University of Southampton

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF MEDICINE

ANTIBODY-BASED VACCINES FOR DELIVERY OF ANTIGEN TO DENDRITIC CELLS *IN SITU*

By

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ABSTRACT

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ANTIBODY-BASED VACCINES FOR DELIVERY OF ANTIGEN TO DENDRITIC CELLS *IN SITU*

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Numerous studies have documented the crucial role of dendritic cells (DC) in the cross-priming of cytotoxic T-lymphocyte responses against exogenous antigens. However, these responses, particularly to tumour specific antigens, are often sub-optimal. Aiming to investigate the effectiveness of vaccines targeting antigen to DC in situ and their ability to potentiate immunity, we generated a panel of conjugates consisting of ovalbumin (Ova) protein linked to monoclonal antibodies (mAb) that target molecules expressed mainly on DC. The effectiveness of the various [FabxOva] conjugates was investigated in in vitro co-culture assays and in an in vivo system where Ova-specific CD8 and CD4 T cells were adoptively transferred into naïve mice. The results revealed that targeting of the integrin CD11c, induced the strongest CD4 and CD8 T cell responses, followed by DEC205 and MHC-II targeting. Co-administration with the adjuvant α-CD40 mAb, prevented the induction of tolerance and generated functional memory effector T cells. In addition, immunization with a single low dose of [α-CD11cxOva] had the unique ability to rapidly generate high titres of α-Ova IgG. Together with data from biodistribution studies, we demonstrate here that delivery of antigen to DC in situ, in particular via CD11c, can efficiently potentiate T and B cell immunity and propose that this molecule plays an important and as yet poorly characterized role in the transfer of intact antigen to B cells and possibly $CD8^{+}$DC. Immunization with [α-CD11cxOva] was observed to enhance the resistance to tumour development and these encouraging results highlight the potential clinical benefit of this strategy in the treatment of cancer.

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"Rather than a universe of static certainty, at the most fundamental level of matter, the world and its relationships are uncertain and unpredictable, a state of pure potential, of infinite possibility"

Lynne McTaggart, The Field

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List of abbreviations

α-	Anti-
5DC	5-aza-2-deoxycytidine
7AAD	7-aminoactinomycin D
Ab	Antibody/ies
Ag	Antigen/s
AP-1	Activator protein-1
APAF-1	Apoptotic protease activating factor-1
APC	Antigen-presenting cell/s
BAFF	B cell activating factor
BcR	B cell receptor
BM	Bone marrow
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDR	Complementarity-determining region
CFA	Complete Freunds' Adjuvant
CFSE	5,(6)-carboxyfluorescein diacetate succinimidyl ester
CHO K1	Chinese hamster ovary K1 cells
CR	Complete response
CRD	Carbohydrate recognition domains
CSR	Class switching recombination
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell/s
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DD	Death domain
DMF	N, N-dimethylformamide
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-Linked Immunsorbant Assay
ER	Endoplasmic reticulum
Fas-L	Fas-Ligand
FCS	Foetal calf serum
FITC	Fluorescein-isothiocyanate
FLT3-L	fms-like tyrosine kinase 3 ligand
FSC	Forward scatter
GM-CSF	Granulocyte macrophage-colony stimulating factor
h	Hours
HEV	High endothelial venule
HLA	Human leukocyte antigen
HPC	Haematopoetic progenitor cell
HPV	Human papilloma virus
HRP	Horse-radish peroxidase
Hsp	Heat shock protein
i.v.	Intravenous
IC	Immune-complex/es
ICAM	Intercellular adhesion molecule
ICOS	Inducible co-stimulator
iDC	Immature dendritic cells

IDO	Indoleamine 2,3-deoxygenase
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
ITAM	Immune tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JNK	c-jun N-terminal kinase
KLH	Keyhole limpet hemocyanin
-L	Ligand
LC	Langerhans' dendritic cell/s
LFA-1	Lymphocyte function-associated antigen-1
LN	Lymph node
LPS	Lipopolysaccaride
mAb	Monoclonal antibody/ies
MBP	Myelin basic protein
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MZ	Marginal Zone
NF-κB	Nuclear factor-κB
NK	Natural killer cell
Ova $_{257-264}$	Ovalbumin peptide 257-264
PAMP	Pathogen-associated molecular pattern molecule
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PI	Propidium iodide
PLC-γ	Phospholipase C-γ
PR	Partial response
PRR	Pattern-recognition receptor
PTK	Protein tyrosine kinase
RAG	Recombination activating gene
RANK	Receptor activator of NF-κB
s.c.	Subcutaneous
SEM	Standard error of the mean
SH2	Tandem Src homology 2
SMCC	Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SSC	Side-scater
TAA	Tumour-associated antigen/s
TAP	Transporter associated with antigen processing
TcR	T cell receptor
TGF-β	Transforming growth factor-β
T_H	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
T_{reg}	Regulatory T cell

Overview of the immune system

The immune system is composed of cells and molecules with specialized roles in defending the host against infection. To establish an infection, the pathogen must first overcome numerous surface barriers, such as enzymes and mucus that are either anti-microbial or inhibit the attachment of the pathogen. However, once inside, the pathogen encounters two further levels of defence. First, the innate and then the adaptive immune systems.

The innate response comprises phagocytic cells (neutrophils, monocytes, and macrophages), cells that release inflammatory mediators (basophils, mast cells and eosinophils), and natural killer cells (NK), together with cytotoxic molecules such as those of the complement system. This response is not Ag-specific and consists of all the immune defences that lack immunological memory, thus remaining unchanged however often the Ag is encountered.

The adaptive system is comprised of Ag-specific B and T cells, which act as cellular effectors of the adaptive system. T cell priming occurs when the surface receptors on these cells interact with antigenic peptides presented on Major Histocompatibility Complex (MHC) class I and/or II molecules, expressed on the surface of professional antigen presenting cells (APC) in combination with a maturation signal.

B cells and macrophages are also considered APC, however, dendritic cells (DC) are thought to be the most efficient and have the unique ability to prime naïve T cells. Immature DC (iDC) act as sentinels, constantly sampling their surrounding microenvironment for pathogens and damaged tissue. DC become fully matured when pattern-recognition receptors (PRR) recognize distinct pathogen-associated molecular patterns (PAMP) on the surface of microorganisms. Endogenous danger signals, such as IFN-α or heat-shock proteins, also lead to DC activation. As the DC mature and migrate to the draining secondary lymphoid organs, the peptides on the MHC complexes can be presented to naïve T cells, which only in the presence of a maturation or danger signal(s), lead to the induction of a

cellular response involving CD4 T helper cells and cytotoxic CD8 T cells. However, if the maturation signal is absent, T cell tolerance is induced thereby preventing functional immunity.

Finally, DC are also important in launching humoral immunity, through their capacity to activate naïve and memory B cells. NK cells and NKT cells can also be activated by DC. Thus, DC can regulate all of the elements of the immune system, as they are the link between innate and adaptive immunity. Not surprisingly, they are a fundamental target and tool for anti-cancer immunotherapy.

Chapter 1 Introduction

1.1 Cancer immunotherapy

In Britain, the lifetime risk of developing cancer is more than one in three, with approximately 65% of cases occurring in those aged 65 or over. Cancer causes seven million deaths every year accounting for 12.5% of deaths worldwide. There are over 200 different types of cancer, including breast, lung, colorectal and prostate, accounting for over half of all cases diagnosed in adults, whereas leukaemia is the most common cancer in children. Treatment may involve surgery, chemotherapy, radiotherapy, bone marrow transplant, hormone therapy, gene therapy or some combination of these depending on the type of cancer.

Nonetheless, these treatments have limited success rates, with relapse often reoccurring as a result of resistance. Furthermore, the traditional treatments are not tumour-specific and consequently cause a range of adverse side effects. Together these factors have led to the investigation of alternative strategies aiming to improve the quality of life of patients on treatment and their long-term survival rates. Out of many new approaches, immunotherapy has been supported by encouraging results from animal models as well as initial human trials, highlighting the exquisite specificity of both antibody (Ab) and T cells, which allows the immune system to distinguish the most subtle of biochemical differences between cancer and normal cells.

Although tumour immunotherapy was attempted over a century ago, when William Coley used extracts of bacteria to cause sporadic anti-tumour responses [1], its success to date has been very limited. Nevertheless, our belief in the potential benefits of tumour immunotherapy has been recently boosted with the success of monoclonal Ab (mAb)-based approaches, such as α-Her-2 (Trastuzumab or Herceptin) and α-CD20 (rituximab or Rituxan), which represent the fastest growing class of cancer therapeutics.

Immunotherapeutic strategies can be categorised as passive or active. Passive immunotherapy includes the administration of Ab, immuno-toxin complexes and adoptive transfer of stimulated cytotoxic cells, which aim to eradicate established

tumours. In contrast, active immunotherapy involves the induction and potentiation of the host's natural immunological response against tumour Ag. This has been shown to be achieved by various strategies including the immunization against tumour associated Ag (TAA) with loaded DC, exogenous cytokine administration and transfection of tumour cell lines with genes encoding costimulatory or inflammatory cytokines.

The current enthusiasm and belief in the potency of immunotherapy is as a result of substantial progress in different areas of immunological research. Examples include the identification of immunogenic tumour-associated Ag, the appreciation that professional APC must be activated to induce immunity and prevent tolerance, the key role played by DC in linking the innate and the adaptive system through recognition of a variety of danger signals, and the involvement of Treg cells in suppressing T cell immunity against self-tissues including tumours.

The best evidence for immunesurveillance of non-hematopoetic tissues comes from studies that show that about 50% of the immunodeficient recombination activating gene 2-deficient ($RAG2^{-/-}$) mice spontaneously develop gastrointestinal malignancies by the age of 18 months and the same phenotype was seen in IFN-γ deficient mice [2] and patients with heritable immunodeficiencies [3]. Together these findings demonstrate that the immune system can recognise tumours and is constantly targeting tumour development through pathways involving lymphocyte effector cells and IFN-γ secretion.

The main obstacle to be overcome in cancer immunotherapy is the lack of reactive T cell responsiveness caused either because the tumour lacks sufficient TAA or the tumour reactive T cells that develop are rendered tolerant or anergic due to lack of immune stimulation [4] [5]. Many TAA are indeed self-Ag and therefore any immune response may be accompanied by the development of autoimmune diseases (e.g. vitiligo after regression of melanoma). In an attempt to prevent self-tissue destruction, the immunological system has developed complex safety mechanisms, which also have an impact on the effectiveness of immunotherapy.

Furthermore, immunotherapy of established tumours often leads to only a transient tumour regression, and it has been proposed that to achieve complete remission danger signals must be maintained. Potential endogenous danger signals that activate DC include the release of intracellular nucleotides, type-I interferon (IFN), CD40-Ligand (CD40-L), Tumour necrosis factor (TNF), interleukin 1-β (IL-1β), Toll-like receptor-ligand (TLR-L), co-stimulatory molecules and many others. Thus, an improved understanding of tumour-elicited danger signals may lead to the identification of potent immunological adjuvants that mimic those signals and generate a robust anti-tumour response. This concept of introducing "adjuvants" at the same site as vaccines with TAA has been tested and the resulting data seem very encouraging [6] [7].

1.1.1 Tumour-associated antigens (TAA)

In the early 1990s, Boon and colleagues [8] reported the first successful cloning of a human TAA, termed melanoma Ag-1 or MAGE-1 (subsequently renamed MAGE-A1), which elicited a spontaneous CTL response in the autologous melanoma patient. Many other TAA recognised by CD8 T cells have since been identified by this direct approach, including MAGE-A1, BAGE-1 and GAGE-1, where complementary DNA libraries from tumour cells were transfected into target cells expressing the appropriate HLA molecule, and then anti-tumour CTL were used to identify transfectants carrying the appropriate target peptide [8]. Another approach has been developed, which is based on the screening of recombinant cDNA expression libraries of human tumours with autologous serum. Sera from a large proportion of cancer patients contain Abs against both surface and intracellular proteins of cancer cells. It is assumed that this Ab can only be produced with the help of a CD4 T-cell response [9]. This technique is known as SEREX (serologic analysis of recombinant cDNA expression libraries) and genes such as SSX-1, SCP-1and NY-ESO-1/LAGE-2 were all identified using this methodology [9].

As a result of an extensive search using these techniques, a large number of sequences potentially coding for TAA have been identified. Depending on their

expression pattern, TAA are subdivided into groups characterised by different specificity and potential clinical relevance. The main subgroups are briefly described below and in table 1.

I. **Tumour-specific Ag**, are expressed only in tumour cells, derived from altered self-proteins that result from gene mutations, translocations, or transcription of alternative reading frames, or from idiotypic proteins. Their high specificity is matched by a clinical usefulness limited in many cases to individual patients. For example, cancers of the haematopoetic system represent unique situations not shared by most solid tumours. B cells and any lymphomas arising from them can express unique idiotypes which can serve as a TAA. Other examples include mutant p221/ras and mutant p53.

II. **Non-mutated shared Ag overexpressed on cancer cells**. There is evidence that over-expressed proteins such as carcinoembryonic Ag, p53 and MUC-1 are TAA.

III. **Viral TAA.** Viruses are associated with approximately 20 % of human malignancies. Examples include Epstein-Barr virus (EBV)-induced lymphoma and human papilloma virus (HPV)-associated cervical cancer.

IV. **Differentiation TAA**, are expressed in tumour cells and their untransformed counterparts, although generally to a lesser extent in the latter. These TAA are largely used in active specific immunotherapy of tumours of discrete histological origin, mainly melanomas (e.g. MART-1 (Melan-A), tyrosinase) and prostate cancer (e.g. prostatic acid phosphatase).

V. **Cancer/Testis (CT)-TAA,** by contrast, are shared Ag that correspond to normal gene products with highly restricted tissue distribution. Many workers in the field feel that they provide the most potential in cancer immunotherapy. CT TAA are not expressed in most somatic tissues, with the exception of the testis, but are often over expressed in tumours of various histological types, including melanomas. Prototypes of this group are the MAGE, GAGE and BAGE families and NY-ESO-1.

Antigen Class	Antigen	Malignancy
Tumour-specific Antigen	Immunoglobin Idiotype	B-cell non-Hodgkin's lymphoma, multiple myeloma
	Mutant p21/ras	Pancreatic, colon, lung cancer
	Mutant p53	Colorectal, lung, bladder, head and neck cancer
Non-mutated, overexpresed Antigen	Carcinoembryonic –Ag	Colorectal, lung, breast cancer
	p53	Colorectal, lung cancer
	MUC-1	Colorectal, pancreatic, ovarian, lung cancer
	Her-2/neu	Breast and lung cancer
Viral Antigen	Human papilloma virus (HPV)	Cervical, penile cancer
	Epstein Barr virus (EBV)	Burkitt's lymphoma, post-transplant lymphoproliferative disorders
Differentiation/ Developmental Antigen	MART-1	Melanoma
	Tyrosinase	Melanoma
	gp100 (pmel-17)	Melanoma
	prostatic-acid phosphatase	Prostate cancer
Cancer/Testis Antigen	MAGE-1	Melanoma, coloreactal, lung, gastric cancer
	BAGE	Melanoma, bladder, breast cancer
	GAGE	Melanoma, head and neck, lung cancer
	NY-ESO-1	Melanoma, bladder, breast, liver cancer

Table 1 Human tumour-associated antigens recognised by CD8 T cells.

List of multiple cancers expressing a few of the TAA antigens discussed above[10, 11].

CT-TAA belong to the most promising family of TAA identified to date, as they meet the key requirements for a potent tumour-rejection Ag, such as specificity and immunogenicity. As previously mentioned, CT-TAA expression is almost restricted to gametogenic tissues mainly testis, and cancer cells. The testis is an immune-privileged organ, suggesting that these Ag would have not been exposed to the immune system during the process of central tolerance and clonal deletion of CT Ag-specific T cells, preventing the development of tolerance to these Ag in cancer patients.

The process of CT-TAA discovery in cancer, by either T cell epitope cloning or by serological analyses, has unintentionally separated them into 2 groups, those defined by T-cell recognition (e.g. MAGE-A, BAGE, GAGE) and those defined by Ab recognition (e.g. SSX, NY-ESO-1,MAGE-C). However, there is increasingly evidence of a coordinated immune response to these Ag in cancer patients and most importantly, both Ab and T-cell (CD4 and CD8) responses have been shown for three of the 20 CT-TAA families: MAGE-A, SSX and NY-ESO-1 (reviewed in [12]) . NY-ESO-1 is the most immunogenic CT-TAA known to date, and serum Ab against it has been detected in melanomas, ovarian, lung and breast cancers [13].

1.1.2 DC-based cancer vaccines

Numerous approaches for the therapeutic vaccination of cancer patients have been developed including the use of autologous and allogenic tumour cells, which are often modified to express various cytokines, peptides or proteins and DNA vaccines [14]. Although some of the results were positive, these strategies rely on random encounter of the injected target with host DC. A lack of encounter with DC or alternatively, an inappropriate encounter (i.e. with iDC) might result in absence of immunity or even worse, to tolerance to the target Ag. Therefore, approaches immunizing with autologous DC or Ag-targeted to DC in the presence of a maturation signal might circumvent such problems. Moreover, DC-based immunization has been shown to induce humoral and cellular immunity, capable of mounting immunological memory for the protection against subsequent tumour challenges.

The rationale for the development of TAA loaded DC as therapeutic vaccines in humans was based on the vast literature reporting the beneficial effects of DC-based vaccines in pre-clinical studies on animal models. Initially it was found that injection of DC-enriched preparations of splenocytes or epidermal cells pulsed with tumour lysate could protect naïve animals from subsequent lethal tumour challenge [15, 16]. Later on, the administration of density-gradient purified splenic DC pulsed with either a soluble protein expressed by a B cell

lymphoma [17] or with a synthetic MHC class I-restricted peptide derived from a tumour model Ag could induce the generation of peptide-specific CTL, which accompanied protective responses. Therefore, these early experiments provided proof of principle that by loading DC with tumour lysate or Ag ex vivo, it was possible to induce tumour-specific immune response in immunized mice, which led to the proposal that this strategy could result in an effective cancer vaccine.

The discovery that granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4 (added to suppress the outgrowth of monocytes) can promote the generation of large numbers of iDC in vitro from murine bone marrow cultures [18, 19] has allowed considerable progress in this field [20]. Clinical trials of DC vaccination were made possible by obtaining large numbers of human DC using similar in vitro culturing techniques. Two general protocols have been routinely exploited: (1) the purification of iDC precursors from peripheral blood and (2) the in vitro differentiation of DC from peripheral blood monocytes or $CD34^+$ hematopoetic progenitor cells in the presence of GM-CSF. After activation of DC precursors by GM-CSF, DC differentiation may be influenced by a variety of cytokines, including IL-4 [21, 22], IL-15 [23] , TNF-α [24] and IFN-γ [25].

In principle, either defined or undefined TAA can be delivered to DC and these can be composed of single-Ag in the form of tumour specific peptides [26], proteins [27], single messenger RNA (mRNA) transcripts [28] or TAA expressed endogenously by tumours transfected by plasmid or viral constructs [29]; or multiple Ag, including the use of peptide mixes [30], whole tumour lysates [31], total tumour mRNA [32], whole tumour cells [33], apoptotic bodies [34], tumour derived heat shock proteins (hsp)[35] and tumour/DC fusions [36] .

In contrast to the multiple Ag approach, loading with single Ag is specific and avoids the possible development of autoimmunity against a normal cellular Ag. Moreover, this strategy is easier to document and quantify, however, on the down side, the single Ag approach suffers from a variety of deficiencies including tumour immune escape, human leukocyte antigen (HLA) specificity in the case of peptides, lack of T cell help and the fact that the system is not allowed to choose the epitopes from the total pool that will be most useful for the

mounting of the immune response. To generate an optimal anti-tumour response it is now thought that in addition to an Ag-specific CTL response, immunization should include helper epitopes for the priming of CD4 T cells, which provide important help signals for the maintenance of potent CTL responses and the development of immunological memory [37]. Peptides with dual major histocompatability complex-I (MHC-I) and MHC-II specificities have been identified for the TAA, NY-ESO, and reported to induce strong CD8 and CD4 T cell responses [38].

Clinical trials

In 1995, Murherji et al [39] reported the results of the first clinical trial that established the feasibility and safety of immunization with MAGE-1 peptide-loaded DC for the treatment of metastatic melanoma. Supporting trial studies followed reporting successful results [40, 41], including a phase I trial demonstrating a clinical response rate of 27 % (9 partial response (PR) out of 33 patients) after administration of prostate-specific membrane Ag (PSMA) peptide pulsed DC to patients with metastatic prostate cancer [40].

In 2001, Banchereau et al [42] published a study reporting very encouraging results, where $CD34^+$ progenitor derived DC were pulsed with 4 HLA-A2 melanoma peptides (MART-1, tyrosinase, MAGE-3 and gp100) in the presence of KLH and TNF-α, and delivered subcutaneously (s.c) to 18 patients with stage IV metastatic melanoma, which is a fatal disease with a survival of 6-10 months. Ag-specific IFN-γ secretion in response to at least 1 of 4 peptides was detected by ELISPOT in 17 of the 18 patients, and in general, the patients who acquired immunity against more than one peptide showed the best clinical outcome. Additionally, this trial produced impressive clinical results, with 3 CR and 4 PR out of 17 patients (41 %).

A follow-up study with the long term outcome of these patients has been recently published and patients who have survived longer are those in whom vaccination with CD34-DC elicited T cell immunity to at least two melanoma peptides. Despite the limitations in correlating clinical responses and T cell immunity in

such a small trial, it was reported nevertheless that 7 of 10 patients with immunity to at least two melanoma peptides survived longer than the median survival of 20 months and as to August 2005, there were 4 surviving disease free patients. Although this trial showed a direct correlation of T cell immunity and clinical outcome, more often than not, clinical data fail to translate the generation of Ag-specific T effector cells detected by ELISPOT, proliferation or CTL lysis assays to the clinical setting [43-48].

Since the initiation of the first DC-based vaccine trials, our general knowledge of DC immunobiology has expanded and strong evidence has been presented demonstrating that the maturation status of DC can affect its ability to mount a potent immune response. Results by Dhodapkar et al demonstrated that administration of iDC could result in the development of Ag-specific tolerance [48-50] and when Jonuleit et al [51] directly compared the immunogenicity of mature DC and iDC, the mature DC were clearly superior inducers of effector T cells. Table 2 identifies some of the trials with DC cultured in the presence of a maturation stimulus reported for the treatment of melanoma.

Culture protocol	DC origin	Route	T-cell response	No. patients	Clinical	Ref.
IL-4/GM-CSF/FCS	moDC	i.n	n.d.	16	2CR, 3PR	[52]
IL-4/GM-CSF/IL-1β/IL-6/TNF-α/PGE2	moDC	s.c.	13	30	8SD, 1CR	[53]
Calcium ionophore/IL-12/IL-2	moDC	i.v./i.d/ i.n.	13	28	3PR	[54]
IL-4/GM-CSF/IL-1β/IL-6/TNF-α/ PGE2	moDC	i.n.	5	11	3SD	[55]
IL-4/GM-CSF/MCM/IL-6/TNFα/ PGE2	moDC	i.d.+i.v.	2	10	4SD, 1PR	[56]
FLT3-L/GM-CSF/TNF-α	$CD34^+$	s.c.	16	17	3CR,4PR	[42]
IL-3/IL-6/SCF/IL-4/GM-CSF/TNF-α	$CD34^+$	i.v.	2	14	6SD,1PR	[57]
IL-4/GM-CSF/TNF-α	$CD14^+$	i.d.	7	13	5SD,2PR	[58]

Table 2 DC vaccine clinical trials in the treatment of advanced melanoma.

Although a great number of clinical trials investigating the effectiveness of cancer vaccines have been published, the great majority of them are phase I/II trials testing the toxicity and dosage of these vaccines in small groups of patients with advanced, non-curable, treatment resistant tumours rather than their clinical effectiveness. Furthermore, even when clinical responses are reported, it is extremely difficult to evaluate and compare the data, mainly because some trials do not specify the exact criteria used to determine clinical response, which taking in consideration that most of these patients have progressive tumours, anything can be categorized as a clinical response, from the stabilisation of the tumour progression to complete remissions.

In addition to the confusion involving the criteria employed for observed clinical responses and immuno-monitoring of the patients, DC based vaccines, in particular, have other areas of extensive variability as a result of the necessity to culture DC progenitors in vitro in the presence of a combination of a wide range of cytokines and growth factors which does not facilitate the comparison between trials. Therefore, the direct comparison of clinical efficacy between DC based and other cancer vaccines strategies remains to be evaluated in larger controlled phase III trials where a consensus exists on the, optimal method of DC generation, the best protocol to assess the immune responses, and clinical outcome in the patients.

Nevertheless, it is clear from the published clinical data that DC vaccines can effectively augment immunogenicity to TAA (refer to the T cell response column in Table 2) and it can result in clinical remission [59]. Although comparison between trials is difficult as previously emphasized, Rosenberg et al [60] selected 35 reports of vaccines trials that included 765 patients whom they believed represented the majority of published trials, and these included multiple cancer types treated with a variety of the most common vaccines. The summary of their analyses suggest that there were 7 (4 %) clinical responses in 175 patients following immunization with peptide vaccines, no responses in 206 patients receiving pox viruses, 6 (4.2 %) responses in 142 patients treated with native or modified tumour cells and 14 (7.1 %) responses in 198 patients receiving DC

vaccines. Thus, this analysis highlights that for at least a portion of the patients, vaccination with DC loaded with 1 or more peptides is an effective intervention with a great potential for the treatment of less advanced cancer patients or as prophylactic treatment in the prevention of future relapse in patients that were successfully treated.

The most remarkable aspect of DC based vaccines is the safety records assembled from the large number of trial performed to date, which report that side effects are uncommon and only mild and temporary adverse effects were reported by patients, such as fever, local reaction, fatigue and vitiligo (among melanoma patients), with no serious or life threatening effects recorded, which at this early stage in the development of a novel cancer treatment may be unparalleled by any of the traditional anti-cancer agents.

As we reached 10 years from the first DC vaccine trial, more follow-up studies are being published reporting the long term effect of these vaccines and in many cases clinical responses were not durable and relapse was common place. The failure to mount effective memory anti-tumour responses in patients when pre-clinical data shows long term protection in animal models is thought to be a result of a number of factors that render the system tolerant to tumour Ag. For instance, TAA generally have low-immunogenicity (as they may be self-Ag), and the development of tumours in humans tends to be very slow which facilitates the tolerization of the system against the Ag thereby enabling the tumour to grow and actively suppress any anti-tumour response (Table 3 summarizes some of these factors).

The large numbers of complex tumour escape mechanisms identified to date also suggest that the effectiveness of DC vaccines can be improved by combining it with therapies aimed at blocking or removing immunosuppressive molecules, cytokine or cells. Promising approaches would include the administration of mAb to deplete or block $CD25^+$ T reg. cells or blockade CTLA-4 signalling both which have been shown to potentiate T cell immunity [61].

Lymphocyte factors
Lack of T cell help
Insufficient number of anti-tumour T cells
Low avidity of T cell for tumours
T cells are anergic
Down-regulation of T cell receptor signal transduction
Apoptosis of T cells function when encountering tumour
Inadequate T cell function (cytokines, lysis)
T cell cannot infiltrate tumour
Active suppression by suppressor lymphocytes (e.g. T reg.)
DC mediate suppressive function and promote T reg. proliferation
Tumour factors
Insufficient TAA expression
Loss of HLA expression by tumour cells
Tumour production of immunosuppressive factors (e.g. TGF-β, IL-10, IL-6)
Tumour resistance to apoptosis or other cell destruction pathways (e.g. p53 mutation, downregulation of Fas receptor, over expression of Bcl-2)
Prevention of cell death by increasing expression of serine protease inhibitor (PI9), secretion of NO, activity of IDO to protect from T cell immunity
Decreased expression of molecules involved in antigen presentation (e.g. TAP-1)
Tumours acquire ability to secrete their own growth factors and upregulate expression of growth factor receptors

Table 3 Summary of factors that contribute to the development of tumours, despite the apparent generation of anti-tumour effector cells.

Abbrv: T regulatory cells (T reg.), Human leukocyte Antigen (HLA), Transforming growth factor-β (TGF-β), Interleukin-10 (IL-10), Protease inhibitor-9 (PI-9), Nitric Oxide (NO), Indoleamine 2,3-deoxygenase (IDO), Transporter associated with antigen processing -1 (TAP-1).

Moreover, although the clinical effectiveness of currently available DC vaccines are to a certain extent disappointing, it should not be interpreted to mean that cancer vaccines are of no therapeutic value, because different types of cancer have unique challenges for successful vaccination [62]. Thus, despite the fact that the majority of the patients were not reported to show complete tumour regression, these data should rather emphasise the need for changes and increased efforts to generate a more potent anti-tumour response. Areas of focus should include: (1), the generation of anti-tumour CD4 T cells that recognise

MHC-II restricted Ag, which would enhance the CTL response and sustain the survival of memory cells; (2), the exploration of improved adjuvants such as TLR-agonists to activate innate immunity; (3), the use of agonistic mAb (e.g. 4-1BB mAb), which stimulate CD8 T cells, or the administration of pro-inflammatory cytokines; and (4), the blockade of secretory immunosuppressive molecules such as TGF-β, IL-10, IL-13, as well as eliminating T reg cells and blockade of CTLA-4 molecules.

Furthermore, there are other attractive practical points associated with this approach, such as the fact that Ag-bearing DC vaccines do not cause significant side effects and can be administered in out-patients, unlike most conventional therapies. Moreover, considering that cancer is a chronic condition, disease control with prolonged survival and good quality of life might be considered a therapeutic success in itself even if complete remission is not obtained. However, the current drawbacks associated with DC based vaccines is related to the cost involved in the isolation and expansion of the DC in vitro to induce their maturation and Ag loading before being injected back into the patient; together with the fact that these vaccines are patient specific and are not easily stored.

Thus, because of these weaknesses, investigators continue to look for new strategies that can increase the efficacy of DC vaccines and reduce their cost, and one of these new strategies rely on the expansion of DC in patients and/ or direct targeting of Ag to endogenous DC in situ, thereby circumventing the need to extract and expand cells in vitro. A potential candidate for expanding human DC numbers in vivo without activating them is pre-treatment with FLT3-L. Whether the dramatic increase in DC numbers seen in vivo following FLT3 ligand treatment enhances immune responses to vaccines remains to be established [63]. Like tumour cells, murine DC have been reported to secrete Ag presenting vesicles of endosomal origin, termed exosomes. Peptide coated DC-derived exosomes were shown to induce potent anti-tumour immune responses [64]. A phase I clinical trial has recently been launched, aimed at vaccinating subcutaneously metastatic melanoma and inoperable lung cancer patients with autologous DC-derived exosomes pulsed with MAGE-3 class I and II peptides [65].

Of more interest to us is the approach that has so far been only tested in mice, which involves the in situ delivery of Ag to DC by targeting Ag to molecules expressed on these cells using mAb. Recently several DC-associated C-type lectin receptors such as DEC205, DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) and CD206 have been shown to efficiently bind and internalise Ag, which was subsequently directed into the endocytic compartments for loading of MHC-II and presentation to CD4 T cells [66, 67]. More importantly, when mice were immunized with Ag complexed with α-DEC205 mAb, a strong response of IFN-γ secreting CD8 and CD4 T cells was generated. This result showed that Ag targeting via DEC205 can be efficiently cross-presented to CD8 T cells and Ag-specific immunity mounted provided a DC maturation stimulus (e.g. α-CD40 mAb) was co-administered with the vaccine, to prevent the induction of tolerance. The therapeutic effectiveness of this vaccine in a melanoma animal model was reported to prevent the development and reduce the progression of established tumours [68, 69].

Apotolopous' group [67] also reported that ex-vivo targeting of the CD206 on macrophages and DC, with oxidized mannan linked to MUC-1 (an adenocarcinoma TAA) gave rise to highly effective CTL production and tumour protection after adoptive transfer, and suggested that this method might be suitable for immunisation of patients as both DEC205 and CD206 have been reported to be expressed in human DC.

Another example of efficient Ag-targeting to DC was reported by Wang et al in 2000 [70], when they showed that by targeting Ag to CD11c molecules, a marker of all subsets of DC with a hamster α-mouse CD11c mAb (N418), enhanced the serum IgG Ab levels in the primary response. A single low-dose immunization with CD11c-Ag, produced high titre IgG Ab in only 7 days, whereas the irrelevant mAb conjugated to the same Ag in the presence of adjuvant produced little measurable serum IgG until day 20. In agreement with these data, Berry et al [71] reported that CD11c conjugated to a synthetic peptide corresponding to an exposed region of DC-SIGN generated a rapid peptide-specific serum IgG1 response, which was also the predominant IgG class recovered by Wang et al

[70]. The molecular basis for this enhanced humoral response remains to be determined.

Therefore, as these few examples suggest, in vivo targeting and loading of DC might be the ideal vaccination strategy, as it is cell free, not as labour intensive as ex-vivo culturing of DC, a vast array of TAA or tumour cells could be easily delivered to the patients regardless of their HLA haplotype, administration is very easy and potentially the Ab-complexes could be manufactured on a large scale resulting in off-shelf reagents. Based on the encouraging data reported in animal models and the potential advantages that this approach would have over the current DC vaccines, we aimed to investigate the effectiveness of targeting our model Ag, Ova, to DC in situ via different molecules and determined the most efficacious target molecule to evoke a robust CD4 and CD8 T cell response with long lived memory responses that could be reflected in a therapeutic model.

1.2 Dendritic cells

1.2.1 DC subsets

The several, and often opposing, roles now attributed to DC cannot be all performed at once by the same cell, suggesting the existence of different sets of DC that perform different functions. Originally, two models were proposed to account for this diversity: (1), the functional plasticity model, which suggested that distinct DC subsets might represent different activation states of a single lineage, the functional differences depending solely on local environmental signals; and (2), the specialized lineage model, which argued that the DC subsets were products of completely separate developmental lineages and that the precursors of the DC would already be separated and functionally committed at an early stage. However, our current understanding suggests a combination of the two models may occur with a large degree of functional plasticity.

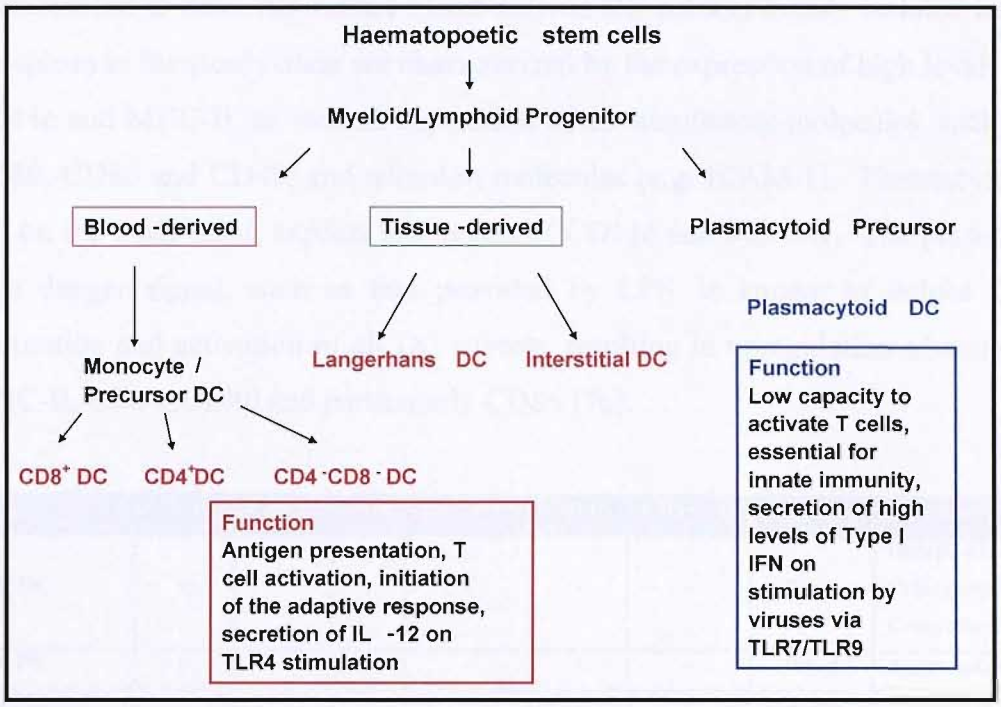


Diagram 1 Summary of the six major DC subsets identified to date and some of their main functional differences.

The first major subdivision of DC is between the plasmacytoid DC (pDC) and conventional subsets. This subdivision was originally made in humans [72] and was later extended to the mouse [73]. The conventional DC can be further subdivided into three subsets of blood-derived DC and two tissue-derived DC (Diagram 1). The conventional DC subsets identified in the spleen are all blood-derived [74], because there is no lymphatic drainage to this organ. There are three blood-derived subsets: $CD8^+CD4^-$ DC, $CD8^-CD4^+$ DC, $CD8^-CD4^-$ DC. These are also found in the lymph nodes (LN) [75], together with the progeny of tissue-derived DC. In the case of the LN draining the skin, there are two distinct tissue-derived subsets, these are the Langerhans' cells (LC) and the dermal or interstitial DC. Non-cutaneous LN do not contain LC, but these organs will have their own interstitial DC.

These six major DC subsets were characterized and distinguished from each other on the basis of the expression of different phenotypic markers including CD11c, CD11b, CD4, CD8, DEC205, CD45RA and langerin. Table 4

summarizes the phenotypic differences associated with the DC subsets characterised to date. All mature blood-derived DC subsets freshly isolated from the spleen in the steady-state are characterized by the expression of high levels of CD11c and MHC-II, as well as expression of co-stimulatory molecules, such as CD80, CD86 and CD40, and adhesion molecules (e.g. ICAM-1). Plasmacytoid DC on the other hand, express low levels of CD11c and MHC-II. The presence of a danger signal, such as that provided by LPS, is known to induce DC maturation and activation of all DC subsets, resulting in upregulation of surface MHC-II, CD40, CD80 and particularly CD86 [76].

