu, 1983) and applied suitable nucleotide changes to match the PCC protein sequence. The PCC fragment encoding PCC protein was designed with an IgG signalling sequence incorporated upstream (Figure 2.3), divided into 20 overlapping oligonucleotides using the Calgene programme and synthesised using a method by Richard Hale (Affymax).

The PCC fragment was assembled region by region in two PCR steps starting with about 8 oligonucleotides at a time. The PCR mixture (100 µl total) for the oligonucleotide assembly step contained 10 µl pfu buffer (Stratagene), 10 µl dNTPs (dATP, dCTP, dGTP, dTTP, each at 10 mM, Boehringer Mannheim), 1 µl of equally mixed oligonucleotides (100 µM, Genosys) and 2 µl pfu Turbo polymerase
Figure 2.1. Map of pVAC1.OVA

Figure 2.2. Protein sequence of chicken cytochrome C. The 3' region (in bold) is the only part that differs from the pigeon cytochrome C sequence. In the latter, this region (shown below) corresponds to the immunogenic epitope PCC\textsubscript{88-104}. 
Figure 2.3. DNA sequence (coding strand 5’>3’) of the PCC fragment (before cloning), showing the signal sequence (underlined orange), the PCC88-104 epitope (underlined purple) and the rest of the PCC protein (underlined red). Start codon (green), stop codon (red) and Xhol sites (blue) are also indicated. Oligonucleotides (pcc1 to pcc20), used for the gene synthesis, are displayed above (coding 5’>3’ strand) and below (non-coding 3’<5’ strand) the sequence.
Chapter 2: Material and Methods

(Stratagene). The reaction was done as follow: 2 mins at 40°C, 10 secs at 72°C and 40 cycles (15 secs at 94°C, 30 secs at 40°C, 2 mins + 2 secs/cycle at 72°C). The PCR mixture (100 μl total) for the gene recovery step contained 10 μl pfu buffer, 10 μl dNTPs, 1 μl of each primer (oligonucleotides that correspond to the 5' end of each strand), 20 μl of assembly product and 2 μl pfu Turbo polymerase. The reaction was carried out in 30 cycles (45 secs at 94°C, 2 mins at 72°C) followed by 10 mins at 72°C. For the recovery of larger fragments, the polymerisation temperature was lowered to 55°C.

The final fragment was then purified and cloned into pVAC1 using two XhoI sites (Figure 2.4) to create pVAC1.PCC (5034 bp). The vector was checked for correct sequence inserted in the right direction. All plasmids were propagated in E. coli DH5α strain and prepared using Endofree Maxiprep kits (Qiagen) for PMDD, resulting in less than 0.1 endotoxin units per μg DNA.

2.3. Cartridge preparation for PMDD

2.3.1. Standard method

The preparation of cartridges was based on previously published method (Eisenbraun et al., 1993; Pertmer et al., 1995). Gold particles (2 μm diameter from DeGussa Metals Group, South Plainfield, NJ, USA) were sonicated in the presence of 0.05 M spermidine (Sigma) 2-3 times for 30 sec. Plasmids were added in required volume to achieve desired DNA loading rate (DLR, typically 2 μg per mg gold), followed by 1 M calcium chloride (American Pharmaceutical Partners Inc., Los Angeles, CA, USA). The volume of spermidine and calcium chloride was slightly higher than that of DNA to ensure efficient precipitation on gold particles. After thorough mixing, the suspension was left to rest for 10 min. The DNA-coated gold particles were washed three times in absolute ethanol and resuspended in absolute ethanol (0.114 ml / mg gold) containing 0.05 M polyvinylpyrrolidone (PVP, Sigma). They were injected and adsorbed onto the inner surface of Tefzel tubing (TFX Medical Inc. Jaffrey, NH, USA) by centrifugal force using a tube turner device (Barnant Co, Barrington, IL, USA). The Tefzel tubing was left to dry and then cut into 0.5 inch (1.27 cm) length.
Gradual assembly by PCR / gel purification cycles

Figure 2.4. Assembly of the PCC fragment and its cloning into pVAC1.
cartridges, which were stored desiccated at 4°C. One cartridge typically contains 0.6-0.8 μg of plasmid DNA, as assessed by spectrophotometric analysis (Genequant II, Pharmacia Biotech.) of DNA eluted from 2-4 cartridges per preparation (50 μl TE / cartridge). In experiments in which the amount of antigen DNA was varied, the total DLR was maintained at 2 μg per mg gold using empty vector.

2.3.2. Linked and unlinked preparations

In experiments involving delivery of two antigens simultaneously (Chapters 6 and 7), the two relevant plasmids were either coated on the same gold particles (linked) or in different gold particles (unlinked) in the same cartridge. A schematic representation of the different preparations described in this section can be viewed in Figure 2.5.

A “full dose linked” preparation was obtained by mixing pVAC1.PCC and pVAC1.OVA in a ratio 1:1 for the DNA number of copies (i.e. 44% and 56% of the DLR respectively) and co-precipitating on gold particles to achieve 2 μg DNA per mg of gold. A “half dose linked” preparation was achieved by mixing in 1:1 volume ratio a full dose linked preparation with one made exclusively with pVAC1. For “unlinked” preparations, first pVAC1.PCC and pVAC1 (44% and 56% respectively) were mixed and co-coated as above. In parallel, pVAC1.OVA and pVAC1 (56% and 44% respectively) were co-coated likewise. After 3 washes and resuspension in the final volume of ethanol/PVP, the two preparations were mixed in a 1:1 volume ratio. Full and half OVA-control preparations were made exactly like the full and half linked preparations, except that pVAC1.PCC was replaced by pVAC1.

2.4. DNA loading onto gold particles

The coating of different DNAs on gold particles was analysed by PCR. Initial calculations, based on the amount of DNA effectively coated, were carried out to determine the number of copies of each DNA species that could be expected on an individual bead (Section 2.4.1). Two methods were then developed (Sections 2.4.2 and 2.4.3) for isolation of single bead for PCR (Section 2.4.4).
Figure 2.5. Schematic representation of various preparations for the co-delivery of linked or unlinked antigens by PMDD.
2.4.1. Preliminary calculations and estimations

With a density of 19.6 g/cm$^3$ and an average diameter of 2 μm, we have calculated that a single gold particle would be 4.19 μm$^3$ in volume, 12.56 μm$^2$ in surface and 8.21x10$^{-8}$ mg in weight. Consequently, 1 mg of gold should contain some 1.22x10$^7$ particles.

Statistics done on several cartridge preparations reveal that effective loading rates were 0.72 ± 0.07 μg DNA per cartridge (n=40) and 0.70 ± 0.05 mg gold per cartridge (n=23), which imply a effective DLR of 1.06 ± 0.13 μg DNA / mg gold (n=23) recovered out of 2 μg DNA / mg gold initially added. Considering a minimal effective DLR of 1 μg DNA / mg gold achieved and that 1 bp equals 660 pg / pmole, the average number of plasmids per bead can be estimated as shown in Table 2.1.

2.4.2. Limiting dilution method

A suspension of DNA gold particles with a minimal effective DLR of 1 μg DNA / mg gold contains at least 8.8 μg DNA /ml ethanol. 456 μl of this suspension, containing approximately 5x10$^7$ beads and at least 4 μg DNA, was centrifuged and resuspended in 150 μl ethanol. Ten-fold dilutions in ethanol were then carried out down to 50 beads/tube. Tubes were thoroughly vortexed before each pipetting because gold particles do not remain in suspension and settle quickly. Suspensions containing between 5x10$^4$ and 50 beads were diluted 1:10 in water to obtain between 5x10$^3$ and 5 beads/tube. Immediately after each dilution in water, 15 μl (a tenth) was transferred into a PCR tube. Tubes (i.e. containing between 500 and 0.5 beads) were heated in order to remove traces of ethanol, before other PCR components were added.

2.4.3. Micromanipulation method

Glass micropipettes were prepared using a pipette puller. Some gold suspension (5 μl) was spread onto a glass slide. After ethanol evaporation, single beads were selected under a microscope, picked up by micromanipulated pipettes and gently
<table>
<thead>
<tr>
<th></th>
<th>pVAC1</th>
<th>pVAC1.PCC</th>
<th>pVAC1.OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (bp)</td>
<td>4627</td>
<td>5034</td>
<td>6502</td>
</tr>
<tr>
<td>pg / pmole</td>
<td>3.05x10^6</td>
<td>3.32x10^6</td>
<td>4.29x10^6</td>
</tr>
<tr>
<td>pmole / mg gold</td>
<td>0.328</td>
<td>0.301</td>
<td>0.233</td>
</tr>
<tr>
<td>mole / particle (x10^-20)</td>
<td>2.688</td>
<td>2.467</td>
<td>1.91</td>
</tr>
<tr>
<td>copies / particle</td>
<td>16190</td>
<td>14859</td>
<td>11504</td>
</tr>
</tbody>
</table>

**Table 2.1.** Estimation of the average number of copies per gold particle for different plasmids used in this study.
transferred into PCR tubes containing 5 µl water (Figure 2.6). A bead was able to stick firmly to the tip of the pipette, which was purposely crushed against the bottom of the tube to ensure that the bead remained in the tube. No suction was involved in the process to avoid mixing the bead with other fluids such as silicon, which may have an effect on PCR. Electric charges may be involved in the attachment of the bead to the pipette.

2.4.4. PCR

Each PCR tube contained 16 µl water (or 6 µl in limiting dilution experiments), 2.5 µl PCR buffer 10x (Clontech), 0.5 µl dNTP mixture (dATP, dCTP, dGTP, dTTP, each at 10 mM, Boehringer Mannheim), 0.5 µl primer mix (each at 10 µM, Genosys or Invitrogen), 0.5 µl Advantage cDNA polymerase (Clontech) and 5 µl template (or 15 µl in limiting dilution experiments) for a final volume of 25 µl. The PCR reaction was executed in 30-35 cycles comprising 30 sec denaturation at 94°C and 3 min annealing/extension at 68°C.

PCR products (12 µl + loading buffer) were run on a 1% (first part) or 3% (second part) agarose gel (Sigma) containing ethidium bromide (Sigma) in TBE buffer at 100 volts. Markers (1 kb ladder) were purchased from Gibco.

2.5. Th1 and Th2 cultures

Spleen from OVA-B10D2 x B10A Fl mice were teased out in Hank's balanced salt solution (HBSS) between two glass slides previously sterilised with 70% ethanol. The resulting suspension was centrifuged at 1400 rpm and resuspended in filtered red blood cell lysis buffer (2.073g NH₄Cl, 0.251g KHCO₃, 0.011g EDTA in 250 ml H₂O, 1 ml per spleen) for 1-2 min. The tube was filled with HBSS and centrifuged again. The pellet was resuspended in 10 ml HBSS, debris left to settle and cells transferred into new tubes. Clumps were washed with 2 ml HBSS and further cells were transferred.
Figure 2.6. Gold particles and their micro-manipulation. Coated gold micro-particle were spread on glass slide. An isolated bead was selected (A) and picked up by pressing the micro-manipulated pipette on it (B). The pipette was then lifted (C) and examined to check that the bead was firmly attached to its tip (D) before transfer to the PCR tube.
Chapter 2: Material and Methods

After centrifugation, splenocytes were treated with 5 ml anti-murine CD8 per spleen (3.168 supernatant, gift from Dr Rose Zamoyska) for 30 min on ice. Cells were washed and resuspended in (per spleen) 2 ml plain RPMI medium + 120 μl reconstituted standard rabbit complement (Cedarlane Laboratories Ltd, Hornby, ON, Canada). They were then incubated for 20 min at 37°C with occasional shaking. After one wash with HBSS, cells were counted and cultured in 25 cm³ flasks at 37°C under 5% CO₂.

For Th1 cultures (per flask), 22x10⁶ cells were stimulated with 200 nM OVA peptide (OVA323-339, sequence ISQAVHAAHAINEAGR, Washington Singer Labs) in 11 ml Th1 medium, which consisted of complete RPMI medium (RPMI 1640, 10 mM L-glutamine, 100 units/ml penicillin/streptomycin, 5x10⁻⁵ M 2-ME), 10% FCS, 20 ng/ml human IL-2 (Peprotech) and anti-IL-4 mAb (11B11 supernatant, 1:6 dilution). Cells were split 1:2 in Th1 medium on days 2, 3 and 4 post-stimulation. Murine IL-12 (5 ng/ml, Peprotech) was added on days 3 and 4.

For Th2 cultures (per flask), 22x10⁶ cells were stimulated with 100 nM OVA peptide in 11 ml Th2 medium, containing complete RPMI medium, 20 ng/ml human IL-2, 20 ng/ml murine IL-4 (X63/IL-4 supernatant, batch concentration assessed by ELISA), anti-IFN-γ mAb (1.5 μg/ml, R&D Systems, on day 0; AN18 supernatant, 1:10 dilution on other days) and anti-IL-12 mAb (3 μg/ml, R&D Systems, on day 0; C15-6 supernatant, 1:10 dilution on other days). Cells were split 1:2 in Th2 medium on days 2, 3 and 4 post-stimulation.

Murine cell lines were gift from the National Institute of Medical Research (Mill Hill, London): X63/IL-4 cell line from Dr Brigitta Stockinger, AN18 and C15-6 cell lines from Dr Jean Langhorne. X63/IL-4 cells were cultured in IMDM medium supplemented with 5% FCS, 10 mM L-glutamine, 100 units/ml penicillin/streptomycin, 5x10⁻⁵ M 2-ME and 1 mg/ml G418.
2.6. FACS analysis & adoptive transfer

Naïve T cells were prepared from spleen as described in Section 2.5. Polarised T cells were washed in HBSS and pooled. All cells were counted and stained (0.4 x 10^6 per tube) for surface markers for 10 min (5 min for streptavidin step). PCC-specific transgenic T cells were stained with anti-\( \nu \beta 3 \)-PE (see Table 2.2 for details) and anti-\( \nu \alpha 11 \)-biotin mAbs / streptavidin-Quantum Red, while OVA-specific transgenic T cells were detected using anti-TCR clonotype KJ1.26-PE and anti-CD4-Tri Color mAbs. Cells were also stained with FITC-conjugated anti-CD62L, anti-CD69, anti-CD45RB mAbs or matching isotype control. Staining was done for 10 min at 4°C (5 min with streptavidin) in staining buffer (PBS, 10% rabbit serum, 0.1% sodium azide), whereas PBS, 0.5% BSA (Albumin bovine, NBS Biologicals), 0.05% sodium azide was used for washes. Analysis was performed on a FACScan (Becton Dickinson) using the Cellquest software (events counted were 5000 TCR-Tg T cells). The frequency of \( \nu \beta 3 +\ \nu \alpha 11 +\) or KJ1.26+ CD4+ within total cells was used to calculate the number of splenocytes required for a given number of transgenic T cells. These cells were injected in 0.2 ml into lateral tail vein of recipient mice (Chapters 5-7). In Chapters 3-4, 2.5x10^7 splenocytes from DO.11.10 or OVA-B10D2, or 1x10^7 splenocytes from 5CC7 rag2^{-/-} (~3x10^6 TCR-Tg CD4^+ T cells) were intravenously injected in 0.1 ml into syngeneic recipients. The frequency of transgenic T cells was checked afterwards by FACS (Coulter XL or FACScan) after staining as above.

In some experiments described in Chapter 5, washed cells were resuspended in warm HBSS containing 5 \( \mu \)M CFSE (CarboxyFluorescein diacetate Succinimidyl Ester, Molecular Probes) and incubated 10 min at 37°C. They were then washed in cold complete RPMI medium (10% FCS) before being resuspended in HBSS and inoculated as in above paragraph (see also Section 2.8.4).

In experiments involving the adoptive transfer of polarised T cells (Chapter 6), naïve, Th1 and Th2 cells were also restimulated for intracellular staining (Section 2.8.3) in order to check their cytokine profile.
<table>
<thead>
<tr>
<th>Specifity</th>
<th>Conjugate / usage</th>
<th>Clone</th>
<th>Isotype</th>
<th>Per test (pg)</th>
<th>Source</th>
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Table 2.2. Antibodies and other reagents used for FACS analysis, ELISA, ELISPOT and in vitro neutralisation in cultures.
2.7. Immunisations

Recipient mice (3 per group) were shaved on their abdomen and given one shot on each side by PMDD (as on Figure 2.7, panel A). Helium pressure, used to propel DNA-coated gold particles at high velocity, was set up at 500 psi. Particle-mediated DNA delivery (typically ~1.5 μg total DNA per mouse) was conducted with either the PowderJect XRI device (formerly Accell, PowderJect Vaccines Inc., WI, USA) in Chapter 3 or the Helios gene gun (Bio-rad; Figure 2.7, panel B) kindly provided by Dr Jeremy Brockes (Department of Biochemistry, UCL).

In some experiments, groups of mice were also immunised with 100 μg ovalbumin (Sigma) emulsified in CFA (Sigma) and subcutaneously injected in 0.1 ml at the base of the tail (Chapter 3).

2.8. FACS analysis

2.8.1. Preparation of lymph node cells

On day 3 and/or 5 post-immunisation, inguinal and periaortic lymph nodes were collected from groups of three mice, pooled and squashed between two sterilised slides. Lymph node cells were prepared as described in section, without the lysis step, and finally resuspended in complete RPMI medium (10% FCS). Apart from a small fraction kept for flow cytometry analysis (Section 2.8.2), the suspension was used for ELISPOT (Section 2.11) and/or intracellular cytokine staining (Section 2.8.3).

In Chapter 3, draining lymph nodes were not pooled and suspensions were made from individual mice. After samples were removed for FACS analysis, suspensions from the same group were pooled before other assays. Cells were finally resuspended in complete RPMI medium with 10% FCS for ELISPOT (Section 2.11) and/or intracellular cytokine staining (Section 2.8.3) or with 0.5% syngeneic mouse serum for T cell proliferation assay (Section 2.9) and ELISA (Section 2.10).
Figure 2.7. Sites of DNA administration on the abdomen of mice (A) and Helios gene gun from Bio-rad (B).
2.8.2. Clonal expansion and activation markers expression

Samples (0.4x10^6 per tube) were assessed for both PCC-specific and OVA-specific T cells in parallel, stained using the protocol described in Section 2.6 and analysed by flow cytometry (100,000 total events counted). The frequency of TCR-Tg T cells, reflecting clonal expansion, is expressed as the percentage of vβ3+ αβ1+ or KJ1.26+ CD4+ within the lymphocyte population (gated according to forward and side scatter). Marker expression (CD69, CD62L, CD45RB) is determined as the percentage of marker positive cells (compared with isotype control) within either the vβ3+ αβ1+ or the KJ1.26+ CD4+ population.

In Chapter 3, individual samples were stained with FITC- or PE-conjugated KJ1.26 (0.2 µg, Caltag), anti-CD4-QuantumRed (0.5 µg, Sigma) and either anti-CD69-FITC (0.4 µg, Pharmingen) or anti-CD25-PE (0.5 µg, Pharmingen) mAbs. Flow cytometry analysis was conducted on a Coulter XL.

2.8.3. Intracellular cytokines

Two protocols for intracellular staining were used, with small differences:

1x10^6 cells from Th1 or Th2 cultures (or from OVA-B10D2 x B10A F1 spleens for naïve cells) were mixed with 1x10^6 cells from B10D2 x B10A F1 spleen in 1 ml complete RPMI medium (10% FCS), containing 1 µM OVA peptide and 2 µM monensin (Sigma). Two tubes of each group were incubated for 4 hours at 37°C under 5% CO₂, then split into 4 tubes and washed. Cells were stained with KJ1.26-PE and anti-CD4-TriColor (as in Section 2.6) for 10 min at 4°C, washed, fixed with 4% formaldehyde for 10 min at 4°C, washed and kept overnight at 4°C. Next, they were washed in saponin buffer (PBS, 0.5% BSA, 0.5% saponin (Sigma), 0.05% sodium azide), stained for 20 min at 4°C with either anti-IFN-γ, anti-IL-4, anti-IL-2 (all 0.5 µg, Caltag) or isotype-matching control, all FITC-conjugated mAbs. Finally, they were washed twice in saponin buffer, once in normal wash buffer and analysed on FACSscan.
For experiments described in Chapter 3, 2x10^6 cells were restimulated for 4 hours at 37°C in 1 ml complete medium containing 10% FCS, 1 µM OVA peptide, 4 µg/ml anti-CD28 (Pharmingen) and either 10 µg/ml brefeldin A (Sigma) for IFN-γ and IL-2 or 2 µM monensin for IL-4. Cells (1x10^6 per tube) were then stained for 10 min with FITC- or PE-conjugated KJ1.26 (0.2 µg, Caltag) and anti-CD4-QuantumRed (0.5 µg, Sigma). They were washed and fixed overnight at 4°C (Caltag fix&perm kit). They were then permeabilised (Caltag fix&perm kit) and stained for 20 min at 4°C with either anti-IFN-γ-FITC (0.2 µg, Caltag), anti-IL-4-PE (0.5 µg, Caltag), anti-IL-2-FITC (1 µg, Pharmingen) or with matching isotype control. Finally, they were washed twice and examined on a Coulter XL.

2.8.4. Measurement of cell division in CFSE-stained T cells

Undivided cells retain a maximal Fl-1 fluorescence, similar to the initial level of fluorescence (Fo), however, since cells tend to lose some fluorescence with time, Fo is measured on undivided cells. Cells that have divided have lost CFSE (1:2 dilution between daughter cells at each division). Cells that have divided n times have a fluorescence reduced to the value F and the number of division is calculated by n = ln(Fo/F) / ln(2).

2.9. T cell proliferation

4x10^5 lymph node cells were restimulated in vitro in triplicate with OVA peptide (concentrations ranging from 0 to 1 µM). After approximately 54 hours incubation at 37°C under 5% CO₂, they were pulsed with tritiated thymidine (33.55 KBq/well) for a further 15 hours before cell harvesting and measurement of cell-incorporated radioactivity by scintillation counting (Wallac Betaplate Counter). Background proliferation, obtained from cultures with no peptide, was subtracted.
2.10. Cytokine secretion (ELISA)

4x10^5 cells were cultured in vitro as above with 1 μM OVA peptide and supernatant was collected from 1 to 5 days after restimulation (one triplicate per day) and frozen. On the day prior to analysis, Maxisorb 96-well plates (Nunc) were coated with 50 μl of capture antibody (anti-IL-2, anti-IL-5 and anti-IFN-γ at 2 μg/ml, anti-IL-4 at 1 μg/ml). On the day of analysis, plates were blocked for at least 1 hour with PBS, 2% BSA. Cytokine standards (Pharmingen) were used in doubling dilution from 2.5 ng/ml (5 ng/ml for IFN-γ). Samples for analysis were thawed and used in triplicate at 1:2 dilution for IL-4 and IL-5, and at 1:5 dilution for IL-2 and IFN-γ. Both standards and samples were incubated for 2 hours. Captured cytokines were detected by incubating with biotinylated antibodies (50 μl at 0.5 μg/ml for IL-4 and IL-5, 1 μg/ml for IL-2 and 2 μg/ml for IFN-γ) for 2 hours. Plates were then treated with streptavidin-conjugated horse radish peroxidase (1:2000 dilution, 50 μl/well, Caltag) for 30 min. Finally, TMB substrate (tetramethyl benzidine, Sigma) was added (50 μl/well) and plates were put in the dark. After 30 min, the reaction was stopped with 50 μl/well sulphuric acid 0.25M and absorbance was read at 450 nm on a SpectraMax 340 reader. Data were analysed by the SoftmaxPro program.

2.11. Cytokine-producing cells (ELISPot)

Multiscreen 96-well filtration plates (Millipore) were coated with capture antibodies for IFN-γ, IL-2, IL-4 or IL-5 (750 ng/well, Pharmingen or eBioscience) overnight. On the day of analysis, plates were blocked with complete RPMI medium (10% FCS), then lymph node cells (density ranging from 1 to 8 x 10^5 cells/well) were added in complete RPMI medium alone or containing either 1 μM PCC peptide (PCC88-104, sequence KAERADLIAYLKQATAK, Avecia) or 1 μM OVA peptide. All the above was conducted under sterile conditions. After 24 hours incubation at 37°C and 5% CO₂, plates were incubated for 2 hours with biotinylated detection antibodies (50 ng/well, Pharmingen), 2 hours with streptavidin-conjugated alkaline phosphatase (Caltag) and finally with substrate (AP conjugate substrate kit, Bio-Rad) until spots clearly appear (~10 min). At least 3 washes with PBS were performed between each step. Spots were counted using the Eli 2.6/2.9 software on AID plate.
reader (Autoimmun Diagnostika GmbH) or an image analysis system developed at GlaxoSmithKline (Chapter 3). The number of spots was normalised per million cells for all densities. Processed data were only those from densities that gave similar optimal results (within standard deviation) and were shown after subtraction of the background (no peptide).

2.12. Statistical analysis

Student’s $t$-test was performed in experiments that have been repeated at least three times for all groups. Symbols: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. 
Chapter 3

CD4+ T cell responses to PMDD in an adoptive transfer model in BALB/c mice
Chapter 3: CD4+ T cell responses to PMDD in BALB/c mice

3.1. Introduction

Although vaccines have proven for more than a century to provide an effective protection against many infectious agents, current developments open new prospects and challenges for the future.