DC type	CD11c	CD8	CD4	DEC205	CD11b	CD45RA	Origin	Functions
$CD8^+$ DC	+	+	-	+	-	-	**Blood**	High IL-12 Cross-priming Cross-tolerance
$CD4^+$ DC	+	-	+	-	+	-	**Blood**	Ag presentation
$CD8^-CD4^-$DC	+	-	-	-	+	-	**Blood**	High IFN secretion
Langerhans' DC	+	-/Low	-	**Very High**	+	-	**Skin**	Traffic from skin to LN Transport/present contact sensitizing Ag
Dermal/ Interstitial DC	+	-	-	+	+/-	-	**Tissue**	In the periphery Traffic to LN Present Ag to T cells
Plasmacytoid DC	**Low**	+/-	+/-	-	-	+	**Blood/ Tissue**	High IFN secretion Involved in viral immunity

Table 4 Phenotypic profiles of the six major murine DC subsets identified to date and their main functional properties.

In conclusion, there are 3 major subsets of murine conventional blood-derived DC: $CD8^+$ $CD4^-$ $CD11b^-$ $DEC205^+$; $CD8^-$ $CD4^+$ $CD11b^-$ $DEC205^-$; $CD8^-$ $CD4^-$ $CD11b^+$ $DEC205^-$ and two tissue-derived ones, $CD8^{low/-}$ $CD4^-$ $CD11b^+$ $DEC205^{high}$ $langerin^+$ and $CD8^-$ $CD4^-$ $CD11b^{+/-}$ $DEC205^-$ $langerin^-$. Plasmacytoid DC are identified as $CD11c^{low}$ $CD11b^-$ $CD8^{+\ (subset)}$ $CD4^{+\ (subset)}$ $DEC205^-$ $CD45RA^+$.

In contrast to the many studies on mouse DC, there are relatively few studies on mature human DC (hDC) freshly isolated from tissue and hDC lack the expression of CD8α, which makes direct comparisons difficult. Nevertheless, at

least 3 subsets of mature hDC are known [77]: interstitial DC, $CD11c^{+}HLADR^{+}CD11b^{+}CD4^{+}$; LC containing Birbeck granules, $CD11c^{+}HLADR^{+}CD11b^{+}$ $CD4^{+}$langerin $^{+}CD1a^{+;}$ and lastly, pDC which in humans lack CD11c, unlike murine pDC, $CD11c^{-}CD11b^{-}HLADR^{+}CD4^{+}$ $DEC205^{-}CD45RA^{+}$.

1.2.2 Developmental origin of the murine DC subsets

There is considerable confusion about the origins of the distinct subsets of DC, if they are products of separate developmental lineages or whether they are different activation states of a single lineage.

Early work suggested that DC were of myeloid origin, because myeloid haematopoietic progenitor cells (HPC) can develop into LC DC and interstitial DC [78]. Furthermore, DC can be generated from bone marrow (BM) cells, cord blood progenitors and peripheral blood in the presence of the myeloid growth factor, GM-CSF in combination with TNF-α, or IL-4 [79] or IL-5 [80]. However, later work provided evidence that certain subsets had lymphoid origins. For instance, in vivo studies showed that adoptive transfer of purified thymic precursors into irradiated recipients resulted in the development of T cells, B cells, NK cells and thymic $CD8^{+}$ DC but not cells of the myeloid lineage [81].

However, direct tests of the lineage segregation model only became possible when both myeloid-restricted and lymphoid-restricted precursor cells were isolated from BM. This confirmed that both precursors could produce all the mature splenic and thymic DC subsets, albeit with some bias in the subset balance [82, 83]. Furthermore, tracking studies with 5-bromo-2-deoxyuridine (BrdU) indicated that none of the DC subsets are products of the others [84]. Thus, a degree of phenotypical and functional commitment must occur downstream of the early haematopoietic precursors.

Although monocytes can differentiate into DC in the presence of inflammatory cytokines, the extent to which they do so in vivo and especially for the turnover

of DC in the steady state remains unknown. Recently, Fogg et al [85] reported that a clonogenic BM progenitor that is $CD117^+$ (c-kit), $CD115^+$ and CX_3CR1^+ has been identified and was demonstrated to gives rise to monocytes, macrophages and resident $CD8^+$ and $CD8^-$ splenic DC. These cells were suggested to be upstream precursors of bona fide monocytes, but they may circulate somewhat independently of monocytes and have a greater plasticity for differentiation than the conventional blood monocyte. Other studies have shown that under severe inflammatory conditions (e.g. local UV irradiation) there is a recruitment of $Gr\text{-}1^{hi}$ monocytes (a monocyte marker also referred to as Ly-6c/G) to the inflamed skin and these cells were able to proliferate locally and differentiate into functional LC or dermal macrophages in vivo [86]. Furthermore, in agreement with these findings, Le Borgne et al [87] showed that depletion of monocytes prevents in vivo priming of $CD8^+$ CTL against an innocuous protein antigen administered with adjuvant. Thus, all together these results support the notion that a subset of monocytes could act as circulating precursors of DC and macrophages during inflammatory conditions when there is a demand for DC and macrophages, however if this is true under steady state condition remains to be determined.

The microenvironment has also been shown to have profound effects on the phenotype and function of DC. Oldston's group [88] showed that virus infection of BM derived pDC induced their differentiation into conventional DC, thereby undergoing phenotypic changes, enhanced Ag presentation capacity and recognition through Toll-like receptors (TLR) mainly expressed by myeloid DC, which exemplifies the degree of plasticity involved in DC phenotypes and function.

1.2.3 Distinct localization and lifespan of DC subsets

Briefly, the $CD8^+$ DC subset has been found to be localized in the T cell areas of the mouse spleen, LN, and Peyer's Patches. In the steady-state, $CD8^-$ DC are found in the MZ of the spleen, the subcapsular sinus and T cell areas of the LN

[89, 90]. Upon stimulation these cells have been demonstrated to rapidly migrate from the MZ to the T cell areas where they can prime naïve T cells [91]. In addition, LC and interstitial DC are found only in the T cell areas of the LN and not the spleen, as these have migrated from epithelial and dermal tissues, respectively [92]. The remaining subset, the pDC, has been described in the MZ of the spleen.

Furthermore, BrdU-labelling studies by Shortman's group et al [76, 93] have showed that in the steady-state the distinct DC subsets also differ in their lifespan. All three subsets of mature DC in the spleen have remarkably short lifespan, with $CD8^+$DC being the first to be generated from bone marrow precursors and having the fastest turnover in the spleen (half-life 1.5 days), so almost all of the cells were replenished by 3 days. The $CD4^-CD8^-$ DC and $CD4^+CD8^-$DC had half-lives of 2 and 2.9 days, respectively. These rates were for mice aged 5-7 weeks old, older mice (20 weeks) showed a 15-20% slower rate of turnover. The continuous and rapid labelling of all three subsets in the spleen also suggested that the dividing precursors are from independent pathways. Both skin derived subsets had longer life-spans (half life >21 days), probably a reflection of long residence time in the skin rather than presence in the LN presenting Ag.

When the turnover of the DC subsets in other lymphoid organs was assessed, the data indicated that as in the spleen, $CD8^+$DC in the LN, had a shorter lifespan than the $CD8^-$DC subsets, however, the turnover rate varied with the location of the LN, as the total time for this subset to be replaced was approximately 3 days in spleen, 4 days in mesenteric LN and 9 days in cutaneous-draining LN. The factors causing these differences remain unclear. Moreover, these studies conclude that the tissue specific DC subsets from the cutaneous LN had longer lifespans than the blood derived subsets found in the spleen and LN, with only 40 % of unlabelled LC being replaced after 21 days and dermal DC preceding them by 2-3 days.

This strikingly low turnover of LC agreed with data from other groups [94, 95] showing that DC that migrate from the skin, and in particular LC, have an

exceptionally long lifespan as measured from the last dividing precursor and their death in the LN. Dermal DC had a faster turnover and movement from the skin than LC, however, once they arrived in the LN, all DC present in healthy, uninfected mice displayed a rapid turnover from short (9 days) to very short (2-3 days), which was shorten by the presence of Ag or microbial products, possibly as a safety mechanism to limit the prolonged priming of DC. These findings contradict the conclusion from Ruedl et al [94] that these DC have long lifespan in the LN, which implied that these DC have a prolonged contact with T cell in the LN than the other subsets. Overall, DC in the spleen and LN turnover rapidly (within 6 days) and the DC in the skin take longer (> 12 days)[76].

1.2.4 Antigen uptake, internalization and presentation

In most tissues, DC are present in an immature state unable to stimulate T cells. Although these DC lack the requisite accessory signals for T cell activation, they are extremely well equipped to capture Ag. Thus, iDC are thought to act as sentinels specialized in the detection, internalization and processing of Ag. They can acquire Ag via different mechanisms, such as phagocytosis, macropinocytosis and receptor-mediated endocytosis.

In iDC, macropinocytosis is constitutive [96], enabling them to rapidly and non-specifically sample large amounts of surrounding fluid. Phagocytosis, in contrast, is initiated by the engagement of specific receptors that trigger a cascade of signal transduction necessary for actin polymerization and effective engulfment. In general, the same receptors mediate both phagocytosis and receptor-mediated endocytosis. Apoptotic and necrotic cell fragments can also be internalised by iDC, in a process reported to involve the scavenger receptor CD36, heat-shock protein binding receptor CD91, phosphatidylserine receptor and vitronecting receptor ($\alpha_V\beta_{3)}$ or $\alpha_V\beta_5$ integrins [97, 98], as well as viruses, bacteria and intracellular parasites[99, 100].

1.2.5 Receptor-mediated endocytosis

Receptor-mediated endocytosis allows the uptake of macromolecules through specialised regions of the plasma membrane, termed clathrin-coated pits comple [101]. Viral and tumour Ag can be taken up by DC as soluble proteins or shed from live tumour cells. The most efficient pathway of viral and tumour Ag loading to DC, however, seems to be the uptake of apoptotic or necrotic tumour cells [102]. Therefore, the development of new Ag delivery strategies applicable for the in vivo loading of MHC-I and -II molecules via the exogenous route is of special interest.

A large number of endocytic receptors are selectively expressed by subpopulations of iDC, but like mouse macrophages, iDC also express non-Ag specific endocytic receptors including FcRI (CD64), FcγRIII (CD16), FcγR II(CD32) [103, 104] and complement receptors CR3 and CR4 [105]. These receptors are involved in the binding and internalization of opsonized Ag, by Abs and/or by complement fragments, to phagocytic cells.

- ***Fc receptors***

Ag complexed with IgG Ab bind to and are taken up by the FcR mentioned above. Internalization of Ag-IgG immune complexes via FcRII (CD32) in human DC was shown to enhance the efficacy of Ag-presentation 100-fold in comparison compared to free Ag [106]. Furthermore, it also resulted in efficient peptide loading of MHC-II molecules and promoted MHC-I restricted cross-presentation at very low Ag concentrations [107].

However, targeting with IgG type Ab can result in opposing effects due to the different intracellular signalling sequences of various FcR. Using human xenografts, both the activation and inhibitory effect of different FcR was demonstrated [108]. Indeed, FcγRIIB contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), which is able to interfere with the stimulatory effect of other FcR upon cross-linking by immune complexes, which suggests that tumour cell-specific IgG-Ab participate in the stimulation of cellular anti-

tumour immune responses by activating DC and by modifying anti-tumour effector functions [109].

- ***Complement receptors***

There are a number of complement receptors but more emphasis will be given to CD11c/CD18, also known as CR4, p150, 90 and $\alpha_x\beta_2$, as it expressed at high levels by DC and has been shown to be an important target in our current research.

CD11c/CD18 is a member of the integrin superfamily, which is comprised of α and β- subunits. Currently, more than twenty different kinds of integrins have been identified, which can be classified in eight subgroups based on the β-subunit. The β_2 (CD18) integrins are expressed mainly on leukocytes: leukocyte function-associated Ag-1 (LFA-1, CD11a/CD18), MAC-1 (CD11b/CD18), p150, 95 (CD11c/CD18) and CD11d/CD18. These integrins have been reported to mediate several leukocyte activities including adhesion, spreading, chemotaxis, migration, phagocytosis and respiratory burst [110].

As transmembrane receptors, each subunit contains a large extracellular domain, a single membrane spanning segment and a short cytoplasmic tail. The cytoplasmic tail of the β-subunit is necessary and sufficient to mediate the linkage of integrins to the actin cyskeleton. Although α-subunits can bind cytoskeletal proteins, current evidence indicates that the major functional role of the α-cytoplasmic tail is to modulate cytoskeletal interactions by directly interacting in a ligand dependent fashion with the cytoplasmic region of the β-subunit [111]. In several types of the α-integrin subunits (αL, αM, αX, αE, α1, 2, 10 and 11), there are characteristic inserted domains (I-domains) which are responsible for the binding of ligands. I-domains have a unique structure containing a metal ion dependent adhesion site (MIDAS) at the top of the domain where the ligand is bound [112]. There are five exposed loops surrounding MIDAS, which have been shown to undergo conformational changes to bind their physiological ligands [113].

CD11c/CD18 has been shown to act as a receptor for the complement C3 cleavage product iC3[114], fibrinogen [115], denatured proteins [116], intercellular adhesion molecule-1 (ICAM-1) [117] and, interestingly, to be involved in Ag capture and delivery to Ag processing compartments [118]. CD11c/CD18 is expressed on monocytes, macrophages, granulocytes, a subset of NK cells and in a small proportion of activated T and B cells. However, it is still considered a DC surface marker because it is expressed at significantly higher levels on these cells and it is the predominant leukocyte integrin on DC. CD11c is expressed on the surface of all subsets of mouse DC [119] and all human DC of myeloid origin [120] .

- ***C-type lectin receptor superfamily***

Together with the TLR family, the C-type lectins represent another group of PRR, which are best known for their ability to bind specific pathogen associated glycosylated Ag. C-type lectins recognise carbohydrate residues in a calcium dependent manner via a highly conserved carbohydrate recognition domain (CRD). C-type lectins are the most abundant Ag receptors on DC and are implicated in immunoregulatory processes, such as Ag capture, DC trafficking and DC-T cell interactions [121]. Based on the location of the amino (N) terminus, two types of membrane-bound C-type lectins can be distinguished on DC. Type I lectins have their N-terminus located outside, while type II have it inside the cell.

Type I lectins include the macrophage mannose receptor (CD206) and DEC205 (CD205), which have been shown to be involved in cellular interactions and Ag endocytosis. Like other type I lectins, DEC205 is internalized from the cell surface by clathrin mediated endocytosis. It has been estimated that constitutively endocytosed receptors, such as those in this family, mediate ten or more rounds of recycling each hour [122].

Investigations on the cytoplasmic domain of these receptors demonstrated that they contain two potential endocytosis motifs based on a conserved tyrosine residue and a dihydrophobic motif [123]. In contrast to CD206, which has been

shown to recycle between the plasma membrane and early endosomes, DEC205, after internalisation passes through late endocytic compartments containing MHC-II molecules, due to a specific molecular motif (EDE) in its cytoplasmic tail [124], thereby enhancing the efficiency of Ag presentation.

To date, no physiological ligands have been identified for DEC205 and therefore the basis of its ligand specificity remains to be determined. CD206 has been shown to bind a wide variety of microorganisms including *Candida albicans* and *Mycobacterium tuberculosis*, amongst others [123]. Regarding their expression profiles, DEC205 has been shown not to be restricted to DC, as it has also been detected on some epithelial cells, B cells, bone marrow stroma and endothelial cells albeit to a lower level [125]. In the case of CD206, which was originally identified as a macrophage receptor, it is now known that its expression is also not restricted to these cells. DC, lymphatic and hepatic endothelium, kidneys have also been shown to express CD206 [123].

Type II lectins include langerin (CD207), which is specifically produced by LC. DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) (CD209) represents another member of this group and it has been demonstrated to act as an endocytic receptor mediating Ag presentation [126]. In humans, DC-SIGN expression is restricted to professional APC and expression levels are high. It is abundantly expressed by DC residing in lymphoid tissues and at mucosal surfaces and by dermal DC, together with macrophages. A recent study has demonstrated that Ab-mediated targeting of Ag to DC via DC-SIGN effectively induces Ag-specific naïve as well as memory T cell responses in humans, making it a promising target for DC based vaccination strategies [127].

Furthermore, there are other receptors on DC that have been reported to mediate Ag endocytosis which are not members of the families mentioned above, including the CD91 receptor (the α_2-macroglobulin, also known as low density lipoprotein-related protein, LRP) that binds heat-shock proteins (e.g. gp96 and hsp70, hsp90 and calreticulin) derived from tumour or infected cells [128]. Scavenger receptors are also implicated in the internalization of various bacteria,

and DC express at least one, CD36 (classB-SR), which is involved in the uptake of apoptotic bodies [129].

1.2.6 DC maturation

iDC are thought to require 2 independent signals in order to become fully activated: the direct recognition of pathogen via a range of PRR and indirect danger signals. The latter signals include inflammatory cytokines (e.g. TNF-α, IL-1β), LPS, intracellular components, and direct signals from viruses and bacteria via TLR. In response to these, DC become activated and initiate the process of maturation, which transforms the Ag-capturing DC into efficient Ag-presenting cells. Maturation is associated with several coordinated events, including downregulation of endocytic and phagocytic receptors, upregulation of co-stimulatory molecules such as CD40, CD80, CD86 and CD58, adhesion molecules, MHC molecules and changes in morphology.

The five main families of surface receptors reported to trigger DC maturation are summarized below.

1) Toll-like receptors (TLR)

Different pathogen-associated pattern molecules are specifically recognized by one or a combination of TLR (64). Different subsets of DC also express distinct overlapping sets of TLR. For example, in mice TLR3 is preferentially expressed by $CD8^+$ DC, while TLR5 and 7 are mainly expressed by $CD8^-$ DC. Plasmacytoid DC express high levels of TLR7 and 9, focusing on the recognition of viral pathogens, whereas conventional DC, express the remaining members of this family, which target mainly bacterial pathogens. Moreover, TLR recognise different motifs, for instance TLR4 binds LPS (GRAM- bacteria), TLR2 peptidoglycan (GRAM+bacteria), and TLR5 flagellin from both types of bacteria and TLR9 unmethylated CpG motifs.

2) Cytokine receptors

DC sense danger through the binding of inflammatory cytokines (e.g. TNF-α and IL-1β) to their respective receptors [130].

3) TNF-receptor family molecules

CD4 T helper cells induce DC maturation and survival via the interaction of members of the TNFR-family, such as CD40/CD40L. Moreover, DC maturation leads to the upregulation of other members (e.g. OX40L and 4-1BBL) in distinct patterns depending on the activation signal received, which allows them to induce and modulate the T cell response as required. Evidence suggest that the interaction of 4-1BBL/4-1BB is more important in a CD8 T cell response, much like OX40L/OX40 in a CD4 T cell response, with both being essential for the accumulation and survival of large numbers of primes T cells over time[131].

4) Heat-shock protein (Hsp) receptors

Cell death can also be sensed via Hsp receptors (e.g. CD91) as hsp are released by necrotic cells [132].

1.2.7 Migration of activated DC

All mediators of maturation trigger peripheral DC migration into the T cell area of lymphoid organs. This migration involves the coordinated action of several chemokines. Like adhesion molecules, the expression of chemokine receptors can be altered as cells differentiate, allowing them to coordinate their migratory route. Ligand binding to their chemokine receptors transmits signals to induce the expression of adhesion molecules, in particular integrins such as $\alpha_4\beta_7$ or $\alpha_4\beta_1$, which mediate the arrest of the circulating lymphocytes by forming strong interactions with adhesion molecules expressed on endothelial cells (e.g. intercellular adhesion molecule-1 and -2 , ICAM-1/-2) at the site of inflammation or lymphoid organs[133] .

After Ag uptake, activation of iDC inhibits their sensitivity to MIP-3α (and other chemokines specific for iDC) through either downregulation or desensitisation of its receptor [134, 135]. Consequently, maturing DC escape from the local gradient of this cytokine. Upon maturation, DC upregulate the expression of a single known chemokine receptor CCR7(116) and accordingly, acquire

responsiveness to CCL19 (also known as MIP-3β) and CCL21 (or secondary lymphoid tissue chemokine-SLC) [134], which allows them to leave the periphery and enter the lymphatic stream. Mature DC are driven into the paracortical area in response to MIP-3β and/or SLC by cells in the T cell zone [134, 136]. The DC may themselves become a source of these chemokines [134-136] allowing them to amplify the chemotactic signal. Furthermore, these two chemokines can also attract naïve T cells and play an important role in bringing the Ag-bearing DC closer to naïve T cells [136] (Diagram 2).

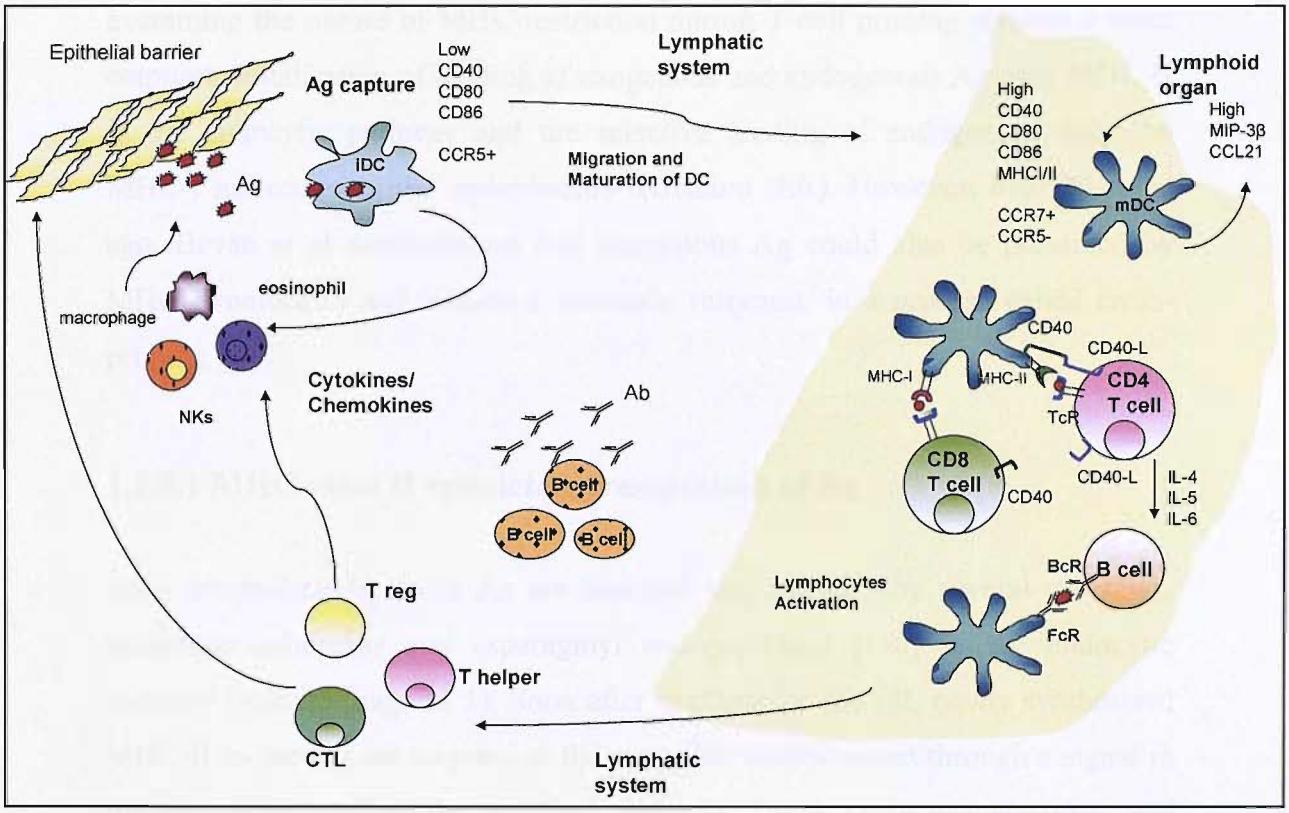


Diagram 2 Life cycle of a dendritic cell.

When immature DC (iDC) circulating the body, encounters antigens (Ag) in the periphery it induces the secretion of pro-inflammatory cytokines (e.g. IFN-α), which in turn can recruit eosinophils, macrophages and natural killer cells (NK) to the site. After Ag capture, iDC expressing low levels of co-stimulatory molecules migrate to the lymphoid organs, as they become insensitive to local MIP-α due to the loss of chemokine receptor CCR5. As DC mature (mDC), the increased expression of CCR7 allows them to respond to the MIP-β/CCL21 chemokines released by resident cells at the lymphoid organ. mDC potentiate their Ag presentation capacity by expressing high levels of co-stimulatory molecules and MHC-peptide complexes, thereby being able to directly present peptides to CD4 T cells and cross-priming CD8 T cells. These activated lymphocytes help DC in their maturation via co-stimulatory molecules (e.g. CD40/CD40-L), which results in lymphocyte expansion and differentiation into effector cells (cytotoxic T lymphocytes (CTL) and T helper cells. Helper T cells also secrete cytokines that permit the activation of macrophages, NK cells and eosinophils. B cells can recognise either native Ag via their B cell receptors or in the form of immune complexes captured on follicular DC. B cells become activated when in contact with Th2 effector cells and their cytokines, which induce them to migrate into various areas where they mature into plasma cells producing Abs, and memory B cells. In the absence of co-stimulation T regulatory cell may develop. It is believed that, after interaction with lymphocytes, DC die by apoptosis. Adapted from [78].

Upon interaction with T cells, DC receive additional maturation signals from CD40L, RANK/TRANCE, 4-1BB and OX40L, which induce the release of IL-8, fractalkine and macrophage derived chemokines that attract lymphocytes to the site [137, 138].

1.2.8 Ag processing and presentation

To trigger an immune response, Ag have to be degraded into short peptides, loaded onto MHC complexes and presented at the cell surface. Early experiments examining the nature of MHC-restriction during T cell priming showed a strict compartmentalisation of loading of exogenous and endogenous Ag onto MHC-II in the endocytic pathway and the selective loading of endogenous only, on MHC-I molecules in the endoplasmic reticulum (ER). However, over 20 years ago, Bevan et al demonstrated that exogenous Ag could also be presented by MHC-I molecules and initiate a cytotoxic response, in a process called cross-priming [139].

1.2.8.1 MHC-class II restricted presentation of Ag

After internalization, most Ag are digested into peptides by several proteases, including cathepsins and asparaginyl endopeptidase [140] in the endocytic pathway (refer to diagram 3). Soon after synthesis in the ER, newly synthesised MHC-II molecules are targeted to the endocytic compartment through a signal in the cytoplasmic tail of the invariant chain (Ii) [141]. Once in endosomes and lysosomes, which have an acidic environment, the Ii chain is degraded, leaving only class II-associated peptide (CLIP) in the peptide binding groove. The non polymorphic HLA-DM and HLA-DO in humans and H2-M and H2-O in the mouse, catalyze the exchange of CLIP for the Ag-derived peptides [142] and the MHC-II-peptide complex is delivered to the surface to present the peptide to naïve CD4 T cells.

Unlike other APC, DC tightly regulate the formation and transport to the surface of MHC-II-peptide complexes during their life cycle, thereby preventing the iDC from presenting self-peptide from engulfed healthy cells to T cells. First, Ag

degradation is inefficient in iDC and internalised Ag can remain intact in lysosomal compartments for several days [143]. Second, in mouse DC, newly synthesised MHC-II molecules have been shown to be retained in lysosomal compartments due to the poor degradation of Ii [144], and were unable to reach the cell surface [145]. Upon maturation of the DC, the degradation of Ii is rapidly increased resulting in efficient transport of the complex to the cell surface [146] together with co-localised co-stimulatory molecule CD86 (B7.2) and MHC-I molecules as clusters involved in T cell stimulation [147]. In addition, anti-inflammatory cytokines can interfere with the regulation of MHC-II processing by DC. For example, IL-10 inhibits the rise in protease activity induced by maturation signals by increasing the endosomal pH causing the long-term inhibition of Ag processing and presentation by mature DC [148].

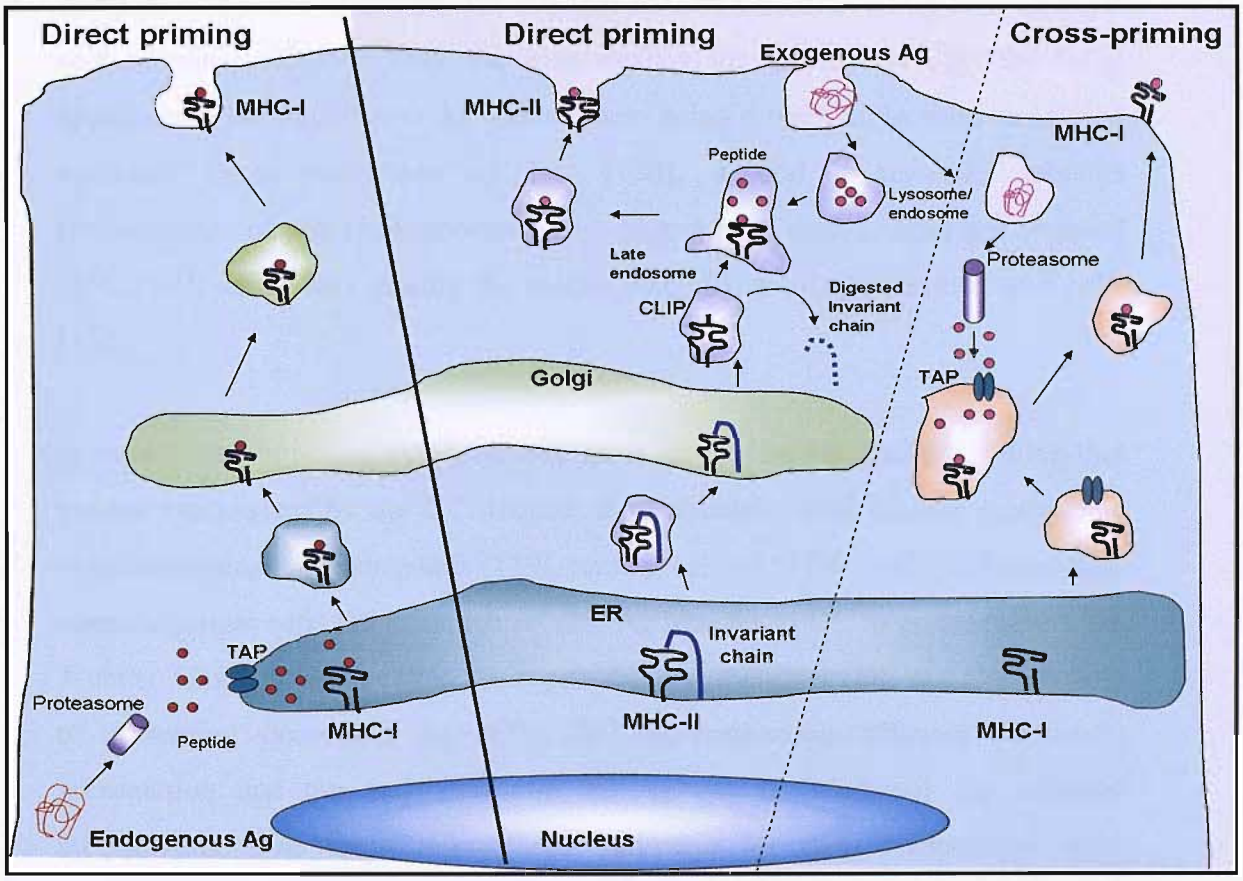


Diagram 3 The three major pathways involved in antigen presentation by dendritic cells.

Grey shaded area of diagram represents the route of exogenous antigen processing and presentation (divided into direct and cross-priming pathways) by dendritic cells; white area, indicates the pathway taken by endogenous antigen (direct priming).

1.2.8.2 MHC-class I restricted presentation of Ag

In contrast to exogenous Ag, peptides derived from endogenous Ag (i.e. from self-components or from virus-derived Ag expressed within the cell) are thought to become associated with MHC-I molecules, which are recognised by CD8 CTL. Thus, CD8 T cells would only be activated by an APC synthesising the Ag within itself.

Endogenous Ag peptides are generated by proteasome degradation of newly synthesised ubiquitinated proteins in the cytosol (refer to diagram 3). The resulting peptides are transferred to the ER by specialised peptide transporters (e.g.TAP) and loaded onto new MHC-I molecules under the control of a loading complex composed of several ER resident chaperones, such as tapasin, calnexin and calreticulin [149]. They are then rapidly transported through the Golgi apparatus to the membrane. As well as there being a marked increase in MHC-I synthesis upon maturation of DC [150], several proteasome subunits characteristic of the immunoproteasome (e.g. LMP2 and LMP7) are induced [151, 152], which may modify the pattern of epitopes being presented to T cells [152].

In most cases, however, cytotoxic responses need to be initiated against Ag that are not synthesised by the DC. Indeed, the immune system mounts a cytotoxic response against allotransplants [139], tumour cells [153] or virally infected non-haematopoietic cells [154], which require Ag transfer to DC to prime naïve CD8 T cells. This process is called cross-priming and therefore, DC are also capable of presenting exogenous Ag [155]. DC are particularly efficient for cross-presentation and the acquisition of the Ag can be mediated via different mechanisms. Two main intracellular pathways for cross-presentation were reported, resulting in the occurrence of either endocytic loading, where low levels of recycling MHC-I molecules are found, or ER conventional loading on newly synthesised MHC-I molecules. Evidence for both sites of peptide loading exists [155].

Castellino et al [156] suggested that internalised Ag may access the cytosol for processing by the proteasome and loading in the ER. The existence of transport from endosomes to the cytosol was initially proposed by Kovacsvics-Bankowski and Roch [157] and such a transport pathway was evidenced in macrophages [157, 158] and DC [159, 160]. Indeed, DC bear a unique endosome to cytosol transport pathway that allows selective delivery of internalised Ag to the cytosol [160]. In vivo cross-priming against tumour Ag requires TAP [153], suggesting that in this case the cytosolic pathway is predominant. In addition to MHC-class I and II, DC express a third class of MHC molecules involved in Ag presentation to T cells and NKT cells, and these are the family of CD1 proteins. However, discussion of this topic is unfortunately beyond the scope of this thesis.

1.3 T and B cell priming

1.3.1 Signals regulating T cell function

Over 30 years ago, the two-signal hypothesis for T and B cell activation was first proposed [161] and it has become widely accepted that T cells require two distinct signals for optimal T cell precursor (TP) cell expansion. Signal 1, is provided by the interaction of the TcR with the MHC-peptide complex on the surface of APC, and signal 2, comes from costimulatory molecules such as CD80 and CD86 on the surface of APC stimulating CD28 on the T cells. Signalling through signal 1, in the absence of costimulation, leads to either the termination of activation and depletion of activated TP cells or a prolonged state of anergy or clonal unresponsiveness [162] (discussed further in section 1.3.6). Costimulation may simply promote the more efficient engagement of TcR molecules to enhance the initial activation, or may provide additional signals to promote cell survival or induce effector functions, such as cytokine secretion or cytotoxicity. Many receptors have now been described to be costimulatory, and these can be divided into two main families: the immunoglobulin superfamily (e.g. CD28/B7) and the tumour necrosis factor receptors (TNFR) superfamily (e.g. CD40/CD40L).

1.3.2 Priming of T cells by DC

The ability to prime naïve CD4 T cells constitutes a unique and critical function of DC both in vitro and in vivo. As certain APC, such as resting B cells that express low levels of co-stimulatory molecules constitutively, are not competent APC and require activation first in order to prime CD4 T cells. In the presence of Ag, T helper cells primed by DC can interact with B cells and stimulate Ag-specific Ab production. In addition, DC are equally important in priming naïve CD8 T cells and induce their expansion [163]. This can happen directly in the absence of T cell help [164], although they often require CD4 T cell help. In the sequential model, help from CD4 T cells is proposed to be transmitted directly to B cells and indirectly to CD8 T cells via CD40L-mediated stimulation of CD40 on DC. This is thought to induce DC licensing and the induction of more potent stimulatory signals to CD8 and presumably other CD4 T cells [165].

Interestingly, CD8 T cells transiently express CD40 following activation, and thus, can also benefit from direct help from CD4 T cells [166]. This finding favours the concomitant "three cluster model" more than the sequential model, previously described. Although concomitant T cell help might not be a strict requirement, it is likely to have an impact on CD8 T cell activation. Therefore, costimulatory molecules are thought to play a key role in the initiation and modulation of the priming and differentiation of T cells.

Nevertheless, it remains to be determined whether the unique ability of DC to prime T cells is a consequence of the expression of costimulatory molecules unique to DC or of the high density of these molecules involved in DC-T cell interactions. The crucial factor that constitutes signal 2 required to sustain T cell activation is the interaction between CD86 on DC and CD28 on T cells (discussed above). T cells can activate DC via CD40-CD40-L signalling leading to increased expression of CD80/CD86 and cytokine release (e.g. IL-1, TNF-α, chemokines, IL-12) [167]. CD40 stimulation on DC also causes the upregulation of OX40-L [168], which subsequently signals activated CD4 T cells to express IL-4 and chemokine receptor CXCR5, whose ligand directs T and B cells into the B cell follicles leading to their encounter at the edges of the follicle.

Furthermore, mature DC also express 4-1BB-L , which complements the function of OX-40L[169]. 4-1BB is a costimulator expressed primarily on activated CD4 and CD8 T cells and its signals preferentially induces CD8 T proliferation and production of IFN-γ, leading to the amplification of CTL responses [170]. Engagement of RANK by RANK-L/TRANCE expressed on activated T cells, stimulates the secretion of cytokines like IL-1, IL-6 and IL-12 by DC, which increases the survival of DC, inhibits DC apoptosis and enhances proliferation of T cell. TRANCE has been suggested to be a key player in the CD40-independent activation of T helper cells [171].

1.3.3 CD4 T helper effector cells

Towards the end of the proliferative phase induced by the secretion of IL-2, which is synthesized and secreted by activated T cells themselves, T cells become effector cells. Mature naïve CD8 T cells are already committed to becoming cytotoxic effector cells should they be activated by Ag (see section 1.3.6). However, the effector options for CD4 T cells are more varied and commitment is made only when the naïve CD4 T cell is first stimulated by Ag. They can give rise to either CD4 T_H1 cells or T_H2 cells. The T_H1 cells bias the immune response towards macrophage activation, via the secretion of macrophage stimulating cytokines such as IFN-γ, GM-CSF, TNF-α, IL-1 and others like IL-3, TNF-β, IL-2 and the expression of CD40-L, Fas-L and TRANCE, thereby leading to inflammatory cell mediated immunity dominated by cytotoxic CD8 T cells and CD4 T_H1 cells.

In contrast, the cytokines secreted by T_H2 cells mainly induce B cell differentiation and production of Abs leading to a humoral response initiated and modulated by cytokines such as IL-4, IL-5, IL-3, GM-CSF, IL-10, TGF-β and the expression of CD40L. Because a continuous inflammatory response controlled by T_H1 cells can be very harmful to the host, cytokines secreted by Th2 cells such as TGF-β, IL-4, IL-10 and IL-13 inhibit Th1 differentiation and

also act on DC to inhibit IL-12 secretion [172], thereby polarizing the system and amplifying the type of response required at non-damaging levels.

The different subsets of conventional DC have been suggested to share the common capacity to present Ag to T cells. However, they differ in the other aspects of DC-T cell signalling that determines the subsequent fate of the T cell they activate. The most striking difference is the ability of the $CD8^+$DC to induce a T_H1-biased cytokine response, whereas $CD8^-$DC tend to induce a T_H2-biased response [173, 174]. Consistent with this, GM-CSF, which preferentially mobilizes myeloid DC in mice, elicits mainly IgG1 Ab in response to soluble Ag, whereas FLT3-L, which mobilizes both lymphoid and myeloid DC subsets also elicits IgG2a Ab, a T_H1 signature [175]. The main factor in this difference has been postulated to be the production of IL-12p70, as in IL-12 deficient mice there is a failure to induce T_H1 responses [174, 176]. Lymphoid $CD8^+$DC but not myeloid $CD8^-$ DC can be induced to make large amounts of IL-12 [174] and IFN-γ [176].

The mechanisms by which myeloid DC induce T_H2 cytokines is not established, and although $CD8^+$DC have the greatest capacity for IL-12 production, different stimuli can change the balance. For instance, LPS from *Escherichia coli* stimulates $CD8^+$DC to produce IL-12 and induce T_H1 responses, LPS from *Porphyromonas gingavalis* does not stimulate IL-12 production and preferentially induces a T_H2 response [177]. Additionally, DC subsets can influence the amount of cytokines these T cells secrete, for example, $CD4^-CD8^-$ DC induce more IL-12, IFN-γ, IL-3 from CD8 and CD4 T cells than do $CD8^+$DC [178].

A further functional difference is that $CD8^+$ DC have been reported to cause early apoptotic death in CD4 T cells activated in culture, unlike $CD4^-CD8^-$DC [179]. The in vivo significance of this may suggest that $CD8^+$ DC could be a specialized subset of tolerogenic or regulatory DC, and there is some evidence of this in in vivo models [180, 181]. Nevertheless, the specialization of the individual DC subsets in the priming of distinct T cell subsets remains unclear.

1.3.4 CD8 T cell cytotoxic function

Naïve CD8 T cells are stimulated to proliferate and to develop into cytotoxic effector T cells after recognition of peptides associated with MHC-I molecules on APC, in particular DC. Endogenous cytosolic proteins are generally the source of MHC-I restricted Ag, however, DC can cross-present Ag in the context of MHC-I molecules. Both direct priming and cross-priming, leads to the activation of naïve CD8 T cells, which differentiate into effector CTL.

Variations in the requirement by CTL for help from CD4 T_H1 cells, appears to be related to the inflammatory nature of the immunogen. Ag such as tumour cells, minor alloantigens and loaded cells are considered to be rather non-inflammatory, thus requiring help to circumvent their poor stimulatory nature. In contrast, highly inflammatory stimuli such as viral or bacterial infection do not require help. In several recent studies, it has been shown that secondary expansion of memory CTL is greatly impaired if help is absent during the priming phase [166], even for those responses previously classified as helper independent. However, the need for "help" in the CTL primary effector phase remains highly controversial, where some reports suggested that CTL responses in general appeared to be helper independent in the primary effector phase but secondary expansion was highly dependent on receiving help during the primary phase. Interestingly, help was not required during secondary expansion once it was received in the priming phase [166, 182]. On the other hand, experimental models using different immunogens suggested that primary expansion of CTL was dramatically reduced in the absence of help [183].

In 1998, three groups provided strong evidence that signalling of CD40 on the DC was the prime means of helper-dependent DC licensing for CTL generation [165, 184, 185]. Schoenberger et al [167] found that CTL priming to a tumour Ag was CD40-dependent and, furthermore, they showed that helpers could be replaced by the CD40-stimulating mAb. Matzinger's group[165] further demonstrated that the use of α-CD40 mAb could prime a normally-dependent CTL response in mice deficient of helper cells. The ability to signal CD40 on the

DC alleviated the need for the helpers and CTL to recognise Ag at the same time, so that T helper cells should be able to license several DC for priming of CTL. There is also evidence that 4-1BB signalling on the CTL can replace the need for help [186].

Another factor determining the efficiency of CTL priming is the subset of DC cross-priming the naïve CD8 T cells. Both $CD8^+$ and $CD8^-$ DC subsets can elicit efficient Ag-specific CTL responses to peptide Ag in vivo and in vitro [187]. However, in the case of particulate or soluble protein Ag, studies have shown that while all subsets could capture, process and present to CD4 T cells, only the $CD8^+$ DC were capable of cross-presenting to Ag-specific CD8 T cells [188].

1.3.5 B cell priming

The "classical model" proposes that activated CD4 T cells expressing CD40L provide signals to B cells that induce proliferation [189], Ig class switching [190], Ab secretion [191] and rescue from apoptosis at different times during the life of a B cell [192]. Moreover, this signal has been shown to have a role in the development of germinal centres (GC) and the survival of memory B cells [193]. Thus, this suggests that a conditioned DC could act as a temporal bridge between a CD4 T helper cell and a B cell by analogy to the previously discussed sequential model, in which DC offer costimulatory signals to CD4 and CD8 T cells.

However, the whole story seems to be more complex than previously understood, because B cells upregulate the expression of CD40L on their surface following contact with Ag [194]. Furthermore, recent data have shown that DC can take up and retain Ag in its native state, and transfer this Ag to naïve B cells to initiate Ab responses [195, 196]. Therefore, these observations lead to the hypothesis that DC could display Ag to both T and B cells in a ménage a trois. Indeed, DC-B cell clusters have been shown to be stable complexes [197], indicating a serious cross-talk between these cells, possibly via the interaction of CD40 molecules on DC and CD40L on activated B cells. DC have also been reported to

interact with B cells to initiate T-independent Ab responses [198] suggesting that CD40L on B cells may play an important role in early B cell activation in T-dependent and T-independent Ab responses.

1.3.6 T cell tolerance

Stem cells continually migrate from the bone marrow to the thymus, where they develop into T cells. Since T cells recognise peptides on MHC molecules and these are highly polymorphic, the desirable immature T cells are those that can recognise self-MHC molecules but that are not autoreactive. This objective is achieved by thymic selection, a process that involves both positive and negative selection.

The induction of central tolerance requires the presence of self-Ag in the thymus, however, not all self-Ag occur in the thymus, which leads to the requirement of the existence of additional tolerance mechanisms to prevent autoimmunity. Mechanisms occurring elsewhere in the body are referred as peripheral tolerance and they complement central tolerance. Tolerance of CD4 and CD8 T cells has been demonstrated to be achieved by either clonal deletion [199] or anergy [200]. In addition, peripheral tolerance can also be generated via immunological ignorance (Diagram 4). Under normal circumstances autoreactive T cells that cannot enter normal tissues other than the lymphoid organs ignore their Ag, thereby maintaining self-tolerance[201].

Efficient tolerance mechanisms were shown to be especially important at sites of infection, where maturing DC process and present both self and non-self Ag. It has been known for some time that maturation is a control point for initiating immunity, but the concept that this carries substantial risks emerged when DC were found to process Ag from dying cells and that DC were continually capturing and presenting self and harmless environmental Ag in the steady state [202]. Early studies suggested that $CD8^+$ DC in mice have a propensity for inducing tolerance [179, 203], but recent literature has also demonstrated the ability of $CD8^+$DC to produce IL12p70 in response to several stimuli. This

paradox suggests that the function of the DC is influenced heavily by external signals such as inflammatory cytokines and cell-cell interactions with CD4 T cells.

This principle was elegantly demonstrated by Steinman's group in an experiment where mice were immunized with α-DEC205 mAb linked to an Ag in the presence or absence of a maturation signal (e.g. α-CD40 mAb). In the absence of the maturation signal, the Ag-specific CD4 T cells underwent initial proliferation, but then were almost entirely deleted, which resulted in the tolerization of the system. In contrast, when the maturation signal was present, immunity occurred, generating high number of Ag-specific CD4 T cells that were not deleted and produced large amounts of IFN-γ [204]. These results indicated that low doses of soluble proteins targeted to DC in the steady state are successfully cross-presented and lead to deletional tolerance if a maturation signal or inflammatory signal is not provided. It is theoretically possible that these different outcomes are a result of presentation by different subsets of DC, but the literature favours the interpretation that the same DC function in immunity and tolerance depending on their state of maturation.

The actual mechanism by which these cells are deleted in the periphery is via the Fas-Fas-L interaction. Engagement of Fas induces apoptosis in Fas-positive cells and since T cells express both Fas and Fas-L on activation, the interaction between the two molecules can induce apoptosis [199]. In addition to clonal deletion, the immune system also mediates tolerance via the action of T regulatory (Treg) cells (Diagram 4). Immune regulation by Treg cells has the additional advantage that as well as preventing autoimmunity as with clonal deletion, it also serves to limit the development of harmful pathology during normal immune responses. Studies in a number of experimental autoimmune disease models provide convincing evidence that specialised Treg cells are an integral part of the T cell repertoire [205].

$CD4^+CD25^+$ T cells, which constitute approximately 10% of the peripheral murine CD4 T cell population, have been shown to possess a potent regulatory activity [206]. These cells show a partially anergic phenotype, in that they

proliferate poorly upon TcR stimulation and their growth is dependent on exogenous IL-12 [207]. $CD4^+CD25^+$Treg cells have been demonstrated to inhibit diabetes in mice [208], induce tolerance to alloantigens [209], impede anti-tumour immunity [210] and regulate the expansion of CD4 T cells [211]. The regulatory function of $CD4^+CD25^+$ T cells has been suggested to involve the inhibition of IL-2, the overexpression of CTLA-4 [212, 213], secretion of TGF-β [214] and IL-10 [215].

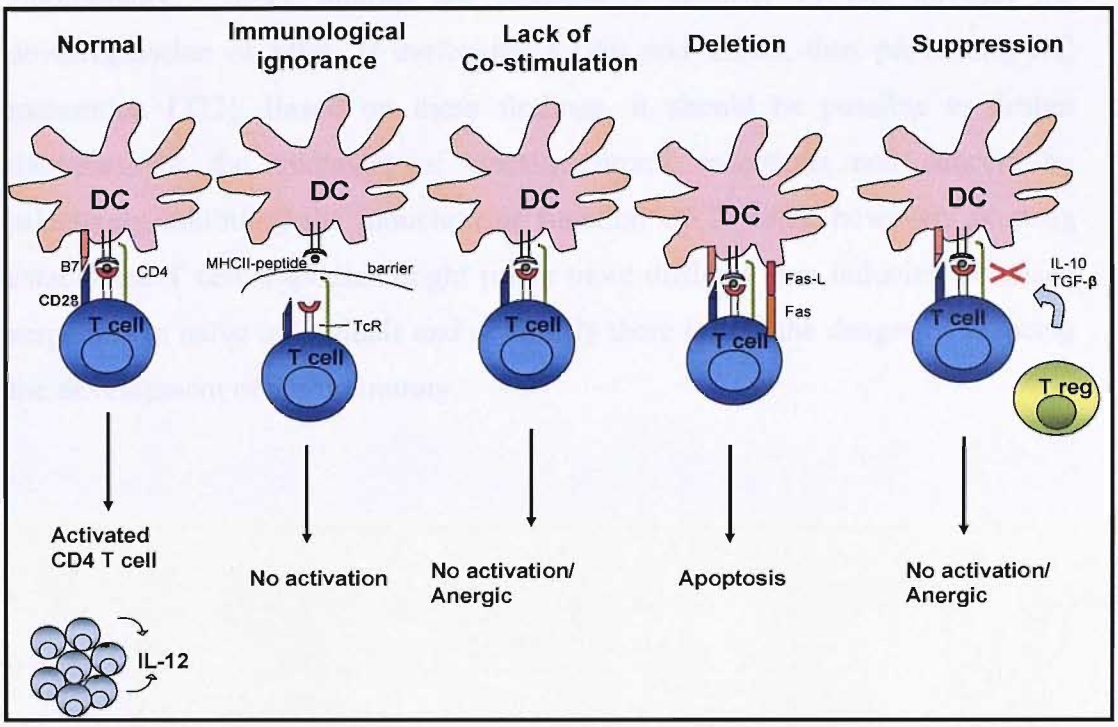


Diagram 4 Major mechanisms associated with peripheral T cell tolerance (Adapted from [216]). **Refer to text for description of the pathways.**

Other subsets of CD4 Treg cells have been identified and these include the type 1 Treg (Tr1) cells, which secrete high levels of IL-10, but no IL-2 and IL-4, and low to moderate levels of TGF-β. Functional studies on Tr1 cells have suggested that their bystander immunosuppressive properties are mediated mainly by the secretion of IL-10, as neutralisation of IL-10 reversed the inhibition of T_H 1 clones regardless of their Ag specificities [217]. T_H3 cells are part of another subset of CD4 Treg cells and contrary to Tr1 cells; they secrete high levels of TGF-β and moderate of IL-10. Recently, certain tumour cell lines were reported to produce TGF-β [218], which may represent another strategy by which tumours suppress the induction of tumour-specific CTL responses [218]. In addition to the

CD4 Treg populations, recent data have identified CD8 Tr cells, secreting IL-10 or TGF-β [219, 220] but not much more is known about their functional properties. Together these data indicate that these cells might use multiple and as yet undefined mechanisms to mediate suppression.

There are reports in the literature suggesting that activation of DC that secrete IL-10, but not IL-12, can direct naïve T cells towards a Tr1 subtype [221]. Additionally, IL-10 inhibits the stimulatory capacity of DC through the downregulation of MHC II molecules, CD80 and CD86, thus preventing DC maturation [222]. Based on these findings, it should be possible to design therapies for the treatment of certain chronic infections and cancers by selectively inhibiting the induction or function of Tr cells, however, skewing established T cell responses might prove more difficult than inducing polarised responses in naïve individuals and obviously there is also the danger of inducing the development of autoimmunity.

Project

There is now convincing evidence from studies in both animals and humans that DC vaccination has distinct potential for inducing immune responses and is a safe and feasible approach for cancer immunotherapy. The results from Phase I and II trials, although difficult to compare, have demonstrated encouraging clinical activity for several tumour types. However, the great majority of the current protocols involve administration of in vitro cultured, loaded and conditioned DC, which makes this strategy labour intensive, expensive and patient-specific.

Aiming to circumvent these limitations, we generated a panel of Ab-based vaccines, which targeted our model Ag, Ova, to APC in situ. Whole Ova protein was chemically linked to mAb specific for surface molecules expressed on APC, in particular DC (listed in table 5).

[FabxOva] conjugate	Target
N418xOva	CD11c
DEC205xOva	DEC205
Mc39-16/ Mc106A5xOva	Bcl1/A31 Idiotype (controls)
3/23xOva	CD40
M5/114xOva	MHC-II
LOB15.1xOva	TLR2
2.4G2xOva	FcRγ II/III

Table 5 List of [FabxOva] conjugates and their targets on antigen presenting cells.

The advantage of this strategy is that by effectively delivering Ag directly to DC in vivo, we aimed to amplify Ag-specific T and B cell immunity without having to expand the DC in vitro, which would eliminate the major costs and drawbacks associated with the current protocols (diagram 5). Furthermore, we investigated the efficacy of the individual [FabxOva] conjugates as potential therapeutic vaccines in cancer immunotherapy by using animal tumour models.

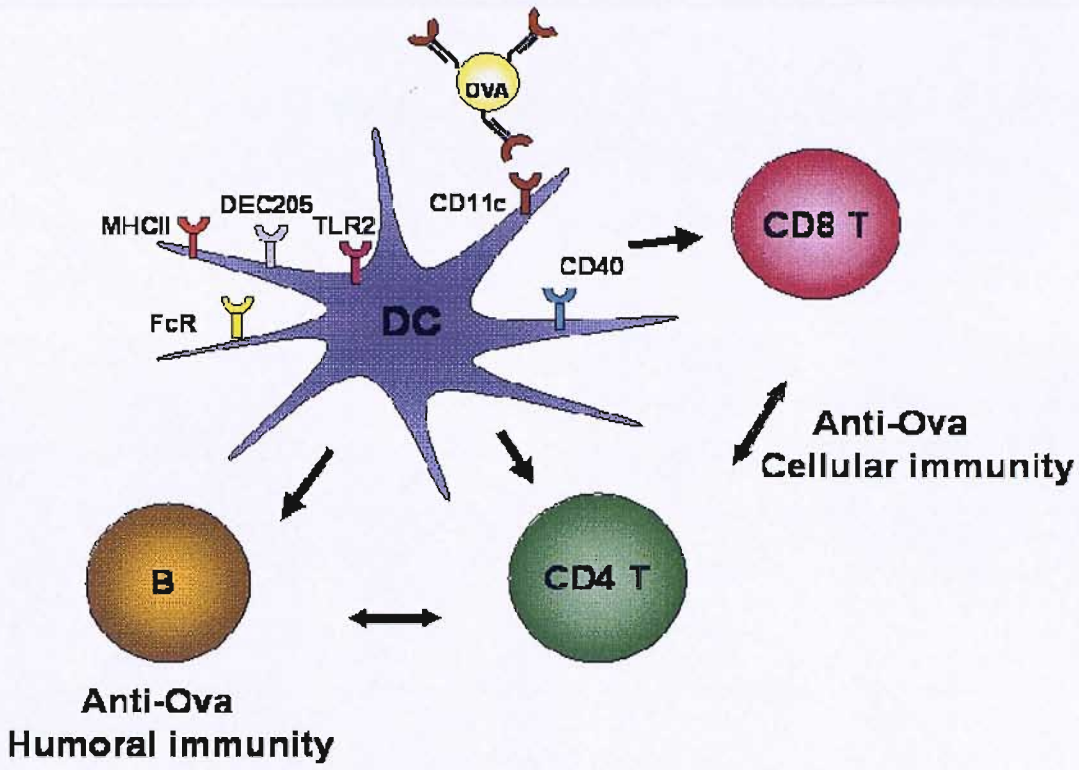


Diagram 5 Diagrammatic representation of the objectives aimed to be achieved in this project.

By delivering ovalbumin directly to surface molecules on dendritic cells in situ, we aimed to potentiate anti-ovalbumin T cell and humoral immunity, and assess the efficacy of these conjugates as potential cancer vaccines.

Chapter 2 Materials and Methods

2.1 Cell culture

Cells were cultured in RPMI 1640 medium (Invitrogen Ltd, Paisley, UK), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 mM pyruvate (Invitrogen) and 10 % (v/v) Foetal Calf Serum (FCS; Myoclone Plus, Invitrogen) and maintained at 37 °C in a 5 % CO_2 humidified incubator. Chinese hamster ovary (CHO) K1 cells stably transfected with the FLT3-L-Fc fusion protein were cultured in GMEM-S (First Link, UK) supplemented with 10 % (v/v) dialysed FCS (First Link, UK), 100 U/ml penicillin and 100 μg/ml streptomycin and 50 μM methionine sulfoximine (MSX) (Sigma). The supernatant was collected and purified using a protein-A column.

2.2 Cell quantitation

Cell numbers were determined using a Coulter Industrial D Cell counter (Coulter Electronics, Bedfordshire, UK), or by manual counting using a haemocytometer (Improved Neubauer).

2.3 Reagents

H-2K^b-restricted OVA $_{257-264}$ peptide (SIINFEKL) and an I-A^b-restricted peptide (LSQAVHAAHAEINEAGR; $OVA_{323-339}$) were obtained from Peptide Protein Research Ltd (Fareham, U.K.) The lyophilised peptide (> 95 % purity) was dissolved in phosphate buffered saline (PBS; 1.37mM NaCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and its concentration measured by BCA assay (Pierce, Rockford, USA). PE-labelled H-2K^b OVA $_{257-264}$ tetramers were obtained from Proimmune (Oxford, U.K.) and Beckman Coulter UK Ltd (Buckinghamshire, UK). LPS from *Salmonella minnesota* was obtained from Sigma (Poole, UK).

2.4 Mice

Ovalbumin (Ova)-specific, OT-I TcR transgenic mice crossed onto C57BL/6 mice were obtained from Dr Matthias Merkenschlager (Imperial College, London) and bred in the in-house animal facility (Tenovus). OT-II TcR transgenic mice were obtained from the University of Edinburgh. Wild-type C57BL/6 mice were bred and maintained in our in house animal facilities, and at few occasions, mice were also obtained from Charles River Laboratories (Harlan, UK). TLR4-deficient mice, C3H/HeJ, and control mice, C3H/HeN, were obtained from Charles River Laboratories (Harlan, UK). All mice were used at approximately 8-12 weeks of age. Animal experiments were carried out according to the UK Home Office licence guidelines, and were approved by the University of Southampton's ethical committee.

2.5 mAb

A list of the mAb used in this project and their sources is given in table 6. All hybridoma lines secreting mAb were expanded in tissue culture and purified from culture supernatant using a Protein-A column (Amersham Biosciences UK Ltd, Little Chalfont, Bucks, UK) according to the manufacturer's instructions and the purity of the mAb produced was routinely assessed by electrophoresis (Beckman EP, Beckman, USA). All mAb used for FACS analysis were fluorescein (FITC) or phycoerythrin (PE)-conjugated in house with the exception of α-mouse CD83:PE (IgG1, eBiosciences), α-mouse $V\alpha_2$ –TcR:FITC (clone B20.1, IgG2a, BD Pharmigen), α-mouse $V\beta_{5.1,5.2}$ TcR:PE (clone MR9-4, IgG1, BD Pharmigen), α-mouse allophycocyanin (APC)-labelled α-CD8α (clone 53-6.7, BD Pharmingen), APC-labelled α-CD4 (clone RM4-5 BD Pharmingen). Rabbit α-Ova (Nordic Immunological Laboratories, The Netherlands), polyclonal rabbit α-Ova-HRP was labelled in house with HRP from Sigma.

mAb	Specificity	Isotype	Source
N418	CD11c	Hamster IgG	ATCC
DEC205	DEC205/CD205	Rat IgG2a	ATCC
M5/114	MHC II	Rat IgG2b	ATCC
2.4G2	FcRγ II/III	Rat IgG	ATCC
3/23	CD40	Rat IgG2a	G. Klaus
LOB15/1	TLR2	Rat IgG	In house
GL-1	B7.1/CD86	Rat IgG2a	ATCC
1610A15	B7.2/CD80	Hamster IgG	ATCC
YTS169	CD8	Rat IgG2a	S. Cobbold
YTA 3.1.2	CD4	Rat IgG2a	S. Cobbold
5D3	MMR/CD206	Rat IgGa	Serotec Ltd.
YN1.4.7	ICAM-1/CD54	Rat IgG2b	ATCC
Mc39-16	A31 lymphoma- idiotype	Rat IgG2a	In house
Mc106A	BCL_1 lymphoma- idiotype	Rat IgG2a	In house
1D3	CD19	Rat IgG2a	D. Fearon
1452C11	CD3	Hamster IgG	In house

Table 6. List of α-mouse mAb, their isotypes and source.

ATCC- American Type Culture Collection, US; , S.Cobbold (Sir William Dunn School of Pathology, Oxford, U.K.);G.Klaus (National Institute of Medical Research, London, U.K.); D. Fearon (University of Cambridge School of Clinical Medicine, Cambridge, U.K.) and Serotec Ltd (Oxford, U.K.).

2.6 Conjugation of Ova to monovalent mAb

In brief, purified IgG was digested with pepsin (1% w/w) and the $F(ab')_2$ fragments collected after passing the mixture through an Ultragel chromatography column (AcA44). The $F(ab')_2$ fragments were reduced by the addition of 1/10 volume of the reducing solution (220 mM 2-Mercaptoethanol and 1 mM EDTA) for 30 min at 30 °C and separated from the reducing agent over a G25 column. The endotoxin-low chicken ovalbumin (Sigma) was activated with the linker succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce) according to the manufacturer's protocol. The unconjugated Ova was removed by passing the mixture through a G25 column and then mixed with the Fab_{SH} fraction overnight at 4 °C. The mixture was separated on an AcA44 column and the [FabxOva] conjugate fraction evaluated by HLPC. To remove contaminating IgG and endotoxin, the conjugate was passed through an α-Fγc immunosorbant column and a polymixin column, respectively. Multi-test Limulus Amebocyte Lysate (Pyrogen Plus, Bio Whittaker), an endotoxin assay, was performed following the manufacturer's instructions, to confirm that the [FabxOva] conjugates were endotoxin low. Conjugates were considered endotoxin low when less than 5 ng of endotoxin per mg of conjugate was detected. [FabxOva] conjugates were characterised by SDS-PAGE and quantification of the Ova content of the conjugates estimated by comparison with known quantities of Ova in an ELISA system. Conjugates were estimated to contain 20-30% Ova. The production of the conjugates was routinely carried out by Alison Tutt.

2.7 Chapter 3: Phenotyping of DC and T cell immunity

2.7.1. Preparation of splenic DC

Spleens were removed aseptically from C57BL/6 mice and digested with Collagenase D (from *Clostridium histotyticum*, Roche Diagnostics GmbH, Germany) (1 mg/ml) for 40 min at 37 °C. A single cell suspension was prepared using a cell strainer and cells were washed in PBS. Red blood cells were lysed by incubating the resuspended pellet for 5 minutes at room temperature in lysis solution (0.83 % (w/v) ammonium chloride, 0.1 % (w/v) $KHCO_3$ in dH_2O), after

which the splenocytes were washed once in PBS, counted and resuspended in 10 % RPMI. $CD11c^+$ splenocytes were purified by MACS® beads.

2.7.2. Standard protocol for staining cell surface antigens

Approximately 1 x 10^5 cells were incubated on ice for 20 min with fluorophore-conjugated antibodies (10 μg/ml final concentration for FITC or appropriately titrated for PE). Cells were then washed in FACS buffer (PBS, 1 % (w/v) Bovine Serum albumin (BSA)-reduced endotoxin; AvonChem, UK and 0.1 % (w/v) sodium azide (Sigma)) and stained with 7-aminoactinomycin D (7AAD) (2 μg/ml) for 5 min before subsequent analysis on a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) flow cytometer. Counts were gated on the viable lymphocyte population of $CD11c^+/7AAD^-$cells. Data were analysed using CellQuest software (BD Biosciences).

2.7.3 Phenotyping of splenic DC

$CD11c^+$splenocytes were pre-treated with α-FcRII/III mAb (2.4G2) (10 μg/ml) to block non-specific binding of fluorescent labelled mAb. DC maturation was induced by culturing DC in the presence of LPS (1 μg/ml), IL-4 and GM-CSF (10 ng/ml) for 24 h prior to surface staining.

2.7.4 In vitro T cell proliferation assays

The proliferation status of the cells was determined by measuring radiolabelled [^{3}H] - thymidine incorporation. Only replicating cells will incorporate [^{3}H]-thymidine, and therefore, an increase in the number of dividing cells will be evidenced by an increase in [^{3}H]-thymidine incorporation compared to controls.

In vitro proliferation assays were performed by culturing OT-I or OT-II T cells from fresh spleens in round-bottomed 96-well plates for a total period of 66-68 h in the presence of gamma-irradiated (2500 rad) total splenocytes. [FabxOva] conjugates or $Ova_{257-264}$ (SIINFEKL)/$Ova_{323-339}$ peptide (used as a positive

controls) were added to the plate in combination with α-CD40 mAb. Cell numbers and concentrations are indicated in the figure legends. Methyl-^{3}H-thymidine (0.5 μCi /well; Amersham, Buckingham, UK) was added to the cultures in the last 16-18 h of culture, before cells were harvested onto glass fibre filters (Unifilter GF/B, Perkin Elmer) using an automated harvester (Filtermate harvester, Packard). [^{3}H]-thymidine incorporation was subsequently determined by liquid scintillation counting.

Ex vivo proliferation assays were performed using spleens from C57BL/6 mice immunized with [FabxOva] conjugates (2.5 μg) and α-CD40 mAb (50 μg) i.v. 24 h prior to performing the assay. Freshly isolated spleens were treated with Collagenase D (1mg/ml) and red blood cells lysed with lysing buffer (as described in section 2.7.1). A splenocyte single cell suspension was prepared, irradiated and cultured with OT-I or OT-II T cells from spleens as described above and [H^{3}]-thymidine incorporation detected via liquid scintillation counting. As an internal control, to demonstrate that splenocytes were viable APC, Ova peptide (0.002nM) was added to a well of each irradiated splenocyte suspension (stock 4x10^{5}cells/well) to give a final concentration of 0.001nM, and co-cultured with the OT-I or OT-II T effector cells.

2.7.5 In vivo priming and proliferation of T cells

In vivo priming and proliferation of CD8 or CD4 T cells were evaluated by adoptively transferring OT-I or OT-II T cells, respectively, into naive C57BL/6 recipients. Twenty-four hours later mice were immunized (i.v.) with [FabxOva] conjugates (2.5 μg) in the presence or absence of α-CD40 mAb (50 μg). For tracking Ag-specific CD8 T cells in vivo, blood samples (50 μl) were stained with titrated amounts of PE- labelled H-2K^{b} Ova$_{257-264}$ tetramers and APC-labelled α-CD8α. For tracking CD4 T cell, cell were labelled with APC-labelled α-CD4, FITC labelled α-Vα_2 TcR and PE labelled α-V$\beta_{5.1,5.2}$ TcR. Cells were washed and red blood cells lysed with BD FACS lysing solution (1/10 volume) (BD Biosciences) before analysis on a FACS Calibur. Endogenous CD8 T cell

responses to [FabxOva] immunization were assessed using the same protocol, but without the transfer of transgenic T cells.

Memory responses were induced by re-challenging the immunized mice with H-2Kb-restricted Ova $_{257-264}$ (SIINFEKL; OT-I) peptide (30 μg) (i.v) for CD8 T cell responses and I-A^b-restricted peptide $OVA_{323-339}$ (OT-II) peptide (90 μg) (i.v.) with LPS (20 μg) for CD4 T cell responses.

When assessing the effects of FLT3-L on T cell proliferation, mice were pre-treated with 10 μg of FLT3-L s.c. daily for 9-10 days before being immunized with the [FabxOva] conjugates.

2.7.6. Effector function of primed T cells

The effector activity of primed T cells was assessed by α-IFN-γ ELISPOT assays. Briefly, 96-well plates (BD ELISPOT plates, BD Biosciences) were coated for 24 h with purified α-mouse IFN-γ monoclonal antibody (1:200, BD Biosciences) at 4 °C. Plates were washed, blocked with complete 10 % FCS/RPMI for 2 h at RT. Cell suspensions of splenocytes from mice immunized with [FabxOva] (2.5 μg) with or without α-CD40 mAb (50 μg) 6 days prior to the assay were prepared and plated at $4x10^5$/well. Cells were restimulated, or not, with Ova $_{257-264}$ peptide (1μM) or $Ova_{323-339}$ (1 μg/ml) and incubated for 24 h. Following washes, biotinylated α-mouse IFN-γ (1:500, BD Biosciences) was added and incubated overnight at 4 °C. Streptavidin–alkaline phosphatase conjugate (1:500, Mabtech AB, Sweden) was added, and the plates were incubated at room temperature for 1 h. Plates were developed using BCIP/NBT substrate (Zymed Laboratories, CA).

The in vivo CTL assay was performed by immunizing them with [FabxOva] (2.5 μg) with or without α-CD40 (50 μg). Seven days later, a 1:1 mixture of OVA $_{257-264}$ peptide loaded (1 μM) and unpulsed syngeneic splenocytes (~$1x10^7$cells each) labelled with 5 μM ($CFSE^{hi}$) and 0.5 μM ($CFSE^{lo}$) carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probe), respectively, was injected i.v. After

18 h, the splenocyte single cell suspensions were evaluated by flow cytometry. Specific killing was measured by the reduction of the $CFSE^{hi}$ population in relation to the $CFSE^{lo}$ population (values in profiles refer to the ratio between populations).

2.7.7 In vivo proliferation of CFSE labelled T cells

C57BL/6 mice were immunized (i.v) with [FabxOva] (2.5μg) and α-CD40 mAb (50μg), or with PBS, for 1, 4, 7 or 15 days prior to the adoptive transfer of OT-I ($4x10^6$cells) or OT-II T cells ($1.5x10^6$cells) labelled with CFSE (5 μM, Molecular Probes). Spleens were removed 3 days later and cell suspensions analysed by flow cytometry. Cells were gated on Ova-specific CD8 and CD4 T cells.

2.8 Chapter 4: Humoral response to immunization with [FabxOva] conjugates

2.8.1 Enzyme-Linked Immunsorbant Assay (ELISA) for the detection of serum α-Ova Ab

C57BL/6 mice were immunized i.v. or s.c. with [FabxOva] conjugates (2.5 μg) in the presence or absence of α-CD40 mAb (50 μg) (see figure legend) on D0 and serum samples were obtained from tail bleeds on D7 and D28. To detect the presence of α-Ova IgM and IgG in the serum of the immunized mice, an ELISA was performed according to the following standard protocol. Primary rabbit α-chicken Ova mAb was diluted in coating buffer (15 mM Na_2C0_3, 28.5 mM $NaHC0_3$, pH 9.6) to 25 μg/ml and 100 μl/well added to 96-well ELISA plates (Nunc Immunoplate; Invintrogen, U.K.) and incubated overnight at 4 °C. Unbound antibody was removed and the non-specific binding sites were blocked by the addition of blocking solution (1 % (w/v) BSA in PBS) with Ova (5 μg/ml) (Albumin from chicken egg white, Sigma, Grade VI) for 1 h at 37 °C. The plates were washed twice with wash solution (PBS + 0.05 % Tween-20) and dilutions of the test sera and standards of known concentrations were added in a final volume of 100 μl/well, with all dilutions being made in blocking solution.

Following incubation for 90 min at 37 °C, the plates were washed (5x in wash solution). Horse-radish peroxidase (HRP)-conjugated rabbit α-mouse IgG antisera was diluted 1:2,000 in blocking solution and added (100 µl/well) for a final 90 min. Following washing (5x), 100 µl of HRP substrate (20 mg o-phenyldiamine free base (o-PD; Sigma) in 100 ml phospho-buffered citrate pH 5.0 + 50 µl of 30 % (w/v) H_2O_2) was added and incubated in the dark at room temperature for 30 min. The reaction was terminated by the addition of 50 µl/well 2.5 M H_2SO_4 and the subsequent colour change quantified by measurement of absorbance at 495 nm on an automatic ELISA reader (Dynatec 400, Dynatec). The serum titre was defined as the highest dilution of serum that gives an OD490 value of double that of serum from naïve mice (NMS).

For the depletion of CD4T cells, mice were pre-treated with three injections of α-CD4 mAb (1mg) i.p. on days -4,-1 and 3. On day 0, the depletion of CD4 T cells was analysed by flow cytometry and mice were immunized with [α-CD11cxOva] (2.5 µg) with or without α-CD40 mAb (50 or 500 µg).

2.8.2 The influence of TLR-L on the mAb production by [α-DEC205xOva].

Mice received $2x10^6$ OT-I T cells i.v. and 24 h later were immunized with [FabxOva] conjugates (2.5 µg) and the different TLR-L (20 µg) i.v.. Blood samples were collected and the number of SIINFEKL-tetramer and CD8 positive T cells measured by flow cytometry. On D7, serum samples were also collected and levels of α-Ova IgG assessed by ELISA. On D33, all animals were boosted with [α-CD11cxOva] (2.5 µg) and serum samples assayed 7 days later.

2.9 Chapter 5: Biodistribution of [FabxOva] conjugates within the spleen

2.9.1 Biodistribution of radiolabelled mAb

$F(ab')_2$ fragments of a panel of mAb were radiolabelled with Iodine-125 (for protein iodination, carrier-free and free from reducing agent; Amersham Biosciences/GE Healthcare, UK) using Iodobeads (Pierce Chemicals, Rockford, IL) according to the manufacturer's instructions and dialysed into PBS to remove unbound iodine. Samples of each labelled mAb were counted using a γ-counter (Wallac) to determine the specific activity of the mAb being administered to the animals.

For the biodistribution studies, groups of C57BL/6 mice were injected (i.v.) with varying concentrations of radiolabelled mAb (refer to legends) and sacrificed 6 or 24 h later. Selected tissues were sampled, weighed and counted. The data is presented as the mean percentage uptake of the injected dose per gram of tissue (% ID/g). The effect of FLT3-L and LPS on the biodistribution of the mAb was also assessed, by pre-treating the mice with FLT3-L (s.c.) (10 μg) daily for 10 days prior to injection with the radiolabelled mAb.