On the one hand, research in the field aims to achieve protection against a variety of new threats such as human immunodeficiency virus (HIV), hepatitis C virus, Ebola, biological weapons (e.g. anthrax) and the resurgent tuberculosis. On the other hand, it also tries to understand how the immune system is regulated and to find new ways to better control those mechanisms. This basic research is important to improve both memory responses (essential for prophylactic vaccines) and effector functions (important for therapeutic vaccines). The complexity of the immune system, particularly with its numerous cell types and the various specialisation paths they can follow, makes the job difficult. However, being able to better control the immune system may offer a more attractive alternative to some current drugs, chemotherapy, radiotherapy and other invasive and unpleasant treatments. New hopes are relying on therapeutic vaccines to reinforce specific immunity against chronic infections and tumours, or conversely to subvert, attenuate or stop the aggressive immunity implicated in autoimmune diseases such as diabetes, arthritis and multiple sclerosis.

In the recent field of DNA vaccines, a lot of questions remain to be elucidated. How is the balance between the cellular versus the humoral arm of the adaptive immunity set up in response to DNA? How can we modulate this balance to make it more appropriate? What key cell types need to be educated for this purpose? At what stage of the course of the immune response should we intervene in order to achieve the desired outcome? What improvements are required so that vaccination efficiency in humans, so far unsatisfactory, would equal that obtained in rodents?

In order to approach some of these questions, an \textit{in vivo} mouse model was developed to document in detail the activation of antigen-specific CD4+ T cells in response to particle-mediated DNA delivery, as described in this chapter. A panel of techniques was used to measure, at a single-cell level, those cytokines that reflect the relative contribution of Th1 and Th2 cells emerging from a pool of naive CD4+ T cells. The
evolution of these cells was followed within the early phase of the response (from day 1 to day 6), since cytokines secreted during this period play a critical role in determining the differentiation of CD4+ T cells. For comparison purpose, the response to a "more conventional" type of immunisation was assessed in parallel, namely antigen (ovalbumin) emulsified in CFA, one of the most potent adjuvants available.

In this model, cells from a donor mouse expressing a transgenic α/β TCR specific for the OVA\textsubscript{323-339} epitope of ovalbumin presented on I-A\textsuperscript{d} class II MHC are transferred into syngeneic recipients (Pape 	extit{et al.}, 1997a). The rational for the adoptive transfer is to dilute the frequency of the TCR-T cells. A frequency of approximately 0.5-1% of lymphocytes in lymph nodes is sufficiently high to allow the unambiguous identification at a single cell level of the TCR clonotype (here, KJ1) by flow cytometry, and to follow with good sensitivity the changes in surface markers and cytokine profile on these cells. At the same time, the frequency is low enough to approximate more closely to a realistic model of a "natural" immune response. Since the TCR-Tg T cells are in any case diluted in the normal cells of adoptive hosts, no extensive steps of purification of the TCR-Tg T cells were carried out prior to transfer as in other studies (De Smedt 	extit{et al.}, 2002; Diehl 	extit{et al.}, 2002), thus minimising any artefactual modification from \textit{ex vivo} handling.

3.2. Results

3.2.1. Rapid up-regulation of activation markers precedes clonal expansion

The frequency (Figure 3.1, panel A) of adoptively transferred KJ1+ CD4+ cells in draining lymph nodes (inguinal and periaortic) was measured at various days after immunisation with DNA or protein/CFA. Following PMDD, the frequency of clonotype positive CD4+ T cells remains at baseline levels (1%) for three days and then increases sharply between days 3 and 4 post-immunisation, reaching a maximum of ~6% of total lymphocytes on days 4-5. Immunisation with ovalbumin/CFA also induces a rapid clonal expansion, starting approximately 1 day earlier and reaching a higher maximum level (~10%). The proportion of KJ1+ CD4+ cells also declines more rapidly in the latter group and is very similar at days 5-6 (~4-
Figure 3.1. Clonal expansion of KJ1+ CD4+ cells in lymph nodes (A) and proportion of them expressing either CD25 (B) or CD69 (C). Three-colour analysis was performed by flow cytometry on inguinal and periaortic lymph node cells at different time points after immunisation. Analysis on day 0 was performed on splenocytes before transfer. Each symbol is the mean from 3-4 mice within an independent experiment, except for the non-immunised group (1 mouse / experiment). Lines join the mean of all experiments for each group and each time point. T-test on day 3: differences between pVAC1.OVA group and ovalbumin/CFA group are significant (p<0.005) in all panels; pVAC1.OVA group is significantly different from pVAC1 and non-immunised groups (p<0.005) in all panels; ovalbumin/CFA group is significantly different from pVAC1 and non-immunised groups in panels A (p<0.005) and B (p<0.05).
6%) in both experimental groups. In both control groups (non-immunised and immunised with empty vector), this proportion of KJ1+ CD4+ cells remains at the baseline level throughout the period measured.

The clonal expansion of KJ1+ CD4+ cells was preceded by up-regulation of both CD25 (IL-2 receptor α chain) and CD69 (an early T cell activation marker). Increased expression of both CD25 and CD69 (Figure 3.1, panels B and C, respectively) could be observed within 24 hours of immunisation for both DNA and protein, with maximal levels (~40% and 65-70% of KJ1+ CD4+ population, respectively) reached on the day prior to significant clonal expansion. The maximum proportion of KJ1+ CD4+ cells expressing each marker was approximately the same for both immunisation protocols, although, as for clonal expansion, the response to DNA appeared to be slower. The levels of CD25 returned to baseline levels within 1-2 days after the peak of expression. In contrast, CD69 expression remained high throughout the time-course of the experiment. The levels of CD69 appeared to be sensitive not only to the immunisation, but also to the adoptive transfer procedure itself, since expression rose and remained high in the controls.

3.2.2. Ex vivo proliferative capacity of in vivo primed TCR-Tg T cells

The proliferation of lymph node cells ex vivo following immunisation with DNA or protein is shown in Figure 3.2. In the absence of peptide (Figure 3.2, panel A), no proliferation was observed in any group, demonstrating that cells undergoing clonal expansion in vivo did not continue to proliferate ex vivo in the absence of further exogenous antigen. However, in vivo activation by antigen was an essential prerequisite for ex vivo proliferation, because despite the high precursor frequency of antigen-specific T cells provided in this adoptive transfer model (~1% of lymph node cells), negligible proliferation occurred in response to peptide in the control vector immunised group. In vivo activation, rather than clonal expansion, was necessary for ex vivo proliferation because, in the presence of 1 or 10 nM OVA peptide (Figure 3.2, panels B and C respectively), considerable proliferation could be measured in both DNA and protein immunised groups as early as day 2 post-immunisation, and before any increase in the number of KJ1+ CD4+ cells could be detected in vivo (Figure 3.1, panel A). In contrast with clonal expansion, the time course of ex vivo
Figure 3.2. Proliferation of lymph nodes cells following restimulation with cognate peptide. Lymph node cells were cultured in the absence (A) or in the presence of 1 nM (B) or 10 nM (C) OVA peptide, without additional antigen-presenting cells, for ~54 hours. Tritiated thymidine was then added, and ~15 hours later, incorporated radioactivity was measured. Each symbol represents an individual experiment (mean of a triplicate culture). Each line joins the mean of all experiments for each group. T-test on day 3 (panels B and C): pVAC1.OVA and ovalbumin/CFA groups are similar, and both significantly different from pVAC1 (p<0.005).
Chapter 3: CD4+ T cell responses to PMDD in BALB/c mice

proliferation following pVAC1.OVA immunisation and protein/CFA was similar. However, proliferation to 1 nM peptide was higher in the DNA-immunised group than in the protein-immunised group. The same pattern was expected with 10 nM peptide, but it was obscured since proliferation reached a plateau (at ~500,000 ccppm), which may have been due to the limitations of culture conditions in the in vitro thymidine uptake assay. Higher concentrations (100 nM or more) did not lead to reliable data also probably because of this limitation (data not shown).

Although the same number of lymph node cells was added to each well, the frequency of KJ1+ CD4+ cells was variable between groups and time points. On day 3 post-immunisation for instance, proliferation was identical for DNA or protein/CFA, although the frequency of responders (Figure 3.1, panel A) and the proportion of CD25+ cells (Figure 3.1, panel B) were reversed between the two groups. Thus, proliferative capability may have reflected a combination of both the frequency of KJ1+ CD4+ cells and, within them, the proportion of CD25+ cells. Proliferation of lymph node cells taken on days 1-3 post-immunisation in response to IL-2 (0.4 ng per 4x10^5 cells), instead of OVA peptide, gave a profile of proliferation similar to CD25 expression (data not shown), confirming the importance of functional IL-2 receptor.

3.2.3. Abundant and prolonged production of interleukin-2

The in vivo clonal expansion (Figure 3.1, panel A) and the ex vivo proliferation (Figure 3.2) are likely to be driven in large part by IL-2. Lymph node cells cultured ex vivo in the absence of antigen were unable to generate any measurable IL-2 (data not shown). Restimulation with antigen, however, revealed that following immunisation, lymph node T cells rapidly acquire the potential to produce and secrete IL-2 (Figure 3.3). After 3 days restimulation, IL-2 concentration fell, presumably because of consumption in culture. Therefore, data shown on Figure 3.3, panel A, are from day 2 post-restimulation. Remarkably, the time course and magnitude of the IL-2 response were almost identical for DNA and protein/CFA immunisations. IL-2 can be readily detected 2 days following immunisation with either immunogen, and therefore precedes clonal expansion. IL-2 secreting capacity was maximal on days 3-5 and then decreased (Figure 3.3, panel A), although the
Figure 3.3. IL-2 production. Panel A: lymph node cells were restimulated in vitro with 1 μM OVA peptide. IL-2 concentration was then measured by ELISA in supernatants collected on day 2 post-stimulation. Panel B: lymph node cells were restimulated in vitro with 1 μM OVA peptide, anti-CD28 and brefeldin A for 4 hours. Three-colour analysis was performed to detect IL-2-expressing KJ1+ CD4+. Each symbol represents a pool of 3-4 mice within an independent experiment (mean of triplicate culture in panel A, measure on pooled suspension in panel B). Each line joins the mean of all experiments for each group. T-test on day 3: pVAC1.OVA and ovalbumin/CFA groups are similar, and both significantly different from pVAC1 (p<0.005) in both panels.
proportion of IL-2-producing cells amongst antigen-specific cells remained constant (~50%) up to day 6 (Figure 3.3, panel B). Background levels of IL-2 detected in the control group (pVAC1) may have been produced from some naïve antigen-specific T cells that encounter the antigen for the first time on restimulation.

3.2.4. Th1/Th2 cytokines are produced predominantly during the early phase of clonal expansion

Th1 and Th2-specific cytokines (IFN-γ and IL-4/IL-5 respectively) were measured at various time points after ex vivo restimulating cells from each in vivo time point. In contrast with IL-2, these cytokines can accumulate in the medium. IFN-γ appeared in culture on day 2 and its concentration remained stable for up to 5 days. IL-5 and IL-4 were produced in very low amounts. In particular, IL-4 appeared transiently on day 3 or 4 after restimulation and its concentration could not be accurately quantified by ELISA, as also reported by Rogers and Croft (2000), presumably because of rapid consumption. Data shown in Figure 3.4, panels A and B represent IFN-γ measured 3 days and IL-5 assessed 4 days post-restimulation, respectively.

Negligible cytokine production was seen, not only in absence of antigen during in vitro restimulation, but also in absence of in vivo priming with specific antigen, suggesting that, in contrast with IL-2, in vivo priming is required prior to any measurement of these cytokines ex vivo. However, following in vitro restimulation of in vivo primed T cells, transient production of IFN-γ and IL-5 was detected (Figure 3.4). For DNA immunisation, maximal production of both cytokines was observed on day 3 post-immunisation, at a time when the rate of clonal expansion and expression of activation markers appeared maximal (Figure 3.1). In contrast, for protein immunisation, maximal IFN-γ and IL-5 production (on day 2) and activation marker expression (on days 1-2) preceded detectable clonal expansion. Thus, similarly to the phenotypic markers, a delay of approximately one day in the production of IFN-γ could be observed with DNA compared with protein/CFA, although the maximal production reached was very similar. A similar delay was observed for IL-5 (Figure 3.4, panel B), although maximal production in response to DNA was lower and somewhat prolonged.
Figure 3.4. Secretion of IFN-γ and IL-5. Lymph node cells were restimulated in vitro with 1 μM OVA peptide. IFN-γ (A) and IL-5 (B) concentrations were measured by ELISA in supernatants collected on day 3 or day 4 post-stimulation, respectively. Each symbol reflects an individual experiment and is the mean of a triplicate culture of cells pooled within each group (3-4 mice/group). Lines join the mean of all experiments for each group. T-test on day 3: in panel A, pVAC1.OVA and ovalbumin/CFA groups are similar, and both significantly different from pVAC1 (p<0.05). In panel B, differences between pVAC1.OVA and the two other groups are significant (p<0.05), while ovalbumin/CFA and pVAC1 groups are similar.
3.2.5. The Th1/Th2 balance

The data presented in Figure 3.4 do not provide any information on the relative size of the Th1/Th2 cytokine-producing populations of KJ1+ CD4+ cells. We therefore compared two assays, which give quantitative data on the number of these cells, namely ELISFOT and intracellular staining measured by flow cytometry. Day 3 post-immunisation was a time point of choice for PMDD, as phenotypic markers expression, IFN-γ and IL-5 release are optimal and both Th1 and Th2 are detectable. Thus, Figures 3.5 and 3.6 show an evaluation of cytokine-producing cells on day 3, by ELISFOT and intracellular staining respectively. Both methods are antigen-specific, since a negligible number of spots was detected in absence of peptide in ELISFOT and detection of cytokine was restricted to the KJ1+ CD4+ cells in intracellular staining. Both methods clearly indicated that large numbers of IL-4-secreting and IFN-γ-secreting cells were present after both DNA and protein/CFA immunisation. However, intracellular staining measured a similar average number of IFN-γ+ and IL-4+ cells, while ELISFOT detected about 3-5 times more IL-4+ cells than IFN-γ+ cells. This difference may reflect either a differential sensitivity of the two detection methods used or the variability in the number of IFN-γ+ cells (as also observed in secreted levels of IFN-γ in Figure 3.4, panel A), since we found a good correlation between the two assays for the IL-4+ cells. Rather few IL-5+ cells were detected, consistent with the low concentration of IL-5 measured in supernatants.

A comparison between Figure 3.3 panel B and Figure 3.6 also indicates that there is a considerable number of KJ1+ CD4+ IL-2+ cells, which are proliferating in response to the antigen without secretion of either IFN-γ or IL-4. A direct comparison between DNA and protein/CFA is difficult, because we focused on the optimal time point for the former (day 3), while that of the latter might be a day earlier (as suggested by data from the above sections).
Figure 3.5. Cytokine expression in OVA-specific T cells on day 3 post-immunisation. Lymph node cells were cultured for 24 hours in absence or presence of 1 μM OVA peptide and cells producing either IFN-γ (A), IL-4 (B) or IL-5 (C) were detected using standard ELISPOT assay. Each symbol (except bars) corresponds to an individual experiment (mean of several cell densities, each density in triplicate). Bars are the mean of all experiments for each group. T-test on day 3: there was no significant difference between pVAC1.OVA and ovalbumin/CFA. Statistical comparisons between pVAC1 and the two other groups are shown on panels.
Figure 3.6. Cytokine expression in KJ1+ CD4+ cells on day 3 post-immunisation, evaluated by intracellular cytokine staining. Lymph node cells were stimulated for 4 hours with 1 μM OVA peptide and either brefeldin A (A) or monensin (B), and then analysed by flow cytometry for the detection of IFN-γ+ (A) or IL-4+ (B) cells among KJ1+ CD4+ cells. Each symbol (except bars) corresponds to an individual experiment (analysis on pool from 3-4 mice/group). Bars are the mean of all experiments for each group. T-test on day 3: there was no significant difference between pVAC1.OVA and ovalbumin/CFA Statistical comparisons between pVAC1 and the two other groups are shown on panels.
3.3. Discussion

This study provides a detailed view of the CD4+ T cell responses that follow PMDD, not only in quantitative terms (magnitudes) but also from qualitative (type of immunity expected from CD4+ T cell differentiation) and temporal (kinetics) points of view. Although adoptive transfer of TCR-transgenic T cells has already been used for the study of in vivo T cell activation using protein antigens (Pape et al., 1997a; Rogers et al., 1997; Gudmundsdottir et al., 1999), this is the first time that such a system has been applied to DNA vaccination. In general, the response to either form of immunisation (DNA vs. protein/CFA) is remarkably similar. Evidence of T cell encounter with antigen can be observed within 24 hours of immunisation, and is reflected both in surface phenotype (increased CD25 and CD69 expression) and a change in the functional properties (the ability to respond in vitro by proliferation and cytokine secretion). These early changes are then followed by substantial in vivo clonal expansion in the draining lymph nodes.

3.3.1. Kinetics of T cell activation and expansion

The most significant difference between protein and DNA immunisation in this model is the relatively smaller and slower rate of clonal expansion seen in response to the latter. One factor that might delay the response to PMDD is the lag phase required for synthesis of protein following gene delivery. However, other factors may also come into play, since there is a much smaller time delay in the IL-2 response for instance. The amount of antigen available may be another parameter that can affect the kinetics and magnitude of the response. It is difficult to measure accurately the amount of protein antigen available for presentation following PMDD, but previous studies have estimated it to be less than 1 μg protein produced (Nicolet et al., 1995; Gurunathan et al., 2000). The amount of antigen following protein immunisation is therefore likely to be orders of magnitude greater than following PMDD, which in turn may drive a faster and larger clonal expansion. Irrespective of the mechanism underlying this difference, the increased clonal expansion in response to protein is transitory and is not accompanied by any increase in cytokine secretion. Furthermore, in vivo clonal expansion is tightly regulated and the number of clonotype positive T cells within the lymph nodes does not significantly increase.
after day 3 post-immunisation (in the case of ovalbumin/CFA, it actually decreases). The number of these cells reflects the net interaction of several variables, including proliferation, death and migration out of the lymph nodes (McHeyzer-Williams and Davis, 1995). Although many factors probably combine to control these parameters, the rapid down-regulation of IL-2 receptor (represented by its α chain CD25) observed within four days is likely to play an important role in limiting expansion. In contrast, the capacity to make IL-2 persists throughout the period measured. IL-2 is required both for proliferation and for sensitising T cells to Fas-mediated apoptosis (Van Parijs et al., 1999), and could therefore contribute both to the initiation of the clonal expansion observed and to the subsequent fall in T cell numbers.

3.3.2. Th1/Th2 differentiation and balance

Immunisation via PMDD resulted in an early expression of activation markers (CD25, CD69) and a subsequent burst of cytokine production, at a time when the number of responders starts to rise, reflecting an active cell division phase. This observation is therefore consistent with the notion that passage of the T cells through the cell cycle is an essential prerequisite for cytokine production (Bird et al., 1998; Richter et al., 1999; Reiner and Seder, 1999). Since the bulk of cytokines is released within the first five days after antigen administration, one can postulate that the education of T cells is completed after this short period of time. Interestingly, there are few IL-5+ cells compared with IL-4+ cells on day 3, but the levels of IL-5 secreted remain stable until day 5 and then fall. It is possible that IL-4+ cells start to express IL-5 later after leaving the lymph nodes or that IL-5+ cells represent a small and stable subset of Th2 cells, independent of IL-4+ cells.

As day 3 post-immunisation coincides with the most active phase of the immune response to PMDD, this time point was the focus for the enumeration of cytokine-producing cells, using both intracellular staining / flow cytometry and ELISPOT / image analysis. The two methods gave broadly similar results in terms of reproducibility, although the ratio of IL-4 / IFN-γ was higher by ELISPOT. Each method offers its own significant advantages. Intracellular staining allows simultaneous phenotyping of the responding cells (e.g. restricting analysis to the TCR-Tg population). ELISPOT has a higher inherent sensitivity for the detection of
a smaller proportion of responding T cells and is more readily adaptable to the analysis of larger numbers of samples. However, a limitation for ELISPOT is the possibility that some larger spots reflect the presence of a cell cluster rather than a single cell.

The balance of the T cell responses to PMDD has been previously examined. It was reported that gene gun delivery favours Th2 cells, with 2.5-3 times more IL-4+ cells than IFN-γ+ cells in spleen (Feltquate et al., 1997). In contrast, intramuscular DNA injection induced an opposite pattern. A Th2-favouring effect of PMDD was thought to be due to a lack of CpG stimulation on appropriate receptors, firstly because the DNA directly enters the cells, and secondly because the DNA dose used in PMDD is much lower than that used in intramuscular injection. Three other groups reported either a Th2-preferential (McCluskie et al., 1999; Tüting et al., 1999) or a mixed Th1/Th2 (Oliveira et al., 1999) profile to PMDD, assessed by the isotype of antibody response (IgG1 levels generally greater than IgG2a levels). Our results are broadly in agreement with these previous studies. For example, IL-4+ cells were either similar in number (as measured by intracellular staining) or 3-5 times greater (as measured by ELISPOT) than IFN-γ+ cells. However, this discrepancy may be explained by our observation that IFN-γ+ spots, although fewer, were somewhat larger than IL-4+ spots. From our observation of in vitro cultures, Th1 cells seem to make larger clusters around antigen-presenting cells than Th2 cells, and IFN-γ+ spots may therefore contain more Th1 cells than an equivalent Th2 spot.

Another factor, which may influence Th1/Th2 bias, is the prior antigenic experience of the T cells. The donor T cells used for these experiments were not in a RAG\(^{-/-}\) background and consequently, a small proportion of them may have been previously activated and committed to a specific cytokine phenotype via a second TCR (Lee et al., 1996; Saparov et al., 1999). The genetic background of the immunised mice may also influence Th1/Th2 bias. Most groups who found a preferential Th2 induction have also performed their experiments on BALB/c strain. The genotype of BALB/c, including the variant of the Tpm1 gene (Guler et al., 1997; Guler et al., 1999), is therefore an important factor explaining Th2 bias.
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Additionally, PMDD induced a majority of IgG1 (an antibody isotype favoured by Th2 responses) in both BALB/c and C57BL/6 (Feltquate et al., 1997), suggesting that despite the genetic background and a good early IFN-γ response, Th2 responses eventually predominate later. This is corroborated by the recent observation that PMDD still favours Th2 responses in spite of the injection of CpG DNA at the same site (Weiss et al., 2002).

3.3.3. Conclusions

The results of this study demonstrate that, at least in terms of CD4+ T cell responses, PMDD can induce a response which is similar in many respects to that induced by protein emulsified in CFA, one of the strongest adjuvant combinations available. Moreover, PMDD generally led to more reproducible immune responses than protein/adjuvant injection. Others have demonstrated that PMDD is also much more reproducible than intramuscular DNA injection (Yoshida et al., 2000).

The in vivo model developed here provides a whole film, rather than just a snapshot, of the early events that occur in draining lymph nodes in response to particle-mediated DNA immunisation. In addition, the relatively mixed Th1/Th2 balance generated makes it a robust and sensitive system, in which to assess, both qualitatively and quantitatively, the early CD4+ T cell responses to DNA antigens, with concomitant use of DNA-encoded adjuvants and immunomodulators that can potentially skew this balance. Indeed, in the longer term, the results offer hope that the incorporation of appropriate immunomodulators into PMDD will allow an extremely flexible approach to the manipulation of the immune response, so as to provide optimum therapeutic vaccination for a wide spectrum of diseases, including chronic infection, allergy, autoimmunity and cancer.
Chapter 4

Effect of genetic background and intracellular DNA dose on CD4+ T cell responses to PMDD
4.1. Introduction

Many factors are known to influence the differentiation of CD4+ T cells upon activation; these include cytokines present early during priming (O'Garra, 1998; Kelso, 1999; Swain, 1999), adjuvants (Cox and Coulter, 1997), antigen dose and TCR/MHC affinity (Hosken et al., 1995; Constant and Bottomly, 1997; Leitenberg and Bottomly, 1999) and genetic background (Hsieh et al., 1995; Beebe et al., 1997).

Two different TCR-Tg T cell populations were required to study interactions between different CD4+ T cells within the same animal. In addition to the ovalbumin (OVA)-specific TCR-Tg T cells described in Chapter 3, the choice was made of another well characterised transgenic T cell population, derived from 5CC7 rag2−/− transgenic mice (subsequently referred to as 5CC7) and expressing a TCR specific for pigeon cytochrome C (PCC). These mice have the B10A genetic background and present CD4-restricted antigens on H-2k class II MHC. In contrast, OVA-specific CD4+ T cells, which were obtained from DO.11.10 mice, are on a BALB/c background and of H-2d class II MHC genotype.

The concomitant use of OVA-specific T cells from DO.11.10 mice and PCC-specific T cells from 5CC7 mice in the same host would require that both alleles of class II MHC (d & k) be expressed in the host and in donors. One possible such donor would be 5CC7 x BALB/c F1, however, this cross resulted in the loss of virtually all CD4+ T cells (Figure 4.1, panel A). Indeed, BALB/c mice possess a superantigen (Woodland et al., 1991; Gonzalez-Quintal and Theofilopoulos, 1992), which delete TCR γδ chain-bearing cells (thus practically all CD4+ T cells since almost all of them express γδ3). To circumvent this problem, DO.11.10 mice were backcrossed repeatedly with mice with a B10 genetic background also expressing H-2d class II MHC (B10D2), retaining TCR-transgenic positive mice (OVA-B10D2) at each generation by screening. In 5CC7 x B10D2 F1, CD4+ T cells are not deleted and the great majority express the γδ3 chain of the transgenic TCR (Figure 4.1, panel B).