2.9.2 Localization of labelled mAb within the spleen by immuno-histochemistry and immuno-fluorescence

Mice were injected i.v. with 10 μg of FITC-conjugated α-CD11c, α-DEC205 or α-MHCII $F(ab')_2$ mAb in combination with unlabelled α-CD40 mAb (50 μg). After 1, 4 or 8 h, the mice were perfused (by Jessica Teeling) with saline solution containing heparin. The fresh spleens were embedded in Tissue-Tek ® Optimal Cutting Temperature (O.C.T.) compound (R.A. Lamb Ltd, UK) and snap frozen in isopentane placed on dry ice. Frozen spleen sections were cut using a Microm HM560 cryostat (8 μm thick; at ~ -15 °C) and dried for 30 min at 37 °C and fixed in 100 % acetone for 10 min at 4 °C, subsequently, sections were washed in PBS (3x) and stained with various labelled mAb (refer to legends). All spleen sections were cut, labelled and analysed by Sonya Martin.

- Immuno-histochemistry

  Fixed spleen sections were treated with ImmunoPuro peroxidase suppressor (as per manufacturer's instructions; Pierce). Non-specific binding sites were blocked in PBS containing 10 % normal goat serum (pH 7.6 for 30 min).

  Sections were stained with the following primary mAb for 2 h at RT or overnight at 4 °C: biotinylated rabbit α-FITC (Molecular Probes), rat α-mouse CD19 (1D3, in house), hamster α-mouse CD11c (N418, in house), hamster α-mouse CD3 (1452C11, in house), rat α-mouse CD3 (KT3, in house), rat α-mouse MHCII (M5114, in house), rat α-mouse DEC205 (in house), biotinylated rat α-mouse MOMA-1 (Insight Biotech). Biotinylated secondary mAb: goat α-rat (Stratech) and goat α-hamster (Serotec). Biotinylated mAb were detected with StrepABCComplex/HRP (as per manufacturer's instructions; DakoCytomation) followed by the substrate, diaminobenzidine (DAB) (DakoCytomation). Secondary and tertiary incubations were for 45 min at room temperature. Sections were washed in water and counterstained with haematoxylin before mounting in aqueous mountant (Vector Laboratories).

- Immuno-fluorescence

  Fixed spleen sections were treated with PBS containing 10 % normal goat serum (30 min) to block non-specific binding sites. All mAb dilutions were prepared in PBS containing 0.1 % Tween20. Sections were stained with primary unlabelled mAb, as mentioned above, with the addition of Alexa Fluor 488 rabbit α-FITC (Molecular Probes). Fluorescent secondary and tertiary mAb included Alexa Fluor 488 goat α-rat; Alexa Fluor 488 goat α-hamster, Alexa Fluor 546 streptavidin (all from Molecular probes) and were incubated for 45 min at room temperature. Sections were washed in PBS between labelling stages and counterstained with TOPRO-3 for 30 min (Molecular Probes) and mounted in Vectashield (Vector Laboratories).

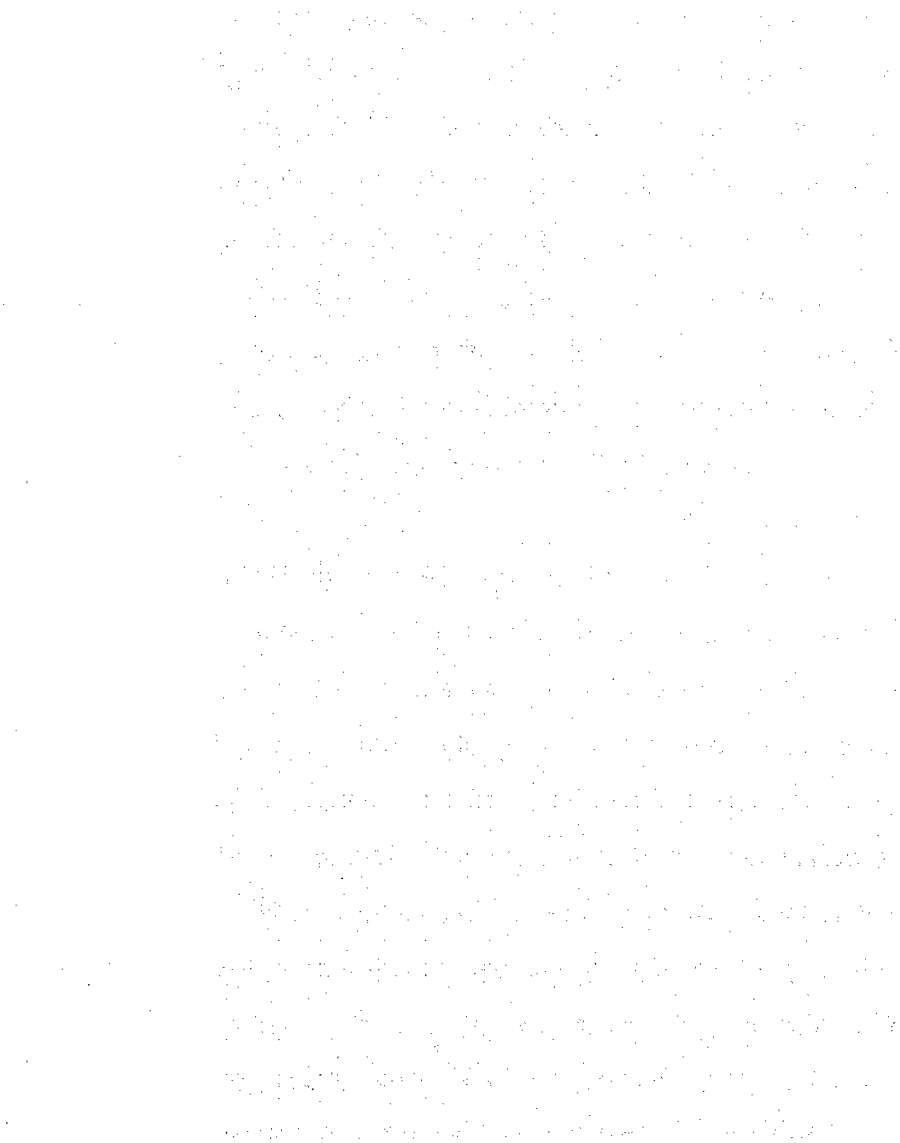
Microscopy: images were collected sequentially on a Leica SP2 CLSM using Argon (488 nm), GreNe (543 nm) and HeNe (633 nm) lasers.

2.10 Chapter 6: Immunotherapy

2.10.1 Prevention of tumour development by immunization with [FabxOva] conjugates.

C57BL/6 mice were immunized with [FabxOva] (2.5 μg) or soluble Ova (0.5 μg or 1 mg) and α-CD40 mAb (50 μg) i.v. 30 days prior to s.c. challenge with Ova-bearing B16 melanoma cells transfected with Ova (B16-Ova) ($4x10^6$) and tumour development was measured over time. Therapy was assessed by monitoring total tumour area and the survival rate of the animals. Some animals had to be culled before reaching the maximum tumour load (above 300 mm^2) due to development of ulceration on the tumour, as required by the Home Office regulations. Seven days after tumour challenge, blood samples were collected and the number of circulating endogenous Ova-specific CD8 T cells was analyzed by flow cytometry as described in section 2.7.5. Natural killer cells were labelled with α-NK1.1 mAb.

Chapter 3 Dendritic cell phenotyping and T cell immunity



3.1 Introduction

Despite its complexity, the adaptive immune system depends on APC to initiate and modulate an acquired response. DC have the unique ability to prime naïve T cells leading to the establishment of immunological memory. These cells are a sparsely distributed, migratory, group of bone-marrow derived leucocytes that are specialized in the uptake, transport, processing and presentation of Ag to naïve T cells. At an "immature" stage of development, DC act as sentinels in peripheral tissues monitoring their environment for the presence of foreign Ag or damaged tissue. They can capture Ag via different mechanisms, such as phagocytosis, micropinocytosis and receptor mediated endocytosis. The latter being mediated by a variety of surface receptors involved in endocytosis of Ag, in particular members of the C-type lectin receptors (macrophage mannose receptor (CD206), DEC205 (CD205) as well as Fc-receptors). Detection and ingestion by organ-resident DC of PAMPs provide a signal for maturation and migration towards the draining secondary lymphoid organs (i.e. LN and spleen), where they are able to present the products of the Ag digestion on MHC molecules to naïve T cells, thereby inducing a cellular immune response that involves $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells.

Immature DC efficiently capture Ag and present Ag derived peptides in the context of MHC molecules. CTL recognize Ag in the context of MHC class I molecules and two general mechanisms have been reported to contribute to how DC process such Ag into this pathway for the activation of naïve T cells. The original hypothesis suggested that only endogenous Ag, that is, Ag synthesized within the cells either by viral infection or tumour transformation, were routinely processed into the MHC I pathway. The second, more recent approach is for the DC to capture Ag from another source and process these captured exogenous Ag into the MHC I pathway, in the same way it has been postulated for exogenous Ag being processed via the MHC II system presenting Ag to CD4 T helper cells. This form of presentation is referred to as "cross-presentation", contrasting with the "direct" or "classic" presentation for endogenously synthesized proteins.

The major APC type known for its capacity to cross present Ag is the DC population and of the three blood-derived DC subsets, the immunological function of the $CD8^+$DC subset has been more well documented [223]. The roles of $CD4^+$DC and $CD4^-CD8^-$DC are poorly understood, but there is some evidence that $CD4^-CD8^-$DC preferentially produced IFN-γ [224], while no dominant cytokine production has been attributed to the $CD4^+$DC population as yet. However, recent evidence suggests that the $CD8^-$DC subsets are involved in priming CD4 T cells to produce T_H2 cytokines, whereas $CD8^+$DC prime naïve CD4 T cells to produce T_H1 cytokines in a process that involves IL-12 [173, 175, 225], which leads to the generation CTL. Furthermore, this polarization into different T cell subsets also depends on the nature of the activation signals received by the DC, for example, LPS from different pathogens can induce distinct T cell repertoires [226] and CD40L-activated monocyte-derived DC have been shown to prime Th1 response whereas pDC activated with IL-3 and CD40L primed T_H2 responses [227].

Encouraged by the body of evidence showing the importance of DC in the induction of different T cell responses, we linked Ova, our model Ag, to Fab' fragments derived from IgG mAb targeting a range of surface receptors on DC that could be involved in Ag uptake and cross-presentation to CTL. This strategy enabled us to identify surface molecules which could be of therapeutic value when targeting a tumour Ag to DC in situ and consequently mounting a potent anti-tumour response.

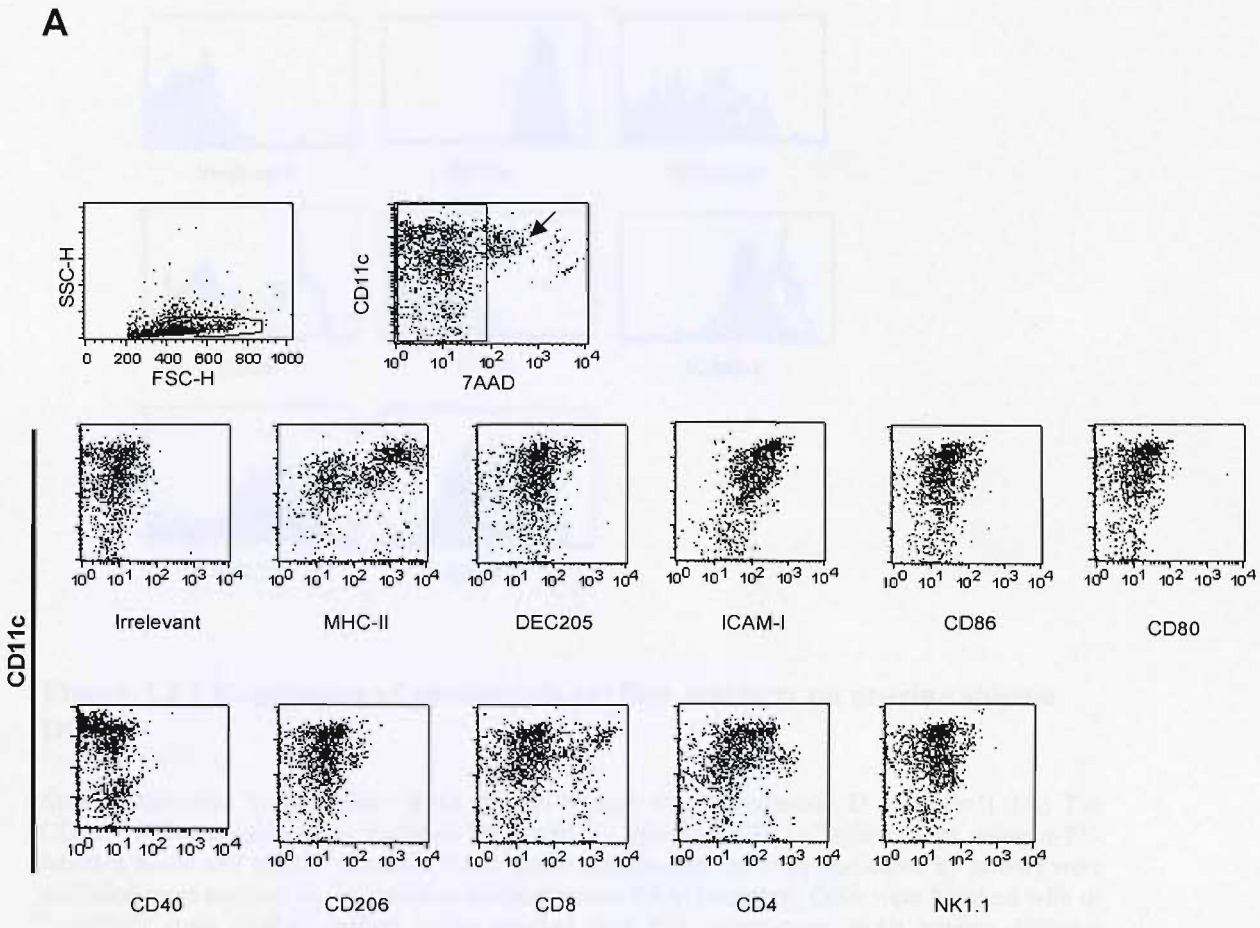
3.2 Results

3.2.1 Phenotypic characteristics of the distinct subsets of splenic DC

The differential expression of certain cell surface markers has already pointed to substantial DC heterogeneity and we wished to assess the surface phenotype of splenic DC, which were subsequently used in some of our functional assays. DC isolation may require many steps depending on the tissue of origin and since viability and function can be influenced by cytokines such as GM-CSF, IL-1, IL-4 and TNF [228], it is important to characterize fresh isolates.

Here, we identified fresh murine splenic DC using a PE-labelled mAb, N418, directed against CD11c. In figure 3.2.1 (A) DC were enriched by positive selection of PE-labelled $CD11c^+$ splenocytes using MACS α-PE beads and MACS columns, and analysed by flow cytometry for their expression of surface markers. Dead cells were stained using 7AAD and excluded from analysis. A small proportion of the splenocytes, probably macrophages [229] , were excluded on the bases of their autofluorescent properties (as indicated by the arrow). To prevent non-specific binding of mAbs via FcR on DC, these cells were first incubated with the α-FcγRII/III mAb, 2.4G2, prior to labelling the positive cells with FITC-conjugated mAbs against different surface markers. In this manner, we were able to identify the distinct subsets of murine splenic DC.

Immature splenic DC, defined as the population expressing high levels of CD11c (< 5 % of total cells), also expressed high levels of MHC II, together with moderate levels of ICAM-1 and low levels of CD80, CD86 and CD40. Furthermore, $CD11c^{Hi}$ cells could be subdivided according to the presence of the T cell markers, CD4 and CD8 (Figure 3.2.1(B)). CD8 expression on DC is in the form of a αα-homodimer, rather than the αβ-heterodimer found on T cells. Our profiles clearly show the existence of two distinct subsets of splenic $CD11c^{Hi}$ DC: $CD11c^{Hi}$ $CD8^+CD4^-$, $CD11c^{Hi}$ $CD8^-CD4^+$, which have been reported to account for 25 % and 50 % of the DC population, respectively, with the remaining 25 % being the double negative $CD11c^{Hi}$ $CD8^-CD4^-$ population [230].


A
SSC-H
FSC-H
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CD11c
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DEC205
ICAM-I
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CD206
CD8
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NK1.1
B
CD11c
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CD8-
CD8+
DEC205

C

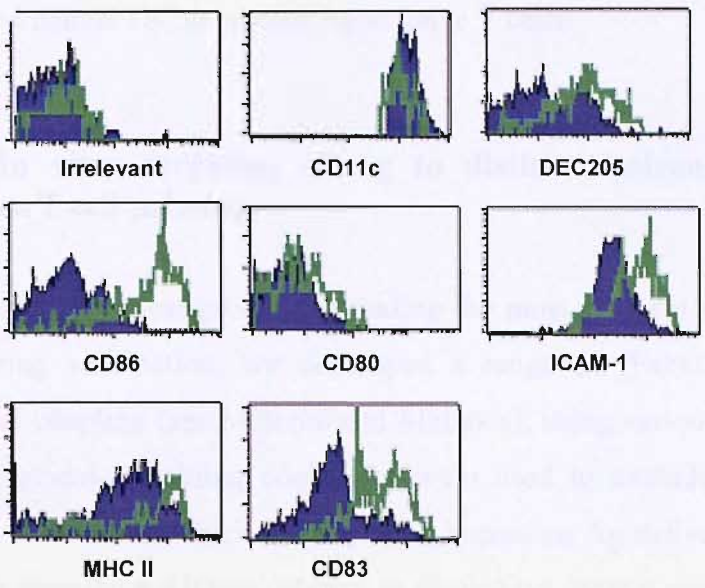


Figure 3.2.1 Expression of phenotypic surface markers on murine splenic DC

Splenocytes were isolated from fresh spleens treated with collagenase D (1 mg/ml). (A) The $CD11c^+$ DC population was enriched by positively selecting $CD11c^+$ splenocytes using α-PE-labelled beads and MACS columns. Dead and/or autofluorescent cells (indicated by arrow) were excluded from analysis by light scatter properties and 7AAD staining. Cells were blocked with α-FcγRII/III mAb (2.4G2) before being labelled with FITC-conjugated mAb against different markers. (B) Splenic DC were prepared as in (A), double labelled with α-CD8 and α-DEC205 mAb. (C) Fresh splenocytes were cultured in the presence of LPS (1 μg/ml), IL-4 and GM-CSF (10 ng/ml) for 24h and matured DC isolated as in (A). PE-labelled $CD11c^+$ matured DC were stained with a panel of FITC-conjugated mAbs. Dead cells were excluded from analysis by light scatter properties and 7AAD staining, and profiles gated on the $CD11c^{hi}$ cells. Filled purple histograms correspond to immature DC and green histograms to mature DC.

To dissect the phenotypic differences between these populations further, three-colour analysis was used and Figure 3.2.1 (B) demonstrates that the expression of DEC205 is restricted to the $CD11c^{Hi}CD8^+$ subset as there was no expression above background levels in the $CD11c^{Hi}CD8^-$ population. To investigate the phenotypic changes associated with DC maturation, mouse splenocytes were cultured in vitro for 24 hours with LPS, IL-4 and GM-CSF. LPS induces the maturation and activation of DC by signalling via TLR4 expressed on these cells. The results in Figure 3.2.1 (C), show that this led to an increase in the surface expression of MHC II molecules, CD80 and most dramatically, CD86, ICAM and CD83 (a DC maturation marker). Maturation also induced a modest increase in DEC205 expression; however, CD11c expression remained mostly unchanged.

In summary, all these features are consistent with the increased ability of activated mature DC to present Ag to naïve T cells.

3.2.2 In vitro targeting of Ag to distinct molecules on APC can enhance T cell priming.

As part of our investigation into finding the most efficient strategy of targeting Ag during vaccination, we developed a range of [FabxOva] conjugates by chemical coupling (see Material and Methods), using various mAb specificities. Fab' fragment-containing conjugates were used to exclude any possibility of Fc:FcR interactions which are known to impact on Ag delivery [231]. The Fab' fraction from the mAb was coupled to whole Ova protein using a chemical linker and the conjugate was subsequently purified on a protein G column. Free unconjugated mAb was removed by passing the conjugate through an anti-Fcγ Immunosorbant column. The Ova component of the conjugates represented approximately 20-30% by weight when measured by ELISA (data not shown). To ensure that our preparations were endotoxin low, all the conjugates were passed through a polymixin column.

To investigate the efficiency with which our panel of conjugates delivered Ova protein to APC and induced its processing and presentation to T cells, we first assessed the proliferation of OVA-restricted TcR transgenic CD8 OT-I and CD4 OT-II T cells in vitro in a coculture system, where freshly isolated and irradiated splenocytes were loaded with the [FabxOva] conjugates together with α-CD40 mAb and OT-I or OT-II T cells.

In these in vitro coculture assays, splenocytes from naïve mice were irradiated and used as the APC population ($2x10^5$ cells per well), of which less than 4 % are thought to be conventional blood-derived DC. Total splenocyte cell suspensions from OT-I or OT-II mice were used as the effector population ($2x10^5$ per well), with 15-20 % shown by flow cytometry (data not shown) to be Ova-restricted CD8 OT-I or CD4 OT-II T cells. A ratio of approximately 1:10 DC to Ova-restricted T cells was used.

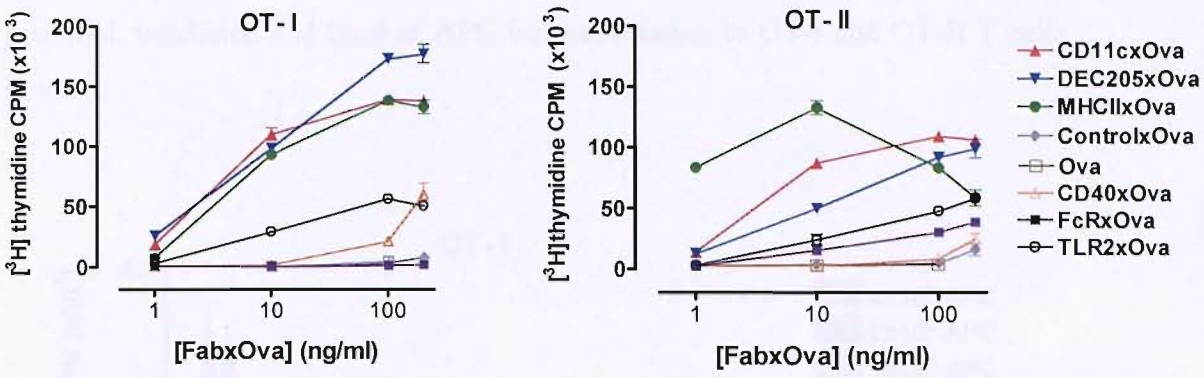


Figure 3.2.2 In vitro targeting of Ova to splenic DC with [FabxOva] conjugates and their effects on the proliferation of T cells.

Spleens were harvested from C57BL/6 mice and treated with collagenase D (1mg/ml). Splenocytes were used as APC ($2x10^5$) and cocultured with [FabxOva] conjugates (1-200 ng/ml) in the presence of α-CD40 mAb (1 μg/ml) and OT-I or OT-II effector cells ($2x10^5$) for 48 h. As positive controls, APC were cocultured with OT-I or OT-II and their respective peptides, H-$2K^b$-restricted $OVA_{257-264}$ (OT-I) peptide (1 μM) or I-A^b-restricted peptide $OVA_{323-339}$ (OT-II) peptide (0.01-0.00001nM) (data not shown). Cultures were pulsed with [^{3}H]-thymidine (0.5 μCi/well) for 16-18hr. Data representative of three individual experiments, MEAN±SEM of triplicates.

Figure 3.2.2 demonstrates that when targeting splenic APC in vitro, the conjugates specific for CD11c, MHC-II and DEC205 were by far the best targets which were equally effective in inducing the proliferation of OT-I T cells. With OT-II T cells, targeting to CD11c and DEC205 showed similar proliferative activity and the [α-MHCIIxOva] conjugate was the most potent, giving effective proliferation at a 10-fold lower concentration. The sharp decline in OT-II T cell proliferation seen at the highest concentration of [α-MHCIIxOva] was probably a result of exhaustion of nutrients in the culture medium. Thus, overall, the data from this in vitro presentation assay with fresh splenocytes as APC, shows that DEC205, CD11c and MHC-II molecules are the most effective targets for Ag delivery.

3.2.3 In vivo targeting of Ova to APC and the induction of T cell proliferation in vitro.

We next investigated the effectiveness by which the conjugates deliver Ag to splenic APC in vivo. Mice were primed i.v. with 2.5 μg of conjugate together

with 50 μg of agonistic α-CD40 mAb, then 24 hours later splenocytes were prepared, irradiated and used as APC for presentation to OT-I and OT-II T cells in vitro.

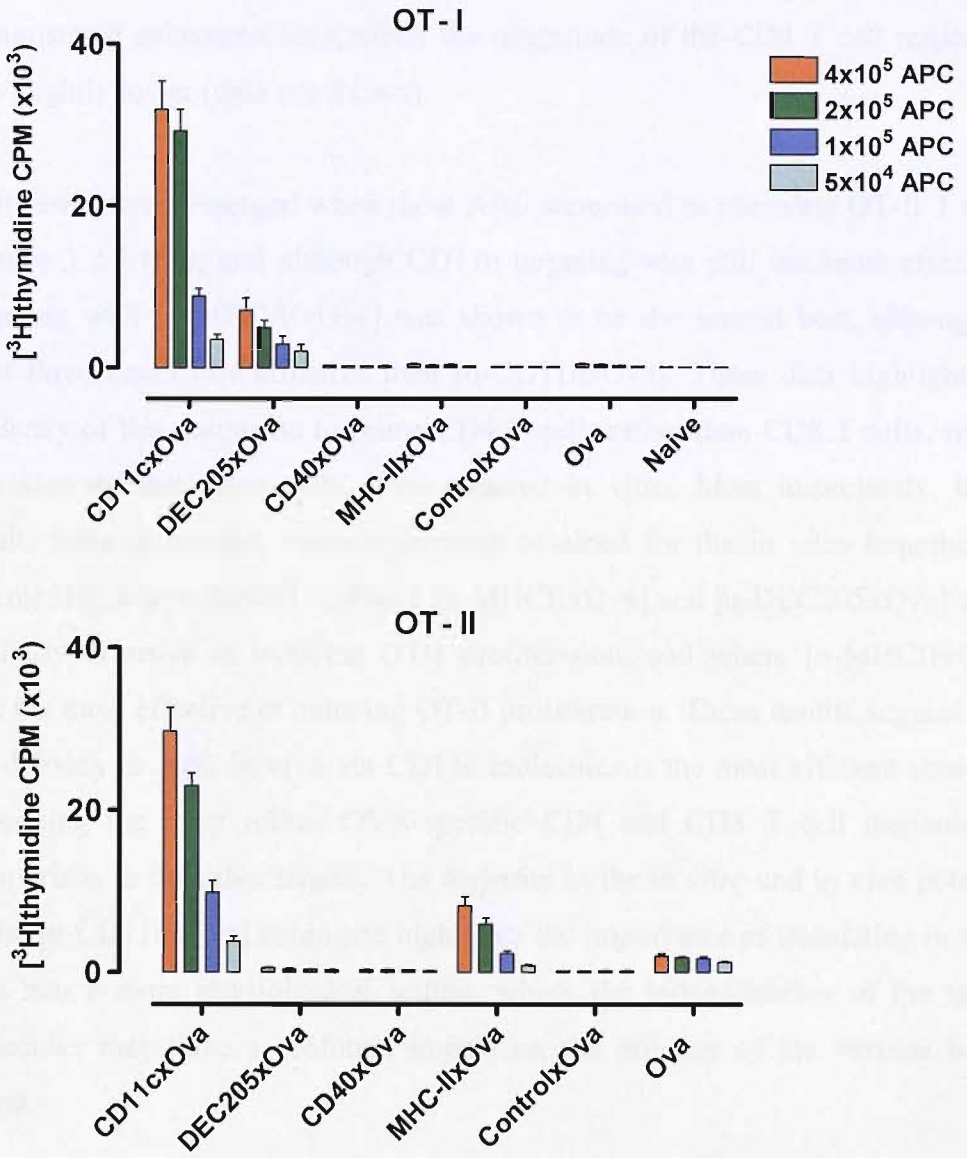


Figure 3.2.3 *In vivo* targeting of OVA to APC and the proliferation of T cells.

C57BL/6 mice were immunized with [FabxOva] conjugates (2.5 μg) and α-CD40 mAb (50 μg). Splenocytes were isolated 24h after immunization and cocultured with OT-I or OT-II splenocytes ($2x10^5$cells/well) for 48h. Cultures were pulsed with ^{3}H-thymidine (0.5 μCi/well) for 16-18h. The mean of each group and the SEM are shown. Data representative of 3 individual experiments.

Pilot experiments determined that 24 hours was the optimal length of time required for the capture and processing of Ag by APC in vivo (data not shown).

Figure 3.2.3 (A) shows that splenic APC from mice receiving [α-CD11cxOva] were by far the most effective at inducing both OT-I and OT-II proliferation. With OT-I cells, targeting with [α-CD11cxOva] was at least four times better than with [α-DEC205xOva], whereas [α-MHCIIxOva] and [α-CD40xOva] were ineffective. A similar pattern was seen when the [FabxOva] conjugates were administered subcutaneously, albeit the magnitude of the CD8 T cell responses was slightly lower (data not shown).

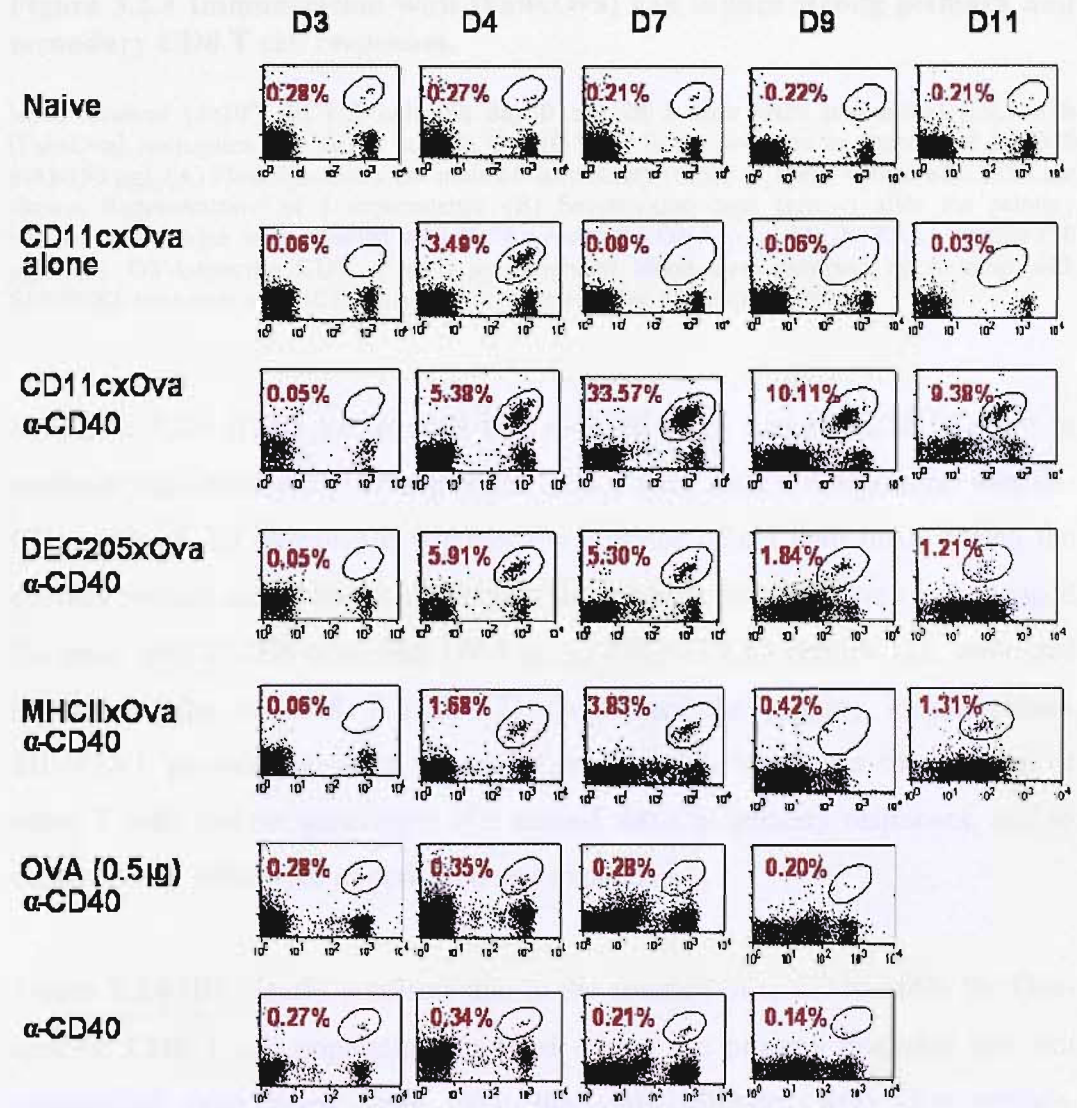
A different trend emerged when these APC were used to stimulate OT-II T cells (Figure 3.2.3 (B)), and although CD11c targeting was still the most effective, targeting with [α-MHC-IIxOva] was shown to be the second best, although at least three times less effective than [α-CD11cxOva]. These data highlight the tendency of this conjugate to prime CD4 T cells rather than CD8 T cells, which was also evident when APC were targeted in vitro. Most importantly, these results were in marked contrast to those obtained for the in vitro targeting of splenic DC, where [α-CD11cxOva], [α-MHCIIxOva] and [α-DEC205xOva] were similarly effective at inducing OT-I proliferation, and where [α-MHCIIxOva] was the most effective at inducing OT-II proliferation. These results suggest that Ag delivery to APC in vivo via CD11c molecules is the most efficient strategy, generating the most robust OVA-specific CD4 and CD8 T cell response in comparison to the other targets. The disparity in the in vitro and in vivo potency of the [α-CD11cxOva] conjugate highlights the importance of translating in vitro data into a more physiological setting, where the bioavailability of the target molecules may have a profound impact on the efficacy of the vaccine being tested.

3.2.4 Immunization with [α-CD11cxOva] generates strong primary and memory CD8 T cells responses

In support of the findings from our in vitro proliferation assays, we next confirmed that immunization with [α-CD11cxOva] generates the strongest CD8 T cell response in vivo. To assess the proliferation of T cells in vivo, we adoptively transferred OT-I or OT-II T cells into naïve mice and 24 hours later

immunized these mice with the various conjugates with or without α-CD40 mAb. The results in Figure 3.2.4 (A/B) show that a single low dose of 2.5 μg of [α-CD11cxOva] is highly effective at stimulating the proliferation of CD8 OT-I T cells, generating 3.5 % tetramer positive cells by day 4. However, only when α-CD40 mAb was given concomitantly did these cells continue to divide and accumulate to give more than 30 % circulating OT-I T cells by day 7. In contrast, in the absence of α-CD40 mAb, the OT-I T cells were practically absent from the blood compartment as their numbers fell below those found in naïve mice by day 7 and were not replenished by the end of the primary response.

A



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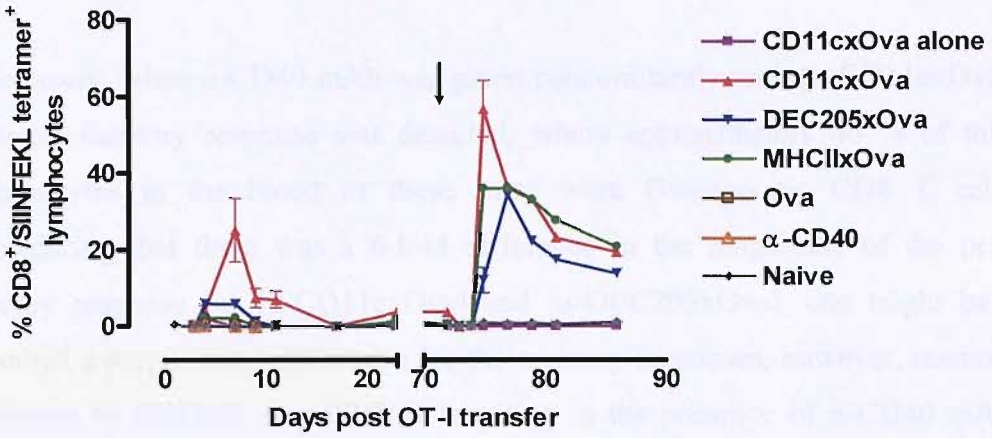


Figure 3.2.4 Immunization with [FabxOva] can induce strong primary and secondary CD8 T cell responses.

Mice received ($4x10^6$) OT-I T cells on day 0 and 24 h later were immunized (i.v.) with [FabxOva] conjugates (2.5 μg) or soluble Ova (0.5 μg) in the presence or absence of α-CD40 mAb (50 μg). (A) Flow cytometry dot plots of the primary response. Mean values with SEM are shown. Representative of 3 experiments. (B) Seventy-one days (arrow) after the primary immunization, mice were boosted with H-2Kb-restricted OVA $_{257-264}$ (SIINFEKL) peptide (30 μg) (i.v). OVA-specific $CD8^+$ T cells in peripheral blood were detected by staining with SIINFEKL tetramers and α-CD8 mAb. Data representative of 2 experiments.

In Figure 3.2.4 (A/B) we showed that α-CD40 mAb was required in order to maintain high numbers of Ova-specific CD8 T cells after immunization with [α-CD11cxOva]. To investigate whether the absence of α-CD40 mAb during the primary immunization had any effect on the memory response, we re-challenged the mice with H-2Kb-restricted OVA $_{257-264}$ (SIINFEKL) peptide (i.e. restricted to the priming of CD8 T cells) 71 days after the primary immunization. SIINFEKL peptide was used instead of soluble Ova to prevent the priming of naïve T cells and the generation of a second wave of primary responses, and so only memory cells were re-activated and expanded.

Figure 3.2.4 (B), clearly confirms that in the absence of α-CD40 mAb, the Ova-specific CD8 T cell population depleted during the primary response was not replenished even when these mice were re-challenged with Ova peptide, suggesting that these cells were deleted and the system tolerized against Ova. The tolerization of the immune system to Ag as a consequence of clonal deletion

has been previously reported on animals which were immunized with [α-DEC205xOva] without the maturation signals provided by α-CD40 mAb [69].

Conversely, when α-CD40 mAb was given concomitantly with [α-CD11cxOva], a strong memory response was detected, where approximately 60 % of total lymphocytes in the blood of these mice were Ova-specific CD8 T cells. Considering that there was a 6-fold difference in the magnitude of the peak primary response for [α-CD11cxOva] and [α-DEC205xOva], one might have expected a proportional difference for the memory responses, however, memory responses to DEC205 and MHC-II targeting in the presence of α-CD40 mAb, almost reached that with [α-CD11cxOva], possibly because it induced such a rapid and potent proliferative response that the splenic compartment may have reached it physical capacity.

Together these findings clearly emphasize that the most efficacious target for a vaccine cannot be predicted based solely on in vitro studies, as the bioavalability of the targets can have a profound effect on the overall physiological response as illustrated by the strong primary response induced by [α-CD11cxOva] in vivo, despite having not shown any outstanding potency when delivered in vitro. Furthermore, the administration of a maturation signal was shown to be crucial in mounting and sustaining of a robust primary and memory response, and avoiding tolerance induction.

3.2.5 Pre-treatment with FLT3-L amplifies T cell responses.

FLT3-L acts by targeting haematopoietic progenitors in the bone marrow, inducing their expansion and differentiation into DC and NK cells [232]. Here, mice were pre-treated with FLT3-L (10 μg) for 10 consecutive days and splenocytes isolated and analysed by flow cytometry. Figure 3.2.5(A), clearly illustrates the effect of FLT3-L on DC numbers, as there was an approximate 6-fold increase in $CD11c^+$ cell number, increasing from 3.2 % to 18.1 % of total splenocytes. However, the treatment has been observed not to interfere with the

developmental and maturation status of the DC (data not shown) as reported by other studies [233].

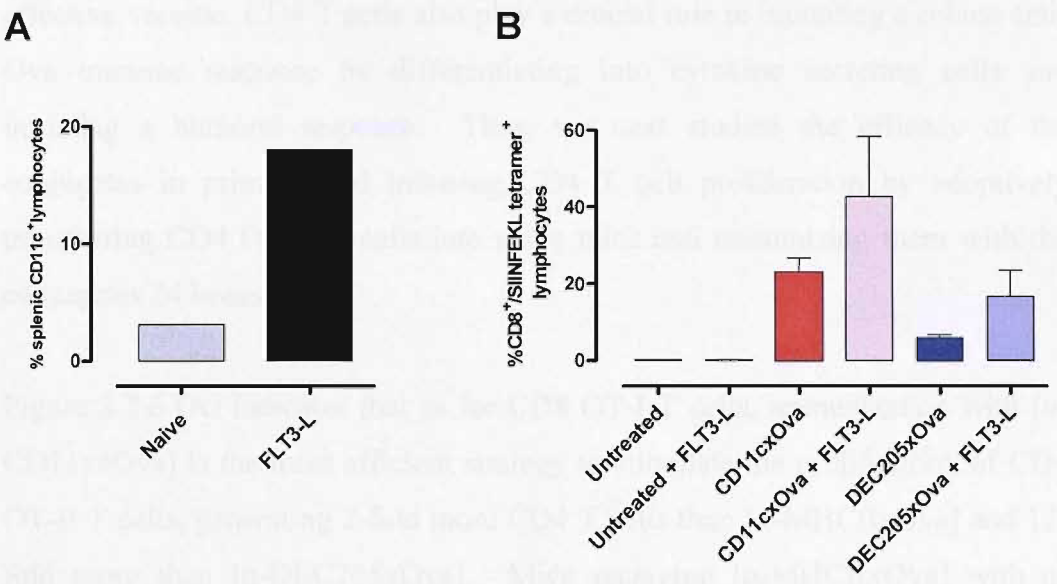


Figure 3.2.5 Pre-treatment with FLT3-L amplifies the T cell response to [FabxOva] conjugates.

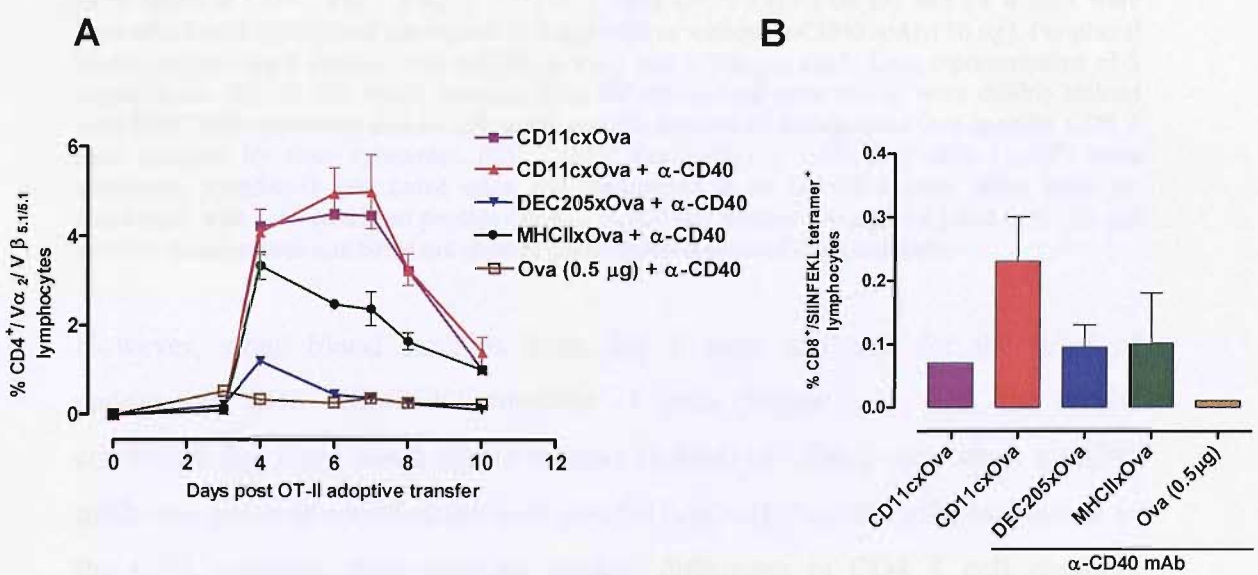
Mice were pre-treated with FLT3-L (10 µg, s.c.) for 10 consecutive days. (A) Naïve and FLT3-L treated spleens were treated with collagenase D (1 mg/ml) and the number of live $CD11c^+$ cells measured. (B) On D10, $CD8^+$ OT-I cells ($2.5x10^6$) were adoptively transferred and 24 h later mice were immunized with [FabxOva] (2.5 µg) with α-CD40 mAb (50 µg) (i.v.). Ova-specific $CD8^+$ T cells in blood samples taken 7 days later were detected by staining with SIINFEKL tetramers and α-CD8 mAb. Mean values and SEM are shown.

Figure 3.2.5 (B) demonstrates that the peak response generated by [α-CD11cxOva] with α-CD40 mAb can be doubled by pre-treating the mice with FLT3-L, when at least 50 % of the circulating lymphocytes on day 7 after immunization were Ova-restricted CD8 T cells. The amplification of the response to [α-DEC205xOva] was slightly higher (~ 2.5-fold). Again, the increase in T cell response to [α-CD11cxOva] was not proportional to the 6-fold increase in DC numbers, which is probably due to the limited physical capacity of the splenic compartment. In addition, these data indicate that the priming of T cells is being performed mainly by DC, as an increase in their number led to a marked potentiation of these responses.

3.2.6 Immunization with [FabxOva] conjugates generate strong primary and memory CD4 T cell responses

The generation of a strong CTL response is not the only important aspect of an effective vaccine. CD4 T cells also play a crucial role in mounting a robust anti-Ova immune response by differentiating into cytokine secreting cells and initiating a humoral response. Thus, we next studied the efficacy of the conjugates in priming and inducing CD4 T cell proliferation by adoptively transferring CD4 OT-II T cells into naïve mice and immunizing them with the conjugates 24 hours later.

Figure 3.2.6 (A) indicates that as for CD8 OT-I T cells, immunization with [α-CD11cxOva] is the most efficient strategy to stimulate the proliferation of CD4 OT-II T cells, generating 2-fold more CD4 T cells than [α-MHCIIxOva] and 12-fold more than [α-DEC205xOva]. Mice receiving [α-MHCIIxOva] with α-CD40mAb, induced a stronger CD4 T cell proliferative response (6-fold higher) than [α-DEC205xOva] with α-CD40 mAb, reinforcing the previous in vitro data suggesting that [α-DEC205xOva] favours a CD8 T cell response and [α-MHCIIxOva] a CD4 T cell response. Interestingly, the addition of α-CD40 mAb had no effect on the proliferation of CD4 T cells induced by [α-CD11cxOva] (Figure 3.2.6 (A)).



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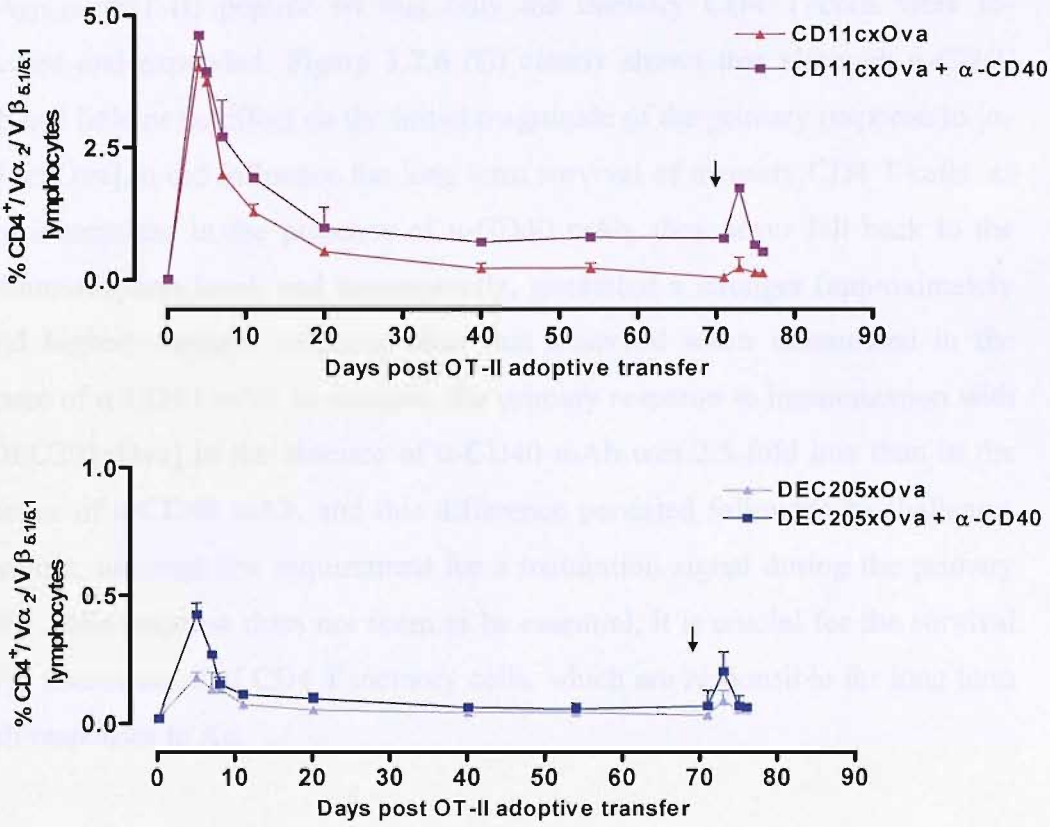


Figure 3.2.6 Primary and memory CD4 T cell responses following immunization with [FabxOva] conjugates.

Mice received $CD4^+$/ $V\alpha_2^+$/ $V\beta_{5.1,5.2}^+$ OT-II T cells ($3x10^6$) (i.v.) on D0 and 24 h later were immunized with [FabxOva] conjugates (2.5 µg) with or without α-CD40 mAb (50 µg). Peripheral blood samples were stained with α-CD4, α-$V\alpha_{2.1}$ and α-$V\beta_{5.1,5.2}$ mAb. Data representative of 3 experiments. (B) On D7, blood samples from the immunized mice in (A) were double stained with SIINFEKL-tetramers and α-CD8 mAb, and the number of endogenous Ova-specific CD8 T cells assessed by flow cytometer. (C) $CD4^+$/ $V\alpha_2^+$/ $V\beta_{5.1,5.2}^+$ OT-II T cells ($1x10^6$) were adoptively transferred into naïve mice and immunized as in (A) 24 h later. Mice were re-challenged with I-A^b-restricted peptide $OVA_{323-339}$ (OT-II) peptide (90 µg) (i.v.) and LPS (20 µg) on D70. Mean values and SEM are shown. Data representative of 2 experiments.

However, when blood samples from day 7 were analyzed for the level of endogenous $CD8^+$ SIINFEKL $tetramer^+$ T cells (Figure 3.2.6 (B)), the results confirmed that there was a higher number (3-fold) of CD8 T cells when α-CD40 mAb was given in combination with [α-CD11cxOva]. Additionally, in contrast to the CD4 response, there was no marked difference in CD8 T cell numbers induced by [α-DEC205xOva] and [α-MHCIIxOva].

Aiming to clarify the long term effect of the presence or absence of α-CD40 mAb on the CD4 T cell memory response, we ensured that the primary response had finished and then re-challenged the mice with I-A^b-restricted peptide $OVA_{323-339}$ (OT-II) peptide so that only the memory CD4 T cells were re-activated and expanded. Figure 3.2.6 (C) clearly shows that although α-CD40 mAb had little or no effect on the initial magnitude of the primary response to [α-CD11cxOva], it did influence the long term survival of memory CD4 T cells, as when immunized in the presence of α-CD40 mAb, they never fell back to the pre-immunization level, and consequently, generated a stronger (approximately 9-fold higher) memory response than that observed when immunized in the absence of α-CD40 mAb. In contrast, the primary response to immunization with [α-DEC205xOva] in the absence of α-CD40 mAb was 2.5-fold less than in the presence of α-CD40 mAb, and this difference persisted following re-challenge. Therefore, although the requirement for a maturation signal during the primary CD4 T cells response does not seem to be essential, it is crucial for the survival and/or maintenance of CD4 T memory cells, which are responsible for long term recall responses to Ag.

3.2.7 Endogenous T cell responses to [FabxOva] conjugates and the characterization of their effector functions.

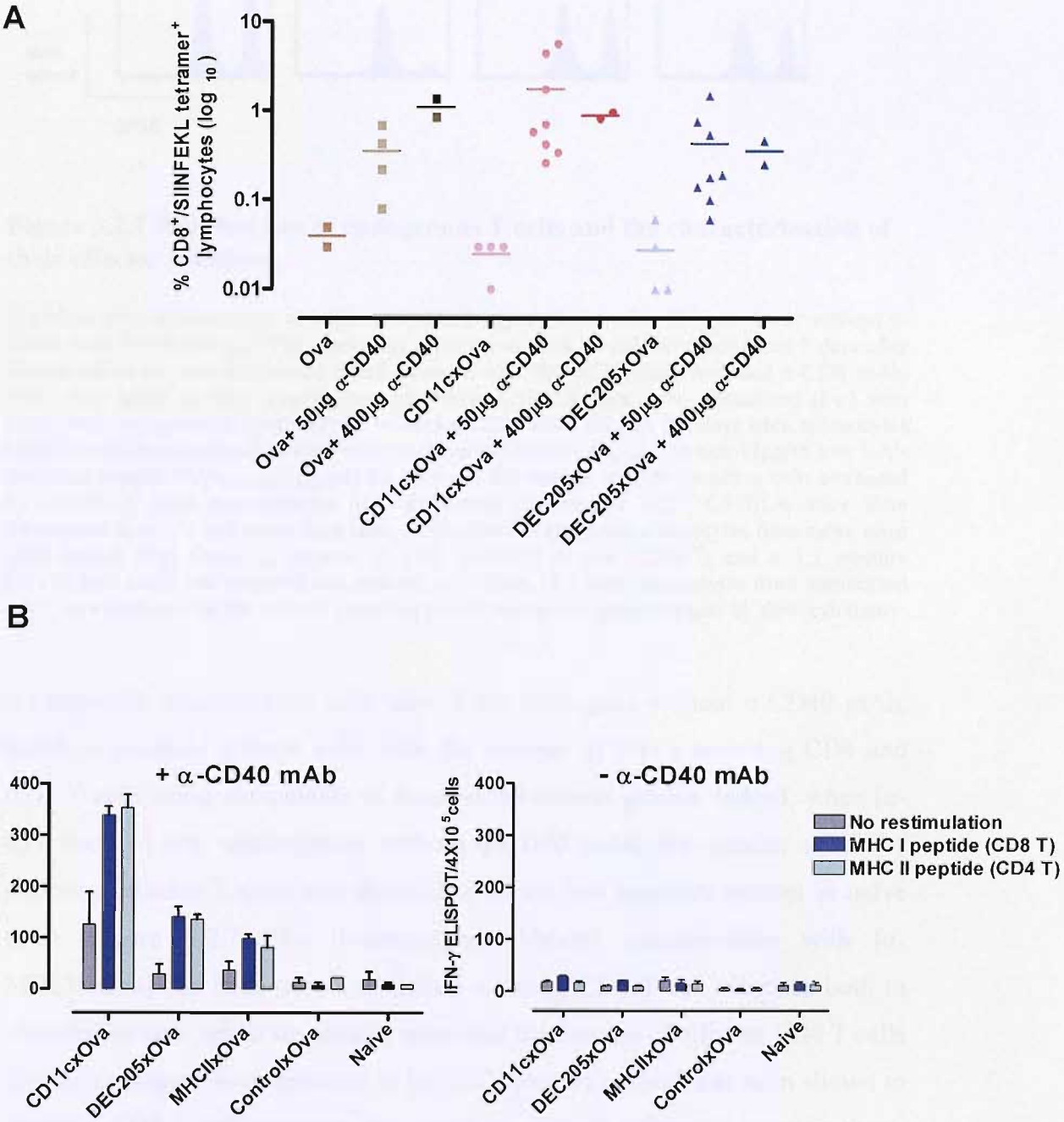
Aiming to assess the potency of [FabxOva] conjugates under more physiological conditions, we measured the number of endogenous Ova-specific CD8 T cells generated following immunization with the conjugates and α-CD40 mAb by flow cytometry. The data in Figure 3.2.7 (A) concur with the in vivo adoptive transfer experiments, as they also demonstrate that a maturation signal, again provided by α-CD40 mAb, is required for the effective priming and expansion of endogenous Ova-specific CD8 T cells. The co-administration of 50 μg of α-CD40 mAb with [α-CD11cxOva], [α-DEC205xOva] or soluble Ova enhanced the CD8 T cell response by approximately 88-, 21- and 9-fold, respectively, when compared to the administration of the conjugates or soluble Ova alone, based on the mean values of the distinct treatments .

Additionally, we noticed that the difference in the strength of the CD8 T cell responses to [α-CD11cxOva] and [α-DEC205xOva] with α-CD40 mAb (50 μg) observed in the endogenous model was not as marked as previously observed in the OT-I T cells adoptive transfer experiments and there was a higher degree of variability associated with these responses. This small discrepancy is probably a result of two factors, firstly, OT-I transgenic CD8 T cells are thought to be more sensitive to stimulation than their endogenous counterparts; secondly, the sensitivity limitations associated with the flow cytometric data for such low numbers of circulating endogenous CD8 T cells also has an impact in the variability of the response. Thus, we attempted to amplify the endogenous response by administering a higher dose of α-CD40 mAb with the conjugates (figure 3.2.7 (A)). However, the results show that 50 μg α-CD40 mAb, the amount routinely used in our in vivo experiments, was the optimal dose for both [α-CD11cxOva] and [α-DEC205xOva], as higher concentrations inhibited the response slightly.

Soluble Ova was also investigated to highlight the efficacy of targeting Ag directly to DC, and in this case, 2 mg of soluble Ova, which contains 4000 times more Ova than that present in 2.5 μg of the [FabxOva] conjugates, together with 400 μg of α-CD40 mAb was needed to generate a similar number of Ova-specific CD8 T cells obtained with 2.5 μg of [α-CD11cxOva] (~ 0.5 μg of Ova) and α-CD40 mAb (50 μg). Overall, immunization with [α-CD11cxOva] and α-CD40 mAb evokes the strongest proliferative response for both endogenous and OT-I CD8 T cells.

Furthermore, in support of the flow cytometric data on the endogenous CD8 T cells responses to the conjugates, we also measured the number of endogenous IFN-γ secreting CD8 and CD4 effector T cells using the more sensitive ELISPOT assay. In Figure 3.2.7 (B), results show mice immunized with a single dose of [FabxOva] (2.5μg) with or without α-CD40 mAb (50 μg). Six days later, spleens were removed and splenocytes re-stimulated in vitro with $H2K^b$-restricted (recognized by CD8 T cells) or $I\text{-}A^b$ restricted (recognized by CD4 T cells) Ova peptide for 24 hours. The results clearly demonstrate that immunization with [α-CD11cxOva] and α-CD40 mAb generated approximately

2.5-fold more endogenous Ova-specific IFN-γ secreting CD8 and CD4 effector T cells than [α-DEC205xOva], and 3-4 fold more than [α-MHCIIxOva]. The number of Ova-specific IFN-γ secreting CD4 and CD8 T cells was comparable for most treatments. In addition, immunization with [α-CD11cxOva] and α-CD40 mAb generated a higher number of total effector cells secreting IFN-γ without re-stimulation with peptide than did all the other treatments.



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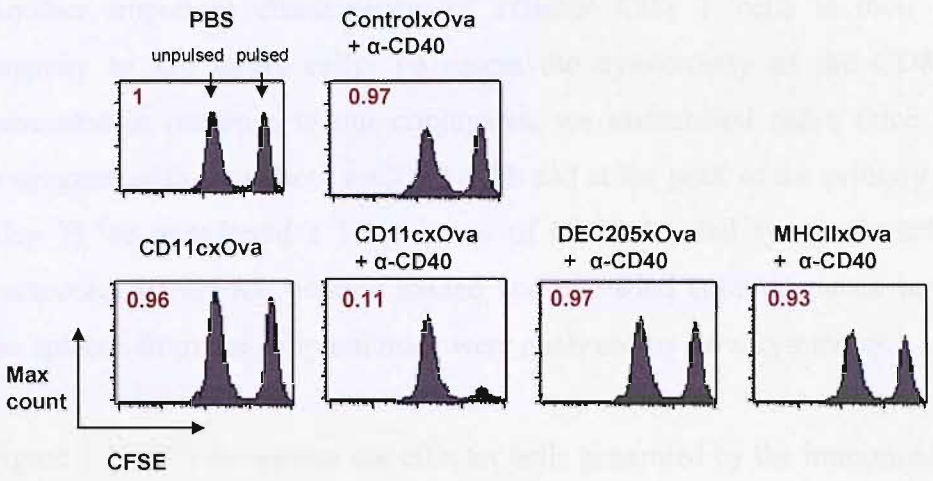


Figure 3.2.7 Proliferation of endogenous T cells and the characterization of their effector functions.

(A) Mice were injected (i.v.) with [FabxOva] (2.5 μg) or soluble Ova (2 mg) with or without α-CD40 mAb (50 or 400 μg). The number of endogenous $CD8^+$ T cells was monitored 7 days after immunization by double staining blood samples with SIINFEKL-tetramers and α-CD8 mAb. Cells were gated on live lymphocytes. (B) Naïve C57BL6 mice were immunized (i.v.) with [FabxOva] conjugates (2.5 μg) with or without α-CD40 mAb (50 μg). Six days later, splenocytes ($4x10^5$) were re-stimulated in vitro with H-$2K^b$-restricted $OVA_{257-264}$ peptide (1μg/m) or I-A^b-restricted peptide $OVA_{323-339}$ (1μg/ml) for 24 h and the number of IFN-γ positive cells evaluated by ELISPOT. Data representative of 2 individual experiments. (C)) C57BL/6 mice were immunized as in (A) and seven days later, CFSE-labelled syngeneic splenocytes from naïve mice were pulsed with $Ova_{257-264}$ peptide (1 μM) ($CFSE^{hi}$) or not ($CFSE^{lo}$) and a 1:1 mixture (~$1x10^7$cells each) was prepared and injected i.v.. Then, 18 h later, splenocytes from immunized mice were analyzed for the ratio of surviving pulsed versus non-pulsed targets by flow cytometry.

As expected, immunization with any of the conjugates without α-CD40 mAb, failed to generate effector cells with the number of IFN-γ secreting CD4 and CD8 T cells being comparable to those in the control groups. Indeed, when [α-CD11cxOva] was administered without α-CD40 mAb, the number of IFN-γ secreting effector T cells was diminished to the low numbers present in naïve mice (Figure 3.2.7 (B)). Interestingly, although immunization with [α-MHCIIxOva] has been shown to induce a strong CD4 T cell response both in vitro and in vivo, when we actually measured the number of effector CD4 T cells for this conjugate in comparison to [α-DEC205xOva], which has been shown to favour a CD8 T cell response, there was no marked difference between them. This suggests that although [α-MHCIIxOva] biases the expansion of CD4 T cell

thereby producing high numbers of Ova-specific CD4 T cells, these cells later fail to become effector cells.

Another important characteristic of effector CD8 T cells is their cytolytic capacity to kill target cells. To assess the cytotoxicity of the CD8 T cells generated in response to our conjugates, we immunized naïve mice with our conjugates with or without α-CD40 mAb and at the peak of the primary response (day 7) we transferred a 1:1 mixture of CFSE labeled syngeneic splenocytes composed SIINFEKL peptide loaded and unloaded cells. Eighteen hours later, the spleens from the recipient mice were analyzed by flow cytometry.

Figure 3.2.7 (C) shows that the effector cells generated by the immunization with [α-CD11cxOva] and α-CD40 mAb, possess Ag-specific cytolytic functions as most of the Ova peptide loaded target cells were eradicated within 18 hours. In contrast, in the absence of α-CD40 mAb, there was virtually no change in the number of target loaded cells in comparison to the unloaded cells. Moreover, although [α-DEC205xOva] has been shown to evoke a strong CD8 T cell response, based on these results, we propose that the number of effector cells generated failed to reach the threshold required to eliminate the target cells, as this treatment and [α-MHCIIxOva], were unable to kill the target cells to a noticeable level.

In summary, these data reinforces the fact that immunization with [α-CD11cxOva] and α-CD40 mAb, is the most effective vaccine as it generates the highest number of CD8 and CD4 T cells and these cells are Ag specific bona fide effector cells, capable of secreting IFN-γ and lysing target cells.

3.2.8 Ag targeted to DC can be presented to naïve T cells for long periods and favour distinct immune responses depending on the target molecule.

We next investigated for how long targeted DC are able to present Ag to naïve CD8 and CD4 T cells in vivo. CFSE-labelled OT-I or OT-II T cells were adoptively transferred 1, 4, 7 and 15 days after immunization with [FabxOva]

conjugates and α-CD40 mAb and their proliferation assessed by flow cytometry (Figure 3.2.8).

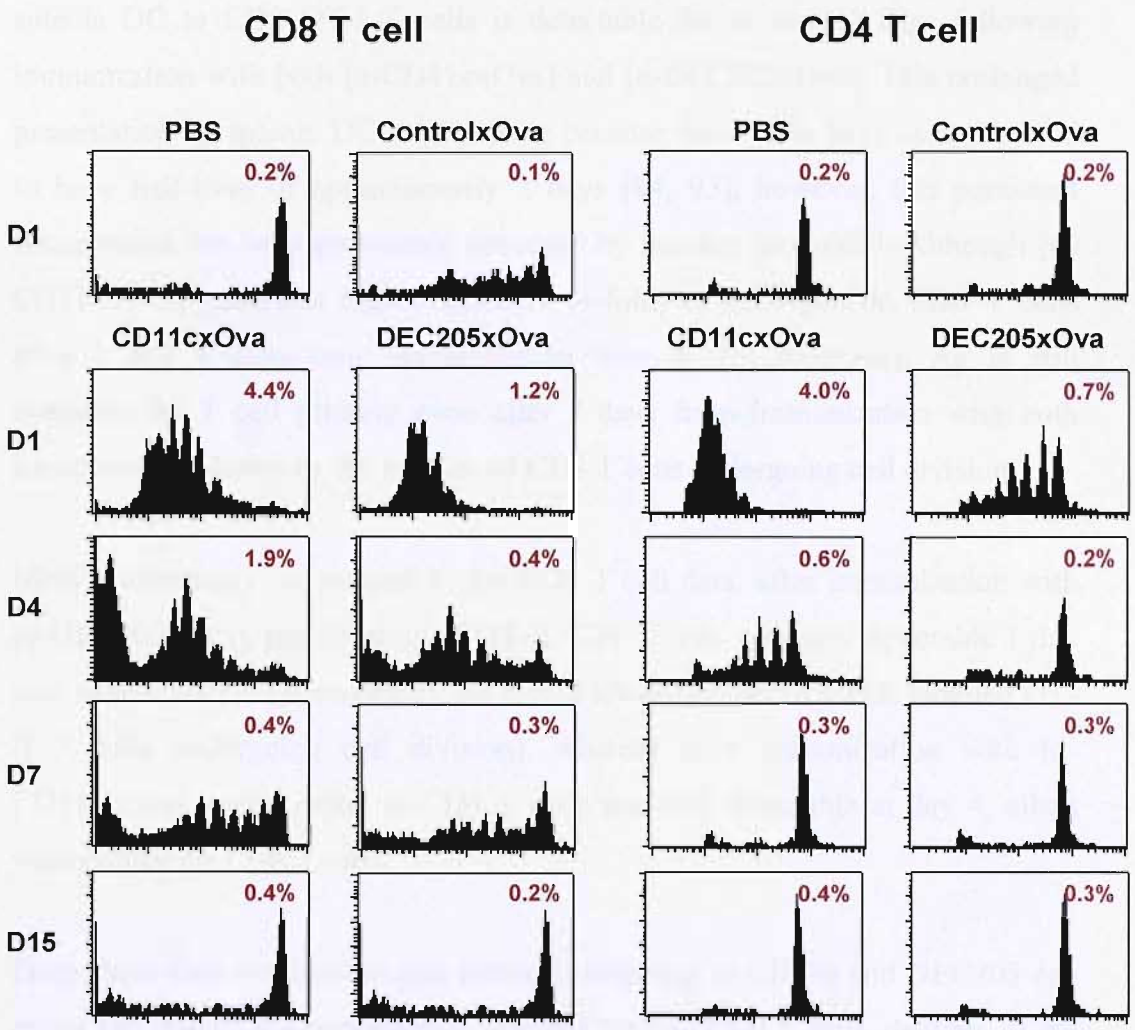


Figure 3.2.8 Antigen delivered to DC via distinct molecules can favour the priming of different T cell subsets

C57BL6 mice were immunized (i.v) with [FabxOva] (2.5μg) and α-CD40 mAb (50μg), or with PBS, for 1, 4, 7 or 15 days prior to the adoptive transfer of CFSE labelled OT-I ($4x10^6$cells) or OT-II T cells ($1.5x10^6$cells). Spleens were removed 3 days later and cell suspensions analysed by flow cytometry. Cells were gated on Ova-specific CD8 and CD4 T cells. Data representative of 3 experiments. Figures (red) represent the percentage of CFSE labelled SIINFEKL tetramer$^+$ CD8 T cells and $V_{\beta 5.1,5.2}{}^+$ CD4 T cells.

The interesting results from this work suggest that there is a difference in the length of time in which Ag derived peptides are expressed on the surface of DC depending on the molecule or DC subset being targeted by the conjugate, and

consequently, different conjugates can favour the priming of distinct T cell subsets

Figure 3.2.8 demonstrates that the presentation of Ova derived peptides by splenic DC to CD8 OT-I T cells is detectable for at least 7 days following immunization with both [α-CD11cxOva] and [α-DEC205xOva]. This prolonged presentation by splenic DC is surprising because these cells have been reported to have half-lives of approximately 2 days [84, 93], however, this persistent presentation has been previously observed by another group[69]. Although [α-CD11cxOva] generates higher numbers (4-fold) of Ova-specific CD8 T cells after 1 and 4 days from immunization than [α-DEC205xOva], Ag is still available for T cell priming even after 7 days from immunization with both treatments, as shown by the profiles of CD8 T cells undergoing cell division.

More interestingly, in contrast to the CD8 T cell data, after immunization with [α-DEC205xOva], proliferation of OT-II CD4 T cells was only detectable 1 day post immunization (as shown by the 6-fold lower number of CFSE labelled OT-II T cells undergoing cell division), whereas after immunization with [α-CD11cxOva], proliferation of CD4 T cells was still detectable at day 4, albeit weaker than for CD8 T cells.

From these data we propose that although targeting to CD11c and DEC205 can prime and initiate the proliferation of both CD8 and CD4 T cells, delivery of Ag by [α-CD11cxOva] induces the presentation of peptides on MHC-II complexes for longer than [α-DEC205xOva], which suggests a bias towards CD4 T cell priming. It is possible that by targeting DEC205 molecules, we are delivering Ag to a small subset of $CD8^{+}$DC that have been reported to efficiently cross-present exogenous Ag to CD8 T cells [234], whereas CD11c molecules, are expressed on both $CD8^{+}$ and $CD8^{-}$ DC subsets, and therefore, the targeting of the latter subset may preferentially generate a stronger CD4 T cell response as reported by some studies [188, 223, 235].

3.3 Discussion

First, we started by identifying the main blood derived DC subsets found in the spleen of naïve mice (i.e. $CD8^+$DC, $CD8^-$DC and $CD4^-CD8^-$DC subsets) and highlighted that all of these expressed high levels of CD11c molecules on their surface, which was unaltered by maturation signals. In contrast, only the $CD8^+$ DC subset was shown to express DEC205, which was upregulated following DC maturation. Consistent with the notion that mature DC decrease their endocytic capabilities and enhance their ability to prime naïve T cell, when immature splenic DC were activated with LPS, they were shown to upregulate the expression of MHC-II and co-stimulatory molecules (e.g. CD80, CD86, ICAM-1) (Figure 3.2.1).

Next, we targeted Ova to splenic APC with our [FabxOva] conjugates in vitro and concluded that of all the conjugates, delivery of Ag via CD11c, DEC205 and MHC-II molecules were the most promising targets for the development of strong Ag-specific CD8 and CD4 T cell immunity (Figure 3.2.2). Furthermore, these results clearly demonstrate that Ag targeted to all three surface molecules is efficiently delivered to both MHC-I and MHC-II processing compartments, as they all primed CD8 and CD4 T cells. Indeed, targeting of MHC-II molecules seemed to favour CD4 T cells priming, probably because Ag delivered via MHC-II can be directed to the MHC-II compartment more readily. Nevertheless, at this point we were unable to define the role of the different DC subsets in the priming of CD8 T cells.

Although, targeting of TLR2 has been reported to amplify T cell immunity [236], the data shown here indicates that this molecule is relatively ineffective in comparison to CD11c, DEC205 and MHCII molecules. Like for CD40 molecules, stimulation of TLR2 seems to be more important for the induction of various signaling pathways associated with the activation and modulation of DC rather than Ag-uptake and processing. Delivery of Ova with [α-FcRxOva], which targets both the inhibitory FcγR-II and the activating FcγR–III, failed to facilitate T cell priming. Many studies have shown that IgG-immune complexes bound to the activating FcγR-I and FcγR–III expressed on APC are efficiently internalized

and processed for the priming of T cells [237, 238]. On the other hand, FcγRIIB is thought to function as an inhibitory receptor for tyrosine kinase initiated cell activation when it is cross-linked with activation receptors such as the TcR, BcR and FcRγI/III [239, 240]. Targeting with [α-FcRxOva] conjugate failed to potentiate T cell priming. This conjugate, composed of 2.4G2 mAb, binds FcγRIIB and FcγRIII equivalently [241], therefore, it is possible that the lack of T cell response was a result of higher expression levels of the inhibitory FcRγIIB on the DC. Indeed, studies by Kalergis et al [238] have demonstrated that wild type iDC derived from BM or from spleen, predominantly expressed FcγRIIB, which accounted for approximately 75 % of the total FcγR expression. It seems that the balance between activating and inhibitory FcγR influences the maturation signals received by the DC, which determines their ability to prime T cells. In this model, IC loaded FcγRIIB-deficient DC were shown to have anti-tumour effects unlike the wild-type [238, 242, 243]. Alternatively, because this conjugate can bind various cells expressing FcγR other than DC, which are particularly efficient in naïve T cell priming, this could limit their availability to these cells and consequently lead to poor T cell responses.

Further studies were performed to examine the effectiveness of the three best conjugates in delivering Ag to APC in vivo, particularly to DC. DC have been demonstrated by numerous studies to be important for the cross-priming of CTL, as these cells were shown to persist in the absence of B cells [167], but were abrogated by depletion of phagocytes [244]. The results from the proliferation assays using ex vivo loaded DC, indicated that although targeting of Ag to CD11c, DEC205 and MHC-II molecules were shown to have comparable efficacies in vitro, when targeted in vivo, [α-CD11cxOva] was strikingly better than any of the other targets (Figure 3.2.3). These findings suggested that the accessibility to the target molecules in the lymphoid organ could be having an impact on the efficacy of [α-DEC205xOva] and [α-MHCIIxOva], or that the cell biology of the CD11c molecule favoured efficient uptake and processing of Ag in this setting.

In addition, it was evident at this point that DEC205 targeting, unlike MHC-II targeting, favoured the proliferation of CD8 T cells rather than CD4 T cells.