The effect of genetic background on immune response has been demonstrated in several systems. Upon in vitro activation, OVA-specific TCR-Tg T cells from a
Figure 4.1. CD4+ T cells are deleted in 5CC7 rag2-/- x BALB/c F1 (A), but not in 5CC7 rag2-/- x B10D2 F1 mice (B). Lymphocytes from blood were analysed by flow cytometry for CD4 and vβ3 expression. Histogramme showing vβ3 were gated on CD4+ cells.
B10D2 or a BALB/c background mount a predominant Th1 or Th2 response respectively (Hsieh et al., 1995), suggesting that these mice have a different genetic environment, which conditions T cells (and presumably other cell types) towards one type of immunity or another. In a model of Leishmania infection, the difference between protective Th1 responses (providing resistance in B10D2 mice) and inappropriate Th2 responses (leading to susceptibility in BALB/c mice) has been attributed to several loci (Beebe et al., 1997). One such locus appears to control IL-12 responsiveness (Guler et al., 1999).

In addition to genetic influences, higher doses of antigen, in the form of peptide or protein, and stronger affinity for TCR/peptide or MHC/peptide interactions both favour Th1 responses whereas increased doses of antigen as a complete live or killed organism seem to better stimulate Th2 cells (Constant and Bottomly, 1997; Leitenberg and Bottomly, 1999). In the case of DNA as antigen, higher doses may also support the generation of Th1 cells (Feltquate et al., 1997), possibly by a CpG-mediated adjuvant effect (Roman et al., 1997).

In this chapter, the early CD4+ T cell response to DNA antigens was assessed in different genetic backgrounds. Thus, the response to pVAC1.OVA between BALB/c and B10D2 background was compared, as well as the response to two different antigens (pVAC1.OVA and pVAC1.PCC) in the same background (B10). Finally, the effect of delivering different amounts of DNA per DC by coating variable amounts of DNA per particle was assessed. All mice were administered the same number of gold particles, so that the number of transfected DC would be similar, but the dose of DNA per DC would vary. The dose response to pVAC1.OVA and pVAC1.PCC in the BALB/c and B10A models respectively was then investigated.
4.2. Results

4.2.1. Influence of the genetic background

Antigen-specific T cells expanded in response to a standard dose of DNA antigens, up-regulated CD25 (Figure 4.2) and secreted IFN-γ and IL-4 (Figure 4.3). The profile of the response to pVAC1.OVA in BALB/c (left panels in Figures 4.2 and 4.3) was very similar to that presented in Chapter 3, although clonal expansion was lower and fewer cytokine-producing cells were detected. These differences may be explained by small changes in protocols, environmental conditions in animal facilities and operating consistency of gene guns, between experiments described in Chapter 3 (carried out at GSK, Stevenage) and in other chapters (carried out at UCL). For instance, when tested for penetrability of gold particles after firing, using a quality control assay developed at Powderject Vaccines and routinely used at GSK (Stevenage), the Helios gene gun (used in experiments described in Chapters 4-7) was found to perform less efficiently than the Accell gene gun (used in experiments mentioned in Chapter 3).

In B10 mice, CD4+ T cells entered cell division faster than in BALB/c mice (Figure 4.2, top panels). In the latter, the TCR-Tg T cells have hardly expanded by day 3, but expanded mainly between day 3 and 5, while in the former, expansion occurred mainly before day 3. In contrast with TCR-Tg T cells from DO.11.10 or 5CC7, those from OVA-B10D2 had a lower frequency baseline in control groups and a more limited extent of clonal expansion in immunised groups (middle panel). In these experiments, \(2.5 \times 10^7\) splenocytes from OVA-B10D2 or DO.11.10 mice were injected into syngeneic recipients, so the difference may be attributed to a slightly lower proportion of TCR-Tg T cells in the former \((\sim 10\% \text{ or } 2.5 \times 10^6 \text{ TCR-Tg T cells})\) than in the latter \((\sim 12\% \text{ or } 3.1 \times 10^6 \text{ TCR-Tg T cells})\). From the 5CC7 mice, \(1 \times 10^7\) splenocytes \((\sim 32\% \text{ or } 3.2 \times 10^6 \text{ TCR-Tg T cells})\) were injected.

Interestingly, the kinetics of CD25 expression appears associated more with the antigen (or the MHC genotype) used than with the genetic background (Figure 4.2, bottom panels). CD4+ T cells responding to pVAC1.OVA down-regulated CD25 between day 3 and day 5 in both BALB/c and B10D2 background, while those
Figure 4.2. Clonal expansion and CD25 expression by TCR-Tg T cells in different genetic backgrounds. Recipient mice received \(2.5-3.2 \times 10^6\) TCR-Tg T cells from syngeneic donors and were immunised the day after with either control vector or antigen vector (standard dose, \(\sim 1.5 \mu\text{g/mouse}\)). Draining lymph nodes were collected on days 3 and 5 post-immunisation. OVA-specific and PCC-specific T cells were detected by flow cytometry as KJ1+ CD4+ and vβ3+ vα11+ cells, respectively. Each symbol represents the mean from 3 mice within an experiment whereas the dash shows the mean of all experiments.
Figure 4.3. CD4+ T cell differentiation in different genetic backgrounds. Recipient mice received 2.5-3.2x10^6 TCR-Tg T cells from syngeneic donors and were immunised the day after with either control vector or antigen vector (standard dose, ~1.5 μg/mouse). Cells from draining lymph nodes collected on days 3 and 5 post-immunisation were restimulated with relevant peptide for 24 hours in ELISPOT assay for detection of IFN-γ+ and IL-4+ antigen-specific T cells. Each symbol represents the mean from 3 mice within an experiment whereas the dash shows the mean of all experiments.

Figure 4.4. Th1/Th2 balance in different genetic backgrounds. IFN-γ / IL-4 ratio calculated from average data shown in Figure 4.3.
stimulated with pVAC1.PCC retained high levels of CD25 expression on day 5. This difference could be attributed to differential affinity between TCR and peptide/MHC complexes (OVA/I-A^d or PCC/I-E^k).

As expected from previous findings (Hsieh et al., 1995), data in Figures 4.3 and 4.4 confirm that the OVA-specific response in BALB/c mice is preferentially Th2 (up to 7 times more IL-4+ than IFN-γ+ cells) while it is slightly skewed toward Th1 in B10 mice (up to two times more IFN-γ+ than IL-4+ cells) for both OVA- and PCC-specific responses. Consistent with the slower kinetics of clonal expansion, the number of cytokine-producing cells in BALB/c mice continues to increase between day 3 and day 5 (Figure 4.3). In contrast, this number falls during the same time in B10 mice, and this, in conjunction with a faster kinetics of expansion, may reflect an escape of effector cells from draining lymph nodes.

4.2.2. Efficiency of DNA coating

To address the question of dose response to DNA, variable amounts of DNA were coated onto gold particles (ranging from 0.4 to 6.0 μg DNA per mg gold). These amounts initially added are referred to as theoretical DNA loading rates (t-DLR). The amount of DNA effectively coated was quantified by spectrophotometry after cartridge elution (see Material and Methods) and expressed as effective DNA loading rates (e-DLR). The e-DLR (μg DNA per mg gold) was obtained by doubling the value measured (μg DNA per cartridge), assuming the loading of 0.5 mg per cartridge as initially established by Powderject.

Table 4.1 shows the e-DLR achieved from various t-DLR in different experiments. Small quantities of DNA are totally precipitated onto the gold, whereas a significant loss of non-bound DNA is observed when more than 1 μg DNA per mg gold is initially added for precipitation. For example, adding 3 times more DNA than the standard dose (i.e. with a t-DLR of 6) result only in a 2-fold increase in the e-DLR. Thus the coating efficiency negatively correlates with the t-DLR. Some values of coating efficiency were over 100%. This may due to an under-estimated value of 0.5 mg per cartridge previously given. By counting the number of cartridges made from
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Table 4.1. Efficiency of DNA loading on gold particles. Within individual cartridge preparation, the effective DNA loading rate (e-DLR) was evaluated from cartridge elutions. The percentage of coating efficiency is (e-DLR / t-DLR) x 100. Values shown in bold represent the standard DLR used in all other experiments.
Chapter 4: Effect of genetic background and intracellular DNA dose

a given amount of gold, an average of 0.7 mg of gold per cartridge was found (see Material and Methods), but this value may be over-estimated because all gold particles cannot be completely transferred into the tubing due to rapid precipitation. The amount of gold loaded per cartridge may be realistically estimated between 0.5 and 0.7 mg, and thus, the actual coating efficiencies may be slightly lower than those shown in Table 4.1. The maximal e-DLR that could be achieved was 3.65 μg per mg gold (i.e. 1.83 μg / cartridge).

4.2.3. Dose response to intracellular DNA antigens

Mice were always immunised with 2 cartridges (1 mg gold particles), therefore the dose of DNA per mouse (μg) is the same value as the e-DLR (μg DNA / mg gold) shown in Table 4.1. All doses tested, from 0.47 to 3.65 μg / mouse (i.e. 0.24 – 1.83 μg DNA / cartridge), induced the same extent of clonal expansion, at least by day 5 after immunisation (Figure 4.5, upper panels). Similarly, the degree of activation of these cells, as measured by CD25 expression, was similar within the dose range tested (Figure 4.5, lower panels). Neither the kinetics of clonal expansion nor CD25 expression was dose-dependent over the range tested.

By contrast, the generation of cytokine-producing cells (IFN-γ+ and IL-4+) was optimal with at least the standard dose (~1.5 μg DNA / mouse); higher doses failed to improve the response further (Figure 4.6). Using the lower dose (~0.5 μg DNA / mouse), although the same number of CD4+ T cells were activated, only about half of them (compared with standard and high doses) secreted cytokines, on both day 3 and day 5. In control animals, which received a standard dose of empty vector (dose 0 of antigen), the number of TCR-Tg T cells remained at baseline level, slightly up-regulated CD25 (compared with before adoptive transfer) and did not secrete effector cytokines (Figures 4.5 and 4.6). Both IFN-γ and IL-4 were enhanced with higher DNA doses and no change in ratio toward Th1 or Th2 was observed between the lowest and the highest dose tested (Figure 4.7). The preferential induction of Th1 or Th2 cells in relation to the genetic background was conserved regardless of the dose.
Figure 4.5. Clonal expansion and CD25 expression by TCR-Tg T cells in response to various doses of DNA. Recipient mice received 2.5-3.2x10^6 TCR-Tg T cells from compatible donors and were immunised the day after with either control vector (standard dose, ~1.5 μg DNA / mouse) or antigen vector (dose ranging from 0.47 to 3.65 μg DNA / mouse). Draining lymph nodes were collected on days 3 and 5 post-immunisation. OVA-specific and PCC-specific T cells were detected by flow cytometry as KJ1+ CD4+ and vβ3+ vα11+ cells respectively. Data plotted as dose 0 are those obtained from control mice immunised with a standard dose of empty vector. Each symbol represents the mean from 3 mice within an experiment.
Figure 4.6. Differentiation of CD4+ T cells in response to various doses of DNA. Recipient mice received 2.5-3.2x10^6 TCR-Tg T cells from compatible donor and were immunised the day after with either control vector (standard dose, ~1.5 μg DNA / mouse) or antigen vector (dose ranging from 0.47 to 3.65 μg DNA / mouse). Draining lymph nodes were collected on days 3 and 5 post-immunisation and cells were restimulated in ELISPOT assay for detection of IFN-γ+ and IL-4+ antigen-specific T cells. Data plotted as dose 0 are those obtained from control mice immunised with a standard dose of empty vector. Each symbol represents the mean from 3 mice within an experiment.

Figure 4.7. Th1/Th2 balance and DNA doses. IFN-γ / IL-4 ratio calculated from data shown in Figure 4.6.
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4.3. Discussion

4.3.1. Effect of genetic background

The genetic variability between strains within the same species is a factor leading to contrasting immune responses. In several models of infection or autoimmune disease, different strains of mice either recover or succumb to the disease. These observations helped to establish the existence of an inherent genetic predisposition to a certain Th1/Th2 bias. The data presented in this chapter corroborate such findings published on the Th1/Th2 balance in BALB/c and B10 mice (Hsieh et al., 1995). Furthermore, the nature of the antigen expressed (PCC vs. OVA) or the dose of DNA administered did not influence such bias. The bias was more pronounced in BALB/c (3-7 times more IL-4+ than IFN-γ+ spots) than in B10 mice (1.5-2 times more IFN-γ+ than IL-4+ spots). However, in subsequent chapters, about 4 times more IFN-γ+ than IL-4+ cells in B10 background was measured. This difference appeared after the ELISPOT protocol was changed, to include a higher cell density (8x10^5 cells per well). In contrast with IL-4, which gives a linear titration (e.g. twice as many spots when adding twice as many cells per well), IFN-γ secretion appeared dependent on the in vitro cell density, so that doubling the density could result in 3-4 times more IFN-γ+ spots. Likewise, it was reported that IFN-γ+ cells are linearly detected only at high cell densities (Power et al., 1999). Since higher densities may better reflect the compact structure of a lymph node, we believe that the ratios obtained in Chapters 5-7 are more representative. Thus, it is possible that the Th1 population was underestimated and that the difference between the number of IFN-γ+ and IL-4+ cells was lower in BALB/c and greater in B10A models. The genetic bias in immune response shows the importance of establishing animal models in several immunologically distinct strains when developing immunomodulators to rectifying inappropriate responses that lead to pathological conditions. For instance, an anti-tumour vaccine that works in a Th1-prone individual may not be efficient in a Th2-prone one, thus requiring the concomitant use of immunomodulators.
4.3.2. Effect of intracellular DNA dose

Variable amounts of DNA were loaded onto the gold particles, resulting in variable numbers of copies per bead, and after transfection, per cell. Nevertheless, the amount of gold delivered was the same between all groups, so that the number of transfected cells remained fairly constant. Increasing the intracellular dose of antigen up to an optimal threshold resulted in a greater proportion of activated CD4+ T cells that differentiated into IFN-γ or IL-4 producers. Thus, it may take stronger TCR signals to induce CD4+ T cell differentiation than that required for stimulating proliferation. This is consistent with the finding that more TCR signals are required for IFN-γ than for IL-2 production (Itoh and Germain, 1997). Furthermore, short TCR stimulation leads to intermediate (i.e. activated non-polarised) T cells, which are committed to proliferation, whereas effector and memory T cells require longer TCR stimulation (Iezzi et al., 1998; Iezzi et al., 1999; Lanzavecchia and Sallusto, 2001). The duration and strength of TCR signals are related, because stronger signals induce more CD40L expression, which in turn induce more adhesion molecules expression on DCs and more prolonged activation.

Nevertheless, the Th1/Th2 balance was not apparently affected within the range of doses tested. The modulating effect of antigen dose & affinity (Hosken et al., 1995; Constant and Bottomly, 1997) and of the overall TCR signal strength (Leitenberg and Bottomly, 1999) on Th1/Th2 balance has been described previously. In these studies, a preferential Th1 induction upon stronger TCR signals was found. Stronger TCR signals may result in a more efficient up-regulation of CD40L on activated cells, leading to further CD40-CD40L ligation and more IL-12 secreted by DC (Ruedl et al., 2000). However, these effects were observed when DCs were incubated with antigens, while DNA directly introduced into DCs has not been tested in this context. Thus, in terms of TCR signal strength, it is plausible that the dose range of DNA tested here was not wide enough to observe a sufficient shift in the strength of the TCR signal, or that the APC transfected by PMDD (mostly Langerhans cells) differ from those used in other studies in numbers and ability to present antigens.
Chapter 4: Effect of genetic background and intracellular DNA dose

As discussed in the previous chapter, when the dose of immunising DNA is increased, one more factor, the number of CpG motifs, comes into consideration. CpG motifs present on bacterial DNA can interact with TLR9 on APCs (Bauer et al., 2001) and act as a Th1-promoting adjuvant (Roman et al., 1997; Leclerc et al., 1997). It is believed that high levels of CpG delivered upon injection of large doses of DNA intramuscularly contribute to the preferential Th1 induction, while PMDD may fail to do so because of 10-100 fold lower doses used (Feltquate et al., 1997). Furthermore, the few CpG motifs introduced by PMDD may not be able to interact with TLR-9, since the DNA is propelled directly into the cytoplasm.

In studies involving protein or peptide, higher dose of antigen injected may result in a greater number of DCs picking up and presenting antigen, whereas with PMDD, even though the total amount of DNA per mouse was increased, a similar number of DCs should have been transfected regardless of the dose. Thus, the effect of protein/peptide dose on Th1/Th2 balance may be a combination of the amount of antigen presented by DCs and the number of antigen-bearing DCs involved. In Chapter 7, this question is further investigated by the delivery of beads with different proportion of gold particles carrying antigen-coding vectors (100% vs. 50%) among the total DNA-carrying particles. Another way to examine this would be to vary the number of cartridges delivered per mouse at immunisation.

These data show that the standard dose (~1.5 μg DNA per mouse) is an optimal and saturating dose for inducing both proliferation and differentiation of CD4+ T cells by PMDD. Furthermore, lowering the dose by up to 2-fold did not appear to compromise either proliferation or differentiation. Thus, the 2-fold dilutions used for the co-expression of two antigens in later chapters should not limit the responses. Moreover, the data demonstrate that both OVA- and PCC-specific TCR-Tg T cells can generate detectable Th1 and Th2 cells in the same genetic background (B10) model, prompting their concomitant use in a combined adoptive transfer model for the study of their in vivo interactions.
Chapter 5

Characterisation of a combined adoptive transfer (CAT) model for PMDD: CD4+ T cell responses and loading of multiple DNA vectors
5.1. Introduction

The antigen-specific responses of CD4+ T cells from OVA-B10D2 and 5CC7 transgenic mice, sharing the same genetic background (B10), have been previously studied (see Chapter 4). The combined adoptive transfer (CAT) model was established by combining together these two transgenic models. Since the two TCRs recognise peptide on different MHCs (I-A^d and I-E^k), F1 donors were produced by crossing 5CC7 and OVA-B10D2 transgenic mice with B10D2 and B10A mice respectively. Likewise, recipient mice were B10D2 x B10A F1. These crossings enable the co-expression of both I-A^d and I-E^k in all donors and recipients, thus allowing the concomitant use of both TCR-Tg T cells within the same model in full histocompatibility for the study of their in vivo interactions. Nevertheless, before any co-transfer of these TCR-Tg T cells, it was important to characterise how each CD4+ T cell population from these new F1 donors behaved in vivo in the absence of the other.

Firstly, the activation of PCC-specific naïve CD4+ T cells was examined by flow cytometry in both draining lymph nodes and spleen. The kinetics of CD69, CD62L and CD45RB expression, measured by flow cytometry, as well as the measure of cell division by CFSE staining provided indications about the fate of naïve CD4+ T cells following immunisation by PMDD.

Secondly, the response of naïve or in vitro pre-polarised CD4+ T cells was analysed. This study was needed in order to verify that in vitro pre-polarised T cells retain their cytokine profile after adoptive transfer and upon restimulation in vivo and that they can migrate to lymph nodes in sufficient number. The latter is an obvious prerequisite to further studies of the ability of polarised T cells to induce changes to other T cells in the lymph nodes.

Finally, the in vivo interactions between CD4+ T cells were investigated in later chapters by the concomitant activation of two populations of TCR-Tg T cells by their respective antigens, either preferentially co-expressed in the same DC (linkage) or rather expressed in different DC (no linkage). Prior to this study, two methods were
used to isolate single beads and qualitatively evaluate the distribution of several co-coated plasmids within and between gold particles.

5.2. Results

5.2.1. Characterisation of donor cells in the CAT model

Before adoptive transfer into syngeneic hosts, donor T cells from 5CC7 x B10D2 or OVA-B10D2 x B10A were analysed by flow cytometry for the frequency of TCR-Tg T cells among spleen lymphocytes (Figure 5.1), and expression of CD62L and CD69 on their surface (Figure 5.2). The cytokine profile upon peptide stimulation of naïve or in vitro polarised TCR-Tg T cells from OVA-B10D2 x B10A was also examined (Figure 5.3).

Lymphocytes isolated from the spleen of 5CC7 x B10D2 or OVA-B10D2 x B10A mice contained ~17% and 8.6% of TCR-Tg T cells respectively (Figure 5.1). These cells were predominantly CD62L\textsuperscript{high} and CD69\textsuperscript{low} (Figure 5.2), which is characteristic of a naïve phenotype. They also failed to produce high levels of cytokine upon a brief in vitro stimulation (Figure 5.3). However, TCR-Tg T cells from OVA-B10D2 x B10A mice also contained a fraction of CD62L\textsuperscript{low} and CD69\textsuperscript{high} cells (up to 20%), which may represent TCR-Tg T cells that have been previously activated via a second TCR (Lee et al., 1996; Saparov et al., 1999). Unlike 5CC7 x B10D2 mice, OVA-B10D2 x B10A mice were not bred in pathogen-free isolators and thus may be exposed to more environmental antigens. Because the PCC-specific T cells from 5CC7 x B10D2 corresponded more closely to a true naïve population, they were always used as a naïve responder population, while the OVA-specific T cells were used to make polarised CD4+ populations.

After 5 days of in vitro stimulation under polarising conditions, OVA-specific T cells massively expanded while most of the other cells died. As a result, the frequency of these cells was dramatically increased (Figure 5.1). Th1- and Th2- polarised T cells exhibited a significant reduction of CD62L and augmentation of CD69 compared with naïve T cells (Figure 5.2). The expression of CD62L decreased significantly more in Th2 cells than in Th1 cells, a feature that has been previously reported (van
Figure 5.1. Frequency of TCR-Tg T cells within donor lymphocytes. The frequency of naïve TCR-Tg T cells within splenocytes from 5CC7 x B10D2 F1 (A) or OVA-B10D2 x B10A F1 mice (B) was measured by flow cytometry. Cells shown in B were also cultured for 5 days under Th1 or Th2 polarising conditions and assessed likewise (C and D respectively). Panel E shows the mean and standard deviation from n independent measurements (n indicated beside each column).
Figure 5.2. Expression of CD62L and CD69 by TCR-Tg T cells with or without polarisation. The percentage of CD62L+ (A,B) and CD69+ (C,D) cells was assessed by flow cytometry and gated on vβ3+ vα11+ cells (PCC naive) or KJ1+ CD4+ cells (OVA naive, Th1 or Th2). Panels A and C indicate the mean and standard deviation from n independent measurements (n indicated beside each column). Stars between columns symbolise the T-test comparison between adjacent columns, those on top of OVA Th1 and OVA Th2 columns (panel C) were compared with OVA naive (*** p<0.001, ** p<0.01, * p<0.05). Panels B and D show representative fluorescence histograms.
Figure 5.3. Cytokine profile of TCR-Tg T cells with or without polarisation. The percentage of IFN-γ+ (A) and IL-4+ (B) cells was assessed by flow cytometry and gated on K.JI+ CD4+ cells (naive, Th1 or Th2). Panels A and B indicate the mean and standard deviation from n independent measurements (n indicated by each column). Stars on top of high columns symbolise T-test comparison with both low columns (*** p<0.01, * p<0.05). Panels C and D show representative fluorescence histograms for expression of both IFN-γ and IL-4 in OVA-specific Th1 and Th2 respectively.
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Wely et al., 1999). Furthermore, Th1 and Th2 cells selectively produce high levels of IFN-γ and IL-4 respectively (Figure 5.3), while production of IL-2 could not be detected in either population (not shown). The percentage of Th2 cells producing IL-4 with polarised populations was more variable than that of IFN-γ-expressing Th1 cells (Figure 5.3, panels A-B). Also, the range of fluorescence intensity indicated that Th2 cells produced lower amounts of IL-4 compared with IFN-γ from Th1 cells (ranges 10 to ~40 and 10 to ~120, respectively; Figure 5.3, panels C-D). This is consistent with published data showing that Th1 populations contain higher percentage of IFN-γ+ cells, compared with IL-4+ cells in Th2 populations, and that IFN-γ+ cells express higher levels of cytokines per cell than IL-4+ cells in a similar TCR-transgenic model (Openshaw et al., 1995).

5.2.2. In vivo behaviour of PCC-specific naïve CD4+ T cells in response to PMDD

Following adoptive transfer of ~3×10^6 naïve vβ3+ vα11+ cells from 5CC7 x B10D2 mice, syngeneic recipients were immunised with a standard dose of pVAC1 (control) or pVAC1.PCC. Responses in draining lymph nodes and spleen were all assessed by flow cytometry at three time points after immunisation.

Activation of PCC-specific cells by pVAC1.PCC is followed by remarkable changes in draining lymph nodes. A 3-fold expansion of these cells was accompanied by a dramatic up-regulation of CD69 (as early as day 1) and a reduction of the CD62L^high population (Figures 5.4 and 5.5). In control groups, the frequency of TCR-Tg T cells remained at baseline level (~1%). Some inflammation caused by administration of the control vectors may be responsible for a limited enhancement of CD69 (as reported also in BALB/c in Chapter 3) and a small and transient decline of CD62L (Figure 5.4). In contrast, there were no changes in these features on PCC-specific T cells in the spleen. The frequency of TCR-Tg T cells in the spleen (~0.5%) was half that in lymph nodes (Figure 5.4) and did not increase up to day 5. Moreover, the expression of CD62L and CD69 in the spleen remained unchanged throughout the period examined, following immunisation with either pVAC1 or pVAC1.PCC (Figures 5.4 and 5.5).
Figure 5.3bis. Experimental procedure associated with Figures 5.4-5.9. Read-out (days 1, 3 and 5) consists is measuring PCC-specific T cells responses by flow cytometry and ELISPOT.
Figure 5.4. Kinetics of PCC-specific CD4+ T cell clonal expansion, CD69 and CD62L expression after PMDD. Draining lymph nodes (left panels) and spleens (right panels) were collected from adoptively transferred mice (∼3×10⁶ vβ3+ vα11+ cells per mouse) on days 1, 3 or 5 after immunisation by PMDD with either control (pVAC1) or antigen (pVAC1.PCC) vector. The percentage of vβ3+ vα11+ cells (upper panels) and their expression of CD69 (middle panels) and CD62L (lower panels) was analysed by flow cytometry. Each circle represents the mean from 3 mice within an individual experiment (2-3 experiments per group) and each dash indicates mean from all experiments within a group.
Figure 5.5. Changes in CD69, CD62L and CD45RB expression on PCC-specific T cells following particle-mediated immunisation with pVAC1.PCC. Lymphocytes from draining lymph nodes (left panels) or spleen (right panels) were analysed by flow cytometry (see Figure 5.4). The specific analysis of CD69, CD62L and CD45RB (top, middle and bottom panels, respectively) was gated on vβ3+ vα11+ T cells. Histograms show representative fluorescence profiles from a single mouse (3 mice / group / experiment; 2 individual experiments). Data obtained with pVAC1 controls are not shown but were identical at all time points to the profile observed with pVAC1.PCC in the spleen on days 1 and 3.
While CD69 and CD62L are markers that can be affected early upon activation, CD45RB represents a late marker, usually used to distinguish between naïve (CD45RB$^\text{high}$) and memory cells (CD45RB$^\text{low}$). CD45RB appeared to be down-regulated at day 5 (Figure 5.5). Thus, the mean fluorescence of CD45RB intensity was compared between day 3 and day 5 post-immunisation (Figure 5.6). CD45RB expression was significantly diminished between day 3 and day 5, but exclusively upon activation (no changes in control groups immunised with pVAC1). Interestingly, this effect was observed in both draining lymph nodes and spleen, suggesting that both tissues already contained some antigen-specific memory cells.

In addition, PCC-specific T cells were also stained with CFSE prior to adoptive transfer. CFSE is a green fluorescent dye that is equally distributed between daughter cells upon cell division, so that the fluorescence intensity is halved after each division. The peak with the highest fluorescence intensity corresponds to the undivided population, cells that have half this intensity have gone through one round of division, cells that have an intensity 4 times lower have divided twice, etc. Control groups in lymph nodes and spleen show that cells failed to divide in absence of antigen stimulation (Figures 5.7 and 5.8, upper panels). In contrast, a considerable fraction of cells undergo cell division upon immunisation with pVAC1.PCC (Figures 5.7 and 5.8, lower panels).

In groups immunised with antigen, first divisions occurred in lymph nodes between day 1 and day 3 post-immunisation, so that by day 3, more than half the PCC-specific T cells have started to divide, and by day 5, only about 20% remained undivided (Figure 5.8, left lower panels). In the spleen, two-thirds of these cells have divided by day 5 but no apparent division was observed on day 3 (Figure 5.8, right lower panels). A small proportion of CFSE- or CFSE$^\text{low}$ cells can be observed in all groups. These may include endogenous vβ3+ $\alpha$11+ (not labelled with CFSE) and/or some autofluorescent cells (staining positive for vβ3, $\alpha$11 and CFSE at moderate intensity).

The peak of fluorescence of undivided cells was not constant throughout the period examined (fell from ~600 to ~400 between day 1 and day 5), perhaps due to loss of the dye (Figure 5.9). The peak fluorescence of divided cells was used to estimate the
Figure 5.6. Down-regulation of CD45RB after 5 days of *in vivo* priming with antigen. Mean fluorescence intensity of CD45RB expression by vβ3+ vα11+ was measured in draining lymph nodes (left panel) and spleen (right panel) on day 3 and day 5 after immunisation with either control or PCC-encoding vector. Mean and standard deviation from 6 mice in two experiments. T-test: ** p<0.01, * p<0.05.

Figure 5.7. *In vivo* cell division of PCC-specific T cells in response to PMDD. Cells from draining lymph nodes (left panels) and spleen (right panels) from mice immunised with control (top panels) or PCC-expressing vector (bottom panels) were analysed by flow cytometry. Representative fluorescence intensity of CFSE on vβ3+ vα11+ cells is shown (one out of three mice). The number of divisions at the peak of divided populations was calculated for each time point where division was apparent (see Materials and Methods for formula) and displayed in relevant panels.
**Figure 5.8.** Cell division of antigen-specific T cells after PMDD. The proportion of non-divided (CFSE<sup>high</sup>) and divided (CFSE<sup>low</sup>) within vβ3+ vα11+ from draining lymph nodes (left panels) or spleen (right panels) was measured by flow cytometry on days 1, 3 and 5 after immunisation with control or PCC-expressing vector. Numbers shown represent the proportion of cells left in the non-divided population (white). Data reflect the average from three mice.

**Figure 5.9.** *In vivo* cell division of PCC-specific T cells. The peak fluorescence of the undivided (CFSE<sup>high</sup>) and divided (CFSE<sup>low</sup>) fractions of vβ3+ vα11+ cells was measured by flow cytometry on days 1, 3 and 5 after immunisation with pVAC1 or pVAC1.PCC. Data show the average from three mice.
number of cell divisions. This number was calculated as described in Section 2.8.4
and displayed on histograms using arrows (Figure 5.7, lower panels). In the draining
lymph nodes, a continuous progression through successive cell cycles could be
observed, with 4 divisions on day 3 and more than 5 on day 5. In contrast, in the
spleen, cells that went through more than 5 divisions also appeared on day 5 but no
cells with an intermediate state of CFSE fluorescence were visible on day 3 (Figure
5.7, right lower panel; Figure 5.9, right panel), suggesting that these cells did not
actually divide in the spleen but presumably originated from elsewhere.

5.2.3. Recirculation and activation of OVA-specific naïve and polarised T cells

Naïve splenocytes from OVA-B10D2 x B10A mice (containing OVA-specific KJ1+
CD4+ T cells) were cultured in vitro under various conditions (peptide and cytokine
concentrations) in order to achieve the best balance between polarisation and cell
yield (data not shown). For Th1 cultures, better results were obtained with 200 nM
OVA peptide and 5 ng/ml IL-12 added on days 3 and 4 after stimulation. With higher
concentration of peptide (1 μM), more KJ1+ CD4+ T cells were obtained, but with a
lower proportion of IFN-γ upon restimulation. In contrast, adding IL-12 from the first
day of culture resulted in rapid differentiation into IFN-γ producers that appeared
refractory to further proliferation, eliciting poorer yields. Best Th2 cultures were
achieved by using 100 nM OVA peptide and 20 ng/ml IL-4. IL-4 seemed to
synergise with exogenous IL-2 (20 ng/ml) to induce extensive proliferation.
Moreover, lower doses of peptide favoured the generation of greater proportions of
IL-4+ cells but led to very poor yields, so that 100 nM was found to be a good
compromise.

Following adoptive transfer of 3x10⁶ naïve or polarised KJ1+ CD4+ cells (cultured 5
days using best tested conditions), syngeneic recipients were immunised with a
standard dose of pVAC1 or pVAC1.OVA. Responses in draining lymph nodes were
assessed by flow cytometry and ELISPOT at three time points after immunisation.
The frequency of naïve T cells remained constant (~1%) in control group or doubled
in groups that were administered antigen (Figure 5.10, row A, left panel). The
frequency of pre-polarised T cells (~0.75% at day 1) progressively fell with time in
Figure 5.9bis. Experimental procedure associated with Figures 5.10-5.12. Read-out (days 1, 3 and 5 post-immunisation) consists in measuring OVA-specific T cells responses by flow cytometry and ELISPOT.
Figure 5.10. Kinetics of clonal expansion, CD62L and CD69 expression by OVA-specific naive or polarised CD4+ T cells after PMDD. Naive (left panels), Th1- (middle panels) and Th2-polarised (right panels) T cells were adoptively transferred on the day prior to immunisation (3x10^6 KJ1+ CD4+ cells per mouse). Draining lymph nodes were collected on days 1, 3 or 5 after immunisation by PMDD with either control (pVAC1) or antigen (pVAC1.OVA) vector. The frequency of KJ1+ CD4+ cells was measured on total lymphocytes (A) and the expression of CD62L (B) and CD69 (C) was restricted to KJ1+ CD4+ cells. Mean of 2-3 individual experiments (3 mice / group / experiment).
absence of priming (Figure 5.10, row A, middle and right panels), while the frequency was either maintained (Th2) or slightly expanded (Th1) upon \textit{in vivo} priming. This lower, and apparently transient, number of polarised T cells compared with the number of naïve T cells, despite equal numbers transferred, suggests that these cells have a reduced lifespan and/or ability to home and remain in lymph nodes compared with naïve T cells.

Despite differential expression of CD62L between naïve, Th1 and Th2 cells (approximately 80%, 40% and 10% respectively) prior to adoptive transfer (Figure 5.2, panels A-B), cells found in draining lymph nodes after transfer showed high levels of expression (about 80%, 70% and 60-70% respectively), which remained unchanged with time in absence of antigen (Figure 5.10, row B). In contrast, expression of CD62L fell in all groups that encountered antigen. Polarised T cells exhibited higher levels of CD69 immediately after transfer compared with naïve T cells (Figure 5.10, row C), which correlated with their profile before transfer (Figure 5.2, panels C-D). CD69 expression on polarised T cells fell after transfer in the absence of antigen. Upon \textit{in vivo} priming, naïve T cells transiently up-regulated CD69, as early as day 1 after immunisation, while polarised T cells down-regulated CD69 less rapidly than in absence of antigen.

As previously shown in Chapter 3, naïve T cells that were not primed \textit{in vivo} can still produce some IL-2 upon \textit{ex vivo} stimulation with peptide (Figure 5.11, row C, left panel), but no IFN-γ or IL-4 (Figure 5.11, rows A-B, left panels). More surprisingly, \textit{in vitro} polarised Th1 and Th2 cells did not produce IFN-γ or IL-4 respectively after \textit{ex vivo} restimulation if they were not restimulated with antigen \textit{in vivo} (Figure 5.11, rows A-B, middle and right panels). When mice were immunised with antigen, a large number of IFN-γ+ and IL-2+ cells, but few IL-4+ cells, were detected after \textit{ex vivo} restimulation of naïve and Th1 populations, while a large number of IL-4+ cells, and few IFN-γ+ and IL-2+ cells, were generated in the Th2 population (Figure 5.11).

The IFN-γ / IL-4 ratio (Figure 5.12) shows that i) naïve T cells elicit a preferential Th1 response upon activation in this model, as anticipated from Chapter 4, ii) the cytokine profile of naïve, Th1 and Th2 cells is retained after their transfer and
Figure 5.11. *Ex vivo* cytokine profile of OVA-specific naive or polarised CD4+ T cells. Cells from the same experimental groups described in Figure 5.10 were restimulated in ELISPOT assay on days 3 and 5 after immunisation with pVAC1 or pVAC1.OVA for detection of IFN-γ- (A), IL-4- (B) and IL-2- (C) producing cells. Columns show the mean of 2-3 individual experiments (3 mice / group / experiment).

Figure 5.12. *Ex vivo* IFN-γ / IL-4 ratio from antigen-specific naïve, Th1 and Th2 cells. The ratio was calculated on the basis of the data shown in Figure 5.11.
priming *in vivo*, and iii) the response progressively shifts from Th1 to Th2 (consistent decrease of the ratio between day 3 and day 5; see discussion in Sections 5.3.2. and 8.1.1).

5.2.4. Co-coating of different DNAs for antigen co-expression by PMDD

The PCR technique was used to check the relative proportion of different plasmids on gold particles after co-precipitation. The conditions for optimal PCR were tested on various vectors expressing different size antigens, mixed at equivalent amounts before precipitation on gold particles (Figure 5.13). One set of primers was used for all plasmids, recognising sequences on pVAC1 upstream and downstream of the insert. All inserts were effectively amplified, but KlenTaq-based polymerases (gel shown) showed better yields than standard Taq polymerases (not shown).

Plasmids expressing ovalbumin (OVA) and secreted EpCAM (sEpCAM) were used in preliminary experiments, before the PCC gene was cloned. When mixed in equivalent amounts and diluted, both antigens could be detected down to levels of 1 fg per PCR reaction (Figures 5.14 and 5.15A, lanes 3-5). The same mixture was precipitated onto gold particles and the suspension was then diluted down to approximately 1 bead by limiting dilution (see Material and Methods for details). Both antigens were still detected at some of the lower dilutions (Figure 5.14, lanes 15-17), but unlike the mixture of purified plasmids, the two plasmids precipitated on gold particles were not equivalently amplified, with the smaller insert yielding more intense bands (Figure 5.14, lanes 12-17). The accuracy of this method was limited by the difficulty in making correct serial dilutions (gold particles settle very quickly) and the uncertainty that each band visualised at limiting dilution represented a single bead.

An alternative method, normally used to isolate cells from tissues, was applied to obtain single gold particles. Glass pipettes with a very fine tip were made using a pipette puller. The two plasmids expressing OVA and sEpCAM were mixed in equal amounts and coated on gold particles. Some of the suspension of DNA-coated gold particles was spread on glass slides and individual particles were picked up with a pipette (one pipette per bead) under a microscope (Figure 2.6) and transferred into
Figure 5.13. Amplification of inserts from a mixture of pVAC1 vectors with different antigens. Each plasmid (lines 3-8) was added at 0.1 pg per reaction, in the presence of dNTPs, KlenTaq polymerase and buffer and primers specific for pVAC1 sequences upstream and downstream of the insert. After 26 cycles, PCR products were loaded and run on a 0.8% agarose gel. Antigens tested were ovalbumin, Flu nucleoprotein PR and secreted EpCAM.

Figure 5.14. Detection of plasmids on single gold particles by limiting dilution. A mixture of plasmids expressing ovalbumin or sEpCAM were either diluted in water to various amounts (lanes 3-5) or co-precipitated on gold particles, which were then diluted in ethanol as explained in Material & methods (lanes 6-17). PCR was carried out using primers specific for pVAC1 sequences upstream and downstream of the insert. After 30 cycles, PCR products were loaded and run on a 1% agarose gel.
Figure 5.15. Detection of plasmids on single gold particles by micromanipulation. A mixture of plasmids expressing ovalbumin or sEpCAM were either diluted in water to various amounts (gel A, lanes 3-5) or co-precipitated on gold particles, which were then either eluted, quantified and diluted in water (gel B, lanes 2-5) or spread on glass slides and individually picked up by micromanipulation (upper gel, lanes 7-17; lower gel, lanes 7-18). PCR was carried out using primers specific for pVAC1 sequences upstream and downstream of the insert. After 30 cycles, PCR products were loaded and run on a 1% agarose gel. A star indicates a case where bead was suspected to be dropped during the micromanipulation process of transfer into PCR tube.
PCR tubes containing water. Both antigens were detected by PCR (Figure 5.15, gel A lanes 7-17 and gel B lanes 7-18). As previously observed (Figure 5.14), the smaller antigen was amplified more efficiently than the longer antigen. This may be due to competition at the precipitation stage (more pVAC1.sEpCAM coated than pVAC1.OVA) or at the PCR level (shorter fragment can be amplified faster than longer fragment). However, some of the same suspension was eluted in TE to recover the DNA that had bound to the gold. After spectrophotometric quantification, the mixed plasmids were diluted down to 1 fg and were successfully detected by PCR with bands of equal intensity produced for each of the two fragments within each dilution (Figure 5.15, gel B lanes 2-5). Thus, it is unlikely that one plasmid is preferentially coated over the other one. Nevertheless, if there is competition at the PCR level, this seems to occur only when DNA is precipitated on gold but not when it is free in aqueous suspension. In addition, some single particles did not generate any PCR product, suggesting that some particles were dropped during the transfer process (as indicated on some lanes by a star symbol; Figure 5.15, gels A-B) presumably because beads were loosely attached outside the pipette. Alternatively, a proportion of beads may not carry any DNA. The heterogeneity of intensity between bands from lane to lane suggests that gold particles carry variable amounts of DNA.

The same method was used to evaluate the coating of pVAC1.OVA and pVAC1.PCC in two of the preparations (half dose linked and unlinked) described in Section 2.3.2 and shown in Figure 2.5. However, since these gold/DNA preparations include empty vectors as well (pVAC1), the vector-specific primers utilised above could not be used because they also amplified a very short fragment on pVAC1. When these primers were used, large amounts of these short products were generated, to the detriment of the OVA and PCC inserts, which were much less efficiently amplified. Thus, new sets of primers were specifically designed for each antigen-coding insert. In addition, plasmids were co-coated in a 1:1 ratio of number of copies rather than weight.

Gels A and B (Figure 5.16) show PCR data from gold beads randomly isolated from a mixture made with half the beads with both PCC- and OVA-containing vectors and the other half with empty vector (half dose linked). Out of 23 beads tested, about half (11) were indeed positive for both antigens while the other half (12) were negative.
Detection of linked and unlinked plasmids on single gold particles by micromanipulation. Gels A-B: a mixture of plasmids expressing OVA or PCC were co-coated on gold particles, which were then mixed on a 1:1 volume ratio with particles coated with empty vectors (linked half dose). The control is a PCR without any bead or DNA. Gels C-D: plasmids expressing OVA or PCC were separately co-coated with empty vectors on gold particles then both preparations of DNA/particles were mixed on a 1:1 volume ratio (unlinked). DNA/gold slurries were spread on glass slides and individually picked up by micromanipulation. PCR was carried out on single beads using mixed primers specific for the OVA and the PCC insert sequence. After 30 cycles (35 cycles for gel D), PCR products were loaded and run on a 3% agarose gel.
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Gels C and D (Figure 5.16) show the results from a mixture, in which half the beads were loaded with OVA-expressing and empty vectors and the other half with PCC-expressing and empty vectors (unlinked). Out of 28 beads tested from this preparation, 11 were positive for OVA only, 12 were positive for PCC only and 5 were positive for both OVA and PCC. Thus, although OVA-carrying and PCC-carrying beads were mixed, the majority of the beads express only the vector with which they were coated. However, a few beads appeared to have been contaminated by vector from other beads. Such contamination may result from free plasmids detaching from beads and contaminating other beads. Alternatively, these double PCR bands may reflect two or more smaller beads stuck together, that were mistaken for a single bead. This is more likely because if free plasmids were present in the suspension, they would have been expected to contaminate the double negative reactions observed in gels A and B, thus altering the expected 1:1 ratio observed. Although variations in intensity can be observed between beads, the shorter fragment no longer generates a band more intense than the longer fragment. The unequal ratio seen in Figures 5.14-5.15 may have been compensated for by changing from a 1:1 ratio in DNA weight to a 1:1 ratio in number of copies or by amplifying fragments with a more similar size.

5.3. Discussion

5.3.1. Recirculation of CD4+ T cells after adoptive transfer

In order to be able to study the interactions between naïve and polarised CD4+ T cells, two requirements needed to be met: i) polarised T cells must retain the ability to migrate to the same anatomical location as naïve T cells and ii) they must also preserve their characteristic phenotype (i.e. type of cytokine secreted) after transfer in vivo. Recirculation through lymph nodes is controlled by at least two parameters, CD62L and CCR7. CD62L binds to CD34 expressed on endothelial cells in HEVs (Baumheter et al., 1993). Ligands for CCR7 (the chemokines CCL19 and CCL21) are produced at HEVs to attract CCR7+ cells into the paracortex of the lymph nodes (Yoshida et al., 1998; Baekkevold et al., 2001). Both CD62L and CCR7 are usually required for lymph node homing and are typically expressed on naïve T cells and mature DC (Bradley et al., 1994; Saeki et al., 1999; Baekkevold et al., 2001).
Activated T cells down-regulate CD62L and CCR7 and leave the paracortex. However, these markers are progressively re-expressed on memory T cells (London et al., 1999; Sallusto et al., 1999), allowing them to re-enter the lymph nodes. The rapidity of re-expression of these markers may depend on the experimental conditions. Here, in vitro pre-polarised T cells showed relatively high levels of CD62L expression (60-70%) in lymph nodes two days after their transfer in vivo. In another study, in vitro pre-activated T cells re-expressed CD62L at 54% after 1 week and at 80% after 10 weeks in vivo (London et al., 1999). CD4+ T cells from efferent lymph, blood and afferent lymph in sheep respectively contain 35%, 59% and 67% CD62L+ cells (Haig et al., 1999), suggesting sequential stages of CD62L up-regulation between the time they exit and the time they re-enter the lymph nodes. After T cell activation, CD62L exhibits a complex and dynamic regulation, with rapid shedding, followed by rapid re-expression (Buhrer et al., 1992; Chao et al., 1997), then a second and more prolonged loss after a few days (Chao et al., 1997), followed by progressive re-expression (London et al., 1999). Furthermore, pre-activated T cells (Willimann et al., 1998) and CD62Llow CD4+ T cells (Debes et al., 2002) retain the ability to respond to CCL19 and CCL21.

The data obtained in this model do not provide clear evidence whether the polarised T cells found in the draining lymph nodes derive from the CD62Lhigh fraction of the injected cells, from CD62Llow cells that re-express the marker in vivo or from a combination of both. Given the very small fraction of CD62Lhigh cells in the Th2-polarised culture, it is likely that some CD62L re-expression was involved. The pre-polarised T cells were found in lower frequency than naïve T cells in the lymph nodes, and unlike the latter, they progressively disappeared after transfer, presumably by tissue redistribution or clonal deletion. Thus, only a subset of these cells was co-localised with naïve T cells and this occurred only for a limited amount of time. In another study (Iezzi et al., 2001), the difference in frequency between adoptively transferred naïve and polarised T cells was much more pronounced (3.1%, 0.9% and 0.2% for naïve, Th1 and Th2 cells respectively). The use of a difference genetic background (BALB/c) may account for a slower re-expression of CD62L after transfer (London et al., 1999) and for the different migration properties of polarised cells (Iezzi et al., 2001). Moreover, the duration of in vitro stimulation may also influence the stability of homing receptors in parallel to that of the cytokine profile.
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(Murphy et al., 1996), such that flexibility may be lost on irreversibly committed T cells after repeated rounds of stimulation.

5.3.2. Fate of CD4+ T cell following PMDD

Clonal expansion in the draining lymph nodes correlated well with the profile of cell division measured by CFSE staining of naïve T cells, with no division observed 24 hours after immunisation despite signs of activation (CD69 up-regulation). Then, after 3 days, both naïve and polarised T cells started to down-regulate CD62L. This change foreshadowed the forthcoming exit of these cells out of the lymph nodes. Finally, after 5 days, some down-regulation of CD45RB was also visible, a trend generally associated with the formation of effector (Bradley et al., 1991) and memory cells (Beverley, 1991). These three steps occur in the draining lymph nodes, to which transfected Langerhans cells are most likely to migrate, but not in the spleen. The total absence of CD69\textsuperscript{high} and CD62L\textsuperscript{low} cells in the spleen, accompanied by no visible clonal expansion, strongly suggest that no antigen presentation occurs in the spleen following PMDD, at least within the first 5 days after immunisation. Some antigen-specific T cells were found in the spleen on day 5. They were similar to those observed at the same time in draining lymph nodes in that they have clearly divided and down-regulated CD45RB, but are different in being CD69- and CD62L\textsuperscript{high}. Moreover, these cells did not appear to have been activated in the spleen, since there was no evidence for an intermediate division stage on day 3, as opposed to the draining lymph nodes.

These data are consistent with a model in which, following particle-mediated DNA immunisation, CD4+ T cells are activated in the draining lymph nodes, and then progressively migrate out of the lymph nodes into the spleen (Iezzi et al., 2001) and other tissues (Reinhardt et al., 2001), where they can perform their normal effector functions. The cells found in the spleen had down-modulated CD69 more rapidly than those remaining in the lymph nodes, and also somehow re-expressed CD62L.

Importantly, upon \textit{in vivo} priming, adoptively transferred polarised T cells exhibited the same cytokine pattern that they had prior to transfer. Th1 cells, like naïve cells, elicited a predominant IFN-γ response, accompanied by a large population of IL-2+
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cells and few IL-4+ cells. The Th1 bias also shown by naïve cells in vivo may be attributed to the genetic background, as discussed in Chapter 4, although in the present chapter, the ratio IFN-γ / IL-4 was higher than that measured previously on the same model, perhaps because the detection of IFN-γ by ELISPOT assay was improved with an increased range of cell densities (see Section 4.3.1). However, differences were observed between naïve and Th1 cells. The number of IFN-γ+ cells generated from naïve cells decreased between days 3 and 5 after immunisation, while this number increased for Th1 during the same time. Also, there were less IL-2+ cells after transfer of Th1 cells compared with naïve cells, which may reflect the difference in cell frequency between these two populations. In contrast, Th2 elicited a preferential IL-4 response, with few IFN-γ+ and IL-2+ cells generated. It is well established that Th2 cells produce no or little IL-2 (Mosmann et al., 1986).