These interesting findings were confirmed by adoptive transfer experiments, where the proliferation of transgenic Ova-specific CD8 and CD4 T cells was monitored during their primary and secondary responses following immunization with the conjugates (Figure 3.2.4).

It was clear from the collective data that immunization with [α-CD11cxOva] evoked the most potent CD8 and CD4 T cell responses, and as previously observed for the ex vivo data (Figure 3.2.3), immunization with [α-DEC205xOva] favoured CD8 T cell priming, whereas [α-MHCIIxOva], the priming of CD4 T cells (Figure 3.2.6). Furthermore, targeting of DEC205 did not seem to permit the presentation of peptides for the priming of CD4 T cells for longer than 1 day following immunization, as observed for CD11c targeting (Figure 3.2.8). The latter in contrast, favoured the presentation of peptides in a MHC-II context for at least an additional 3 days compared to [α-DEC205xOva], suggesting that immunization with [α-CD11cxOva] biases the prolonged priming of CD4 T cells unlike [α-DEC205xOva] (Figure 3.2.8). Unfortunately, the data for [α-MHCIIxOva] is not available at this time as it is currently being investigated, but one would expect it to resemble [α-CD11cxOva] in favouring CD4 T cell priming as it has always been shown to generate higher numbers of these cells than [α-DEC205xOva].

The ability of distinct DC subsets to cross-prime CD8 T cells is still highly controversial as many reports suggest that splenic $CD8^+$ and $CD8^-$ DC do not exhibit significant differences in their ability to stimulate naïve CD8 T cell in vitro and in vivo [187]. In contrast, there is also strong evidence suggesting that when fully activated, $CD8^+$DC, are the most efficient subset for MHC-I cross-presentation, which produces high levels of IL-12 and strongly bias CD4 T cells towards an T_H1 response with extensive IFN-γ secretion, which results in the induction of a potent CD8 T cell response [175, 224, 225]. Moreover, $CD8^+$DC have been suggested to express lower numbers of MHC-II molecules than $CD8^-$ DC [245] and have a lower capacity to stimulate CD4 T cell proliferation and production of IFN-γ in culture, compared with either of the $CD8^-$DC subsets, which in contrast, have been shown to favour MHC-II-restricted presentation [179, 188, 223]. Thus, as clearly demonstrated in our phenotypic analyses

(Figure 3.2.1 (B)), only the $CD8^+$ DC subset expresses DEC205 and therefore, Ag delivered via [α-DEC205xOva] selectively targets these cells, whereas [α-CD11cxOva] and [α-MHCIIxOva] are not restricted to a specific subset.

The controversy associated with the specialization of the distinct DC subsets in their ability to prime and modulate CD4 and CD8 T cell immunity, has been very recently investigated in more detail by Shortman's group. In one study [235], they demonstrated that both $CD8^+$ and $CD8^-$ DC can provide activating signals to the CD4 T cells in culture and, in addition, the overall death rate of these CD4 T cells before entering the first cell division was not significantly different nor was the time taken to enter the first cell cycle. However, when CD4 T cells were primed by $CD8^+$ DC, a larger proportion each CD4 T cell division was lost before the next division. The nature of the death signal causing the loss of these cells remains undefined [235].

Even more recently, another study by this group assessed the ability of both $CD8^-$ and $CD8^+$DC to cross-present exogenous Ag administered in vivo [234]. They conclude that the unique ability of $CD8^+$DC to cross-present Ag is not dictated by Ag capture but by the ability to deliver the Ag to the cross-presentation pathway, a capacity found primarily and constitutively in this subset. The nature of the exogenous Ag was shown to have an effect on the efficiency by which these cells were able to cross-present to CD8 T cells. The cross-presentation of Ova bound to latex beads administered in vivo, was completely dependent on the $CD8^+$DC, even though $CD8^+$and $CD8^-$DC had captured exactly the same amount of Ag. Similar results were obtained for cellular Ag (cell associated Ova). In the case of soluble Ova, however, it was endocytosed and presented on MHC-II complexes by all subsets, but $CD8^+$DC were by far the most efficient at cross-priming [234].

These recent findings suggest that targeting soluble Ag to $CD8^+$DC is the main, if not the only way, to induce potent cross-priming of exogenous Ag to CD8 T cells. When we delivered Ag to DC in vitro, we recorded comparable effectiveness in the priming of CD8 T cells by all three conjugates [α-DEC205xOva], [α-CD11cxOva] and [α-MHCIIxOva], but we were unable to

distinguish the involvement of the different DC subsets at this stage because the latter conjugates could be interacting with all subsets. However, when Ag was delivered in vivo, the CD8 T cell priming response to [α-DEC205xOva] and [α-MHCIIxOva] was markedly lower than [α-CD11cxOva] (Figure 3.2.4). The distinct distribution of the DC subsets within the lymphoid organs has been previously reported [89, 91], and our data presented in chapter 5, confirmed that $CD8^{+}$DC are found mainly within the deep regions of the T cell zone of the white pulp, whereas the $CD8^{-}$DC subsets are more easily accessible to blood borne Ag as they are scattered in the marginal zone (MZ) of these organs. This suggests that the lower CD8 T cell response to [α-DEC205xOva] and [α-MHCIIxOva] might be a result of the inability of these to physically reach the DC subset (i.e. $CD8^{+}$DC) required for potent CD8 T cell priming.

More importantly, the observation that Ag delivery by [α-MHCIIxOva] has the capacity to generate a strong CD4 T cell response but fails to prime CD8 T cells effectively (Figure 3.2.6), in contrast to [α-CD11cxOva] that efficiently primes both CD8 and CD4 T cells, raises a few important points: firstly, there are three types of APC in the lymphoid organs that express MHC-II molecules (i.e. DC, B cells and macrophages) which have the potential to be involved in the activation of these CD4 T cells. However, macrophages are located mainly in non-lymphoid tissues (e.g. liver), the red pulp of the spleen and the subcapsular and medullary sinuses of LN, and not in the T cell areas where they would interact with naïve T cells [246], which suggests that these cells are more important for the activation of effector CD4 T cells that migrate into non-lymphoid tissues later in the response [247].

Although B cells have been shown to prime naïve CD4 T cells [248-251], the current view is that naïve CD4 T cells are primed by DC and only interact subsequently with B cells [252]. The main arguments supporting this position include: (1) the frequency of B cell clones bearing a receptor for a particular Ag is too low (1 in 10^4–10^5 cells) for them to take part significantly in T cell priming [253]; (2) in vivo proliferation of Ag-specific CD4 T cells after immunization is normal in B cell-deficient mice [254]; (3) studies show interactions between Ag-bearing DC and Ag-specific CD4 T cells in situ within 12-24 h following Ag

administration [255, 256], which is supported by data from real-time microscopic studies showing that DC-T interaction occur before B-T interaction [257-259]; (4) studies comparing the Ag-presenting capacity of B cells and DC showed that B cells are less efficient than DC [260, 261]; (5) resting B cells do not express the costimulatory molecules needed to activate naïve CD4 T cells, however, they are capable of activating memory and effector CD4 T cells due to their lower costimulatory requirements [249].

Moreover, the activation and expansion of Ag-specific B cells requires two distinct signals, like for T cell activation. Signal (1) is provided via the engagement of the BcR by antigen, and (2), by the ligation of CD40 by CD40L, which is expressed by activated CD4 T cells. In addition to recognizing Ag, the cross-linking of BcR by Ag provides intracellular signals essential for B cell proliferation and survival. Furthermore, it induces signals for BcR internalization [262], the direction of the BcR–Ag complex through the endocytic pathway towards MHC-II rich compartments (MIIC) [263] and the upregulation of costimulatory molecules necessary for CD4 T cell engagement, such as CD86 molecules [264]. Thus, in view of all these data and the fact that by targeting Ova directly to MHC-II molecules we are bypassing the critical stimulatory signals provided by BcR ligation, it seems unlikely that B cells are the predominant APC inducing the strong CD4 T response observed following immunization with [α-MHCIIxOva] (Figure 3.2.6).

Secondly, although immunization with [α-MHCIIXOva] induces a stronger CD4 T cell response than CD8 (Figure 3.2.6), it is not a result of its failure to successfully deliver the Ag to the MHC-I compartment, because when targeted in vitro [α-MHCIIxOva] was almost as effective as [α-CD11cxOva] and [α-DEC205xOva], therefore they can all process Ag and present it on MHC-I complexes. Thus, although Ag is being efficiently targeted to DC by [α-MHCIIxOva], it is clear that the anatomical structure of the spleen is preventing the delivery of Ag to possibly the only DC subset capable of effectively cross-priming CD8 T cells (i.e. $CD8^{+}DC$), and therefore, since [α-CD11cxOva] can generate a potent CTL response, one suggests that Ag bound to CD11c molecules are transported and transferred to $CD8^{+}$ DC located in the T cell zone

of the white pulp, whereas MHC-II molecules fail to do so. If this hypothesis is correct, one would expect that the ability of [α-CD11cxOva] to evoke strong CD4 and CD8 T cell responses is a result of Ag delivery to a large number of $CD8^-$DC located in the MZ, which in the presence of a maturation signal migrate into the T cell zone where they can efficiently prime CD4 T cells and by transferring a proportion of the bound Ag to $CD8^+$DC they can also effectively prime the CD8 T cells.

More interestingly, these findings raise an important question regarding the function of CD11c molecules in the transfer of Ag to other DC subsets. A proposal for inter-DC Ag transfer is not without precedent; in 1998, Inaba et al [265] proposed the MHC-II-restricted Ag transfer between migrating DC and a separate subset within the T cell zone (i.e. $DEC205^+CD8^+$DC). Surprisingly, this was found to be a highly efficient way of spreading relatively low amounts of injected Ag over a large proportion of lymphoid resident DC leading to the amplification of the immune response against Ag entering the periphery.

Moreover, a study by Shortman and Carbone's group [266] reinforced this hypothesis by demonstrating that CTL responses to herpes simplex virus was dependent on the migration and transfer of Ag by skin emigrating DC to lymphoid resident DC to mount a robust cytotoxic response, and these latest findings support other previous studies highlighting the possible existence of inter-DC Ag transfer [267-273]. Proposed mechanisms for the transfer of Ag include the release of exosomes or apoptotic bodies [245, 274] by migratory DC. Thus, various mechanisms for Ag presentation may exist, including: (1), the conventional pathways of immigration into the lymphoid tissue by DC originally present at the site of Ag challenge; (2), lymphoid resident DC ($CD8^+$DC) might have access to Ag in the lymph and blood contents, in addition to the capture of Ag transferred by migratory DC; and (3), the possible ingestion of dead DC; all of which result in the amplification of Ag-specific adaptive responses.

Nevertheless, we are still unable to explain how [α-DEC205xOva] can reach their targets on $CD8^+$DC (discussed further in chapter 5), but this result supports the idea that this subset of DC is highly efficient in the capture of the low

amounts of [α-DEC205xOva] that had access to this area and that specifically targeted the Ag to these cells specialized in the cross-priming of CD8 T cells.

In addition to the effective delivery of Ag to DC by the different conjugates, the data here also highlighted the crucial role of a maturation signal, which we provided by α-CD40 mAb, to prevent the system from becoming tolerized to the delivered Ag. As previously reported for DEC205 targeting [69], when a low dose of Ag was delivered to DC in vivo in the steady state via CD11c, these cells failed to maintain a strong CD8 T cell response even when they were effectively presenting Ag and inducing extensive T cell proliferation initially (Figure 3.2.4). The expanding T cells seemed to be unable to maintain their proliferation and differentiation, which resulted in their deletion after approximately 5 days unless a maturation signal was provided concomitantly with [α-CD11cxOva]. These findings are consistent with the sequential model that supports the notion that DC in the lymphoid organs constitutively present Ag on both MHC I and II molecules leading to peripheral tolerance, and, unless they are exposed to cellular signs of danger, for example in the form of factors released from damaged cells, TLR-ligands from pathogens or maturation signals (e.g. CD40-L/ α-CD40 mAb) from CD4 T cells which license them to be converted from cross-tolerant to cross-priming APC, CTL immunity will not be induced.

For some time now it ha been known how strongly CD8 T cells can respond to infection with viruses or bacteria. A naïve T cell can rapidly undergo up to 15 divisions to produce more than 10^4 progeny within 7 days of antigenic stimulation [275], but for constant protection, it is essential that these expanding cells become memory cells capable of dealing rapidly with the reappearance of a pathogen, whether it is by re-emerging from a latent or chronic site of infection or by a new infection. The role of CD4 T cells and of activation, growth and survival signals provided by other cells, such as DC, in the CD8 T cell response is not entirely understood. The sequential model, proposes that for non-inflammatory cellular Ag (e.g. tumour cells, dead cells), the priming of CD8 T cells required help from CD4 T cells and both of these cells had to recognize helper and CTL epitopes on Ag presented by DC but not necessarily on the same DC.

Furthermore, many reports [165, 167, 185] have shown that the function of Ag-specific CD4 T cells recognizing Ag on the DC could be replaced, at least partially, by activating DC with an agonist α-CD40 mAb as we have used in our system. These groups demonstrated that CD40 ligation on DC, results in the secretion of cytokines such as IL-1, IL-6, IL-12, TNF-α together with the upregulation of costimulatory molecules (e.g. ICAM-1, CD80 and CD86), which enable these activated DC to prime CD8 T cell in the absence of CD4 T cell help. Our findings are in accordance with this model because when mice were immunized with a non-inflammatory Ag, they required an adjuvant to induce the activation of DC and priming of CD8 T cells.

One weakness of this sequential model was that a strong primary response by CTL to many infectious agents can be readily measured in animals lacking CD4 T cells [276]. This lack of requirement for CD4 T cell licensing was later explained by proposing that the DC could be directly activated by the infectious pathogens, which unlike tumour cells and necrotic cells, express PAMPs that can be recognized by TLR expressed on DC and circumvent the need for CD4 T cells. In addition, another model was proposed by Matzinger et al [277], suggested that factors released from damaged and transformed cells could also provide strong danger signals.

Two sets of studies have questioned this sequential model and turned this field around. The first set suggested that primary CD8 T cell responses to non-inflammatory cellular Ag is in fact help independent, and help during the priming phase is required solely to programme the CTL to become effector and memory cells capable of rapidly responding to restimulation [166, 278]. However, other groups have shown that not all CTL responses to non-inflammatory Ag are independent of CD4 T cell help [279].

The other set of data includes results indicating that primary CTL responses to pathogens are independent of CD4 T cells, but there is in fact a requirement for these cells to allow the generation of long-lived, functional memory CD8 T cells able to rapidly respond to rechallenge with pathogen [280]. Our ELISPOT and IFN-γ intracellular staining data support the idea that the CD8 T cells had to be

primed in combination of a maturation signal (i.e. α-CD40 mAb) to generate IFN-γ secreting effector cells and as expected, immunization with [α-CD11cxOva] induced the highest number of effector CTL, followed by [α-DEC205xOva] and [α-MHCIIxOva] (Figure 3.2.7).

In addition, the effector cells generated after immunization with [α-CD11cxOva] and α-CD40 mAb, were shown to have potent in vivo cytolytic functions as most of the Ova-loaded splenocytes transferred into the immunized mice were rapidly eradicated (Figure 3.2.7). All the other treatments were unable to kill the Ag-loaded cells, probably as a result of the lower number of effector cells generated after immunization. When mice were rechallenged with MHC-I-restricted Ova peptide, only those primed in the presence of α-CD40 mAb generated functional memory cells, whereas mice immunized with [α-CD11cxOva] alone were tolerized to Ova.

Potential mechanisms for the maintenance or programming of memory CTL by CD4 T cells are still being debated but they suggest a new model involving a more complex conversation between the three cells involved in CTL responses. One suggested cross-talk mechanism includes the possible interaction between CD4 and CD8 T cells, mainly because the maintenance of CTL memory cells has been shown to be independent of the continued interaction with APC [281] and CD4 T cells may provide direct signals to the CTL or indirectly by the secretion of growth factors [282].

In contrast to CD8 T cells, the priming of CD4 T cells with [α-CD11cxOva] alone, evoked a primary response comparable to when α-CD40 mAb was present, but when [α-DEC205xOva] was administered alone, it was shown to be half as effective as when given with α-CD40 mAb (Figure 3.2.6). The reasons for the discrepancy in the requirement of a maturation signal for the priming of CD4 T cells is unclear, however, it is possible that in the case of [α-CD11cxOva], the conjugate delivers a large amount of Ag to the DC, which may affect the threshold of the costimulatory signal required for the effective priming of CD4 T cells and this lowered requirement can be met by the higher number of CD4 T cells present in the system following the adoptive transfer of OT-II T cells. The

fact that CD8 T cell proliferation and expansion following immunization with [α-CD11cxOva] in the same mice was dependent on the adjuvant, suggests that the costimulation threshold for these cells is higher than for CD4 T cells, which is not surprising as these cells have harmful cytolytic functions that need to be constantly monitored to prevent unnecessary tissue damage.

Nonetheless, signalling via CD40 was required for the mounting of a potent memory and effector CD4 T cell response, as only the animals immunized with α-CD40 mAb were able of produce IFN-γ secreting CD4 T cells and resulted in strong memory responses. Even though [α-MHCIIxOva] was demonstrated to evoke the expansion of CD4 T cells, most of these cells failed to differentiate into effector cells as a lower number was shown to secrete IFN-γ upon restimulation of Ova peptide than when targeted to DEC205, implying that although Ag can be efficiently presented to CD4 T cells via MHC-II molecules, the intrinsic function of these molecules is not of an endocytic receptor and this is reflected on their inability to generate effector CD4 T cells (Figure 3.2.7).

In conclusion, of all the [FabxOva] conjugates investigated here, [α-CD11cxOva] generated the most potent CD4 and CD8 T cell responses in vivo, in contrast to when splenic DC were targeted in vitro, which suggest that the anatomical distribution and availability of the DC subsets being targeted by the different conjugates have an impact on its overall effectiveness. Thus, it is proposed here that CD11c molecules may be involved in the transfer of intact Ag to other DC subsets and it is thereby capable of evoking strong CD4 and CD8 T cell responses. Co-administration of [α-CD11cxOva] with α-CD40 mAb prevented the establishment of tolerance against Ova and allowed the proliferating T cells to acquire bona fide effector and memory cell functions.

Chapter 4 Humoral response to immunization with [FabxOva] conjugates

4.1 Introduction

Targeting of Ag to DC not only initiates T cell mediated immunity but it also activates the other arm of the adaptive system, which is comprised of memory B cells and plasma-cells secreting Ag-specific Abs. Ag-specific B cells recognise soluble Ag in its native form unlike T cells, and the origin of these Ag can be blood-borne or transported by DC (e.g. in the form of immune-complexes). B cells can proliferate and secrete IgM in response to the binding of Ag to its Ag-specific B cell receptor (BcR) but to generate memory B cells help from Ag-specific CD4 T cell is required.

As demonstrated in vivo in the rat, DC can capture and retain unprocessed Ag to be presented to B cells in its native form [195], in addition to their ability to efficiently process Ag into peptides and present them to CD4 T cells. This ability to present Ag to both B and T cell at the same time was the basis for the "ménage a trois model", which proposes that once CD4 T are primed by the DC they upregulate the expression of CD40-L on their surface, which can bind to CD40 constitutively expressed on B cells. However, to prevent the generation of auto-antibodies, these activated CD4 T cells also need to recognise peptides derived from the same Ag presented on MHC-II complexes on the surface of these B cells, which are also professional APC like DC. Together with the secretion of immunostimulatory cytokines secreted by effector CD4 T cells, the differentiation of naïve B cells into memory B cells characterized by Ab isotype switching and affinity maturation can occur.

Indeed, the interaction between CD40-L on activated CD4 T cells and CD40 on B cells has been shown to be crucial for their differentiation process, including rescue from apoptosis, differentiation into germinal centre cells, isotype switching, selection and maturation into memory cells [283]. During this ménage a trois, there is evidence of a more direct DC-B cell dialogue; including the possible interaction between CD40 molecules on activated DC and CD40-L on B cells that is upregulated upon activation [284]. Furthermore, CD40-activated DC can secrete soluble factors, including IL-12 and soluble IL-6rα gp80, which binds IL-6 secreted by the B cells and some DC, and leads to the differentiation of

CD40 activated B cells into plasma cells secreting IgM. DC also orchestrate Ab isotype-switching of activated B cells, where DC alone can induce the expression of IgA on the B cells but, IL-10 and TGF-β is required for further differentiation into IgA secreting cells [285].

In addition, DC have been reported to express B cell activating factor belonging to the TNF family (BAFF/Blys-L) at high levels at the steady-state, which is upregulated upon maturation with bacterial products. BAFF receptor appears to be restricted to B cells and induces both proliferation and Ig secretion by B cells, suggesting that BAFF may represent an important costimulator through which DC regulate B cell proliferation and function [286]. However, one also needs to consider that a conditioned DC can be only a temporal bridge between a CD4 T helper cell and B cells in analogy to the sequential model in which DC interact with CD4 T cells and CD8 T cells sequentially rather than at the same time, for instance when B cell bind soluble Ag rather than Ag presented by DC.

In our previous chapter, we concluded that targeting of CD11c evoked robust CD4 and CD8 T cell responses, and in addition, it was shown to prime CD4 T cells for longer than CD8 T cells, unlike DEC205. Here, we demonstrate the effectiveness of our conjugates in the initiation of a humoral reponse.

4.2 Results

4.2.1 Immunization with [α-CD11cxOva] generates high titres of anti-Ova antibodies

Based on the most promising targets highlighted by the T cell proliferation assays, we immunized mice with 2.5 μg of conjugates targeting CD11c, DEC205, MHC-II and CD40 with or without α-CD40 mAb. Serum samples were collected at different time points and anti-Ova mAb titres measured by ELISA.

Figure 4.2.1 clearly shows the striking ability of immunization with a single low dose of [α-CD11cxOva] to induce the secretion of high titres of Ova-specific antibodies at a very early stage. By day 7, immunization with [α-CD11cxOva] generated titres approximately 120-fold higher than any of the other conjugates, with a mean titre of 1: 6,000. Targeting to DEC205, MHC-II and CD40 failed to generate anti-Ova antibodies above background levels. As seen for the proliferation of CD4 T cells following immunization with [α-CD11cxOva], antibody production was also independent of α-CD40 mAb. The antibody response was maintained at high levels beyond day 27, but the titres were halved reaching a mean titre of approximately 1: 3,000, which was still around 30-60-fold higher than the other conjugates.

Importantly, the comparison between the antibody responses generated by targeted Ag and soluble Ova demonstrated that immunization with soluble Ag using an amount at least 8,000-fold more than that present in 2.5 μg of conjugate, failed to evoke titres comparable to [α-CD11cxOva] at day 7, as the response was approximately 10-fold lower. Although by day 27, immunization with soluble Ova at the highest concentration reached its peak and antibody titres were twice as high as those with [α-CD11cxOva]. The ELISA assay used to assess the antibody titres in these experiments measured both IgM and IgG antibody isotypes but IgM production is restricted to early antibody responses before the process of isotype-switching is concluded. Therefore, these data suggest that immunization with [α-CD11cxOva] generates high levels of IgG antibodies as

the response was maintained high for nearly one month after immunization. Further analysis using an isotype-specific ELISA showed that the responses to Ag targeted to CD11c contained a mixture of IgM and IgG, with a predominance of IgG1 (personal communications with Alison Tutt).

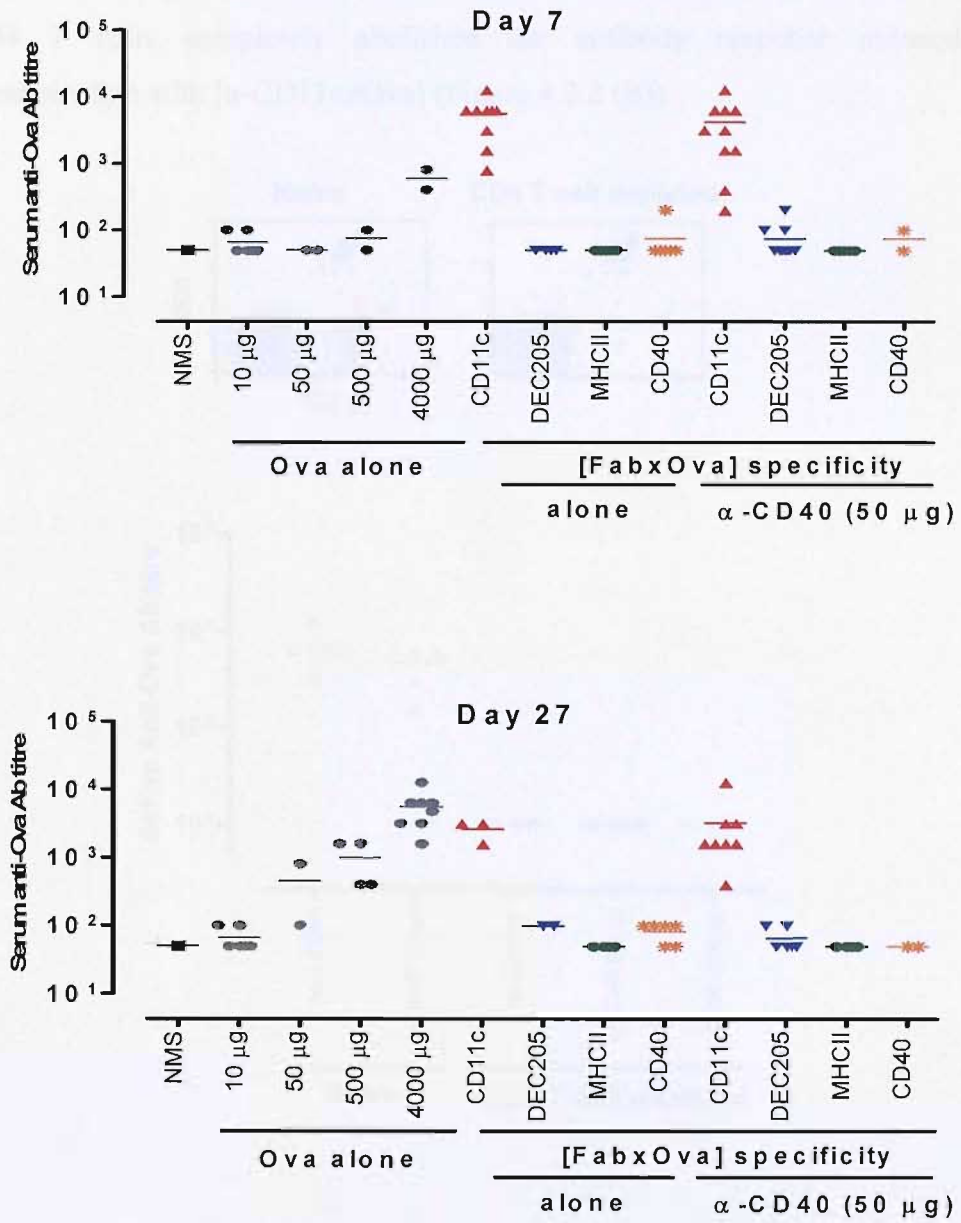


Figure 4.2.1 Immunization with [α-CD11cxOva] generates a potent α-Ova antibody response.

C57BL/6 mice were immunised with soluble Ova or [FabxOva] conjugate (2.5 µg) i.v. with or without α-CD40 mAb (50 µg), and serum samples collected on days 7 and 28. ELISA plates were coated with rabbit α-OVA mAb and blocked in the presence of soluble Ova (10 µg/ml). Mouse serum IgG Ab was detected with an α-mouse IgG-HRP conjugate. The serum titre was defined as the highest dilution of a serum that gives an OD490 value of double that of serum from naïve mice (NMS). Symbols represent individual mice and horizontal lines indicate the mean value of each group.

4.2.2 The role of CD4 T cells in antibody production

We next investigated the production α-Ova antibody in mice depleted of CD4 T cells. Figure 4.2.2 (A) indicates that after three injections of depleting α-CD4 mAb, circulating CD4 T cells were reduced by more than 95 %. The depletion of CD4 T cells completely abolished the antibody response induced by immunization with [α-CD11cxOva] (Figure 4.2.2 (B)).

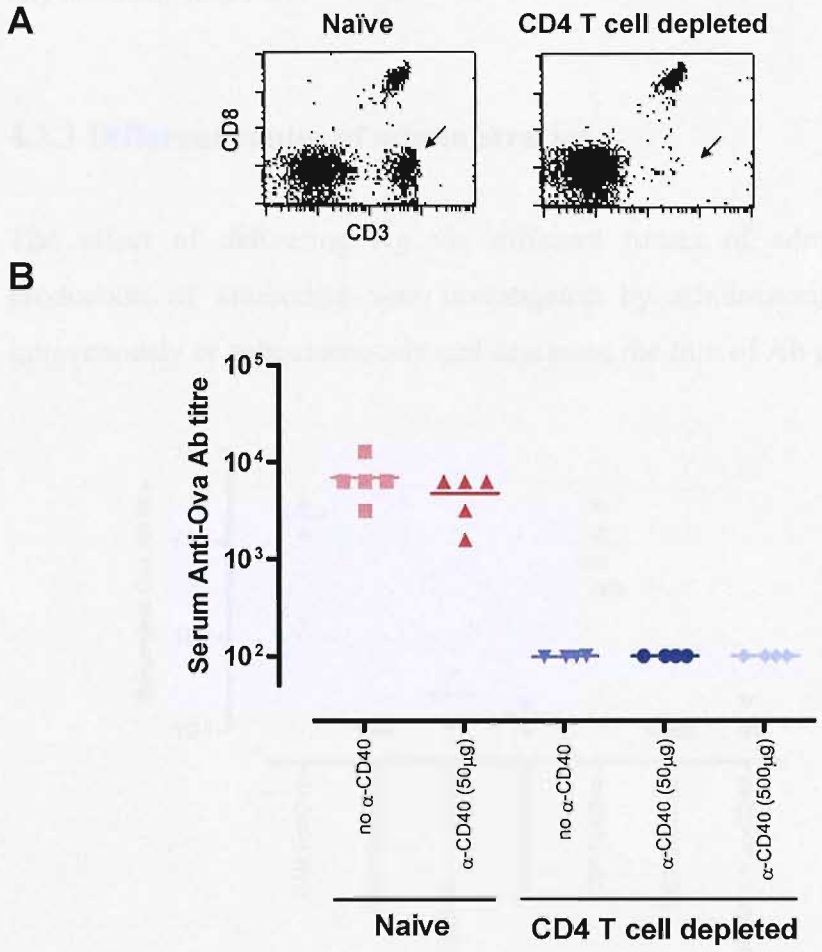


Figure 4.2.2 The role of CD4 T cells in antibody production in response to [α-CD11cxOva]

Mice were pre-treated with three injections of α-CD4 mAb (1mg) i.p. on days -4,-1 and 3. On day 0, the depletion of CD4 T cells was analysed and mice were immunized with [α-CD11cxOva] (2.5 µg) with or without α-CD40 mAb (50 or 500 µg). (A) FACS profiles of CD4 T cell depletion. (B) Anti-Ova antibody production was measured by ELISA. The serum titre was defined as the highest dilution of a serum that gives an OD490 value of double that of serum from naïve mice.

In addition, the role of CD4 T cells could not be replaced by the presence of α-CD40 mAb. Anti-CD40 mAb has been used as a standard adjuvant and it is thought to act by mimicking the signal provided by activated CD4 T cells expressing CD40-ligand (CD40-L), which provides help to B cells and DC by interacting with CD40 molecules on their surfaces. Therefore, these results suggest that the help provided by CD4 T cells is not dependent on the CD40:CD40-L interaction as even 500 μg of the adjuvant was unable to induce any antibody response.

4.2.3 Different routes of administration

The effect of delivering Ag via different routes of administration on the production of antibodies was investigated by administering the conjugates intravenously or subcutaneously and assessing the titre of Ab produced.

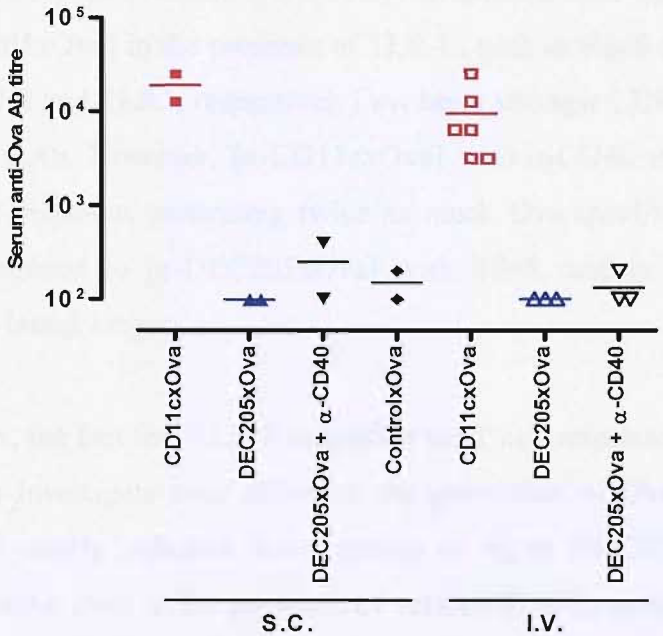


Figure 4.3 Different routes of administration and its effect on antibody production

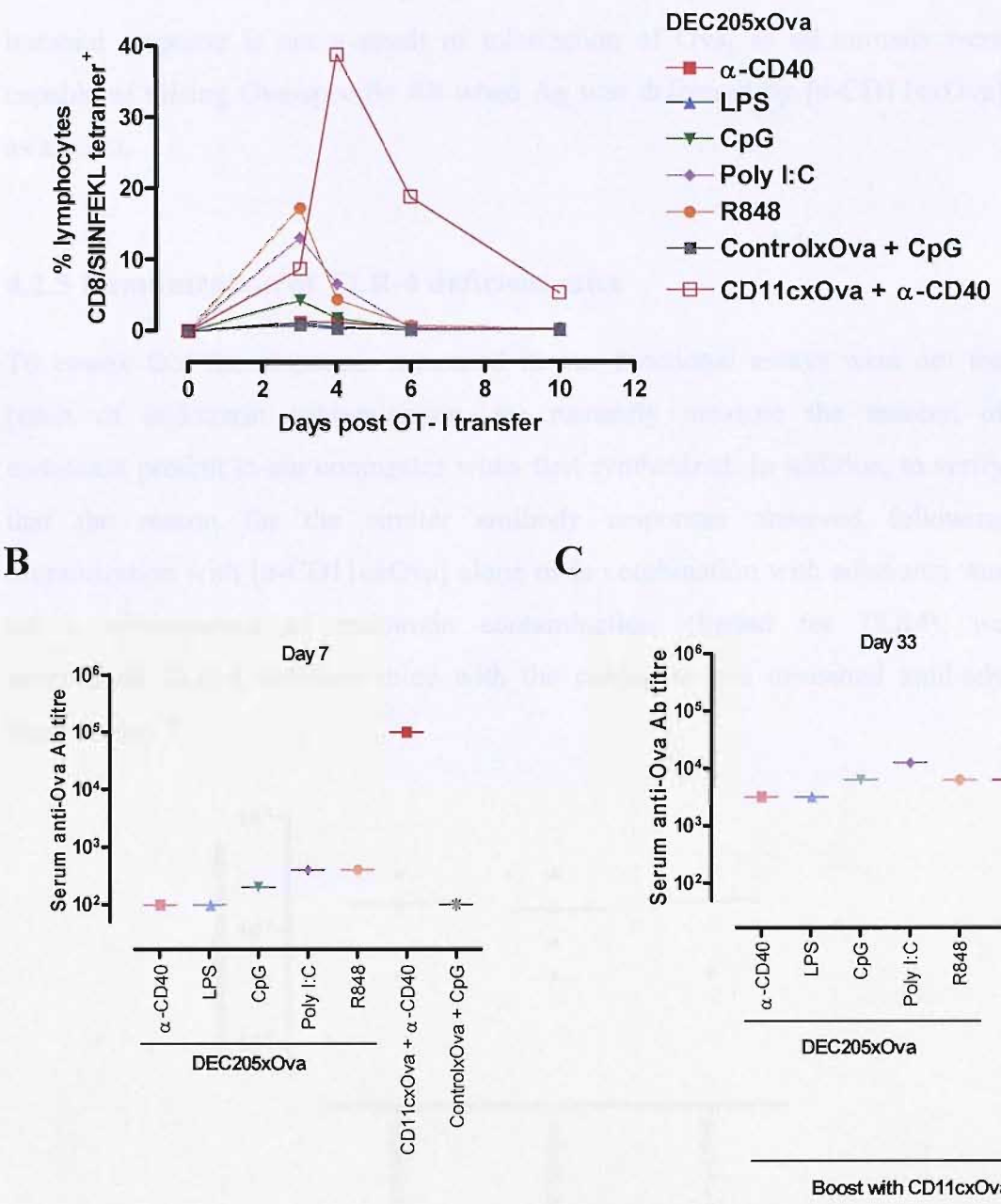
Mice were immunized with [FabxOva] conjugates (2.5 μg) with or without α-CD40 mAb (50 μg) subcutaneously (s.c.) or intravenously (i.v.) and seven days later serum α-Ova IgG titres were measured by ELISA. The serum titre was defined as the highest dilution of a serum that gives an OD490 value of double that of serum from naïve mice.

Figure 4.2.3 suggests that delivery of Ag via CD11c is the only treatment capable of generating high titres of α-Ova antibody. Although the titres generated by subcutaneous immunization were slightly higher than the intravenous injections (approximately 2-fold), there was no significant difference between the two routes of administration ($p > 0.05$ unpaired t-test). Interestingly, we must assume that the delivery of Ag to Langerhans' DC (LC) resident in the skin, which express high levels of DEC205, failed to facilitate the production of mAb following immunization with [α-DEC205xOva].