Before transfer, in vitro antigen stimulation was sufficient to drive Th1 and Th2 cells to express high levels of IFN-γ and IL-4 respectively. Surprisingly, after transfer in vivo, these cells required further in vivo priming in order to express cytokines ex vivo after isolation. Cells from lymph nodes of mice that were immunised with control vector after transfer were very poor producers of these cytokines compared with the same cells extracted from immunised mice. It should be noted that before transfer, all the TCR-Tg were conditioned with appropriate polarising signals but only a fraction (40-80%) expressed the relevant cytokine in each population by intracellular staining. Thus, it is possible that after transfer, a segregation occurs, with the higher producers populating non-lymphoid tissues and the others homing back to the lymph nodes, so that TCR-Tg T cells taken from these lymph nodes fail to produce much cytokine. It has been shown in humans that central memory cells (CD45RA-CCR7+), which can relocate to the lymph nodes, are poor producers of effector cytokines (IFN-γ, IL-4) in contrast with effector memory cells (CD45RA- CCR7-), which migrate to peripheral tissues (Sallusto et al., 1999). Although CD45RB was only measured on naïve cells, pre-polarised T cells found in the lymph nodes may have been CCR7^{high} (hinted by the fact they were CD62L^{high}) and may have been expressing lower levels of CD45RB than naïve T cells. Although this question was not investigated further, these data raise the important issue of what factors determine the fate of activated T cells in becoming either effector memory or central
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memory cells in vivo. T cells cultured under various in vitro conditions may have different abilities for migration after adoptive transfer, resulting in variable ratios of T cells in the lymph nodes versus the periphery (which may explain differences observed between published studies). In order to address these questions, the central / effector memory T cell ratio may be investigated in mouse strains with deficiencies in molecules, which may regulate this ratio (e.g. chemokines and their receptors, \gamma \text{c} chain receptor family and related cytokines, pro- and anti-apoptotic factors) after transfer of cells activated under different in vitro conditions. The identification of the factors controlling effector vs. central memory formation has significant implications in the design of vaccines for therapeutic vs. prophylactic purposes (Kaech et al., 2002).

Finally, on both days 3 and 5 after immunisation, Th1 cells exhibited an increased IFN-\gamma / IL-4 ratio compared with naïve T cells, while in Th2 cells this ratio was less than 1, reflecting that Th1 and Th2 maintained the cytokine phenotype that was expected. Interestingly, the IFN-\gamma / IL-4 ratio decreased in all groups between day 3 and day 5, suggesting a progressive replacement in the draining lymph nodes of early IFN-\gamma-producing cells by late IL-4-secreting cells and corroborating the trend already observed in the BALB/c model (Figure 4.4). This trait was not seen previously in B10 model (Figure 4.4), probably due to non-optimal IFN-\gamma ELISPOT assay. The sequential activation of Th1 and Th2 cells is discussed in details in Section 8.1.1.

5.3.3. Control of linkage vs. non-linkage by PMDD

The combined adoptive transfer model was designed for the simultaneous activation of two antigen-specific populations in vivo, and PMDD was used to control the spatial expression of the antigens. Thus, the characterisation of the CAT model would not be complete without validating the feasibility of PMDD to either favour the co-expression of the two antigens on the same APC (linkage), or on the contrary, their segregation (non-linkage). An initial step in validation of the system was to determine whether the method described for preparing “linked” or “unlinked” gold preparations really yielded populations of beads carrying one antigen only or both antigens.
In preliminary studies, gold particles coated with both pVAC1.OVA (6502 bp) and pVAC1.sEpCAM (5441 bp) were isolated and PCR was used to amplify the inserts that were present on a single bead. Although beads within a homogenous size range were selected, some variation in intensity was observed between beads, and some of them appeared not to be coated at all. This observation confirms other studies (L. Thomsen; personal communication), in which DNA tagged with fluorescent molecules was examined on gold particles by microscopy. In addition, more product was obtained from the smaller insert (sEpCAM, 814 bp) than with the larger one (OVA, 1875 pb), but only when the plasmids were bound to the gold particles and not when they were free in suspension, before coating or after elution from the gold. The size difference between the two inserts as well as the fact that they were mixed in a 1:1 ratio in weight (so that the smaller plasmid is present in a greater number of copies) complicated the interpretation of these experiments.

Later, linked and non-linked DNA/gold preparations were made for the co-administration of pVAC1.PCC (5034 bp) and pVAC1.OVA. In order to compensate for the size difference between these plasmids (even greater than between pVAC1.OVA and pVAC1.sEpCAM), a 1:1 ratio in number of copies rather than weight of DNA was used (representing 43.5% pVAC1.PCC and 56.5% pVAC1.OVA by weight). In addition, primers were designed to specifically amplify a partial fragment of each insert, both fragments with a similar size but distinct enough to be separated on a gel. Using these conditions, the two fragments were amplified with the same efficiency when they were co-coated on beads. When these two plasmids were separated on different beads within the same DNA/gold mixture, most of the beads indeed expressed only one antigen or the other. Nevertheless, some beads (which in fact may well represent two or more smaller particles clumped together) tested positive for both plasmids. It is unlikely that plasmids can detach and re-attach to other beads (as mentioned above in Results). Clumps of two or more beads, on the other hand, can be observed. Such clumps may disaggregate within the high velocity helium stream used to propel the beads or upon impact with the skin. However, a rare event of DC transfected with more than one bead cannot be ruled out. As a consequence, the physical delivery of two different plasmids into different cells may not be completely achieved with unlinked preparations.
Chapter 6

Polarised CD4+ T cells participate in the instruction of naïve CD4+ T cells within DC clusters
6.1. Introduction

Upon activation, T cells can establish positive or negative interactions in vivo. Positive interactions are demonstrated when the activation of one T cell clone is better in the presence of another activated clone than in its absence (cooperation) or if the two clones mutually improve one another's response (synergy). Negative interactions are reflected by a limited activation of a clone in the presence of another activated clone and may be caused by competition (when two clones compete for a limited number of APC or a limited amount of antigen/MHC complexes available) or by inhibition (when one clone secretes inhibitory cytokines such as IL-10). Cooperation (or synergy) between T cells can be mediated by at least three different pathways: (i) the epicrine pathway, which operates indirectly between lymphocytes through signalling via a DC, (ii) the paracrine pathway, which is mediated by a direct and localised cross-talk between lymphocytes by means of cytokines, in an environment such as the DC cluster, and (iii) the endocrine pathway, which involves cross-talk between cells at remote distance from each other.

Cooperation between T cells is best understood in the case of CD4+ T cell help on CD8+ T cell activation. In most cases, CD8+ T cell-mediated CTL responses require a contribution from CD4+ T cells (hence termed helper T cells) (Keene and Forman, 1982; Bennett et al., 1997), although under acute viral infection, CD4+ T cell help may be dispensable (Buller et al., 1987; Liu and Mullbacher, 1989). DCs that have been sensitised by CD4+ T cells via CD40 stimulation (a typical epicrine signal) become very potent in activating CD8+ T cells (Schoenberger et al., 1998; Bennett et al., 1998; Ridge et al., 1998), because their expression of MHC, costimulatory and adhesion molecules is greatly enhanced (Yang and Wilson, 1996; Celia et al., 1996). Although signalling through CD40 is necessary, it may not always be sufficient. In some models, CD40 ligation was found to restore CD8+ T cell responses only partially in the absence of CD4+ T cells, and other CD40-independent (epicrine) signals, as well as soluble mediators (paracrine signals), are also involved (Lu et al., 2000). Other possible epicrine signals include 4-IBB (Shuford et al., 1997) and OX40 (De Smedt et al., 2002), while cytokines supporting CD8+ T cells are likely to include IL-2 and IFN-γ, which are mainly produced by Th1 cells. Th1 cells are generated by the release of IL-12 from DCs, which can be induced by activated
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helper T cells via CD40 stimulation (Cella et al., 1996; Koch et al., 1996). Conversely, Th1 cells may receive help from activated CD8+ T cells in the form of IFN-γ (Mailliard et al., 2002), because IFN-γ enhances the secretion of IL-12 induced by CD40L-expressing activated CD4+ T cells (Snijders et al., 1998) and the responsiveness of CD4+ T cells to IL-12 (Smeltz et al., 2002).

Cooperation between CD4+ T cells or between CD8+ T cells has received much less attention. It has been reported that CD8+ T cells can collaborate with each other in the absence of CD4+ T cell help and in a CD40-independent manner if they are present in sufficient numbers in vivo (Wang et al., 2001; Mintern et al., 2002). Other in vivo studies on CD4+ T cells (Smith et al., 2000b; Laouar and Crispe, 2000) or CD8+ T cells (Kedl et al., 2000) have shown that competition occurs between cells that share the same antigen specificity. Whether this is still true between clones of different antigen/MHC specificity remains controversial in the case of CD8+ T cells (Kedl et al., 2002; Probst et al., 2002). It is likely that naïve CD4+ T cells can benefit from the up-regulation of costimulatory and adhesion molecules induced on DC via CD40 by helper T cells to the same extent than naïve CD8+ T cells. Also, like CD8+ T cells, CD4+ T cell responses are enhanced by signalling via OX40 (De Smedt et al., 2002). A population of CD4+ T cells responding to a strong epitope has been found to enable the development of otherwise undetectable response from another population responding to a weak or cryptic epitope, and this cooperation was shown to be partially mediated by CD40 and OX40 (Gerloni et al., 2000). This effect was observed upon antigen linkage, in other words, upon the co-expression of their respective antigen by the same cell, in this case B cells.

Antigen linkage is required for any epicrine and paracrine cooperation between T cells. Such requirement was already reported 20 years ago for T cells in general (Tucker and Bretscher, 1982), subsequently between helper and cytotoxic T cells (Mitchison and O'Malley, 1987) and more recently between CD4+ T cells (Gerloni et al., 2000). Paracrine versus endocrine cooperation has also been examined in vitro by measuring the ability of polarised CD4+ T cells (termed inducers) to skew the response of naïve T cells of different antigen specificity (termed targets). This has been done in two situations, where DC were either pulsed with both peptides (linkage) or pulsed separately and then mixed (no linkage) (Schuhbauer et al., 2000).
In this in vitro system, inducer cells were able to influence the target cells in both linked and unlinked situations, but with a greater efficiency in the former situation, suggesting that paracrine help is more effective than endocrine help.

In this chapter, the in vitro model described above has been extended to its in vivo context, in order to examine whether previously polarised T cells can alter the cytokine balance of T cells of different antigen/MHC specificity upon concomitant activation. The CAT model described in Chapter 5 was used to explore this question, in conjunction with PMDD for the administration of linked or unlinked antigens as described also in Chapter 5.

6.2. Results

6.2.1. Experimental conditions

For simplification, the same terms of “inducer” and “target” cells, previously used in the in vitro studies (Schuhbauer et al., 2000), were employed in this chapter. Here, PCC-specific naïve T cells (vβ3+ vα11+) were used as target cells, while OVA-specific naïve or polarised T cells (KJ1+ CD4+) were used as inducer cells. The responses of the two cell populations, independently of each other, were characterised in Chapter 5. This chapter deals with the co-transfer and co-activation of these cells in the same mouse.

Two days before immunisation, B10D2xB10A F1 recipient mice received $3 \times 10^6$ naïve PCC-specific CD4+ T cells from syngeneic donors. Then, on the day prior to immunisation, $3 \times 10^6$ naïve or Th1- or Th2-polarised OVA-specific (inducer) T cells were injected. In a fourth group, no inducer cells were transferred, so that the response measured against OVA could only be attributed to endogenous OVA-specific T cells. Finally, recipient mice were immunised with both pVAC1.PCC and pVAC1.OVA, either coated on the same bead (i.e. all beads linked, Figure 2.5) or on separate beads administered as a mixture (i.e. unlinked, Figure 2.5). Linkage was to be favoured with the former, so that individual DC would express and present both antigens on their surface, or avoided with the latter, so that individual DC would express and present only one or other antigen. Responses to OVA and PCC were
assessed in cells from draining lymph nodes on day 5 after immunisation (Figures 6.1-6.2 for inducer cells and Figures 6.3-6.4 for target cells) by flow cytometry (Figures 6.1 and 6.3) and ELISPOT (Figures 6.2 and 6.4). Some complementary data, obtained on day 3 after immunisation, are also shown in Figure 6.5.

6.2.2. Responses of naïve and polarised inducer cells

The clonal frequency of OVA-specific (KJ1.26+ CD4+) T cells in the presence of PCC-specific T cells on day 5 after immunisation with pVAC1.OVA (Figure 6.1, row A) was very similar (~2%) to that observed in the absence of PCC-specific cells (Figure 5.10, row A left panel), although only half the dose of pVAC1.OVA per bead was used because of the 1:1 mixture with pVAC1.PCC or pVAC1. Thus, this dosage had no effect on clonal expansion, consistent with data from Chapter 4 showing that the dose of DNA per bead used is higher than that required to achieve maximum clonal expansion. Naïve OVA-specific T cells expanded approximately 3-fold upon in vivo priming compared with non-immunised control mice. Furthermore, OVA-specific T cells gave similar responses whether they were stimulated by the “linked” or the “unlinked” DNA/particle preparation (Figure 6.1, row A). The observation that OVA-specific T cells proliferated to the same extent, in the presence or absence of linked or unlinked PCC-specific T cells, suggest that the latter had no inhibitory or competitive effect on the clonal expansion of the former.

Naïve and pre-polarised OVA-specific CD4+ T cells were found to follow the same trend of CD69 expression in the absence or the presence of PCC-specific T cells (compare Figure 5.10, row C and Figure 6.1, row B), with levels on day 5 back to baseline for Th2 cells. Likewise, the levels of CD62L after activation of OVA-specific naïve, Th1 or Th2 cells were very similar in the absence or the presence of PCC-specific T cells (compare Figure 5.10, row B and Figure 6.1, row C).

Both Th1 and Th2-polarised populations retained their characteristic skewed cytokine profile upon restimulation in vivo (Figure 6.2, row D), with levels of IFN-γ and IL-4 respectively similar whether they were activated in the absence (Figure 5.11, rows A-B) or the presence of PCC-specific T cells (Figure 6.2, rows A-B). Finally, the differential expression of IL-2 by naïve, Th1 and Th2 cells (compare
**Figure 6.0.** Experimental procedure associated with Figures 6.1-6.5. Read-out (days 3-5) were PCC- and OVA-specific responses by both flow cytometry and ELISPOT.
Figure 6.1. Flow cytometry analysis of the response of OVA-specific inducer cells. B10D2 x B10A F1 recipient mice received $3 \times 10^8$ naïve PCC-specific target cells and $3 \times 10^6$ OVA-specific inducer cells prior to immunisation. Inducer cells were either naïve, Th1- or Th2-polarised, while a fourth group received no inducers. Finally, recipient mice were immunised with both pVAC1.PCC and pVAC1.OVA, either linked (i.e. coated on the same particle, shown as grey bars) or unlinked (i.e. on separate particles and administered as a mixture, shown as striped bars). Lymph nodes were harvested on day 5 after immunisation and flow cytometry was used to measure the clonal expansion (row A) of OVA-specific endogenous responders (column 1), naïve (column 2), Th1 (column 3) or Th2 (column 4) TCR-Tg T cells (KJ1+ CD4+). The expression of CD69 (row B) and CD62L (row C) on the KJ1+ CD4+ cells was measured in parallel. Each bar represents mean ± SEM from at least three individual experiments (three mice / group / experiment). Panels B and C: grey/striped and white bars represent the expression of CD69 on responder (KJ1+ CD4+) and bystander cells (KJ1- CD4+) respectively. Right panels (black bars): analyses on control mice that received $3 \times 10^6$ of both naïve populations but were not immunised (mean ± SD from 4 mice in 2 separate experiments). T-test: **, p<0.01; *, p<0.05; ns, non significant. Stars on top of columns are for statistical comparison with adjacent white columns.
Figure 6.2. ELISPOT analysis of the response of OVA-specific inducer cells. Cells harvested on day 5 post-immunisation, from the experiments described in Figure 6.1, were restimulated in vitro for 24 hours with 1μM OVA peptide to detect IFN-γ+ (row A), IL-4 (row B) and IL-2 (row C) producing cells. Each bar represents mean ± SEM from at least three individual experiments (three mice / group / experiment). Row D: IFN-γ / IL-4 ratio calculated from data on panels A and B. T-test: ***, p<0.001; **, p<0.01; *, p<0.05; ns, non significant.
Figure 5.11, row C and Figure 6.2, row C) and the Th1 bias from the naïve inducer cells (compare Figure 5.12 and Figure 6.2, row D) were also conserved. Since Th1 and naïve cells generate similar levels of IFN-γ-producing cells (Figure 6.2, row A), the higher IFN-γ / IL-4 ratio observed in Th1 cells compared with naïve cells (Figure 6.2, row D) was mainly attributed to lower IL-4 production by the former (Figure 6.2, row B, significant in the linked condition). It should be noted, however, that cytokine-producing cell numbers in rows A-C of Figure 6.2 have not been adjusted for the frequency of KJ1+ CD4+ T cells in the different populations. For example, a similar number of IFN-γ+ spots were obtained by a number of responders 3-fold lower in the Th1 population compared with the naïve one. The frequency of both IFN-γ+ and IL-4+ cells in the Th1 and Th2 inducer populations are therefore considerably higher than in the naïve population, as might be expected. This factor does not affect the cytokine ratio calculated in row D. In contrast to naïve OVA-specific inducers, endogenous OVA-specific T cells demonstrated an unbiased Th1/Th2 balance (ratio ~1, Figure 6.2, row D).

Most importantly, the OVA-specific inducer cells responded identically whether they were primed under the linked (Figure 6.2, left panels) or the unlinked conditions (Figure 6.2, right panels), with no significant differences. The endogenous OVA-specific responders, however, responded differently under these two conditions, with a 10 to 20-fold higher number of OVA-specific cytokine spots elicited upon linkage (p≤0.05 for IFN-γ and IL-4, p<0.001 for IL-2) compared with no linkage. The low number of spots observed in the unlinked condition was similar to that measured in non-adoptively transferred mice (data not shown).

6.2.3. Responses of naïve target cells in presence of inducers

In parallel to the inducer cells and within the same experiments, the responses of PCC-specific target cells were also assessed on day 5. Within each immunisation condition (i.e. linked and unlinked), these cells expanded to the same extent (3 to 4-fold compared with non-immunised mice) in the presence of either naïve or pre-polarised inducers as in the absence of any exogenous OVA-specific inducer cells (Figure 6.3, row A). The expression of CD69 (Figure 6.3, row B) was dramatically
Figure 6.3. Flow cytometry analysis of the response of PCC-specific target cells. Cells harvested on day 5 post-immunisation, from the experiments described in Figure 6.1, were analysed by flow cytometry to measure the clonal expansion (row A) of naive PCC-specific (vβ3+ vα11+) in the presence of endogenous responders (column 1), naïve (column 2), Th1 (column 3) or Th2 (column 4) OVA-specific T cells, and the expression of CD69 (row B) and CD62L (row C) on these vβ3+ vα11+ cells. Each bar represents mean ± SEM from at least three individual experiments (three mice / group / experiment). Rows B and C: grey/striped and white bars represent the expression of CD69 on responder (vβ3+ vα11+) and bystander cells (vβ3- vα11+) respectively. Right panels (black bars): control mice that received 3x10^6 of both naïve populations but were not immunised (mean ± SD from 4 mice in 2 separate experiments). T-test comparison between responder and bystander cells: ***, p<0.001; **, p<0.01; *, p<0.05; ns, non significant.
up-regulated in activated $\nu \beta 3^+ \nu \alpha 11^+$ cells, compared with non-primed $\nu \beta 3^+ \nu \alpha 11^+$ cells or bystander $\nu \beta 3^- \nu \alpha 11^+$ cells. Similarly, the expression of CD62L (Figure 6.3, row C) was only down-regulated on activated $\nu \beta 3^+ \nu \alpha 11^+$ cells. Levels of both CD69 and CD62L were unaffected by the presence of naïve or polarised inducers. Clonal expansion and CD69 expression appeared slightly lower in groups immunised with the unlinked preparation but this difference was not significant.

Nevertheless, the presence of polarised inducer cells clearly affected the cytokine pattern of the responding PCC-specific target cells, when the two antigen genes were delivered on the same particle (Figure 6.4, left panels). As for the OVA-specific naïve T cells (Figure 6.2, row D), the response of PCC-specific T cells in the absence of any inducers was biased towards a Th1 phenotype (Figure 6.4, row D), with a 4:1 ratio of IFN-$\gamma$ / IL-4. Such bias was not altered by the presence of naïve OVA-specific inducers, with similar numbers of IFN-$\gamma^+$, IL-4+ and IL-2+ cells, compared with the absence of inducers, upon immunisation with either linked or unlinked antigens (Figure 6.4). In contrast, Th1 inducers almost doubled the number of IFN-$\gamma$ within the PCC-specific target cells (Figure 6.4, row A, left panel), while Th2 inducers doubled the number of IL-4+ cells and reduced the number of IL-2+ cells within these target cells (Figure 6.4, rows B-C, left panels). As a result, the IFN-$\gamma$ / IL-4 ratio on target cells was strongly altered by pre-polarised inducers (Figure 6.4, row D, left panel), with the Th1 inducers increasing the ratio to almost 7:1, while the Th2 inducers reduced the ratio to close to 1.

The ability of the inducer cells to influence the target cells as described was dependent on linkage. If the two antigens were delivered on separate particles (Figure 6.4, right panels), the inducer population had no significant effect on the targets in any group, even though the production of cytokines by inducers was similar after both linked and unlinked immunisations (Figure 6.2). The IFN-$\gamma$ / IL-4 ratio was almost the same in all four experimental groups (Figure 6.4, row D, right panel).
Figure 6.4. ELISPOT analysis of the response of PCC-specific target cells in the presence of inducer cells. Cells harvested on day 5 post-immunisation, from the experiments described in Figure 6.1, were restimulated in vitro for 24 hours with 1μM PCC peptide to detect IFN-γ+ (row A), IL-4 (row B) and IL-2 (row C) producing cells. Each bar represents mean ± SEM from at least three individual experiments (three mice / group / experiment). Row D: IFN-γ / IL-4 ratio calculated from data on panels A and B. T-test: ***, p<0.001; **, p<0.01; *, p<0.05; ns, non significant. Stars on top of columns are for comparison with all other columns of the panel.
6.2.4. Kinetics of the generation of cytokine-producing cells

In some of the experiments involving antigen linkage, the response was assessed on both day 3 and day 5 after immunisation, in order to examine the changes in the different populations generated. The number of IFN-γ+ cells declined between day 3 and day 5, in those populations with a high frequency of IFN-γ-producing cells (naïve and Th1 inducers, and naive target cells), and rose slightly in OVA-specific endogenous and Th2 cells (Figure 6.5, row A). In contrast, the number of IL-4+ cells was augmented in all groups during the same period (Figure 6.5, row B). Overall, and as reported in previous result chapters, the IFN-γ / IL-4 ratio consistently decreased between days 3 and 5 for all inducer and target populations (Figure 6.5, row D). The differential expression of IL-2 observed between naïve, Th1, Th2 inducers or endogenous OVA-specific T cells was conserved, with an increased number of IL-2+ cells in all groups during the time examined (Figure 6.5, row C, left panel), while this number appeared to decrease in the target population. This difference may reflect slightly different kinetics of PCC- and OVA-specific T cells, as was observed in Chapter 4 for the expression of CD25.

The influence of inducer cells on target cells was more pronounced on day 5 than on day 3. In the presence of Th1 inducers, the number of IFN-γ+ PCC-specific T cells did not decrease in contrast with the other groups (Figure 6.5, row A, right panel). Therefore, the larger number of IFN-γ+ target cells found on day 5 (Figure 6.4, row B, left panel) reflects a Th1 population that is sustained at high frequency rather than augmented.
Figure 6.5. Kinetics of the generation of cytokine-producing cells within the inducer and target populations. Cells harvested on days 3 and 5 post-immunisation, from the "linked" experiment described in Section 6.2.1, were restimulated in ELISPOT assay for 24 hours with 1μM cognate peptide (OVA peptide on left panels or PCC peptide on right panels) to detect IFN-γ+ (row A), IL-4+ (row B) and IL-2+ (row C) cells and calculate the IFN-γ / IL-4 ratio (row D). Each symbol represents mean from two (day 3) or three to four (day 5) individual experiments (three mice / group / experiment). T-test analysis on day 5 is displayed on Figure 6.2 (inducer cells) and Figure 6.4 (target cells).
6.3. Discussion

After their independent characterisation in the CAT model (Chapter 5), both OVA- and PCC-specific CD4+ T cells were used in conjunction in order to study their interaction in vivo upon concomitant activation by particle-mediated DNA delivery of linked or unlinked antigen-expressing plasmids. In this chapter, we assessed the ability of a naïve or a polarised clone (inducer) to influence the differentiation of a naïve clone (target) specific for a different antigen presented by a different MHC on the same APC.