4.2.4 TLR-ligands and antibody responses

Here we attempted to induce a humoral response with [α-DEC205xOva] by using strong TLR-L as adjuvants. Figure 4.2.4 (A), first highlights the advantage of immunization with [α-CD11cxOva] over [α-DEC205xOva] in the generation of a strong CD8 T cell response. Secondly, it demonstrates that immunization with [α-DEC205xOva] in the presence of TLR-L, such as R848 and Poly I: C (ligands for TLR7/8 and TLR3, respectively) evokes a stronger CD8 T cell response than α-CD40 mAb. However, [α-CD11cxOva] with α-CD40 mAb still induces the strongest response, generating twice as much Ova-specific circulating $CD8^+$ T cells compared to [α-DEC205xOva] with R848, and in addition, this robust response lasted longer.

Moreover, the fact that TLR-L amplified the T cell response to [α-DEC205xOva] led us to investigate their effect on the generation of Ova-specific Ab. Figure 4.2.4 (B) clearly indicates that targeting of Ag to DEC205 fails generate high titres of mAb even in the presence of various TLR-L, as the Ab titres remained around background levels after 7 days from immunization. To ensure that the mice were not tolerized following immunization with [α-DEC205xOva], mice were boosted thirty-three days later with [α-CD11cxOva] alone and this resulted in the secretion of high titres of Ova-specific IgG Ab seven days later by all animals, including the control animal primary immunized with [ControlxOva] and CpG (Figure 4.5 (C)).



4.2.4 The influence of TLR-L as adjuvants on CD8 T cell proliferation and antibody production in response to [α-DEC205xOva]

Mice received $2x10^6$ OT-I T cell i.v. and 24 h later were immunized with [FabxOva] conjugates (2.5 µg) and the different TLR-L (20 µg) i.v.. (A) Blood samples were collected and the number of SIINFEKL-tetramers and α-CD8 mAb positive cells measured by flow cytometry. (B) On D7, serum samples were isolated and α-Ova IgG levels were assayed by ELISA. (C) On D33, all animals were injected with [α-CD11cxOva] (2.5 µg) and 7 days later serum samples collected and α-Ova IgG titres measured as in (B).

These results suggest that targeting to DEC205 does not generate an Ab response even when a modest T cell response can be detected. In addition, this lack of humoral response is not a result of tolerization of Ova, as all animals were capable of raising Ova-specific Ab when Ag was delivered by [α-CD11cxOva] as a boost.

4.2.5 Immunization of TLR-4 deficient mice

To ensure that the responses measured in our functional assays were not the result of endotoxin contamination, we routinely measure the amount of endotoxin present in our conjugates when first synthesized. In addition, to verify that the reason for the similar antibody responses observed following immunization with [α-CD11cxOva] alone or in combination with adjuvants was not a consequence of endotoxin contamination. (ligand for TLR4), we immunized TLR-4 deficient mice with the conjugate and measured antibody titres on day 7.

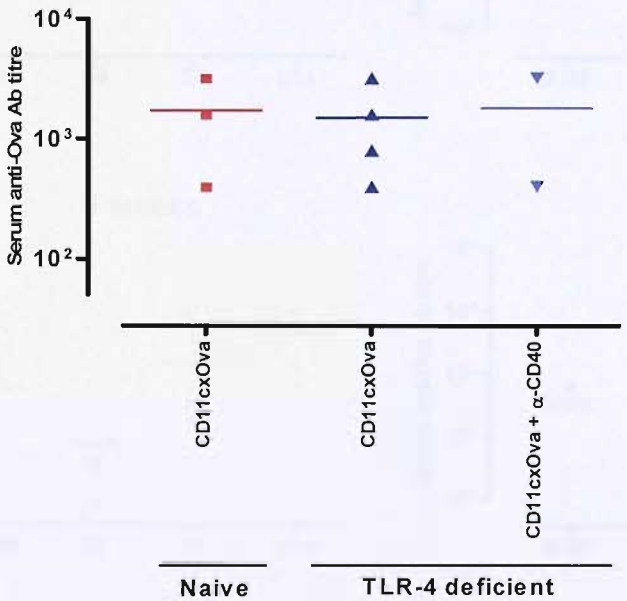


Figure 4.2.5 The generation of antibody responses in TLR-4 deficient mice

Wild-type C3H/HeN or TLR-4 deficient C3H/HeJ mice were immunized with [α-CD11cxOva] (2.5 μg) and α-CD40 mAb (50 μg) i.v.. Serum samples were collected on day 7 and serum α-Ova antibody titres measured by ELISA.

Figure 4.2.5 indicates that the generation of high titres of antibody with [α-CD11cxOva] is independent of endotoxin, as there was no significant difference between the antibody titres by wild-type C57BL/6 mice and the TLR-4 deficient C57BL/6 mice.

4.2.6 Immunization with [α-CD11cxOva] induces long term immunity

To determine whether immunization with [α-CD11cxOva] conjugate generates long-lasting humoral immunity, we next primed the mice with [α-CD11cxOva] and boosted them with soluble Ova (2 μg) 4, 6, 8 or 10 weeks after primary immunization (Figure 4.2.6).

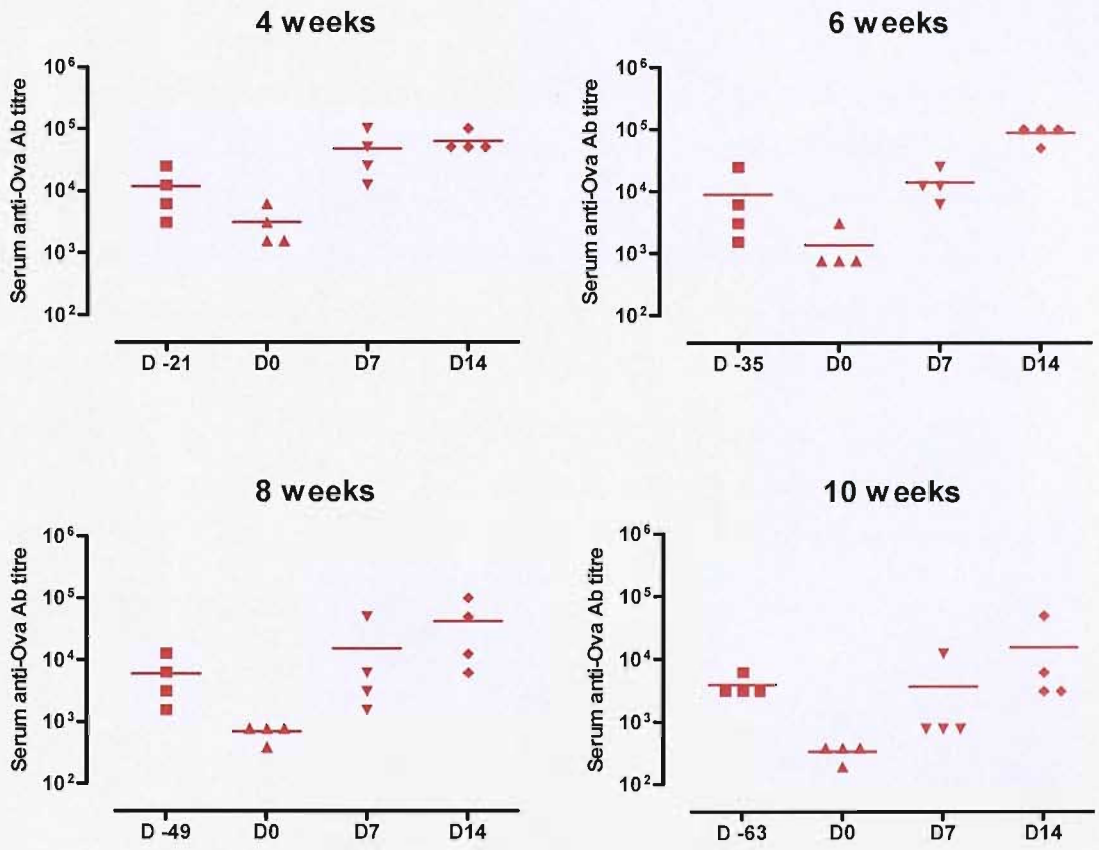
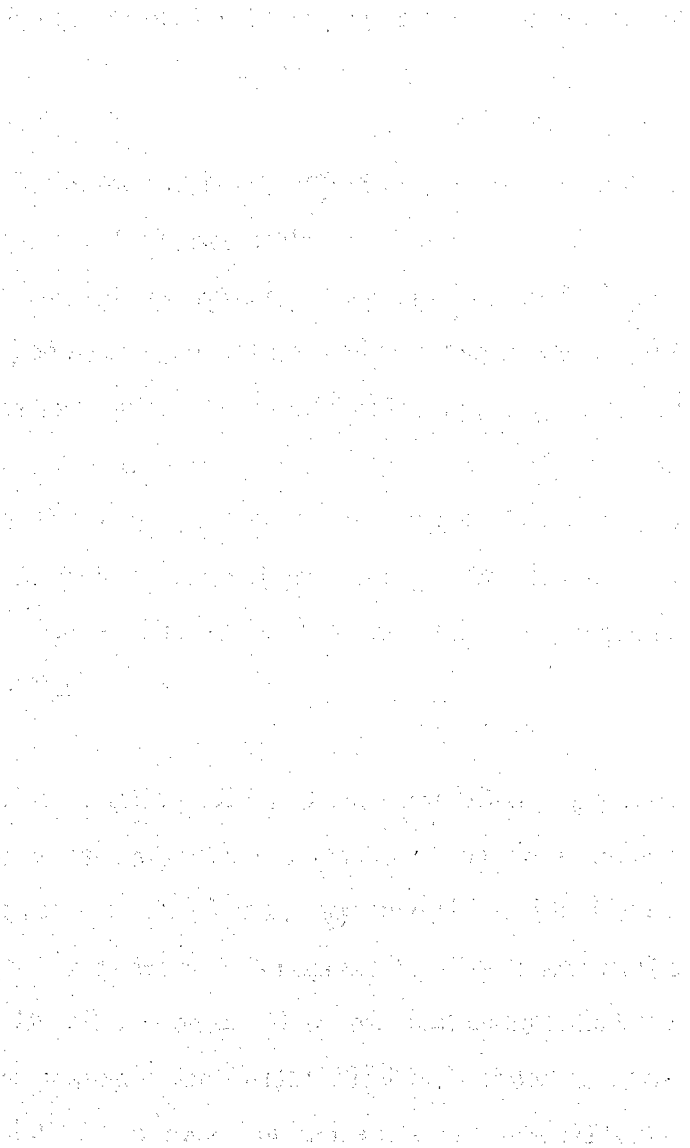


Figure 4.2.6 Antigen targeting to DC evokes long-lasting antibody production

Anti-Ova antibody responses measured by ELISA in mice immunized i.v. with [α-CD11cxOva] (2.5 μg) 4, 6, 8 or 10 weeks before i.p. boost with soluble Ova (2μg) (on D0). IgG titres were measure 7 days after primary immunization, before boost, 7 and 14 days after boost. Symbols represent individual mice and horizontal lines indicate the mean value of each group.

High antibody titres were detected 7 days after primary immunization (average of 1: 7,000) and after the Ag boost, these titres were amplified even further. A robust antibody response was still detected in response to the boost after 10 weeks from the primary immunization, the longest period tested, which indicates the persistence of functional memory B cells long after immunization with a single low-dose of conjugate without adjuvant.



4.3 Discussion

In this chapter, we demonstrate that with a single low dose treatment, [α-CD11cxOva] is the only conjugate capable of generating a rapid and potent Ova-specific humoral response. The data also highlighted the efficiency with which this conjugate targets Ag to DC compared to soluble Ova, which even at a concentration 8,000 times higher than that present in the injected conjugate, was unable to produce such high titres of Ab (Figure 4.2.1). This robust response was maintained over a month and further analysis using an isotype-specific ELISA (data not shown, personal communications), indicates that the majority of the response was composed of the IgG1 subtype, in line with the published data [70, 71].

The observation that immunization with [α-CD11cxOva] evokes the secretion of IgG1 Ab as early as day 7 (i.e. before the development of germinal centres where class-switching occurs) was surprising, but many studies including a report by Wykes et al [287] demonstrated that immunization with Ag-pulsed DC produced consistently higher titres of IgG than IgM, which suggests that DC might actually directly interact with B cells and provide a signal for isotype switching soon after Ag transfer, before T helper cells have been recruited. The strong Ab response to immunization with [α-CD11cxOva] was completely abolished when animals were depleted of CD4 T cells, which indicates this is a T cell dependent (TD) response (Figure 4.2.2).

In contrast to CD11c targeting, DEC205 targeting failed to generate an antibody response. This is consistent with the results of previous studies [288, 289]. Ruprecht and Lanzavecchia [290] proposed that TLR-L like CpG can provide a powerful signal that, in synergy with cognate T cell-help and BcR cross-linking, induces primary B cell responses. Here we demonstrate that the addition of various TLR-L as adjuvant for [α-DEC205xOva], failed to evoke a humoral response (figure 4.4). In contrast, immunization with [α-CD11cxOva] produced comparable titres of α-Ova mAb when injected alone or in the presence of any of the TLR-L adjuvants confirming that this humoral response is independent of adjuvant help.

Moreover, our data indicate that immunization with [α-DEC205xOva] is unable to generate high titres of Ab even when administered subcutaneously, which would target Ag to the skins resident DC, LC, known to express high levels of DEC205 (Figure 4.2.3). In conclusion, these collective findings indicate that the inability of DEC205 targeting to generate a robust humoral response is either characteristic of the function of this endocytic receptor and/or of the subset of DC on which they are expressed.

Targeting of MHC-II was previously demonstrated to favour CD4 rather than CD8 T cell responses (Figure 3.2.3, 3.2.4, 3.2.6), but it failed to generate α-Ova Ab even in the presence of α-CD40 mAb, despite this adjuvant being reported to activate DC and upregulate the expression of MHC-II molecules [291]. Unlike DEC205, all conventional DC subsets express high levels of MHC-II on their surfaces, suggesting that the inability of [α-MHCIIxOva] to evoke an Ab response is not due to subset specialization but rather a functional characteristic of this molecule. Early experiments were focused on the targeting of MHC-II [292, 293], but the increasing awareness of the highly specialized role of DC, resulted in comparative studies which indicated that DC targeting was a more effective strategy to produce a strong Ab response [294]. The explanation that DEC205 and MHC-II targeting fails to generate high titres of Ab as result of lower amounts of these conjugates reaching their respective DC subsets must be considered, but it seems unlikely, as the targeting of DEC205 and MHC-II can evoke strong CD8 and CD4 T cell responses, respectively, suggesting that the Ag does indeed reach their target cell, however, they are unable to prime B cells.

Ag can arrive in the secondary lymphoid organs (e.g. spleen) as soluble Ag, immune-complexes (IC) or in association with DC. The Ag is first encountered by the B cells, macrophages and DC residing in the marginal zone (MZ), which are strategically positioned to respond rapidly to blood borne Ag by priming naïve T cell and follicular B cells passing through the MZ en route to the white pulp. Whereas small molecules appear to gain access to lymphoid follicles, others appear to be transported by Ag-transport cells (ATC). Although ATC remain poorly defined, a population of circulating immature DC have been reported to be involved in the effective capture of circulating Ag and transfer to

MZ B cells once they migrate into the spleen [198]. Thus, it is possible that by targeting CD11c, we are delivering Ag to these cells which are subsequently transferring it to MZ B cells capable of rapidly producing Ag-specific IgG before the formation of germinal centres.

Furthermore, recent work shows that MZ B cells can also act as ATC themselves, by binding IC, possibly from DC, and transporting them to follicular DC (FDC), which are responsible for the Ag transfer to follicular B cells. Similar to follicular B cells, Ag-engaged MZ B cells have been shown to migrate to the B-T boundary zone and be able to transfer Ag directly to naïve B cells [295]. In addition, a recent study has shown that unlike follicular B cells, Ag-engaged MZ B cells are capable of priming naïve CD4 T cells and induce their differentiation into effector cells in the T cell zone [296]. In this respect, MZ B cells are thought to behave more like DC than follicular B cells. Therefore, it is possible that some of the early IgG production observed here is due to the transfer of Ag to MZ B cells, which respond by rapidly generating Ag-specific IgG.

A recent study has shown that unlike macrophages, which contain high levels of lysosomal proteases and rapidly degrade internalized proteins, DC and B cells are protease-poor, resulting in limited lysosomal degradation [297] which explains their ability to retain Ag in its native form for long periods of time. Thus, it is possible that, similar to this model, a proportion of the Ag bound to CD11c is internalized and processed for T cell presentation while some of the bound Ag is recycled back to the surface in its native form to allow B cell activation. Alternatively, CD11c molecules might be slowly internalized by DC, which would enable the direct presentation of opsonized particles, and our conjugate, to be presented to B cells whilst on their surface.

In addition, Clynes R et al [298] have identified an Ag retention compartment accessible by recycling FcγRIIB receptors, and shown that antigen can be re-presented to B cells for at least 48 hours. Antigenic persistence was shown to be long lived, lasting more than 48 hours, suggesting that Ag acquired in the periphery by migrating DC would be available for the activation of lymphocytes in the secondary lymphoid organs. These data supports a model in which

opsonized Ag can enter two complementary FcR pathways: one that where activating FcγR generate T cell epitopes, and another where FcγRIIB, enables preservation and concentration of B cell epitopes.

Importantly, CD11c is expressed on all conventional DC subsets and consequently, can lead to the activation of distinct signalling pathways. Although the DC subsets functional differences remain unclear, many studies have demonstrated that $CD8^+$DC tend to induce a T_H1-biased cytokine response in reactive CD4 T cells, whereas $CD8^-$DC favours a T_H2-biased response [173, 175, 225]. In the last two years, two studies targeting molecules expressed only on $CD8^-$DC have reported their ability to produce high titres of Ab unlike targets on $CD8^+$ DC. In 2005, Corbet and Shortman [299] demonstrated that by targeting Ag without any adjuvants to FIRE (F4/80-like receptor) or CIRE (C-type lectin receptor) only expressed on immature $CD8^-$DC in the mouse, generated Ab titres 100-1000-fold over a non-targeted control. In contrast, DEC205 targeting to $CD8^+$DC failed to generate Ab.

In addition, Carter el al [288] reported that by targeting DC-associated C-type lectin-1 (Dectin-1) on mouse $CD8^-$DC, they were able to evoke strong T cell and humoral responses. Again, the production of Ab was not detected when Ag was delivered via DEC205. Thus, these results suggest that the $CD8^-$DC subset is specialized in the generation of a potent CD4 T cell response which biases towards the production of Ag-specific mAb, whereas $CD8^+$DC favour a CTL response. Whether CD11c has unique properties in the retention of Ag for B cells and/or it simply delivers Ag to a DC subset that is specialized in providing signals to evoke a robust humoral response remains to be determined.

The data presented here highlights the critical role of CD4 T cell in the generation of Ova-specific Ab in our system and suggest that other factors besides the interaction between CD40 and CD40-L are provided by these cells to modulate the production of Ab by B cells, as the presence of α-CD40 mAb could not rescue the response in CD4 T cell depleted mice. Candidate molecules that have been reported to be involved in this process include OX40/OX40L [300], BAFF and APRIL and their receptors (BAFFR, TACI, BCMA) [301],

CD95/CD95L [302], ICOS/ICOSL, which has been suggested to act upstream of CD40-CD40L interactions[303].

In view of the recent published findings mentioned above and the unique ability of [α-CD11cxOva] to rapidly generate high titres of Ab in our system, a model may be proposed (refer to diagram 6). In this model, DC expressing CD11c would bind and endocytose the [α-CD11cxOva] conjugate (diagram 6 (section *A)), causing the degradation of a proportion of them for the generation of peptides to be presented to CD8 and CD4 T cells resulting in potent T cell responses (Figure 3.2.4 and 3.2.6) (section *B). These primed CD4 T cells were shown to persist and provide help to memory B cells for more than ten weeks from primary immunization (Figure 4.2.6) (section *C). Moreover, the subset of DC targeted by the conjugate could influence the outcome of the T cell response, with $CD8^-$DC favouring a T_H2 response and $CD8^+$DC a T_H1 driven response.

Additionally, in analogy to MZ B cells, which have been described to be able to prime naïve CD4 T cells and also transfer intact Ag to FDC or follicular B cells by the cleavage of CR2 [304] (section *D), these targeted DC might also retain some of the conjugate molecules bound to CD11c in its native form as a result of a slower internalization rate or a recycling mechanism (section *E). Therefore, one of the functions of CD11c molecules on DC may be to retain intact Ag to be transferred to B cell and $CD8^+$DC located in inaccessible areas within the lymphoid tissue.

In contrast, Ag delivered to DEC205 has been reported to be rapidly endocytosed and targeted to late lysosomal compartments upon ligation, as a result of its EDE cytoplasmic tail sequence [305] and, therefore, would not be retained intact to activate B cells through their BcR and evoke an Ova-specific Ab response, instead a strong T cell response would be generated.

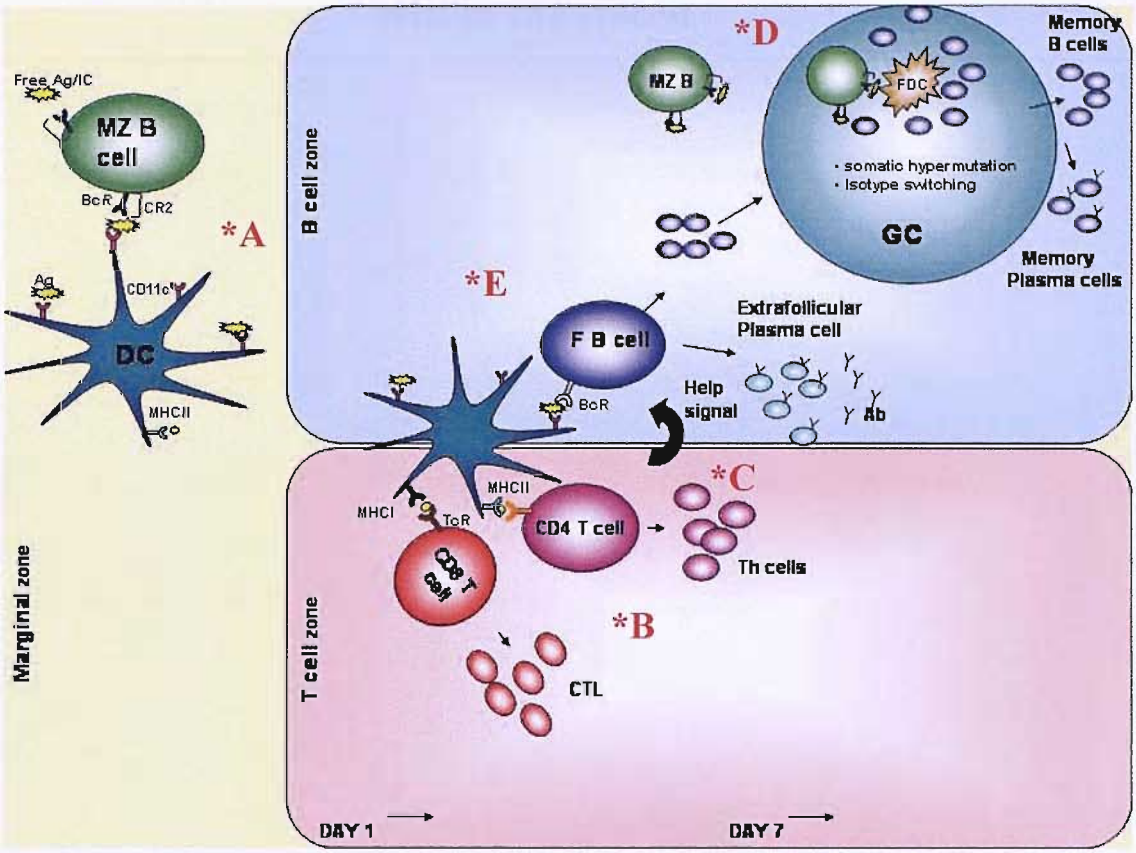


Diagram 6 Proposed model for the function of CD11c in the generation of a robust humoral response and cellular immunity.

Refer to text for description of the proposed model. Abbreviations: marginal zone B cell (MZ B cell), follicular B cell (F B cell), germinal centre (GC), follicular DC (FDC), CD4 T helper cell (T_H cell), cytotoxic T lymphocytes (CTL), immune-complex (IC).

Chapter 5 Biodistribution of [FabxOva] conjugates within the spleen

5.1 Introduction

Peripheral lymphoid organs, such as the spleen, draining lymph node and Peyer's patches, are the major sites for the generation of primary immune responses. These organs provide the meeting place where lymphocytes circulating in the blood encounter pathogens and their products brought from a site of infection. Lymphocytes can recirculate through the body in both the blood and lymph. When they reach the blood capillaries running through a lymph node they can encounter pathogens and become effector cells. However, pathogens can also enter the blood directly and in this case, the spleen is the lymphoid organ responsible for their removal and the activation of lymphocytes like in the lymph nodes with the only difference that both pathogen and lymphocytes enter and leave the spleen in the blood rather than the lymph. Thus, not surprising, the anatomical structure of such lymphoid organs has the important function of facilitating the interaction between APC and naïve lymphocytes, which results in the priming of an Ag-specific immune response.

The spleen is composed of white pulp regions and the red pulp, where blood is filtered and cleared of aged red blood cells by resident macrophages. However, the important site for the initiation of the adaptive response is the white pulp, which has distinct regions, named the marginal zone (MZ), B-cell and T-cell zones, through which naïve B and T lymphocytes continually recirculate.

This diagrammatic representation (Diagram 7) of the spleen helps us to highlight the structures studied in this chapter. Blood from the central circulation flows through the afferent splenic artery that branches into the central arteriole and drains into the MZ from which it percolates into the red pulp. These central arterioles are sheathed by white-pulp areas consisting of the T cell zone area (also know as the periarteriolar lymphoid sheath, PALS) and B cell follicles. In the spleen, the MZ is an important transit area for cells that are leaving the bloodstream and entering the white pulp. Thus, it contains a large number of resident APC, which are involved in Ag capture and presentation to lymphocytes in the white pulp. The MZ macrophages form the outer ring of the MZ and the MZ metallophillic macrophages (labelled with α- MOMA-1 mAb) form the inner

ring close to the white pulp. Staining with α-MOMA-1 mAb was used to enable the identification of the border of the white pulp.

Located between these two macrophage subsets are the MZ B cells (also labelled with α-CD19 mAb) and DC (labelled with α-CD11c mAb). DC are also found in the T cell zone which is visualized by the staining of T cells with α-CD3 mAb.

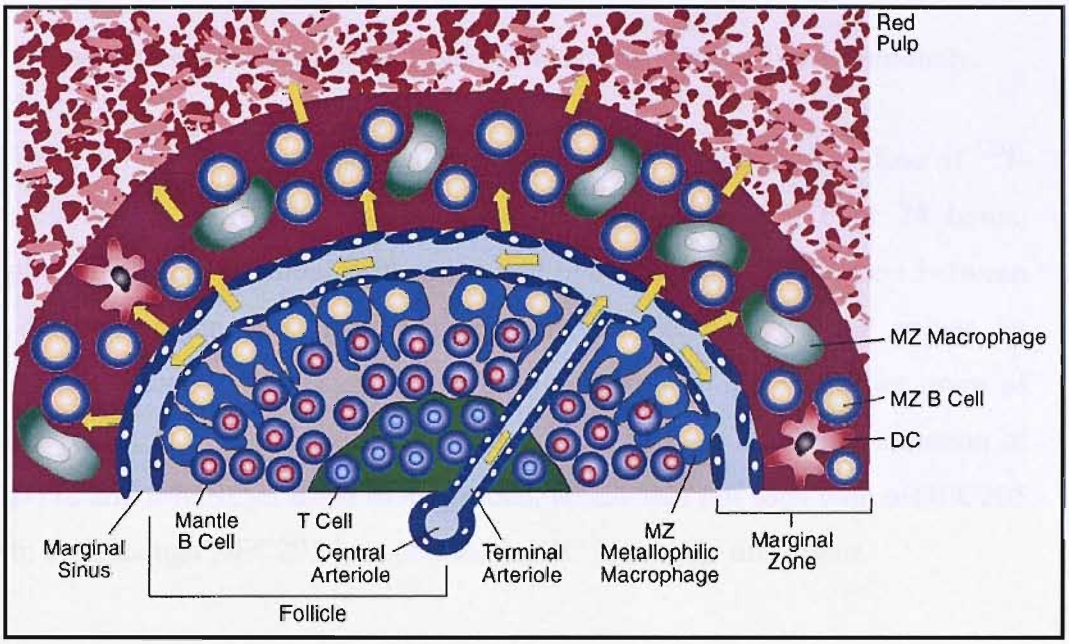


Diagram 7 : Different cell populations within the spleen [306]

During recirculation, the lymphocytes can interact with DC, and other APC, located in the MZ and T cell zone where they become activated and differentiate into effector cells which migrate back to the peripheral to fight the pathogen.

In this chapter, we studied the anatomical distribution and bioavailability of the target cells as this would have profound effect on the overall effectiveness of a vaccine and cannot be predicted using in vitro assays. Hence, our aim is to relate the effectiveness of our individual [FabxOva] conjugates with their physiological distribution within the spleen and the different lymphocyte subsets that are involved in mounting a strong immune response.

5.2 Results

In parallel with our functional assays described in chapters 3 and 4, we wished to investigate the biodistribution of our most potential mAb targeting vaccine candidates over a period of time. To achieve this we radiolabelled the targeting mAb with ^{125}I-iodine, which enabled us to determine the anatomical localization of these mAb over a period of time.

5.2.1 Anatomical localization of F(ab')$_2$ mAb administered intravenously.

In Figure 5.2.1, mice were intravenously injected with a single low dose of ^{125}I-labelled F(ab')$_2$ mAb (2.5 µg). Tissues were collected after 6 or 24 hours, weighed and their γ-radioactivity measured. To allow the comparison between the different organs and their varied sizes, we standardised the values by expressing them as the percentage of the injected dose recovered per gram of tissue. The most outstanding feature of this work was the rapid accumulation of α-CD11c and α-MHC-II mAb in the spleen, which was not seen with α-DEC205 mAb, even though DEC205 is expressed on DC located in this organ.

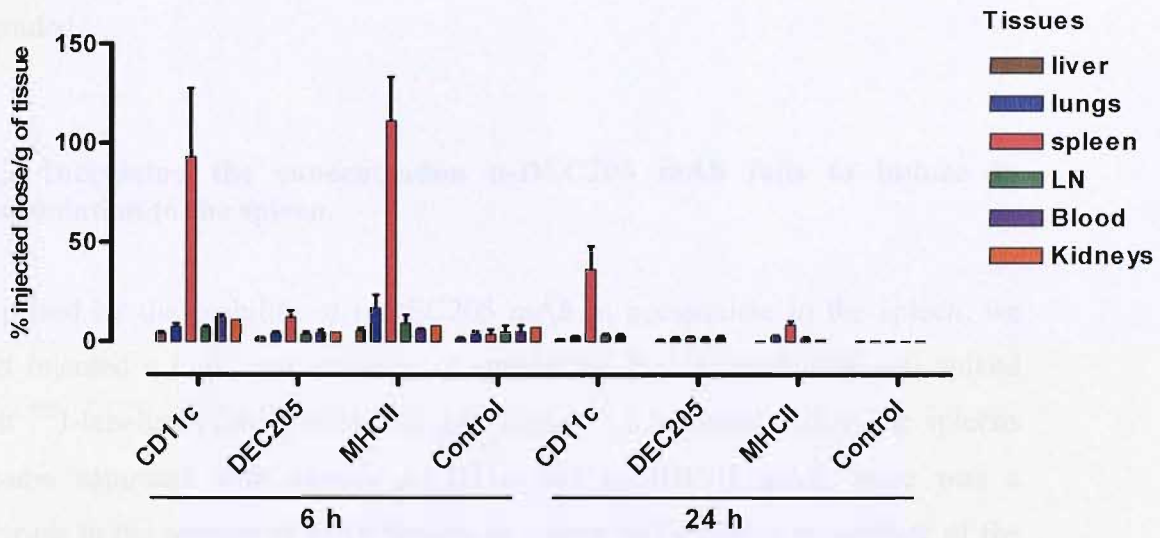


Figure 5.2.1 Anatomical localization of F(ab)$_2$ mAb administered intravenously.

Naïve C57BL/6 mice were injected i.v. with 2.5 µg of ^{125}I-labelled F(ab)$_2$ mAb. After 6h or 24 h, tissues were collected, weighed and γ-radiation measured using a γ-counter. Values are expressed as % of injected dose recovered per gram of tissue. n = 3 individual experiments.

The biodistribution of α-CD11c and α-MHC-II IgG mAb indicated that approximately 100 % of injected dose per gram of tissue (equivalent to ~ 10 % of injected dose) is in the spleen within 6 hours, which represents approximately 25-fold and 8-fold higher accumulation than found with control mAb and α-DEC205 mAb, respectively. This pattern was seen as early as 1 hour after injection (data not shown) and persisted for at least 24 hours. To confirm that all mice received radiolabelled mAb, we assessed their total body count using a Geiger counter (data not shown). In addition, by using $F(ab')_2$ fragments rather than whole IgG in these experiments, we assured that the observed distribution was not a result of non-specific FcR interaction.

Interestingly, although after 6 hours the levels of α-CD11c and MHC-II $F(ab')_2$ mAb accumulated to the spleen were comparable, at 24 hours, the amount of α-CD11c mAb remaining was 3-4-fold higher than for α-MHC-II mAb. It seems likely from these data, that the accumulation of α-CD11c and α-MHC-II mAb in the spleen facilitates the delivery of Ag to APC residing in this organ. Furthermore, the fact that α-MHC-II mAb is not retained in the spleen for as long as α-CD11c mAb, suggests that its specificity for APC cell types other than DC, such as B cells and macrophages, may have an affect on the rate that this mAb is degraded.

5.2.2 Increasing the concentration α-DEC205 mAb fails to induce its accumulation to the spleen.

Surprised by the inability of α-DEC205 mAb to accumulate in the spleen, we next injected a high concentration of unlabelled $F(ab')_2$ mAb (100 μg) spiked with ^{125}I-labelled $F(ab')_2$ mAb (10 μg). Figure 5.2.2 shows that as the spleens became saturated with excess α-CD11c and α-MHC-II mAb, there was a decrease in the amount of mAb present in spleen and a higher percentage of the radiolabelled mAb was detected in other compartments, such as the blood for α-CD11c mAb and the lymph nodes for α-MHC-II mAb. Accumulation of α-MHC-II mAb to the spleen was still detected after the high dose of unlabelled mAb, suggesting that because this mAb binds all professional APC cell types, it

requires a higher dose of unlabelled mAb to saturate this compartment and cause its complete displacement to other organs. More importantly, these results demonstrate that even when a high dose of α-DEC205 mAb was administered, it failed to increase its accumulation to the spleen or LN. In addition, because it did not accumulate in any of the other organs assessed, it is possible that it was eliminated. Therefore, it seems that what limits the accumulation of α-DEC205 mAb to the lymphoid organs is not the availability of free mAb.

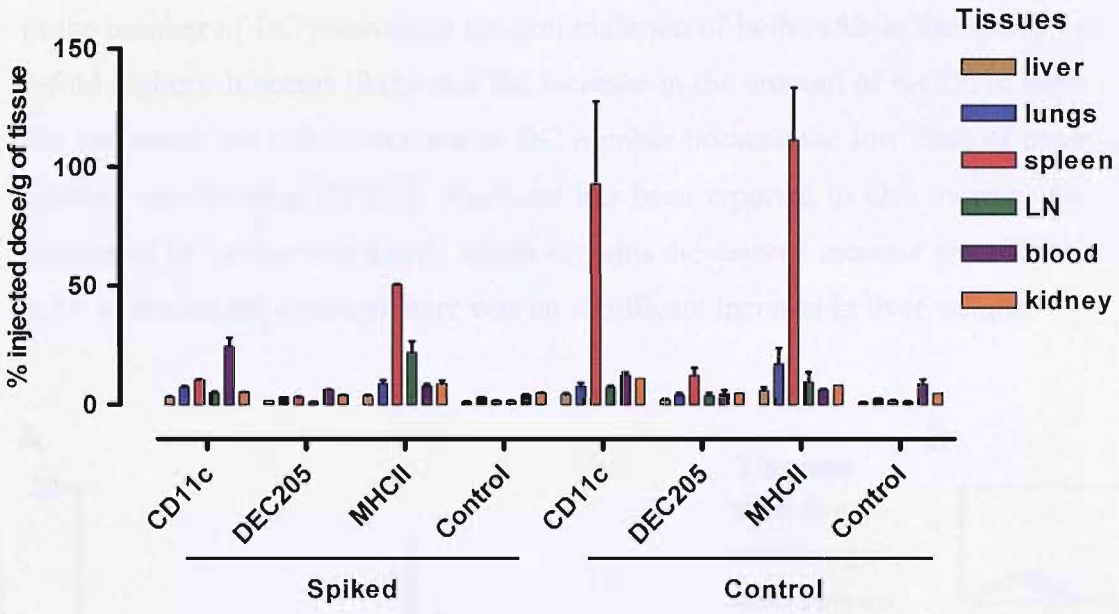


Figure 5.2.2 High concentration of α-DEC205 F(ab')$_2$ mAb fails to accumulate in the lymphoid organs.

Mice were injected i.v. with 100 μg of unlabelled F(ab')$_2$ mAb spiked with 10 μg of ^{125}I-labelled F(ab')$_2$ mAb or 2.5 μg of ^{125}I-labelled F(ab')2mAb as controls. Six hours later, tissues were collected and γ-radiation measured. Values are expressed as % of injected dose recovered per gram of tissue.