6.3.1. Polarised T cells influence their naïve counterparts upon linked antigen expression

Naïve inducer cells had no influence on the target cells, when compared with target cells alone. By contrast, polarised inducer cells markedly altered the differentiation of target cells, confirming and extending previous in vitro studies of CD4+ T cells (Schuhbauer et al., 2000). On the one hand, Th1 inducers led to a sustained high frequency of IFN-γ+ cells on day 5, while in absence of Th1 cells, this frequency dropped between day 3 and day 5. These Th1 inducers may operate by increasing the local concentration of IFN-γ, which is known to synergise with CD40 ligation for the release of IL-12 by DC (Snijders et al., 1998; Mailliard et al., 2002) and to enhance the expression of IL-12 receptor on T cells (Smeltz et al., 2002). As a result, there may be an accumulation in the DC environment of Th1 cytokines, which last longer and affect more naïve cells. Alternatively, this sustained high frequency may also reflect a retention of IFN-γ+ cells in the lymph node, by a mechanism such as that mediated by CXCL10 (Yoneyama et al., 2002). The Th1 inducers did not reduce the IL-4+ population of target cells on day 5.

On the other hand, in the presence of Th2 inducers, the IL-4+ target population was doubled compared with the absence of any inducer or the presence of naïve or Th1 inducers. In the absence of an exogenous source of IL-4, Th2 differentiation is inefficient (Ben-Sasson et al., 2000). However, an initial, yet limiting, amount of autocrine IL-4 may be sufficient to drive Th2 differentiation (Demeure et al., 1995;
Noben-Trauth et al., 2000; Noben-Trauth et al., 2002). By providing a local source of paracrine IL-4 upon activation, Th2 inducers may facilitate the emergence of IL-4+ cells among the naïve target population. Furthermore, Th2 inducers significantly reduced the number of IL-2+ target cells. A possible mechanism for this may be by their secretion of IL-10, which is known to inhibit IL-2 production (de Waal Malefyt et al., 1993). Despite the reduction in IL-2, the expansion of PCC-specific T cells was unaffected, presumably counter-balanced by other factors, including the proliferative properties of IL-4. These interactions are more extensively discussed in Section 8.2.3.

6.3.2. Cytokine-mediated cooperation is mainly confined to cells within a DC cluster

Interestingly, none of the above effects were observed when antigens were preferentially expressed on different APC (i.e. unlinked situation). Thus, it appears evident that the signals involved act in a paracrine or epicrine manner, confined to the DC cluster. However, in this system, the epicrine signals (CD40-mediated DC sensitisation) are supposedly optimal given the high frequency of activated antigen-specific T cells, which should up-regulate CD40L. The fact that naïve PCC-specific T cells were not influenced by naïve OVA-specific T cells upon their concomitant activation suggests that their numbers are sufficient for optimal epicrine signals and do not require further epicrine-mediated help. Likewise, when CD8+ T cells are present in sufficient numbers, they do not require help from CD4+ T cells (Wang et al., 2001; Mintern et al., 2002). Therefore, it is more likely that the effects observed in these experiments are mainly attributed to cytokines acting in paracrine manner. Previous studies have demonstrated the highly directional and localised nature of cytokine secretion in the context of a T cell-B cell synapse (Kupfer et al., 1991), which provide further support for the concept of DC cluster autonomy and DC as a privileged microenvironment, where T cells may interact in a paracrine and epicrine manner.

In addition to the instruction of naïve cells by polarised cells, the data show at least two other factors that influence the Th1/Th2 balance. One is the precursor frequency, because cells present at low frequency (endogenous OVA-specific T cells) exhibited
a mixed Th1/Th2 balance (IFN-γ / IL-4 ratio ~1), while cells present at high frequency (3x10^6 OVA-specific TCR-Tg T cells per mouse) elicited a preferential Th1 response (IFN-γ / IL-4 ratio was 3-4). This feature is further investigated and discussed in Chapter 7. The second factor is the timing, which sees a progressive shift from Th1 to Th2, apparently because of a non-synchronised or sequential generation of these two populations. This feature, already observed in precedent chapters, will be further discussed in Section 8.1.1 of the general discussion.

6.3.3. Antigen transfer has no apparent contribution in the generation of CD4+ T cell responses by PMDD

The demonstration of a linkage effect, by co-delivering two antigens on one bead using PMDD, has important implications in terms of the mechanisms that underlie particle-mediated DNA immunisation. The two antigens used may be synthesised and secreted by both Langerhans cells and the other surrounding cell types (e.g. keratinocytes, fibroblasts). Thus, priming may result either from directly transfected Langerhans cells presenting endogenously synthesised antigen, or from uptake by "bystander" Langerhans cells (or other migrating DC) of antigen secreted by other cells such as keratinocytes in the epidermal immunisation site (i.e. cross-priming or antigen transfer). In the case of CD8+ T cells, the role of cross-priming remains controversial, with data both against (Condon et al., 1996; Porgador et al., 1998) and in favour (Cho et al., 2001).

The data obtained in this study argue against a major contribution for antigen transfer in the activation of CD4+ T cells by PMDD. Indeed, if antigen transfer was very effective, unlinked antigens would become progressively linked, and no significant difference between the two conditions would have been observed. One plausible explanation is that antigen secretion at the site of immunisation occurs too late, at a time when directly transfected DCs have presumably already started to prime T cells, thus providing the major contribution in shaping the immune response. Evidence for this hypothesis are given and discussed in Section 8.1.3 of the general discussion.
6.3.4. Absence of competition between CD4+ T cells of different specificity

Competition has been demonstrated within clones of CD4+ (Smith et al., 2000b; Laouar and Crispe, 2000) and CD8+ T cells (Kedl et al., 2000). Because they share the same antigen/MHC specificity, they compete if they are present at high frequency or if the number of APC or MHC/peptide complexes is limiting. This intraclonal competition has the advantage of allowing the highest affinity clones to be selectively activated over those with lower affinity (Smith et al., 2000b; Kedl et al., 2002). While only partial competition (Kedl et al., 2002) or no effect (Probst et al., 2002) was reported between CD8+ T cells of different specificity, nothing was known about interclonal competition between CD4+ T cells. Some of the data obtained in this study shed some light on this aspect.

It was previously evaluated that the number of transfected DC in the draining lymph nodes following PMDD is low (50-100 per lymph node) (Porgador et al., 1998), which may constitute a limitation for the activation of numerous adoptively transferred TCR-Tg T cells. Nevertheless, in the context of antigen linkage, a large number of PCC-specific CD4+ T cells were primed as efficiently in the presence as in the absence of a large number of OVA-specific CD4+ T cells, potentially competing for the same DC. Even more strikingly, endogenous OVA-specific CD4+ T cells, present at very low frequencies, could be efficiently primed in the presence of an overwhelming number of transferred PCC-specific CD4+ T cells activated on the same DC. This activation of endogenous OVA-specific T cells was, in fact, much more efficient in the presence of the potentially competing PCC-specific T cells (i.e. linked situation) than when competition should be minimised by favouring the segregation of the two antigens on different DC (i.e. unlinked situation).

Unlike in previous studies (Kedl et al., 2000; Kedl et al., 2002), we did not inject peptide-pulsed DC, so the number of antigen-presenting DC in lymph nodes is likely to be even more limiting than in these studies. In addition, at least in terms of the number of APC involved, our model may be more representative of the physiological situation during normal immunisation. Thus, it appears evident that the physical access to APC is not a limiting factor leading to competition (at least for CD4+ T cells). Some competition was reported between CD8+ T cells specific for different
antigens displayed by the same MHC molecules (Kedl et al., 2002), and it could not
be ruled out that some competition may occur at the level of antigen/MHC complex,
but if such competition occurs between CD4+ T cells of different antigen and MHC
specificity, it is largely overshadowed by the beneficial effect of cooperation.

The understanding of cooperation and competition has very important implications
for the design of multivalent vaccines. Components of multivalent vaccines
(proteins, peptides, glycoconjugates or DNA) have sometimes been reported to either
synergise or interfere between each other, but the mechanisms underlying this
phenomenon is not fully understood (see Section 8.3). The model established in this
research may help to better clarify the causes leading to synergy or interference in
vaccines. However, such studies can only be applied to a limited number of model
antigens at present, since the panel of available TCR-Tg mouse strains is restricted
and the cooperation or competition between two endogenous populations is more
difficult to measure. The concepts of cooperation and competition are further
investigated in Chapter 7.
Chapter 7

Cooperative rather than competitive interactions between CD4+ T cells of different antigen / MHC specificity
7.1. Introduction

Some of the observations that were discussed in Section 6.3.4 suggested that cooperation rather than competition occurs between endogenous and TCR-Tg CD4+ T cells of different specificity, when their antigens are presented by the same cells. High precursor frequency was previously reported to be a major factor driving intraclonal competition within CD4+ (Smith et al., 2000b; Laouar and Crispe, 2000) or CD8+ T cell clones (Kedl et al., 2000). Moreover, among CD8+ T cells recruited by a DC, those with the highest affinity are preferentially selected and expanded (Kedl et al., 2002). As a result, the immune CD8+ T cell repertoire is shaped towards immunodominant epitopes (Grufman et al., 1999). However, high affinity T cells are also subject to greater down-modulation by CTLA-4 (Egen and Allison, 2002), thus avoiding the danger of mono- or oligoclonal responses. Competition between CD8+ T cells of different antigen specificity was found to be more limited (Kedl et al., 2002) or insignificant (Probst et al., 2002). Interestingly, interactions between dominant and subdominant epitopes were found to be competitive in the case of CD8+ T cells (Grufman et al., 1999) or cooperative in the case of CD4+ T cells (Gerloni et al., 2000). Grufman et al. (1999) showed that the inhibition of CD8+ T cell responses to subdominant epitopes did not involve CD4+ T cells. On the contrary, this inhibition was stronger when CD4+ T cells were depleted, suggesting that CD4+ may cooperate rather compete with other clones, either CD4+ or CD8+.

Despite these observations, the nature of the in vivo interactions between CD4+ T cells of different specificity has not been fully characterised. In particular, the relative precursor frequency of each T cell clone is likely to determine the extent of cooperation, competition or the absence of any effect. As in Chapter 6, a combined adoptive transfer model was used, in which two TCR-transgenic CD4+ T cell populations, which have different antigen/MHC specificity (i.e. OVA expressed on I-A^d and PCC expressed on I-E^k), were co-injected as naïve into recipient mice. The relative clonal precursor frequency of the two populations was varied and their response was examined under different activation conditions, using PMDD. The OVA-specific CD4+ T cells were activated either alone in the presence of non-activated PCC-specific CD4+ T cells, or concomitantly with PCC-specific T cells. In the latter case, both antigens were either expressed by the same DC or by different
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DCs. The analysis was restricted to day 5 post-immunisation, because the enhancement of endogenous responses observed on day 5 (Chapter 6) was not detected on day 3.

7.2. Results

7.2.1. Correlation between clonal frequency, clonal expansion and Th1/Th2 balance

Variable numbers of PCC-specific naïve TCR-Tg T cells (3x10^3, 3x10^5) were injected into recipient mice. On the following day, these mice were immunised by particle-mediated delivery of linked pVAC1.PCC and pVAC1.OVA (“all beads linked”, group 1, Figure 2.5). On day 5 post-immunisation, the in vivo expansion of the transferred PCC-specific CD4+ T cells and their CD62L expression were assessed by flow cytometry (Figure 7.1, panels A-B) and the cytokine response was analysed on both PCC-specific and endogenous OVA-specific T cells by ELISPOT (Figure 7.1, panels C-E and F-H respectively).

A 10-fold difference in the number of PCC-specific T cells transferred resulted in only a two-fold difference 5 days after immunisation (Figure 7.1, panel A), indicating that the cells at lower frequency expanded up to 5 times more than cells at the higher frequency. In contrast, the decrease in CD62L expression was greater at the higher precursor frequencies (Figure 7.1, panel B). Furthermore, ~80% of TCR-Tg T cells at the highest frequency had divided at least once by day 5, as judged by CFSE staining (Figure 5.8). Thus, the less efficient expansion at the highest frequencies did not reflect a less efficient activation.

In the absence of TCR-Tg T cell transfer, no PCC-specific cytokine responses were observed for IFN-γ, IL-4 and IL-2 (Figure 7.1, panels C-E, first column). After transfer and immunisation, the number of cytokine-producing cells increased. As for clonal expansion, the number of cytokine-secreting cells did not rise in proportion to the precursor frequency (Figure 7.1, panels C-E, columns 2-4). The inverse relationship between the extent of the immune response and precursor frequency is clearly demonstrated in Figure 7.2, panels A-D, where the relative clonal frequency
**Figure 7.0.** Experimental procedure associated with Figures 7.1-7.2. Read-out (day 5) consists of measuring both PCC- and endogenous OVA-specific T cells responses by flow cytometry and ELISPOT.
Adoptively transferred naive PCC-specific T cells

Figure 7.1. Responses of PCC-specific T cells at different precursor frequencies and of endogenous OVA-specific T cells upon linked co-activation. Recipient mice were adoptively transferred with increasing numbers of PCC-specific TCR-Tg T cells and immunised with linked PCC/OVA DNAs by PMDD. Five days later, the percentage of vβ3+ γα11+ cells was determined by flow cytometry on lymphocytes from draining lymph nodes (panel A). The expression of CD62L on the vβ3+ γα11+ (PCC-specific, grey bars) and on the vβ3- γα11+ cells (bystander, white bars) is shown in panel B. Due to the low frequency of vβ3+ γα11+ cells in the absence of transfer, the expression of CD62L could not be accurately determined (ND). Lymph node cells were restimulated with 1μM of either PCC (panels C-E) or OVA peptide (panels F-H) for 24 hours and the number of IFN-γ+ (C, F), IL-4+ (D, G) and IL-2+ (E, H) spots per million lymph node cells was enumerated by ELISPOT. Each column represents mean ± SEM from 3 individual experiments (3 mice / group / experiment). T-test comparison was done between adjacent bars (panel A, C-H) and between grey and white bars within each group (panel B); ***, p<0.001; **, p<0.01; *, p<0.05.
Figure 7.2. Intracinal competition and Th1/Th2 balance depends on precursor cell frequency. Panels A-D show the immune responses to PCC described in Figure 1, panels A, C-E (clonal expansion, IFN-γ, IL-4 and IL-2 respectively), divided by 1, 10 or 100 to normalise to the precursor frequency (reflected by the total number of TCR-Tg cells injected, i.e. $3 \times 10^4$, $3 \times 10^5$ and $3 \times 10^6$ respectively). The endogenous responses were not used because the number of endogenous precursors could not be accurately estimated. Panel E shows the IFN-γ / IL-4 ratio as calculated from Figure 1 (panels C-D for PCC-specific responses, panels F-G for OVA specific responses). The ratio in absence of TCR-Tg T cells transfer was not calculated because of very low cytokine response.
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and cytokine production are plotted against initial precursor frequency (relative output in relation to cell input).

Additionally, when the precursor frequency was reduced from $3 \times 10^6$ to $3 \times 10^4$, the number of IFN-γ+ cells decreased more dramatically than that of IL-4+ cells (Figure 7.1, panels C-D). The IFN-γ / IL-4 ratio therefore declined as precursor frequency decreased (Figure 7.2, panel E, grey circles). This indicates that a greater clonal frequency may favour Th1 more than Th2 responses. By increasing frequency 10-fold from $3 \times 10^4$ to $3 \times 10^5$, the response was shifted from Th2 bias (ratio <1) towards a Th1 bias (ratio >1).

7.2.2. Endogenous CD4+ T cell responses become detectable upon concomitant activation with another CD4+ T cell clone present at high frequency

Within the same set of experiments, the cytokine response from endogenous OVA-specific T cells was also assessed by ELISPOT, as this technique is sensitive enough to detect low frequency cytokine-producing cells and only CD4+ T cells were activated by the class II MHC-restricted peptides used for restimulation. Since mice were immunised with both OVA and PCC antigens co-delivered into the same cells in vivo by PMDD, we were able to document whether the presence of PCC-specific CD4+ T cells, present at different frequencies and concomitantly activated by the same DC as the OVA-specific T cells, would improve the response of the latter, as observed with $3 \times 10^6$ PCC-specific cells in Chapter 6.

In the absence of transferred PCC-specific TCR-Tg T cells, the response of endogenous OVA-specific T cells was virtually undetectable (Figure 7.1, panels F-H, first column), as was observed also for the endogenous PCC-specific T cells (Figure 7.1, panels C-E, first column). However, in the presence of $3 \times 10^4$, $3 \times 10^5$ and $3 \times 10^6$ PCC-specific TCR-Tg T cells, IFN-γ-, IL-4- and IL-2-producing OVA-specific T cells were generated in detectable numbers (Figure 7.1, panels F-H, columns 2-4). Moreover, the IFN-γ / IL-4 ratio of endogenous OVA-specific T cells also increased with greater numbers of PCC-specific TCR-Tg T cells (Figure 7.2, panel E, white circles). In complementary experiments, the number of IL-2-expressing endogenous
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PCC-specific T cells was also greatly enhanced upon concomitant activation with 3x10^6 OVA-specific TCR-Tg T cells and this enhancement depended on linkage (Figure 7.3).

7.2.3. Presentation of two antigens favours cooperation

Recipient mice received 1x10^6 naïve PCC-specific CD4+ T cells (vβ3+ vα11+) and a variable number of naïve OVA-specific T cells (KJ1+ CD4+): 1x10^4, 1x10^5, 1x10^6 or none. The recipients were immunised either with OVA alone or with OVA and PCC co-coated on the same beads (“all beads OVA”, group 4 and “all beads linked”, group 1; Figure 2.5). Five days later, draining lymph nodes were collected for analysis of both PCC- (Figure 7.4, panels A-B and Figure 7.5, panels A-C) and OVA- (Figure 7.4, panels C-D and Figure 7.5, panels D-F) specific responses by flow cytometry (Figure 7.4) and ELISPOT (Figure 7.5).

In the absence of PCC DNA, PCC-specific T cells remained at the same baseline frequency (~0.5%) as in non-immunised mice (Figure 7.4, panel A). These cells were also CD69^low^ (Figure 7.4, panel B) and failed to express IFN-γ (Figure 7.5, panel A) or IL-4 (Figure 7.5, panel B). A few of them produced IL-2 upon subsequent peptide stimulation in the ex vivo assay (Figure 7.5, panel C). When mice were immunised with PCC as well as OVA, a strong PCC-specific (clonal expansion and cytokine production) was evident. The flat trend of the lines shows that the number of PCC-specific T cells migrating through the lymph nodes and their response were not affected by the presence of different numbers of transferred OVA-specific T cells, whether naïve or activated.

Clonal expansion of the OVA-specific T cells, and their CD69 expression, were also similar, whether they were activated alone or concomitantly with PCC-specific T cells (Figure 7.4, panels C-D). As for the PCC-specific responses described in Figure 7.2 (panel A), the clonal frequency after immunisation did not rise in proportion to precursor frequency of transferred cells (Figure 7.4, panel C). In addition, when the OVA-specific T cells were present at high frequency, a greater proportion of them expressed CD69 (Figure 7.4, panel D), corroborating the frequency-dependent decrease in CD62L expression seen for PCC-specific T cells (Figure 7.2, panel B). In
Figure 7.3bis. Experimental procedure associated with Figures 6.1-6.5. Read-out (day 5) were PCC- and OVA-specific responses by both flow cytometry and ELISPOT.
Figure 7.3. Generation of IL-2-producing cells from endogenous or transferred TCR-Tg T cells after immunisation with both PCC and OVA, either unlinked or linked. Some mice were adoptively transferred with $3 \times 10^6$ PCC- or OVA-specific TCR-Tg T cells. Transferred and non-transferred mice were then immunised with linked ("all beads linked") or unlinked PCC/OVA DNAs by PMDD. Five days later, draining lymph nodes were collected and lymph node cells were restimulated with 1μM of either PCC (grey columns) or OVA peptide (white columns) for 24 hours and the number of IL-2+ spots per million lymph node cells was enumerated by ELISPOT. Panel B represents a magnification of panel A on low numbers. Each column represents mean ± SEM from 3 individual experiments (3 mice / group / experiment). T-test results are shown on graphs.
Figure 7.4. Activation and expansion of OVA-specific T cells is not affected by concomitant priming with PCC-specific T cells. Recipient mice were adoptively transferred with $10^6$ PCC-specific T cells ($v\beta^3+ v\alpha 11^+$) and either no, $10^4$, $10^5$ or $10^6$ OVA-specific T cells (KJ1+ CD4+). The following day, they were immunised with cartridges made with "all beads OVA" or "all beads linked" preparations. Five days later, draining lymph nodes were collected and TCR-Tg T cells were examined by flow cytometry. Clonal expansion was measured as the proportion of clonotype-positive cells within lymphocytes (panels A and C for PCC- and OVA-specific response respectively). Clonal frequency in the absence of immunisation, at six days after transfer, is also shown. CD69 expression on $v\beta^3+ v\alpha 11^+$ and KJ1+ CD4+ cells is shown in panels B and D respectively. Graphs represents mean ± SEM from 3 independent experiments (3 mice / group / experiment), except for non-immunised mice, where mean ± SD from 5 mice in two separate experiments was used. Statistical comparison on the OVA-specific responses is shown in Table 7.1.
Figure 7.5. Enhancement of OVA-specific cytokine responses by concomitant activation with PCC-specific T cells. Lymph node cells from the same experiments described in Figure 7.4 were restimulated with 1μM of either PCC (panels A-C) or OVA peptide (panels D-F) for 24 hours and the number of IFN-γ+ (A, D), IL-4+ (B, E) and IL-2+ (C, F) spots per million lymph node cells was enumerated by ELISPOT. Graphs show the mean ± SEM from 3 independent experiments (3 mice / group / experiment). Statistical comparison on the enhancement of OVA-specific cytokine responses is shown in Table 7.1.
contrast, cytokine secretion was strongly enhanced upon concomitant activation of PCC-specific T cells (Figure 7.5, panels D-F). The enhancement was most dramatic at the lower numbers of transferred OVA-specific T cells (Table 7.1, fourth row for statistics). Even in the absence of OVA-specific TCR-Tg transfer, the concomitant activation with PCC-specific responses revealed detectable OVA-specific IFN-γ, IL-4 and IL-2 responses from endogenous OVA-specific T cells, as observed in Figures 6.2 and 7.3.

7.2.4. T cell cooperation requires linkage at low cell numbers

In order to explore whether the cooperative interaction between the two T cell populations required linkage, mice where immunised with OVA alone, OVA/PCC unlinked or OVA/PCC linked (groups 5, 3 and 2 respectively; Figure 2.5). The number of OVA-coated beads was half that used previously (Figures 7.4-7.5) to allow comparison with the "unlinked" preparation. The dosage effect of doubling the number of beads with antigen (between Figures 7.4-7.5 and Figure 1.6-1.1) is analysed in the two first rows of Table 7.1 for OVA-specific responses. Doubling the number of linked PCC/OVA-coated beads resulted in significant increase of IL-4 and IL-2 in the OVA-specific population (Table 7.1, second row). In contrast, doubling the number of beads carrying OVA alone did not significantly improve the OVA-specific responses (Table 7.1, first row). Doubling the number of linked PCC/OVA-coated beads also led to significant increase of clonal expansion and IL-2 in the PCC-specific population (p<0.05).

The OVA-specific response, in terms of the number of IFN-γ+, IL-4+ and IL-2+ cells, but not the overall clonal expansion, was strongly enhanced in the presence of activated PCC-specific T cells (Figures 7.6-7.7, right panels and Table 7.1, third row), confirming results on Figures 7.4-7.5. Delivery of the two antigens on separate beads ("unlinked") showed an intermediate degree of enhancement. At high precursor frequencies, unlinked and linked deliveries of the two antigens were not significantly different (Table 7.1, last row). In contrast, at the lowest precursor frequency of OVA-specific T cells compared with PCC-specific T cells (1:100 ratio or endogenous cells), unlinked help (for IL-4 and IL-2 production) was significantly lower than linked help and induced no or little improvement compare with OVA.
Table 7.1. T-test comparison of OVA-specific responses shown in Figures 7.4-7.7. Symbols: *** p<0.001, ** p<0.01, * p<0.05, ns
Figure 7.6. Activation and expansion of OVA-specific T cells is not affected by concomitant priming with PCC-specific T cells. Same protocol as Figure 7.4 was used, except that recipient mice were immunised with cartridges made with "half beads OVA", "unlinked" and "half beads linked" preparations. Clonal expansion (panels A and C) and CD69 expression (panels B and D) were assessed for PCC- and OVA-specific responses respectively, as in Figure 7.4. Graphs show the mean ± SEM from 3 independent experiments (3 mice / group / experiment), except for non-immunised mice, where mean ± SD from 5 mice in two separate experiments was used (same values as Figure 7.4). Statistical comparison on the OVA-specific responses is shown in Table 7.1. The responses of PCC-specific T cells to OVA/PCC unlinked and linked were not significantly different.
Figure 7.7. Enhancement of OVA-specific cytokine responses is dependent on linkage at low precursor frequency. Lymph node cells from the same experiments described in Figure 7.6 were restimulated with 1μM of either PCC (panels A-C) or OVA peptide (panels D-F) for 24 hours and the number of IFN-γ+ (A, D), IL-4+ (B, E) and IL-2+ (C, F) spots per million lymph node cells was enumerated by ELISPOT. Graphs show the mean ± SEM from 3 independent experiments (3 mice / group / experiment). Statistical comparison on the enhancement of OVA-specific cytokine responses is shown in Table 7.1. The responses of PCC-specific T cells to OVA/PCC unlinked and linked were not significantly different.
alone group (Table 7.1, rows 5 and 6). The number of IFN-γ+ cells was more
variable between experiments and the effect of linkage was not found to be
significant.

7.3. Discussion

The main objective of this study was to explore the interactions between CD4+ T
cells of different antigen specificity, at different precursor frequencies. Titration of
precursor frequencies (between approximately 1:10^2 down to 1:10^6) for both PCC-
specific and OVA-specific T cells showed strong evidence of homeostatic regulation,
presumably mediated by intracloinal competition as demonstrated previously for both
CD4 and CD8 T cells (Smith et al., 2000b; Kedl et al., 2000) or other mechanisms
(Laouar and Crispe, 2000). Clonal expansion, for example, was ~20 times greater
after transfer of 3x10^5 transgenic PCC-specific T cells than after transfer of 3x10^6.
Interestingly, the maximum clonal frequency (approximately 2-3% and 1% for PCC-
and OVA-specific T cells respectively) is within the range seen in vivo after acute
infection (Waldrop et al., 1997; Whitmire et al., 2000), suggesting that this may
reflect a physiologically relevant, but poorly understood, homeostatic mechanism
able to prevent over-dominance of individual T cell clones. However, the
mechanisms that limit T cell expansion remain incompletely understood. The
proportion of cells being activated (as indicated by the down-regulation of CD62L or
the up-regulation of CD69) increases with their precursor frequency. Furthermore,
most of antigen-specific CFSE-labelled T cells at high precursor frequency undergo
between 1 and 5 divisions. The limited clonal expansion observed at high precursor
frequency is therefore not attributed to a limited activation, but rather may reflect
reduced rounds of cell division (Smith et al., 2000b; Laouar and Crispe, 2000), or
increased death or emigration.

The ratio of IFN-γ / IL-4 (reflecting the Th1/Th2 balance) consistently increased with
higher precursor frequencies of PCC-specific T cells. Furthermore, endogenous
OVA-specific T cells (present at much lower frequency) exhibited a lower IFN-γ /
IL-4 ratio than the PCC-specific TCR-Tg T cells, which also increased upon
concomitant activation with increasing numbers of linked PCC-specific T cells. For
example, the endogenous OVA response, normally strongly Th2 biased in this model, was converted into Th1 by transfer and activation of $3 \times 10^6$ PCC-specific T cells. Thus, a high frequency of responding cells favours a Th1 response. There may be at least two explanations for this. First, since a higher proportion of activated T cells is obtained with higher precursor frequencies, it is possible that DCs become better activated by a greater number of CD40L-expressing T cells, thus secreting more IL-12 and driving Th1 differentiation more efficiently (Roy et al., 1993; Celia et al., 1996; Koch et al., 1996). Second, if up to three cell divisions are required for IL-4 production but none for IFN-$\gamma$ production (Laouar and Crispe, 2000; Bird et al., 1998), then the proportion of IL-4+ cells may be reduced at high precursor frequency when T cells undergo fewer cell divisions (Smith et al., 2000b). The apparent lack of dependence of IFN-$\gamma$ production on proliferation (Laouar and Crispe, 2000) may explain the distinct pattern in the homeostasis of IFN-$\gamma$+ cells compared with IL-2+ and IL-4+ cells, which appeared more connected with clonal expansion (Figure 7.2, panels A-D).

The extent of T cell activation at a clonal level can be regulated in principal either by antigen-specific competition (e.g. by the number of available MHC/peptide complexes), or by antigen-independent competition (e.g. by availability of a cytokine or by space on the surface of a DC). Our study provides strong evidence for antigen specificity as the major factor driving competition. The presence of a high number of activated PCC-specific cells within the lymph node does not inhibit, but rather enhances, a concomitant OVA-specific response, even when the precursor frequency of OVA-specific cells is 100-fold lower, or even more in the case of endogenous OVA-specific response, confirming previous observations (Chapter 6). Antigen presentation is unlikely to be a restricting factor, since previous experiments have shown that the amount of DNA per bead used in this study is not limiting. The number of DC transfected and expressing antigen, which has been estimated as little as 50-100 per lymph node following PMDD (Porgador et al., 1998), may limit the total number of peptide/MHC complexes. Some evidence for this is provided by the dosage effect seen when doubling the number of antigen-coated beads/cartridge ("half beads" versus "full beads" groups). However, this effect resulted at best in only a very modest increase in the responses. This supports previous suggestion that
APC and antigen/MHC availability may not be the main factor that drives competition between CD4+ T cells (Laouar and Crispe, 2000). Perhaps the duration of antigen expression, as determined by the stability of peptide/MHC complexes or DC lifespan, provides a more stringent limitation, but this requires further investigation.

Previous studies on cooperation between CD4+ T cells have shown that clones responding to a strong epitope were able to increase/initiate the response of clones specific for a weak/cryptic epitope, a response that would otherwise be low or absent (Gerloni et al., 2000). Likewise, we demonstrate here that a clone present at higher frequency can significantly amplify the responses from a clone present at much lower frequency upon antigen linkage. Thus, in contrast to the competition observed only in the context of a single clone of T cells, there is strong cooperation between T cells of different specificity. When the precursor frequencies of the two populations were both high, the responses of OVA-specific T cells were generally not significantly improved by the presence of activated PCC-specific T cells. In contrast, when the frequency of OVA-specific T cells was reduced (100-fold or more compared with PCC-specific T cells), cytokine responses (IFN-γ, IL-4 and IL-2) were much better in the presence than in the absence of the activated PCC-specific T cells. However, neither clonal expansion nor CD69 expression was affected. The signal delivered by the “helper” T cells was therefore required for cytokine production (i.e. differentiation) but not initial priming or proliferation.

The nature of the signal(s) mediating T cell cooperation remains to be determined, but the release of cytokines in the context of a DC cluster is an attractive possibility. The delivery of DNA either on the same or different beads was used to probe the nature of help. A number of factors, such as co-transfection of one DC with two beads, or ectopic expression of antigen in skin followed by reprocessing and presentation, could theoretically interfere with this experimental design. In practice, however, these factors did not appear to be of major significance and at low precursor frequencies in particular, an absolute requirement for linkage was observed. As T cells numbers increased, linkage became less important, perhaps because cytokine cross-talk between DC clusters became more prominent. It is interesting that the requirement for linkage was lost more readily for IL-2 and IFN-γ,
than for IL-4, perhaps reflecting the lower quantities of this latter cytokine secreted per cell, and hence its more localised activity (Figure 5.3; Openshaw et al., 1995). The role of cytokines in the cooperation between CD4+ T cells is further discussed in Section 8.2.3.2.

In conclusion, two opposing forces shape the emergence of the T cell repertoire following immunisation. Competition, acting at the level of antigen/MHC limits the response to each epitope specifically. Cooperation, acting between all T cells activated within an individual DC cluster, whether specific for the same epitope or not, enhances T cell differentiation, and can permit the detection of responses even when the T cells are present at very low precursor frequencies as in a naïve animal. This cooperation shapes the nature (Th1/Th2 balance) of the immune response to related antigens (i.e. presented as linked on the same DC) and aims to retain consistency in the type of response to these related antigens, in contrast to the responses to unrelated antigens, which may be independent (Ismail and Bretscher, 1999). Thus, this cooperation is likely to be most effective when the phenotype of the T cell responses required (Th1 versus Th2) is the same and may be exploited in multivalent vaccines for the treatment in particular of individuals with reduced responsiveness (see Chapter 8 for further discussion).
Chapter 8

General discussion
8.1. New insights on the biology of particle-mediated DNA immunisation

8.1.1. Kinetics of CD4+ T cell responses

This research has led to several interesting observations regarding the timing of CD4+ T cell responses to PMDD. The first was that PMDD induces helper T cell activation with approximately one-day delay compared with a conventional protein/adjuvant formulation, as illustrated by the different kinetics of CD25 and CD69 up-regulation using these two methods. The clonal expansion, and the secretion of IFN-γ and IL-5 were also delayed. Slower kinetics of activation in PMDD may be due to lower amounts of antigen produced and secreted, a longer time needed for antigen expression from DNA, a more limiting number of APC involved and/or less potent inflammatory signals than those delivered by the adjuvant, resulting in slower migration of DCs.

The second observation was that the kinetics also varied within PMDD immunised groups, depending on the genetic background and the antigen. Effector responses were faster in B10 than in BALB/c regardless of the antigen used, with the IFN-γ and IL-4 responses already declining between days 3 and 5 in the former, while still increasing in the latter. In contrast, proliferative responses appear more dependent on the antigen used rather than the genetic background. The response to pVAC1.OVA was characterised by a transient expression of CD25 with a significant drop between day 3 and day 5, while the number of IL-2+ cells increased over the same period. In contrast, in response to pVAC1.PCC, the expression of CD25 was sustained and the number of IL-2+ cells decreased between day 3 and day 5. It is possible that the prolonged expression of CD25 renders CD4+ T cells more susceptible to IL-2-induced apoptosis (Van Parijs et al., 1999), resulting in a fall of T cell numbers, or alternatively, to other IL-2-mediated signals that induce a more rapid exit from the lymph nodes. Moreover, PCC stimulated a larger clonal expansion and absolute number of IL-2+ cells compared with OVA. The differential regulation of T cell proliferation observed between OVA and PCC may be attributed to the strength of the signals transmitted via these antigens to the respective specific TCR-Tg T cells. The strength of the stimulation may be controlled by the affinity of the interaction between peptide and either MHC or TCR, or the density of peptide/MHC complexes.
displayed by DCs. In a comparative study, the same amounts of plasmid DNAs were used for both antigens, thus a slightly greater number of pVAC1.PCC copies was administered compared with pVAC1.OVA (due to differential size), which may possibly result in different amount of peptide/MHC complexes being presented. However, no apparent bias in the number of IFN-γ+ and IL-4+ cells was detected between the two antigens, although Leitenberg and Bottomly (1999) reported that stronger TCR signals favour IFN-γ production over IL-4. Thus, if T cell activation was more efficient with PCC because of stronger TCR signals, the relative difference in strength between PCC and OVA was probably not sufficient for a more pronounced Th1 bias with PCC (see also Section 8.1.2).

The third observation was an apparent unsynchronised generation of Th1 versus Th2 cells. In most cases, the number of IFN-γ+ cells declined between day 3 and day 5, while the number of IL-4+ cells increased. Even in those experiments where the number of IFN-γ+ cells also increased, the overall IFN-γ / IL-4 ratio usually decreased. It seems that Th2 populations emerge with a delay compared with Th1 cells in this model. This observation is in accord with many previous studies. First, Th1 differentiation can occur rapidly after TCR stimulation in the presence of IL-12 while Th2 differentiation requires prolonged TCR stimulation, even in the presence of IL-4 (Iezzi et al., 1999). More recently, Langenkamp et al. (2000) have reported that DCs may sequentially stimulate Th1 and Th2 cells, within two phases. In the active phase, LPS-activated DCs secrete IL-12, which induces Th1 differentiation. In the subsequent “exhausted” phase, Th2 may then differentiate because IL-12 is no longer produced. This sequential hypothesis is further supported by the observation that the bulk of IFN-γ secretion occurs earlier than that of IL-5 after in vitro (Rogers and Croft, 2000) and ex vivo restimulation (Chapter 3). Surprisingly however, the secretion of IL-13, another Th2 cytokine, is an early event similar to that for IFN-γ (Rogers and Croft, 2000). Finally, DCs sensitised with GM-CSF in vivo 3 days before immunisation induced a Th2 response, while DCs freshly sensitised by this cytokine at the time of or after immunisation, preferentially elicit a mixed Th1/Th2 or a Th1 response respectively (Kusakabe et al., 2000). Thus, when DCs were matured early, they may have been close to “exhaustion” at the time they received the antigen. Given the mutually inhibitory effect that these two populations have on
each other, the cytokine balance may be initially in favour of Th1, due to the early release of IL-12, but also of IFN-γ compared with IL-4. It was indeed reported that IFN-γ requires one or no cell division for its production (Bird et al., 1998; Laouar and Crispe, 2000), while IL-4, needs between 1 and 4 divisions (Bird et al., 1998; Richter et al., 1999). In addition, this research and that of others (Openshaw et al., 1995) have shown that the amount of IL-4 produced per cell by polarised Th2 cells is considerably lower than the amounts of IFN-γ expressed by Th1 cells, at least in these transgenic models. Thus, unequal production of these cytokines may initially favour the differentiation of Th1 cells. In this research, it has also been observed that when the number of IFN-γ+ cells was initially high (i.e. in the case of naïve and Th1 TCR-Tg cells), this number subsequently declined between day 3 and day 5. In contrast, when the number of IFN-γ+ cells was low (i.e. in the case of Th2 TCR-Tg cells and endogenous naïve T cells), this number rose with time. Thus, the elicited IFN-γ+ population may be subject to a stringent regulation that regulates its size.

Finally, the changes in Th1 and Th2 cell ratio in draining lymph nodes during the early period of a primary response may reflect the differential ability and/or speed with which these cells leave the lymph nodes. Following polarisation, Th1 and Th2 cells selectively express a distinct panel of chemokine receptors; however, a subset of these cells up-regulate CXCR5 after activation (Sallusto et al., 1998). Subsets expressing CXCR5, termed follicular B helper T cells (Tfh), specifically migrate to the germinal centres, where they activate B cells and stimulate antibody production (Breitfeld et al., 2000; Schaerli et al., 2000). Although both Th1 and Th2 cells may support B cell activation (Rotteveel et al., 1988; Abbas et al., 1990; Smith et al., 2000a), it is generally accepted that Th2 cells are the most potent in stimulating B cells (Whalen et al., 1988; Abbas et al., 1990). Thus, Th2 cells may reside longer in the lymph nodes, which may explain the more prolonged presence of IL-4+ cells compared with IFN-γ+ cells.

Overall, the kinetics of CD4+ T cell primary responses after PMDD appears to follow the same trends as in other models, with the exception of the delay compared with protein/adjuvant inoculation. PMDD may indeed reflect the sequence of events that follow real infections. The absence of synchronicity between Th1 and Th2
responses, for example, is generally observed in several models of infection with intra- and extracellular pathogens (Rhodes and Graham, 2002) and of autoimmunity (Segal et al., 1997).

8.1.2. Parameters affecting the Th1/Th2 balance

Genetic background is a multifactorial parameter, which was found to have the most dramatic effect on the Th1/Th2 bias. The preferential induction of Th1 and Th2 responses in B10 and BALB/c mice respectively is striking. The different types of responses evoked between mouse strains or human individuals are largely attributed to genetic polymorphism in key genes of the immune system. For instance, the outcome of many infectious or autoimmune diseases, in terms of susceptibility or resistance, is linked to genes that control, among others, IL-12 responsiveness (Gorham et al., 1997; Guler et al., 1999), the level of some transcription factors (Yagi et al., 2002) and MHC expression (Guardiola et al., 1996). The haplotype and level of expressed MHC have consequences on peptide/MHC affinity and peptide/MHC complex density respectively, two parameters that are positively correlated with Th1 differentiation (Murray, 1998).

Other parameters also influence the Th1/Th2 balance, and these include time (see Section 8.1.1. above), method of immunisation and T cell precursor frequency. Several groups have previously reported that PMDD is a method that favours Th2 responses (Feltquate et al., 1997; McCluskie et al., 1999; Tüting et al., 1999), or at least a mixed Th1/Th2 response (Oliveira et al., 1999). This was again recently confirmed by a study which also indicated that this effect is associated with the technique itself and not to a lack of CpG as adjuvant (Weiss et al., 2002). In B10 mice, endogenous responses to PMDD were also Th2-biased, despite a genetic environment that facilitates Th1 responses. In contrast, the responses of adoptively transferred CD4+ T cells were clearly Th1-biased in the same animal model, suggesting that Th1 responses improve with higher precursor frequencies. This observation was also corroborated by the fact that the formation of IFN-γ+ spots in ELISPOT assay is optimal at highest in vitro cell density (personal observation; Power et al., 1999). The effect of higher precursor frequency resulting from adoptive transfer may explain why, in the adoptive transfer model in BALB/c mice, the
response was not strongly Th2-biased and the magnitude of IFN-γ production was comparable to that obtained with protein/CFA.

Surprisingly, the dose of antigen, tested both in terms of amount of DNA per DC (Chapter 4) and of number of antigen-bearing DCs (Chapter 7), did not apparently alter this balance. It was presumed that doubling the number of beads carrying antigen, but with a constant total number of beads, might lead to twice as many antigen-presenting DCs, which would then activate more T cells. A slight increase in all the responses was indeed observed, but this was usually not statistically significant and certainly not double. Furthermore, in the light of evidence that stronger TCR signals favour Th1 responses (Leitenberg and Bottomly, 1999), it was predicted that greater amounts of DNA delivered per DC would result in more antigen being presented by DCs and hence favour a Th1 response. However, the effect of increasing the amount of DNA per bead was a better induction of helper T cell differentiation without a predilection towards Th1 or Th2. It was previously shown that, with increasing amounts of DNA per bead, the production of protein also increases up to a plateau (Eisenbraun et al., 1993). Beyond this plateau, more DNA per cell does not augment the expression of protein. Thus it is possible that the cellular machinery of the cell is able to cope with the transcription of increasing numbers of DNA vectors, up to a certain limit above which it becomes saturated. Thus, the increase of protein expression, below the limit, may result in a more effective helper T cell differentiation and cytokine production. However, it is possible that the dose of protein antigen required for a preferential Th1 differentiation is above this limit and may be achieved with high doses of extracellular protein/peptide (Constant and Bottomly, 1997) but not with high doses of intracellular DNA. It is difficult to compare the amount of protein/peptide given extracellularly (in particular the amount required to alter the Th1/Th2 balance) with the amount produced per cell (e.g. 17-25 ng luciferase per cell in vitro using 0.5 µg DNA / mg gold; Thompson et al., 1993). Eisenbraun et al. (1993) demonstrated that the number of beads carrying antigen, but not the amount of DNA per bead, was a parameter that significantly influences levels of total IgG to hGH antigen, but the isotype of these antibodies was not assessed. Beyond the effect of antigen dose, there was no preferential stimulation of Th1 responses with higher doses of DNA via the adjuvant effect of CpG either. This is consistent with the lack of TLR-9 stimulation
(Bauer et al., 2001) if most of the DNA is efficiently introduced directly into the cytoplasm of cells. Moreover, the response to PMDD appears to be uninfluenced by CpG stimulation, even if DNA is present both intra- and extracellularly (Weiss et al., 2002).

8.1.3. Role of antigen transfer (cross-priming)

It is well recognised that peptides loaded onto class II MHC derive from extracellular antigens (exogenous pathway), while those loaded onto class I MHC come from intracellular antigens (endogenous pathway). However, DCs do not have to be infected to use the endogenous pathway. They have a unique ability to process extracellular antigens also onto class I MHC, a phenomenon termed cross-priming and dependent on the transporter associated with antigen processing (TAP) and the proteasome (Huang et al., 1996; Brossart and Bevan, 1997). Thus, the term "cross-priming" usually refers to the induction of CD8+ T cell responses and implies that intracellular antigens are not sequestered inside infected cells but are acquired by DCs by pinocytosis of secreted proteins (Brossart and Bevan, 1997) or from phagocytosis of apoptotic bodies (Albert et al., 1998).

The notion of cross-priming has been extended to the acquisition of exogenous antigens by DCs for presentation to both CD4+ and CD8+ T cells. Indeed, the induction of CD8+ T cells by cross-priming is inefficient if the same DCs do not simultaneously present peptides to CD4+ T cells (Bennett et al., 1997). In the case of DNA vaccination, the antigen may be in the form of DNA or protein subsequently expressed and secreted by cells. Whether DCs preferentially take up DNA or protein may depend on the conditions of delivery, including the route / tissue and the dose (Corr and Tighe, 1997; Takashima and Morita, 1999; for reviews). Corr et al. (1999) showed that CTL responses evoked by saline injection of DNA depend more on antigen transfer from non-APCs to APCs than on direct transfection of APCs. However, two other studies suggested that CTL responses rely more on DNA uptake rather than uptake of secreted protein, while antibody responses require protein secretion (Whitton et al., 1999; Sbai et al., 2002). Similar controversy applies to immunisation by PMDD, with arguments in favour of a major role of directly transfected DCs (Condon et al., 1996; Porgador et al., 1998) or of antigen transfer
from non-APCs such as keratinocytes, for the bulk of the CD8+ T cell responses elicited (Cho et al., 2001). None of these studies has really examined the CD4+ T cell responses in detail.

Gene gun is an efficient tool to achieve direct transfection of DCs. Studies described in Chapters 6 and 7 provide further evidence that these directly transfected DCs do not efficiently exchange antigens, despite several natural mechanisms that may allow them to do so (Section 1.4.3). Although both PCC and OVA antigens may be secreted, this secretion did not result in the effective reunion of the two segregated antigens in unlinked groups, as judged by the differences observed between linked and unlinked conditions. The lack of antigen transfer despite secretion may be attributable to the timing of DC activation and presentation. Upon transfection by PMDD, Langerhans cells may receive potent inflammatory signals and rapidly leave the targeted area of the epidermis. These DCs may play the major role in the induction of CD4+ and CD8+ T cell responses. The fact that the pool of responding T cells is mainly constituted by T cells that are recruited early for activation (Bousso et al., 1999) further supports this idea. Many non-transfected DCs also leave the skin (Porgador et al., 1999; Bot et al., 2000), limiting the number of DCs in the skin available for cross-priming. Thus, by the time antigens are subsequently synthesised and secreted by transfected keratinocytes, the remaining DCs may not contribute significantly to CD4+ and CD8+ T cell responses, but the secretion of antigens remains an important prerequisite for antibody responses, since removal of the immunisation site within 24 hours abrogates the latter (Torres et al., 1997). The opportunity for the subsequent antigen transfer may also be limited by the transient expression for only 48 hours of antigen in the immunisation site after PMDD (L. Thomsen, unpublished observation).

In some experiments (Chapter 7), some degree of enhancement of OVA-specific responses by PCC-specific T cells was observed in the absence of linkage. At least three possibilities may be put forward to explain this effect. First, some DCs may be directly transfected by more than a single bead, so that two antigens on different beads may be ultimately co-expressed by the same DC on rare occasions. Second, antigen transfer may occur between DCs and non-APCs in the skin; and this cannot be neglected even though it may be a slow process making only a small contribution
to the early priming of T cells, as discussed above. The down-regulation of processing upon DC maturation, which has been extensively documented in vitro, may also contribute to a failure to cross-present secreted antigen within the lymph node. It cannot be ruled out that some antigen exchange may occur via exosomes and/or apoptotic bodies; however, Corr et al. (1999) suggested that antigen transfer is more likely to occur in the site of immunisation rather than in secondary lymphoid tissues. Third, the enhancement observed in absence of linkage may in part reflect a real endocrine effect. This effect was variable and seen most clearly at high precursor frequencies, presumably reflecting the greater amount of cytokine released.

8.1.4. Lessons and prospects for PMDD

Under the conditions used, the highest total amount of DNA that could be effectively precipitated was \( \sim 3.65 \, \mu g \, DNA / mg \, gold \). A minimum of \( 0.1 \, \mu g \, DNA / mg \, gold \) was found sufficient to induce optimal protein secretion and antibody responses to hGH (Eisenbraun et al., 1993), however, a minimum of \( \sim 1.5 \, \mu g \, DNA / mg \, gold \) was required to optimally stimulate both activation and differentiation of OVA- or PCC-specific CD4+ T cells in the present study. This means that at least two different plasmids can be concomitantly inoculated on the same bead for optimal responses, provided that no interference occurs between the two vectors during expression. These parameters are important to consider for optimal co-expression of a second antigen or genetic adjuvant without jeopardising the response to the main antigen. Furthermore, if synergy rather than interference is proven between two encoded genes, then the amount of DNA per mg of gold may be reduced accordingly so that more antigens can be co-expressed (see also Section 8.3).

The choice of promoter strength and specificity as well as the control of antigen localisation are two other important parameters. Studies using APC-specific promoters demonstrated poor cellular responses (Corr et al., 1999; Cho et al., 2001), a result which was interpreted in terms of a dependence on antigen transfer. In contrast, DCs were efficient by themselves in inducing cellular responses after direct transfection or DNA uptake if a strong viral promoter such as CMV was used (Condon et al., 1996; Porgador et al., 1998; Whitton et al., 1999; Sbai et al., 2002; and this study). Thompson et al. (1993) compared the activity of several promoters
after PMDD transfection and concluded that tissue-specific promoters (e.g. mouse mammary tumour virus (MMTV), hepatitis B virus) drive the expression of considerably lower levels of transgene compared with promoters that can be expressed in many tissues (e.g. CMV, SV40, RSV). Finally, it was clear from all these studies that antigen secretion is essential for humoral responses. Thus, depending on the type of immune response required, antigen secretion may be reduced or enhanced by removing or adding a secretion sequence, such as the IgG signal sequence (this study; Svanholm et al., 1999).

The behaviour of CD4+ T cells following PMDD appeared comparable to the responses to other types of immunisation. The magnitude of the responses was similar to that obtained with protein/CFA, but the kinetics were slightly slower (Section 8.1.1). Evidence of activation, however, was observed as early as 24 hours after immunisation. Activated helper T cells underwent clonal expansion, which was visible after 2-3 days and appeared to take place exclusively in draining lymph nodes. From 5 days onward, effector/memory T cells could be detected in the spleen, and presumably also reached other tissues in the periphery.

8.2. Interactions in the DC cluster environment

8.2.1. Naturally related antigens and the role of DC

Ismail and Bretscher (1999) elegantly demonstrated that the Th phenotype of the simultaneous response to two unrelated antigens is independent. They immunised mice with non-cross-reacting red blood cells from sheep (SRBC) or chicken (CRBC). High doses of such antigen induced a mixed Th1/Th2 response while low doses preferentially stimulated a Th1 response. When the two antigens were co-injected, one at high and the other one at low dose, the type of response that they induced was the same as when they were injected alone. However, upon severe infection, the type of response induced may non-specifically alter an ongoing response of a different type (Actor et al., 1993). Such general shift in Th1/Th2 balance appears to also occur during pregnancy when Th1 responses are reduced and Th2 responses favoured, presumably under the influence of the hormones released at that time (Shurin et al., 1999).
It would be detrimental if the immune system induces opposing responses to different determinants of the same antigen/pathogen, given the fact that Th1 and Th2 responses may potentially counter-regulate one another. Thus, it was proposed here that another important role of DC was to orchestrate a homogenous and relevant response from the pool of T cells to which they present different related epitopes derived from a common entity. It was postulated that DC clusters constitute relatively closed microenvironments, within which T cells influence each other to homogenise their response, and between which there is no or little influence. In this model, T cells integrate two types of signals that may control their differentiation. First, they receive numerous signals according to (i) the nature of the DC, the way it has been conditioned by PAMPs and its maturity stage, which determine the nature and quantity of costimulatory molecules and cytokines expressed, and (ii) the nature of antigen, its dose and the number of determinants. Second, they also assimilate information from neighbouring activated T cells. T cells may communicate this information (i) indirectly via the DC by modulating its function, or (ii) directly by cytokines. This intra-cluster collaboration is even more relevant if some of the T cells involved have previously experienced the antigen (e.g. memory T cells) and may be pre-biased. Such experienced memory T cells may contribute importantly in the instruction of naïve T cells. Direct contact-dependent interactions may occur between activated T cells, thus bypassing the requirement of DC, such as CD70-CD27 interaction or T-T presentation. However, these two signals have no effect on the Th1/Th2 bias, they either enhance T cell proliferation or inhibit T cell function, respectively. Thus, a DC should not be regarded simply as a cell presenting an antigen to a T cell, but as a central component that orchestrate a multitude of interactions within a network (Figure 8.1).

8.2.2. Access to the DC cluster

Not all antigen-specific T cells are able to access or remain in a DC cluster. At least two key parameters control this access. Firstly, the TCR affinity determines the strength and the duration of the contact with the DC (Kedl et al., 2002). Secondly, the relative timing of the recruitment of a T cell clone by the DC compared with other clones determines the contribution that this clone will have within the total pool of responding T cells (Bousso et al., 1999). This suggests that a T cell clone that is
Figure 8.1. Positive and negative interactions between CD4+ and CD8+ T cells \textit{in vivo}.

- Dominant epitope
- Subdominant epitope
attracted late may find the DC totally surrounded by other T cells, which have established strong contacts. Such T cells may eventually move on in the search for another less saturated DC, however, such an opportunity is restricted by the limited presence (up to 48 hours) of antigen-presenting DCs in lymph nodes (Ingulli et al., 1997). The number of DCs migrating to the lymph nodes presumably depends on the route by which the antigen is introduced. For instance, PMDD was reported to result in 50-100 antigen-bearing DCs per draining lymph node (Porgador et al., 1998), but whether this represents a high or a limiting number is difficult to establish. It is clear that T cells compete if the number of DC is limiting, because such competition may be circumvented by the injection of mature DCs (Kedl et al., 2000). The same group showed that adoptively transferred OVA-specific CD8+ T cells inhibit an endogenous response specific to the same peptide or a related peptide presented by the same MHC (Kb) by the same DC. The authors concluded that the access to DC was limiting, but also acknowledged that the competition at the antigen level (intraclonal competition) was more efficient than at the DC level. These data supported the view that access to DC is not a major factor in driving competition between T cells. Grufman et al. (1999) suggested that CD4+ T cells do not compete with CD8+ T cells. In the studies reported in this thesis, endogenous OVA-specific CD4+ T cells activation was not inhibited but rather enhanced in the presence of a much greater number of PCC-specific CD4+ T cells when both OVA/A and PCC/Ed complexes were presented on the same DC. Similarly, adoptively transferred TCR-Tg CD8+ T cells specific for lymphocytic choriomeningitis virus (LCMV) gp33-41/D inhibited the response of endogenous CD8+ T cells with the same specificity without affecting the response of other endogenous CD8+ T cells specific for LCMV gp34-41/Kd and gp276-286/Dd (Probst et al., 2002). As these epitopes are related (i.e. derived from the same antigen), they are presented as linked determinants by DCs. Our data and those by Probst et al. (2002) demonstrate that access to DC is not controlled by crowding but rather by the availability of a given peptide/MHC complex on the surface of DC, consistent with a mathematical model by Borghans et al. (1999). However, it seems that the temporal availability of peptide/MHC complexes is more critical than the number of these complexes. Data from Probst et al. (2002) even suggest that MHC by itself may not be a limiting factor as long as the peptides presented are different.
8.2.3. Interactions between T cells

8.2.3.1. DC sensitisation / epicrine signals

In the introduction of this thesis, the role of T cells in back-signalling to DCs has been reviewed. Two pairs of ligands from the TNF-R family appear to play an important role. Activated T cells up-regulate CD40L and TRANCE, which bind to CD40 and RANK on DCs respectively. CD40 signalling enhances the expression of class II MHC, B7.1, B7.2 and ICAM-1 (Cella et al., 1996). Thus activated T cells promote the recruitment and effective activation of further T cells. CD40 ligation also up-regulates RANK (Anderson et al., 1997). Both CD40 and RANK signalling result in IL-12 secretion, which in conjunction with ICAM-1, favours Th1 differentiation (Yang and Wilson, 1996; Cella et al., 1996; Josien et al., 1999; Salomon and Bluestone, 1998; Luksch et al., 1999). TRANCE ligation also enhances IFN-γ production by T cells (Chen et al., 2001). Finally, the expression of CD40L is sustained longer in Th1 than Th2 cells (Lee et al., 2002b). These reports are supported by data from Chapter 7, which show that, in neutral in vivo conditions (that do not involve injection of polarised T cells), the proportion of Th1 compared with Th2 cells increases with the number of activated cells. Although CD40L initially favours the development of Th1 cells, both CD40L expression (Roy et al., 1993) and IL-12 secretion (Langenkamp et al., 2000) are limited in duration. CD40L also initiates Th2 differentiation by the up-regulation of B7 molecules on DCs, because costimulation via CD28 is essential for Th2 development (King et al., 1995; Rulifson et al., 1997). This signal may progressively counterbalance the strong Th1 stimulus initially delivered by CD40L. Finally, CD40 signalling also up-regulates 4-1BBL (Diehl et al., 2002) and OX40L (Brocker et al., 1999) expression on DC, both of which provide survival signals to T cells. Although OX40 and ICOS, both induced on activated T cells, have been implicated in the maintenance of Th2 functions more than Th1 functions, it is unclear, for example, whether OX40 is important for the early development of Th2 cells; it has been reported to costimulate either Th1 (De Smedt et al., 2002) or Th2 (Ohshima et al., 1998; Tanaka et al., 2000) in a primary response. B cells, however, deliver a strong Th2 polarising signal via OX40 stimulation, which enhances IL-4 and represses IFN-γ expression (Flynn et al., 1998).
DC activation by CD40L is the key epicrine signal in cooperation between activated and naïve/activated T cells. The overall contribution of CD40L on Th1 versus Th2 commitment may be evaluated in our model by using T cells from CD154^{+/-} TCR-Tg mice. The relative contribution of each CD40L-induced epicrine signal may be more difficult to dissect because some of these signals (e.g. IL-12) may also be induced by PAMPs or play a role in processes other than just T cell differentiation (e.g. B7, OX40L). Defining the role, both quantitatively and temporally, of these epicrine signals will allow a more rational choice for immunotherapeutic targets. Help given by T cells at high frequency to naïve T cells at low frequency progressively disappeared when the number of those at low frequency was increased (Chapter 7). If two naïve T cell populations were both at high frequency, no or little help was observed between them (Chapters 6 and 7). This help is likely to be a combination of epicrine and paracrine signals, and their relative contribution needs to be addressed. In contrast to naïve T cells, polarised T cells were able to deliver to naïve T cells further signals, which appeared to have a greater paracrine component (Chapter 6). These polarised T cells, present at lower frequency than naïve T cells, are indeed less likely to influence naïve T cells via CD40L expression. Although CD40L expression is only transient, it would be interesting to check whether polarised T cells retain other markers such as TRANCE, which can signal back to DCs.

8.2.3.2. Cytokines

Cytokines secreted by activated T cells may directly affect other T cells. Such cytokines may support T cell proliferation (e.g. IL-2, IL-4), modulate T cell differentiation (e.g. IFN-γ, IL-4) or inhibit T cell proliferation and/or function (e.g. IL-10). For example, activated CD4+ T cells that have acquired a polarised phenotype influence naïve CD4+ T cells to adopt a similar profile, both in vitro (Schuhbauer et al., 2000) and in vivo (Chapter 6). The production of IFN-γ by activated CD8+ T cells, in addition to that secreted by Th1 cells, also importantly contributes to the generation of more Th1 cells (Mailliard et al., 2002). These data all suggest that DC play an important role for two reasons: (i) DC gather T cells in a more confined area so that cytokines secreted by some T cells may benefit others, (ii) DC function may be modulated by cytokines produced by some T cells so that they adjust the activation of other T cells accordingly.
IL-2 is a potent T cell growth factor, which may act in an autocrine or a paracrine manner depending on the amount secreted per cell. Paracrine IL-2 may contribute importantly in the help from CD4+ to CD8+ T cells (Keene and Forman, 1982; Malek, 2002). In the CAT model, the help provided by other activated cells resulted in enhanced cytokine production, but IL-2 was the least enhanced cytokine. Moreover, the clonal expansion was not affected by help, presumably because clonal expansion, for each precursor frequency group, has reached levels that cannot be boosted further.

Th1 cells were found to express high levels of IFN-γ, which correlated with large spots in IFN-γ ELISPOT assays. However, Th1 cells (OVA-specific) did not augment the number of naïve T cells (PCC-specific) that express IFN-γ, presumably because at the precursor frequency examined (3x10^6 per mouse) the propensity to differentiate into Th1 is already high and close to a maximum. Usually, the number of IFN-γ+ T cells in the lymph nodes drops quite rapidly because these cells either leave the tissue or die. Interestingly, OVA-specific Th1 cells sustained the number of IFN-γ+ PCC-specific cells, either by stimulating the differentiation of more IFN-γ+ cells that replace those which disappeared, or by preventing emigration or death of Th1 cells. IFN-γ can enhance both the secretion of IL-12 by DC (Snijders et al., 1998) and the responsiveness to IL-12 by T cells (Smeltz et al., 2002). However, at the time when the higher number of IFN-γ+ cells were observed (day 5), it is unclear whether DCs have all disappeared (Ingulli et al., 1997), or no longer produced IL-12 (Langenkamp et al., 2000), or on the contrary, had their function and survival sustained, for instance by RANK signalling (Wong et al., 1997; Josien et al., 1999). Th1 cells may maintain DCs function better, because they preferentially express TRANCE (Chen et al., 2001). This hypothesis merits further investigation, because TRANCE may be a useful target to sustain Th1 responses in tumour models. At similar precursor frequency, polarised Th1 cells, but not naïve cells, were able to facilitate Th1 differentiation in naïve T cells of different specificity upon concomitant activation. However, naïve clones that were activated in higher numbers were able to enhance Th1 differentiation in other clones at low precursor frequency. Whether this help resulted from the accumulation of IFN-γ in the vicinity or a better sensitisation of DCs to produce IL-12 requires further study. However, the role of
IFN-γ was suggested by the observation that linkage was not always necessary for help, perhaps because high levels of IFN-γ produced may diffuse between DC clusters.

Early studies demonstrated the role of accumulating autocrine IL-4 after sufficient exposure to antigen in inducing Th2 differentiation (Croft and Swain, 1995; Demeure et al., 1995). This autocrine IL-4 production is sufficient to promote Th2 development in the absence of any other source of IL-4 (Noben-Trauth et al., 2000), but this production may remain very modest (Ben-Sasson et al., 2000). Repetitive stimulation or exogenous IL-4 appears to be required for eliciting a more vigorous Th2 response. CD4+ T cells, in the model used in this thesis, express lower levels of IL-4 than IFN-γ and this limited production may explain why Th2 cells constitute a smaller population than Th1 cells (a fact also associated with the genetic background). It is unclear whether CD4+ T cells are repeatedly stimulated during a primary response in vivo. Alternatively, these cells may rely on IL-4 secreted by other cells. Other freshly activated CD4+ T cells have an important role as a source of IL-4 in this respect (Gollob and Coffman, 1994; Croft and Swain, 1995). If these cells remain within a confined microenvironment, then limiting autocrine IL-4 may act as a paracrine stimulus, by accumulating over time. Th2 cells also constituted a source of paracrine IL-4 in this model and significantly improved Th2 differentiation in naïve T cells (Chapter 6). The requirement for a local concentration of IL-4 may explain why linkage was preferred, since Th2 cells had no effect if they were not recruited to the same DC as the naïve T cells (Chapter 6). As was observed for IFN-γ, IL-4 production by naïve T cells of one antigen specificity was not enhanced by the presence of naïve T cells of another, unless the difference in frequency between the two was high (Chapter 7). Thus, the local concentration of IL-4 is more limited if few cells are activated, but may be increased to some extent in the presence of a larger population, which is concomitantly activated. Finally, a study by Ismail and Bretscher (2001) also reflected the need for close CD4+ T cell cooperation for efficient Th2 development. Irradiated mice reconstituted with low numbers of splenocytes elicit an exclusively Th1-type of response after immunisation with xenogeneic red blood cells. Th2 cells progressively emerged after reconstitution with higher numbers of splenocytes, resulting in a mixed Th1/Th2 response.
Th2 cells not only enhanced the number of IL-4+ cells, but also diminished the number of IL-2+ cells. The presence of Th2 cells surprisingly did not significantly affect the number of IFN-γ+ cells. Thus, this effect may well be mediated by IL-10, which is reported to inhibit the transcription of IL-2 without affecting that of other cytokines such as IFN-γ or IL-4 (de Waal Malefyt et al., 1993). Confirmation of this hypothesis may be obtained, for example, by measuring by ELISPOT the number of IL-10+ cells within the Th2 inducer population after in vivo restimulation.

The relationship between DC / T cell ratio and CD4+ T cell differentiation is complex. An in vitro study showed that 1:4 and 1:300 DC-T cell ratios favour Th1 and Th2 responses respectively (Tanaka et al., 2000). An earlier report from the same group suggested that a high number of DC per T cell favoured Th1 differentiation because of more IL-12 per T cell from these DCs (Ohshima and Delespesse, 1997). These results indicate that the DC-T cell ratio may subtly modulate Th1/Th2 response depending on the signal that DCs receive. On the one hand, more T cells may have to compete for the utilisation of IL-12 secreted by DCs, so that T cells at lower number benefit more from IL-12. On the other hand, more T cells may also induce more IL-12 secretion by DCs via CD40L to compensate. The amount of IL-4 produced by T cells after activation may also alter this balance, because IL-4 can inhibit IL-12 production (Koch et al., 1996; Ohshima and Delespesse, 1997; Ria et al., 1998). Thus, if T cells are low IL-4 and high IFN-γ producers, such as in our mouse in vivo model, a high number of T cells per DC may favour Th1 differentiation. In contrast, if T cells are high IL-4 and low IFN-γ producers, such as in a human in vitro model (Ohshima and Delespesse, 1997; Tanaka et al., 2000), then the secretion of IL-12 may be more limited and Th1 differentiation may preferentially occur at low number of T cells per DC.

The contribution of the IL-2, IFN-γ, IL-4, IL-10 and other cytokines in the cooperation between T cells may be further investigated in the CAT model, where the inducer/helper population would come from TCR-transgenic cytokine-deficient mice. This may be useful in estimating the contribution of epicrine signals and in understanding why cooperation between CD4+ T cells leads to better differentiation into cytokine-producing cells without affecting activation and proliferation.
8.2.3.3. Optimising the function of DCs

Dendritic cells provide a site for both competition and cooperation between T cells (Figure 8.1). The ability to express regulatory genes as well as antigens in DCs may also be exploited to improve vaccination efficacy. For example, the recruitment and activation of T cell clones may be limited by the length of time DCs remain in lymph nodes. Thus, signals that prolong survival or delay apoptosis may be used in conjunction with vaccines. Sustaining Bcl-2 expression in DCs, for instance, resulted in greatly enhanced CTL responses (Nopora and Brocker, 2002). PMDD is a method of choice with which to introduce both antigen and regulatory genes inside the same cell. Vaccination using ex vivo manipulated and antigen-pulsed DCs is a method that has also attracted considerable attention, in particular for the treatment of cancer (Jefford et al., 2001; Steinman and Pope, 2002).

The cooperative effect of linked epitope presentation by APCs may be exploited to enhance the response of low responsive individuals. The greater ability of pre-activated/memory over naïve T cells to improve the response of other naïve T cells was evidenced by the study described in Chapter 6 and by another study by Cheng et al. (1998), where anti-tumoral immunity has been successfully boosted by in vivo priming of OVA-specific CD4+ T cells prior to injection of OVA-pulsed tumour cells. The linkage between OVA and the tumour antigens thus allowed the development of an effective anti-tumour cytotoxicity that was not achieved with the immunisation with tumour cells alone. Multivalent vaccines may offer significant advantage over monovalent vaccines by increasing cooperation between T cells. However, such epitopes should be carefully selected and tested in order to avoid possible interference. The influence that different antigenic determinants may have on each other is discussed in the last section below.

8.3. Implications for multivalent vaccine design

Multivalent vaccines may be subdivided into two groups: combination vaccines and poly-epitope vaccines. A combination vaccine usually refers to a mixture of several different components aiming at protecting an individual against several pathogens at once. Its main practical advantage is the reduction in the number of injections into a
single one. The use of combination vaccines requires extensive tests in order to ensure that the immunogenicity and the safety of the mixture are not reduced compared with each component administered individually. A special issue of Clinical Infectious Diseases (Vol. 33, Sup. 4, 2001) is dedicated to different aspects of combination vaccines from immunology to safety regulations. Poly-epitope vaccines are designed to elicit a polyclonal response against several determinants of a given antigen/pathogen. At present, most of these vaccines are aimed at protecting against one pathogen at a time and are also being widely investigated as prospective therapeutic vaccines against cancer. Some examples of positive and negative interactions between components of combination and poly-epitope vaccines will be discussed in this section.

The relative immunogenicity of each component of a combination vaccine may be reduced, enhanced or unchanged compared with monovalent vaccines. This variability may reflect the complexity of the interactions between the different responding T and B cells within an individual and the genetic variation (polymorphism) between populations. It may also depend on the formulation and treatment schedule of the vaccines tested. This may be illustrated by the two following examples. First, in comparative studies between hepatitis A/B combination and monovalent vaccines, three groups reported a similar immunogenicity (Joines et al., 2001; Van Damme and Van der Wielen, 2001; Abraham et al., 2002). One group found that anti-hepatitis A titers were higher with the combined vaccine or simultaneous injection (i.e. at two separate sites) of the two monovalent vaccines than with the monovalent vaccine alone, but anti-hepatitis B titers were higher with the two simultaneous injections than with the combined formulation (Czeschinski et al., 2000). Another group observed that the anti-hepatitis A response was at least as good in the combination vaccine as in the monovalent vaccine, but the anti-hepatitis B response was reduced (Frey et al., 1999). The authors attributed this effect to "immunologic interference", since HAV appears more immunogenic than HBV and thus its response may reduce the anti-HBV response by competition. This is consistent with observations by Czeschinski et al. (2000), where inoculation of a combined vaccine (favouring linkage and thus competition) resulted in lower anti-HBV response than simultaneous but separate inoculation (presumably limiting linkage and competition).
The second example concerns combination of *Haemophilus influenzae* type b (Hib) vaccine with pertussis-diptheria-tetanus (± poliovirus) vaccine. All the responses to a five-component pertussis-diptheria-tetanus vaccine were enhanced when the vaccine was given combined with a tetanus toxoid (TT)-conjugated Hib vaccine compared with separate injections at different sites (Lee *et al.*, 1999). Good levels of anti-Hib were also obtained. However, lower anti-Hib responses have been reported in other similar studies (Eskola *et al.*, 1999) with combined but not simultaneous inoculation. Different concentrations of active antigens or incompatible adjuvants (e.g. that have opposing effects) have been proposed as possible causes of this interference. In another study, a TT-conjugated diptheria-tetanus-pertussis-poliovirus-Hib vaccine was used in combination with a tetravalent pneumococcal vaccine (Dagan *et al.*, 1998). No interference was observed, apart from a significantly lower anti-Hib response with the pneumococcal vaccine that was TT-conjugated. In contrast, no interference was detected if the latter was diptheria toxoid (DT)-conjugated. The authors thus attributed the interference to a common protein carrier. Finally, a pentavalent vaccine (Pentavac, diptheria-tetanus-pertussis-poliovirus-Hib, TT-conjugated) was combined with a hepatitis B vaccine (H-B-Vax II) to form Hexavac. The response to Hexavac was similar in most aspects to the response to simultaneously injected but not combined Pentavac and H-B-Vax II, except that with Hexavac, antibody titers to Hib and HBV were 2-fold lower and titers to poliovirus were enhanced (Mallet *et al.*, 2000).

Despite the interference sometimes observed in these studies, it has been emphasised that the levels of antibody obtained with combination vaccines were still adequate for protection in most cases. The mechanisms underlying either enhancement or reduction of the response to one component within a combination vaccine remain unclear and may depend on factors such as the relative amount and immunogenicity of each component. Furthermore, it is well established that the conjugation of an antigen with an immunogenic carrier protein such as TT or DT improves the response, an effect attributed to linkage (Mitchison, 1971). However, the number of carrier-specific T cells may be limiting and the overloading of antigens conjugated to the same carrier protein would then lead to competition between each antigen-specific population for help from a limited number of carrier-specific T cells, resulting in decreased responses (Dagan *et al.*, 1998; Fattom *et al.*, 1999).
In the case of poly-epitope vaccines against a single pathogen, it is possible that less interference and more synergy occur, because determinants are related and may induce similar rather than antagonising responses. For example, in a tetravalent DNA vaccine against tuberculosis compared with monovalent immunisations, IgG titers were either unchanged for two components, enhanced 4-fold for one component or reduced 2-fold for the fourth component (Morris et al., 2000). In a divalent vaccine against borreliosis, the level of protection achieved against *B. burgdorferi* challenge in mice was 100-fold higher than with only one of the two components (Hanson et al., 2000).

Several DNA vaccines have been designed for the delivery of multiple epitopes, but most studies have so far been carried out only in animal models. Anti-malaria vaccines, for instance, have received considerable attention because protection against malaria requires immunity to the different stages of the infection. In a mouse model, immunisation with plasmids encoding four antigens of *Plasmodium falciparum* induces antibody levels similar or higher than single-plasmid injection (Grifantini et al., 1998). The Th1/Th2 balance obtained (measured as IgG2a/IgG1 ratio) was similar between the antigens and was not affected by the plasmid combination. In monkeys, a trivalent DNA vaccine induced, after four immunisations, 3 to 12-fold higher antibody titers against *P. falciparum* than single vaccines, suggesting absence of interference and possible synergy between these antigens (Jones et al., 2002). Some antigens of this parasite, such as CSP, contain polymorphic dominant epitopes, with limited cross-reactivity for them in humans and mice (Zevering et al., 1998). In this context, immunisation with several epitope variants seems mandatory.

When epitopes are well characterised, it may be more practical to use “minigene” constructs, which express only the immunodominant epitopes from one or several antigens/pathogens (Gurunathan et al., 2000). Efficient CTL induction may require both class I and class II MHC-restricted epitopes (Maecker et al., 1998) or class I MHC-restricted epitopes only (Thomson et al., 1998). Moreover, protection may be achieved with a B cell-specific epitope, rather than with the whole intact protein antigen using such construct (An and Whitton, 1997). In mice, detectable responses, sometimes conferring complete protection, were mounted after minigene
immunisation with a string of epitopes from various model, viral, bacterial and tumour antigens (An and Whitton, 1997; Thomson et al., 1998).

The studies presented in this section offer only a few examples that illustrate the complexity of the positive and negative interactions induced by the co-delivery of several related or non-related antigens. Data on multiple antigen DNA vaccination in humans are not yet available; however, by comparing studies on animal models, it seems that DNA vaccines have better prospects than conventional vaccines when it comes to combining antigens. This reflects a simpler formulation, better control of antigen expression and presentation and the option of expressing immunomodulatory molecules. In addition, the use of PMDD may provide extra advantages, including a more cost-effective vaccine, a greater efficiency in direct cell transfection and the possibility of co-expressing epitopes known to synergise, or conversely, segregating those that are suspected of interfering with each other, as demonstrated in this study. However, no general conclusions can yet be drawn due to the lack of data regarding DNA vaccines in humans. More research in this field will be required before all the advantages of DNA vaccines may be exploited.
Chapter 9


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