5.2.3 Pre-treatment with FLT3-L and LPS facilitates α-DEC205 mAb accumulation in the spleen.

The previous results suggested that the failure of α-DEC205 mAb to accumulate in the spleen was not a result of a low concentration of free circulating mAb. Consequently, this led us to investigate the possibility that the limiting factor was associated with the number of target cells or molecules available for binding by the free mAb, and therefore, by increasing the number of CD8$^+$DC and/or

DEC205 molecules on these cells, one aimed to increase its accumulation to the spleen. To achieve this, we pre-treated the mice with subcutaneous FLT3-L (10 μg) for 10 consecutive days prior to injection with α-CD11c and α-DEC205 mAb. Treatment with FLT3-L has been shown to increase the number of DC in the spleen by approximately 6-fold (refer to Figure 3.2.5), which had an effect on the size and weight of the spleens of treated mice as it almost doubled in size, hence, the values for the biodistribution in figure 5.2.3 are represented as the percentage of injected dose for the total tissue weight. As expected, the increase in the number of DC potentiated the accumulation of both mAb in the spleen (~ 2-fold higher). It seems likely that the increase in the amount of α-CD11c mAb did not match the 6-fold increase in DC number because the low dose of mAb injected was limiting. FLT3-L treatment has been reported to also increase the number of DC in the liver [307], which explains the marked increase of α-CD11c mAb in this organ, although there was no significant increase in liver weight.

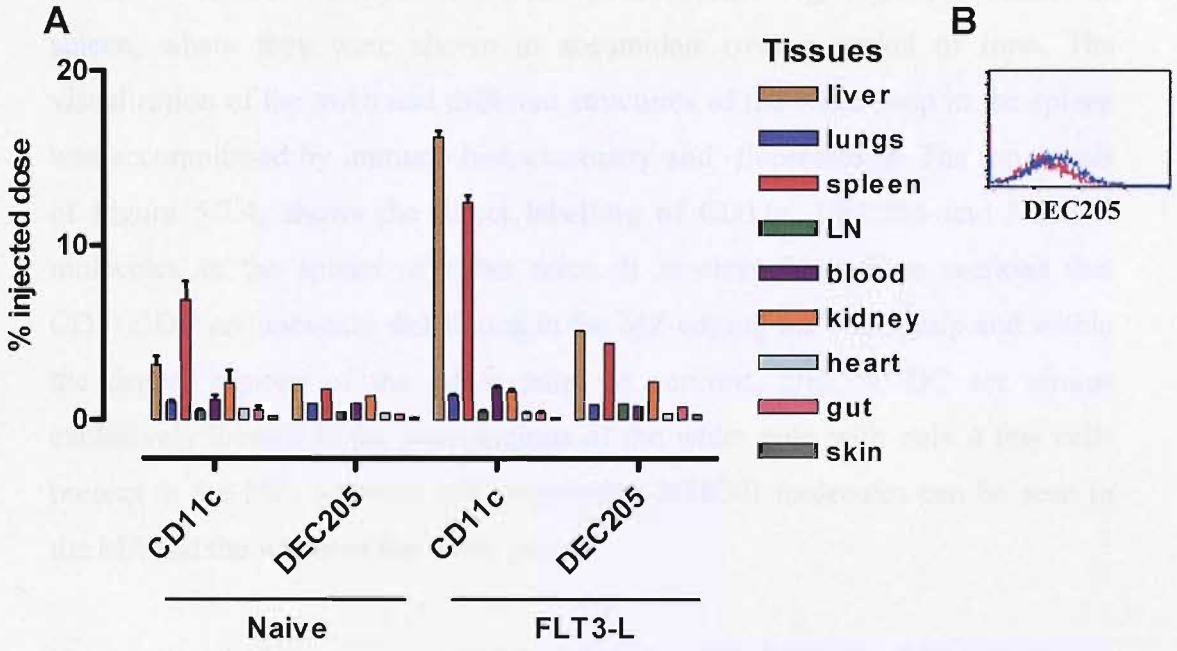


Figure 5.2.3 Pre-treatment with FLT3-L affects the biodistribution of the ^{125}I-labelled F(ab')2mAb

Mice were pre-treated with s.c. injections of FLT3-L (10 μg) for 10 consecutive days prior to i.v. injections with ^{125}I-labelled F(ab')2mAb (2.5 μg). Tissues were collected 6 h later and γ-radiation measured. Biodistribution of labelled mAb is expressed as % of injected dose recovered in total tissue weight due to different spleen sizes. (B) Profile shows the expression of DEC205 on splenic CD11c$^+$ splenocytes in naïve (pink) and FLT3-L treated mice (blue).

Although the percentage of mAb accumulated in the spleen was amplified, the overall difference between α-CD11c and α-DEC205 mAb remained unchanged, with α-CD11c reaching a 3-4-fold higher accumulation than α-DEC205 mAb. Nevertheless, these results indicate that by increasing the number of DC we were able to increase the accumulation of α-DEC205 mAb in the spleen. Furthermore, preliminary studies have shown that the accumulation of α-DEC205 mAb in the spleen is also increased (approximately by 2-fold) when mice are pre-treated with LPS (data not shown). LPS has been shown to induce DC maturation and the upregulation of DEC205 expression on these cells (Figure 3.2.1 (C)), in contrast to FLT3-L (Figure 5.2.3 (B)).

5.2.4 Visualization of the movement of the targeting mAb in the spleen over a period of time.

Next, we wished to determine the movement of the targeting mAb within the spleen, where they were shown to accumulate over a period of time. The visualization of the mAb and different structures of the white pulp in the spleen was accomplished by immuno-histochemistry and -fluorescence. The top panels of Figure 5.2.4, shows the direct labelling of CD11c, DEC205 and MHC-II molecules in the spleen of naïve mice. It is clear from these sections that $CD11c^{+}$DC are unevenly distributed in the MZ edging the white pulp and within the deeper regions of the white pulp. In contrast, $DEC205^{+}$DC are almost exclusively located in the deep regions of the white pulp with only a few cells present in the MZ, whereas APC expressing MHC-II molecules can be seen in the MZ and the whole of the white pulp.

Next, we tracked the movement of the targeting mAb following their intravenous administration. Mice were injected with 10 μg of FITC labelled α-CD11c, α-DEC205 or α-MHC-II $F(ab')_2$ mAb together with unlabelled α-CD40 mAb and spleens collected 1, 4 or 8 hours later. Figure 5.2.4 shows that within 1 hour of injection with FITC labelled α-CD11c $F(ab')_2$ mAb, the mAb was drained into the MZ of the spleen where it was detected mainly on DC scattered in these regions but not to the subset found within the deeper regions of the white pulp. It

is also possible that circulating DC targeted whilst in the blood compartment have also drained into the MZ and positioned themselves in the clusters seen around the white pulp. Within 4 hours, these labelled DC started to migrate from the MZ into the deeper regions of the white pulp (refer to arrows) and by 8 hours, there was an even higher accumulation of these cells inside the white pulp with low numbers of DC still present in the MZ clustering around specific points on the edges of the white pulp.

In contrast, FITC labelled α-DEC205 $F(ab')_2$ mAb could not be detected in any region of the spleen (dark dots are non-specific staining), even though the direct staining of naïve spleens demonstrated that there are DEC205 positive cells present (Figure 5.2.4). This suggests that the mAb is unable to reach the DEC205 positive cells located within the white pulp and is consequently quickly eliminated, which supports the radiolabelled biodistribution data, where α-DEC205 failed to accumulate in the spleen unlike α-CD11c and α-MHC-II mAb.

Anti-MHCII mAb was detected on macrophages in the red pulp and MZ, together with DC and B cells leading to the strong staining of the MZ APC and B cells within the white pulp (refer to arrows, Figure 5.2.4). As time progressed, there was less staining in the red pulp, which suggests that either macrophages rapidly degrade the mAb bound to their surfaces or they also migrate to the MZ and deeper regions of the white pulp. Importantly, cells carrying α-CD11c mAb migrated to the centre of the white pulp, whereas only a small proportion of the cells bound to α-MHC-II were detected in this area and most of the positive cells were found in the outer regions of the white pulp.

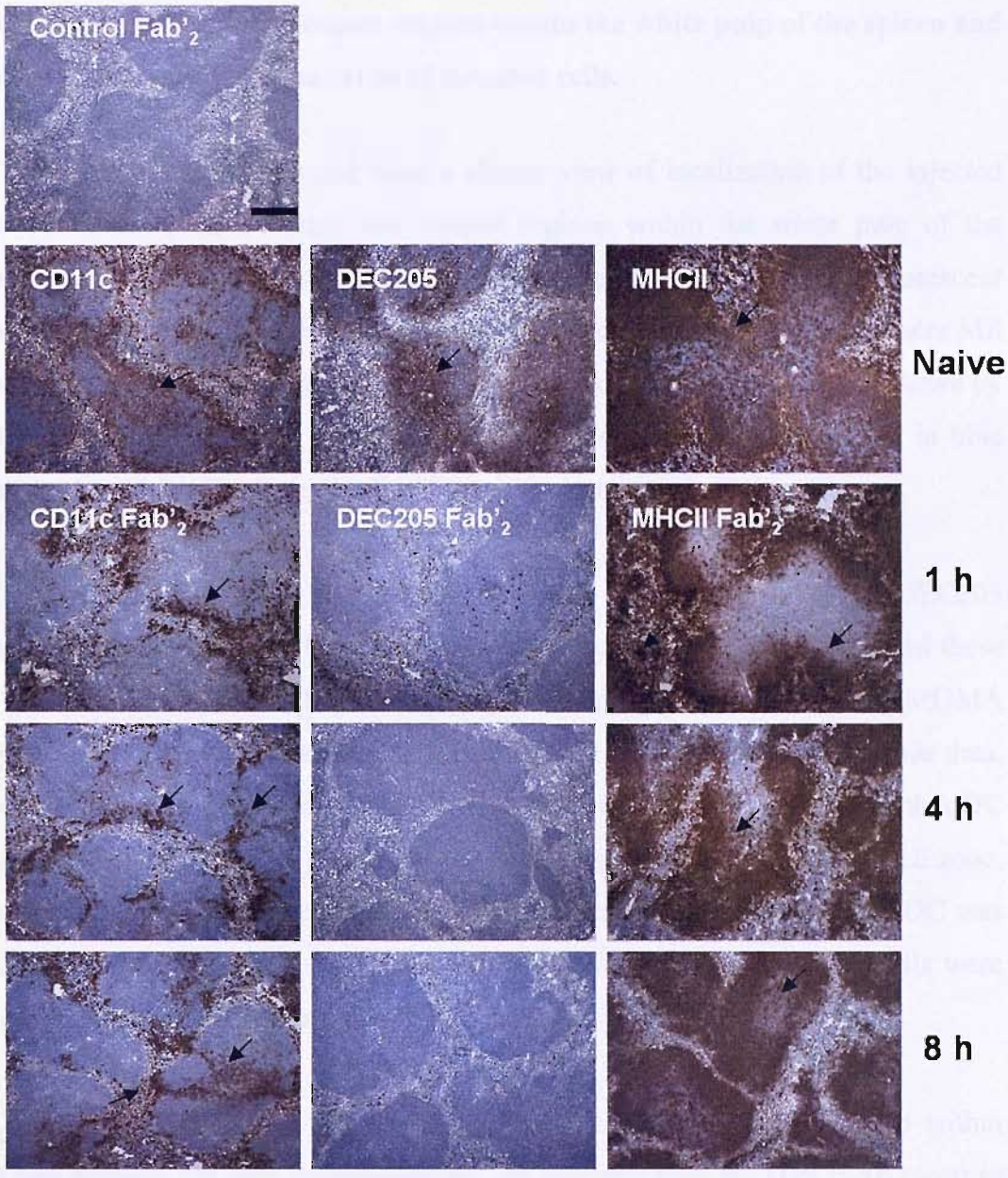


Figure 5.2.4 Immuno-histochemistry of spleen sections following injection with F(ab')$_2$ mAb.

Top panels: spleen sections from naïve mice were stained with α-CD11c, α-DEC205 or control mAb. Lower panels: mice were injected i.v. with 10 μg of FITC-conjugated α-CD11c, α-DEC205 or α-MHC-II F(ab')$_2$ mAb in combination with unlabelled α-CD40 mAb (50 μg). Spleens were removed 1h, 4h or 8 h later and spleen sections were stained with biotinylated α-FITC mAb. Biotinylated Ab were detected with streptavidin complexes. Data representative of 2 experiments. Sections were cut and processed by Sonya Martin. Arrows show cells labelled with the individual mAb and their movements within the regions of the spleen. Bar 200μm.

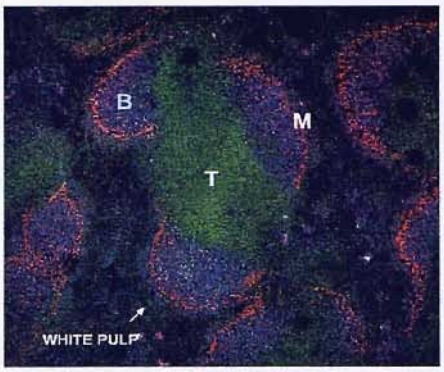
5.2.5 Identification of distinct regions within the white pulp of the spleen and the distinct migration patterns of targeted cells.

To confirm these results and have a clearer view of localization of the injected FITC labelled mAb within the distinct regions within the white pulp of the spleen, we labelled the spleen sections from the same animals with fluorescent mAb. Figure 5.2.5 (A) identifies the distinct regions of the white pulp, where MZ metallophillic macrophages form an even ring around the white pulp as shown by the cells stained in red. The B cells forming the follicles are here seen in blue staining and the T cells in the T cell zone in green.

The top panels of figure 5.2.5 (B) indicates the positioning of CD11c, DEC205 and MHC-II positive cells, however, in this experiment we double-labelled these spleen sections with α-CD19 mAb (red) to visualize B cell follicles or α-MOMA (red) which stains MZ metalophillic macrophages. Confirming the previous data, direct staining of DC with α-CD11c mAb (green) in naïve spleen, show that DC aggregate in the MZ but preferentially on the edges and inside of the T cell zone, avoiding the edges of the B cells follicles. The small subset of $DEC205^{+}$DC was mainly found within the T cell zone of the white pulp and $MHC\text{-}II^{+}$ cells were present in the MZ and throughout the white pulp.

When analysing the movement of the FITC conjugated targeting mAb within these spleens, we also double-labelled the sections with α-CD19 mAb (seen in red) to highlight the B cell follicles and an α-FITC mAb to track the movement of the mAb (seen in green). Figure 5.2.5 (B) supports the data in Figure 5.2.4, as the injection with FITC labelled α-CD11c $F(ab')_2$ mAb stained only the DC in the marginal zone, which aggregated around the T cell zone and there was no detectable staining of the DC within the T cell zone. As time progressed, more DC accumulated on the edges of the T cell zone and started their migration into this region (4 h and 8 h). Twenty-four hours later the staining was weak (data not shown), suggesting that the FITC labelled mAb have been endocytosed and are being digested by the DC.

A



B

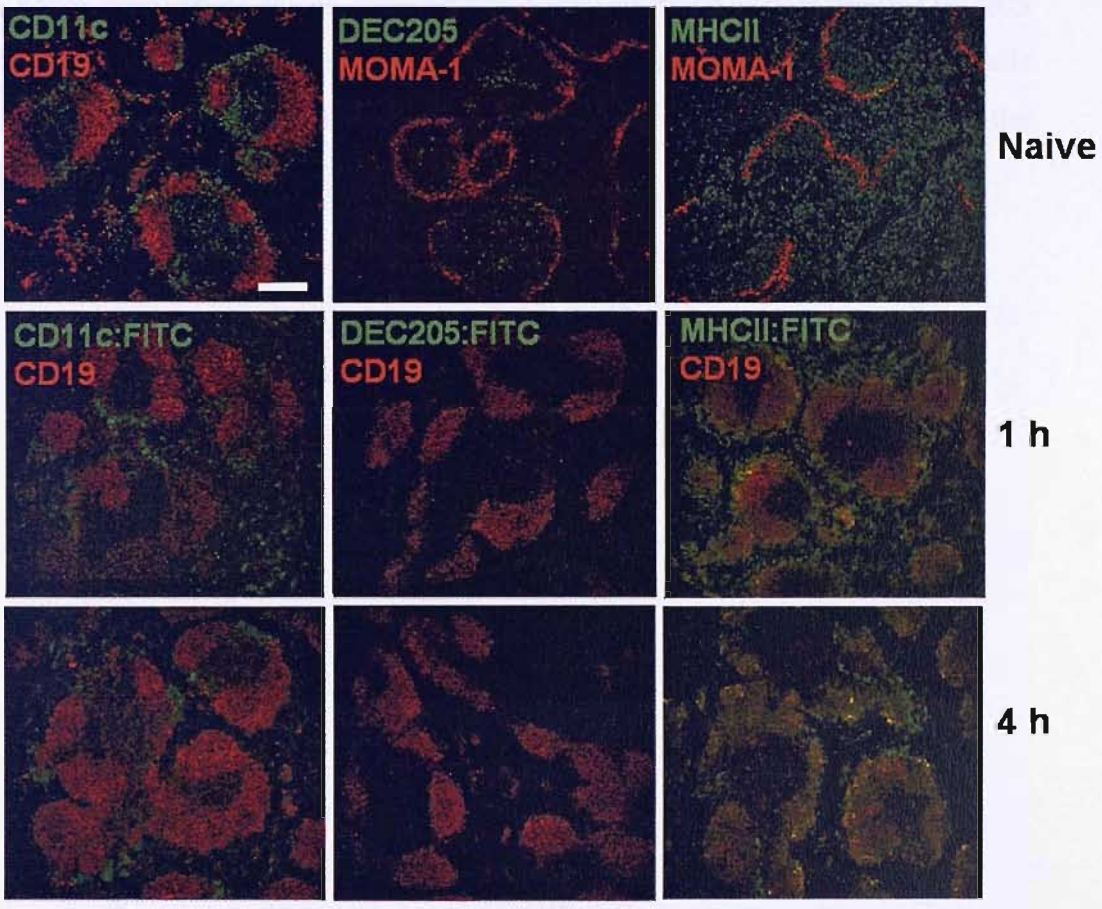


Figure 5.2.5 Fluorescence microscopy of spleens following injection with F(ab')$_2$-FITC mAb alone

(A) Sections of spleens from naïve mice were labelled with α-CD3 (T cells, green), α-CD19 (B cells, blue) and α-MOMA mAb (marginal zone metallophillic macrophages, red). Sections were analysed by immunofluorescence microscopy. (B) Top panels: sections of naïve spleens were double-stained with α-CD11c, α-DEC205 or α-MHC-II mAb (green) and α-CD19 mAb (B cells, red) or α-MOMA (metallophillic macrophages, red). Lower panels: mice were injected i.v. with 10 μg of FITC-conjugated α-CD11c, α-MHC-II, α-DEC205 or α-Control F(ab')$_2$ mAb in combination with unlabelled α-CD40 mAb (50 μg). Spleen were harvested at different time points and sections double-stained with α-CD19 mAb (B cells, red) and Alexa-Fluor α-FITC Ab (green). Spleen sections were cut and processed by Sonya Martin. Bar 200μm.

One hour following the injection with FITC-labelled α-MHC-II mAb, the staining clearly highlights the APC in the MZ, B cells at the edge of the follicles (yellowish colour), and macrophages in the red pulp. Three hours later, most of the labelled cells are found either within the B cell follicles or migrating into the T cell zone from the MZ.

Lastly, we looked at the distribution of FITC labelled α-DEC205 mAb and as before, there was no staining of any cell type within the spleen after 1 or 4 hours, even though the $DEC205^{+}$DC cells were clearly visible within the T cell zone of the white pulp when spleen sections were stained directly with the α-DEC205 mAb, which emphasizes the previous data where injection with α-DEC205 fails to accumulate in the spleen, most probably because the mAb cannot reach the target cells in the deeper regions of the T cell zone.

5.3 Discussion

We have demonstrated here that intravenously injected α-CD11c and α-MHC-II mAb rapidly accumulate in the spleen, in contrast to α-DEC205 mAb (Figure 5.2.1). Moreover, the data from our attempts to enhance the accumulation of α-DEC205 mAb, suggest that the concentration of injected mAb was not the limiting factor (Figure 5.2.2), but rather, the number of targets available for the mAb.

Administration of FLT3-L has been reported to increase the number of splenic DC by approximately 5-7-fold (Figure 3.2.1 (C)) [308], without altering their maturation status. Pulendran B et al [89] demonstrated that the spleens from mice treated with FLT3-L were enlarged by approximately 2 to 3-fold (as observed here) and had the integrity of their structure perturbed and characterized by the shrinkage of the B cell follicles and an expansion of the MZ and T cell zone in the white pulp. In addition, this study highlighted that $CD8^-$DC were mainly found in the MZ, whereas $CD8^+$ $DEC205^+$DC were in the deep regions of the T cell zone, in agreement with our findings (Figure 5.2.4 and 5.2.5). Therefore, it is not surprising that by increasing the number of all subsets of splenic DC with FLT3-L, including the $CD8^+$ $DEC205^+$ DC in the T cell zone, resulted in the increase of α-DEC205 mAb retention in the spleen. Furthermore, the disruption of the distinct compartments within the spleen may also have increased the accessibility of the mAb (Figure 5.2.3) to this area.

In addition to the biodistribution data, we also traced the movement of the individual targeting mAb once they entered the spleen (Figure 5.2.4 and 5.2.5). Not surprisingly, administration of α-CD11c and α-MHC-II mAb resulted in the intense staining of splenocytes. Within one hour from α-CD11c mAb administration with α-CD40 mAb, there was strong labelling of MZ DC and/or Ab-bound circulating DC drained into the MZ. With time, these cells clustered around the "doors" of the T cell zone and started to migrate towards the deeper regions where they can interact with naïve T cells and initiate adaptive immunity [91, 309].

Similarly, α-MHC-II mAb first labelled APC residing in the MZ, red pulp and B cells on the edge of the B cell follicles. Within four hours, most of the staining of macrophages in the red pulp had diminished, as they rapidly catabolized the mAb. As with α-CD11c mAb, α-MHC-II mAb did not stain the deeper regions of the white pulp, discounting the possibility that the T and B cell zones of the spleen are freely accessible to soluble Ag after intravenous injection as first proposed by tracer studies [310], though it is possible that very small amounts of free Ag enter this compartment but are not detectable in our assays. These results were challenged by evidence that the accessibility of lymph [311] and blood borne particles [312] to the lymphocyte areas in the lymph node and spleen, is restricted on the basis of size by the conduit system in these organs.

Nolte et al [312] reported that large molecules, such as mAb (~150 KDa), are unable to reach the white pulp in the spleen, whereas small molecules (< 70 KDa) like chemokines, can directly permeate this compartment. In accordance with our data, when they intravenously injected a mAb against B220 (mainly expressed by B cells), only the cells found in the red pulp and marginal zone of the spleen were accessed and follicular B cells were excluded. Our data show that four hours after injection there is a gradual increase in number of labelled B cells within the follicles, which indicates that recirculating B cells that had encountered the α-MHC-II mAb as they entered the MZ or on the edge of the follicle, initiated their migration towards the deep regions (B - T cell zone boundaries) of the white pulp for the acquisition of T cell help [313, 314]. The migration of lymphocytes is thought to be mediated by their distinct chemokine receptors profiles following activation. Most importantly, these results emphasise the importance of DC in their ability to capture blood borne Ag in the periphery or MZ and efficiently transport them to lymphocytes residing in the deeper regions of the white pulp.

Moreover, the most significant result from these experiments was the lack of DC staining following the administration of α-DEC205 mAb, even though $DEC205^{+}DC$ were detected within the deep regions of the white pulp (Figure 5.2.4 and 5.2.5). These findings support our biodistribution data, which showed no accumulation of this mAb to the spleen at any time point. Clearly, the fact that

DEC205$^+$ DC were located mainly in the deep regions of the T cell zone, suggest that the main reason for the failure of this mAb to accumulate in this organ is due to the restrictive location of the subset of DC expressing the target molecule.

Nevertheless, it is puzzling why we have observed an increase in the accumulation of α-DEC205 mAb to the spleen of mice pre-treated with FLT3-L (Figure 5.2.3) or LPS (data not shown) in the biodistribution studies, since the increase in DC number or expression of DEC205 on activated DC would not affect the ability of the mAb to reach the target cells within the T cell zone. Although FLT3-L treatment has been reported to affect the integrity of the local structure of the spleen [89], the responses to LPS and FLT3-L treatment raise the possibilities that DEC205$^+$DC have either encountered the mAb (or any soluble Ag) outside the T cell zone, or that the free mAb has access to the deep areas of the white pulp. In both cases, an increase in the number of DC or on the expression of DEC205 on these cells would lead to the accumulation of this mAb to the spleen.

Ascertaining the factors contributing to these results is difficult, because many aspects of iDC migration to specific compartments of the lymphoid organs and the influence of the anatomical structure on the availability of Ag to resident cells remain unclear. When considering the possibility that mAb or soluble Ag may have access to the deep regions of the T cell zone, there are studies suggesting that only small soluble molecules, such as chemokines, can permeate the white pulp via the conduit system [312] (as previously discussed) but the molecular weight cut-off for the entry of these molecules has not been precisely determined. However, recent work on the conduit network in the lymph nodes suggest that even though large amounts of soluble molecules do not pass from the subcapsular sinus or conduits into the T cell area, leakage of soluble Ag into these areas may actually occur in some circumstances [245, 315].

This idea is also supported by studies of the lymph node structures, which showed that collagen sheaths surrounding the high endothelium venules (HEV) contain small pores, and so it is reasonable to assume that the cells nearest to the conduits would have access to these Ag [316]. More recently, Sixt M et al [317]

reported the strategic positioning of scattered resident DC along the basement of the conduits in the lymph nodes that were able to take up and present soluble Ag to T cells before the migrating DC reaches this organ. Migrating DC that had already encountered Ag in the periphery did not associate with the conduit system, probably as a result of morphological changes associated with their maturation status.

Most importantly, these findings indicate that the conduit network is not only important for the transport of small molecules that affect the recruitment of lymphocytes to the lymphoid organs but also plays a role in the direct sampling of soluble Ag by DC residing in close association with the conduits, and in this way, DC and other lymphocytes can be rapidly informed of the status of the periphery. Therefore, based on the recent data which may also be relevant for the spleen, we cannot rule out the possibility that a small amount of circulating α-DEC205 mAb may have had access to the T cell zone but at very low levels which could not be detected in our assays until the number of DC or DEC205 molecules on the DC was increased and overcame the sensitivity limitations of the assays.

Alternatively, the other possibility is that immature circulating $DEC205^{+}$DC might have encountered the mAb in the blood or MZ compartment before they reached the white pulp and this small number of cells was enough to efficiently prime naïve T cells once they reach the T cell zone. When spleen section were directly labelled with α-DEC205 mAb, there was a small number of positive cells scattered around the MZ and possibly the red pulp, as well as the dense area in the T cell zone, which indicates that these cells may have had access to the soluble Ag prior to their migration into the white pulp and, therefore, an increase in the number of DC or DEC205 would increase the binding of the mAb to these cells.

Despite our limitations in determining how the Ag actually reaches the $DEC205^{+}$ DC subset, an important point to be raised from these experiments is that only a very small amount of Ag needs to actually reach these cells to effectively induce the priming of T cells, as shown by the strong CD8 T cell response observed

following immunization with [α-DEC205xOva]. Based on these results, $CD8^+$ DC have to be extremely efficient in the capture of Ag and cross-priming of CD8 T cells, which agrees with the literature suggesting that this subset favours the cross-priming of CD8 T cells conversely to $CD8^-$ DC that favour CD4 T cell priming [223, 318].

Thus, in this case, targeting via CD11c induces a stronger CD8 T cell response than DEC205 mainly because of its easy accessibility to a large number of $CD11c^+$DC expressing high levels of CD11c, which overall can compensate for its lower efficiency cross-priming CD8 T cells. Or alternatively, and more likely, in view of the recent discoveries that $CD8^+$DC are the main cells capable of efficiently priming CD8 T cells, it is possible that the Ag bound to CD11c on MZ $CD8^-$DC are involved in the transfer of intact Ag to the $CD8^+$DC subset and B cells once they migrate into the T cell zone. Therefore, it is likely that the strong immune response seen with [α-CD11cxOva] is not only a result of target availability, as this molecule has been shown to have unique properties that could be of therapeutic value in a clinical setting, such as the fact that Ag delivered via CD11c is available for the priming of CD4 T cells for longer periods than when DEC205 is targeted, and CD11c is the only target capable of rapidly generating high titres of Ab in response to a single low dose of Ag without adjuvant.

Chapter 6 Immunotherapy

6.1 Introduction

Significant progress in the understanding of the molecular characterization of immune responses against tumours, as well as, advances in DC immunobiology has opened up new opportunities for the development of effective immunotherapies for cancer patients.

There is now unequivocal evidence, from both animal and humans, that the immune system can recognize and destroy tumour cells. However, the response against TAA is usually weak and this has provided a major stimulus for the development of new immunotherapies capable of amplifying anti-tumour immunity leading to effective treatment.

While both humoral and cellular effector mechanisms can contribute to tumour lysis, the latter are felt to be responsible for tumour regression in the majority of the cases. $CD8^+$ cytotoxic T cells, in particular, have been proposed to recognize and kill tumour cells in various tumour models [319, 320]. Studies on the Ag specificity of tumour-reactive T cells derived from patients and from in vitro models have enabled the identification of an array of potential targets for the therapy of different cancers. These TAA include proteins with tumour-specific mutations, tissue-specific differentiation Ag, reactive embryonic Ag, viral proteins, and a range of self-proteins. Unfortunately, despite the recent advances in this field, the development of clinically effective cancer vaccines has been limited by the weak immune responses generated against these proteins, as they are generally self-proteins to which the immune system has been tolerized.

Since 1973, when Steinman and Cohn first described a new cell type named DC [321], a vast number of studies have highlighted the crucial role of DC in priming T cells against all classes of pathogens and tumours. In addition, DC are also crucial for the generation of immunological memory and humoral responses. Taken together these findings indicate that DC are important candidates for the targeting of Ag to induce potent and effective adaptive responses against tumours. Although now many clinical trials exploiting the efficacy of DC-based vaccines are under way and have been reported to show promising results, there

is still many unanswered questions that need to be clarified, including which are the most effective DC, the most effective protocol design, how to immunomonitor and which patient population should be included.

In this chapter, we investigated the therapeutic effectiveness of our [FabxOva] conjugates as potential cancer vaccines and attempted to determine the effector cells responsible for the therapeutic effects observed in our B16 melanoma animal model.

6.2 Results

6.2.1 Immunization with [FabxOva] conjugates can prevent tumour development.

Our functional assays have assessed the effectiveness of the [FabxOva] conjugates and the results show that targeting Ag to CD11c, DEC205 or MHCII molecules evokes the strongest anti-Ova immune responses. To evaluate if these results could be translated to a clinical setting, we performed preliminary experiments investigating their effectiveness as anti-tumour vaccines in a subcutaneous B16 melanoma model.

Mice were vaccinated with a single low dose of [FabxOva] conjugates (2.5 μg) and α-CD40 mAb (50 μg) intravenously and one month later, they were challenged at a distal site with B16 melanoma cells stably transduced with Ova. The homogenous expression of soluble Ova within these cells (seen in green) was confirmed by fluorescence microscopy (Figure 6.2.1 (C)). The results shown in Figure 6.2.1 (A) demonstrate that the therapeutic performance of the conjugates reflected the trends observed in our functional in vivo experiments, in particular, immunization with [α-CD11cxOva] induced the longest protection against tumour development. Although immunization with [α-DEC205xOva] was shown to evoke a weaker CD8 T cell response than [α-CD11cxOva] (refer to Figure 3.2.3 and 3.2.4), this difference was not translated to a major difference in therapeutic efficacy.

By day 14, the tumours in the control groups (untreated and α-CD40 mAb alone) had reached the maximum size permitted under Home Office regulations, whereas the tumours in the animals vaccinated with [α-CD11cxOva] or [α-DEC205xOva] had only just started to develop. Immunization with 1 mg of soluble Ova protein, which has a 2000-fold higher concentration than that present in the conjugates, showed comparable protective effects to those observed following treatment with [α-MHCIIxOva]. Nonetheless, both were less efficacious than [α-CD11cxOva] and [α-DEC205xOva]. In addition,

immunization with soluble Ova at comparable amounts to that present in the conjugates, failed to enhance tumour resistance as tumour progression was similar to that seen in the control groups.

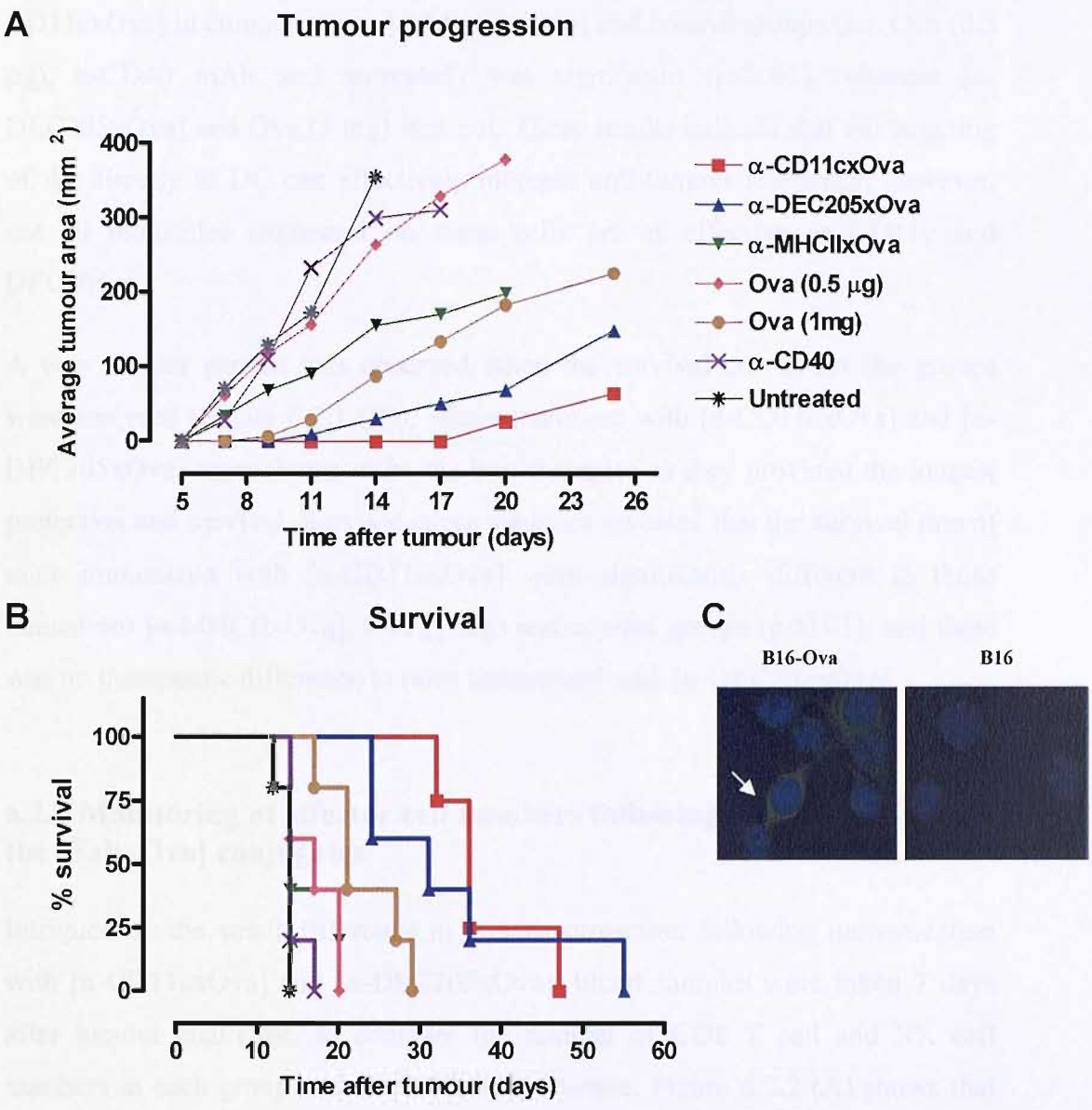


Figure 6.2.1 Therapeutic activity of [FabxOva] conjugates as anti-tumour vaccines in a melanoma model.

Mice were immunized with [FabxOva] (2.5 μg) or soluble Ova (0.5 μg or 1 mg) and α-CD40 mAb (50 μg) i.v. 30 days prior to s.c. challenge with B16-Ova melanoma cells ($4x10^6$). Tumour development was monitored. (A) Average tumour area. (B) Survival curves, mice were culled when the tumour area was above $300mm^2$. Data representative of 2 individual experiments (n = 5 mice/group). (C) Expression of intracellular Ova by stably transduced B16-Ova cells. B16-Ova melanoma cells were permeabilized with 0.5 % TritonX-100, labelled with a FITC-conjugated primary α-Ova Ab and stained with the secondary Alexa-488 conjugated α-FITC Ab (green). The cytospins were counterstained with Sytox-Orange (nucleus, blue).

The data in Figure 6.2.1 is presented as average tumour sizes for clarity purpose, however, t-test statistical analyses of individual tumour sizes measured at day 14, indicate that the difference in tumour development after immunization with [α-CD11cxOva] in comparison to [α-MHCIIxOva] and control groups (i.e. Ova (0.5 μg), α-CD40 mAb and untreated) was significant ($p<0.05$), whereas [α-DEC205xOva] and Ova (1 mg) was not. These results indicate that the targeting of Ag directly to DC can effectively increase anti-tumour resistance; however, not all molecules expressed on these cells are as effective as CD11c and DEC205.

A very similar pattern was observed when the survival curves for the groups were analysed (Figure 6.2.1 (B)), where treatment with [α-CD11cxOva] and [α-DEC205xOva] were shown to be the best therapies as they provided the longest protection and survival. Survival curve statistics revealed that the survival rate of mice immunized with [α-CD11cxOva] were significantly different to those treated wit [α-MHCIIxOva], Ova (1 mg) and control groups ($p<0.01$), and there was no therapeutic difference to mice immunized with [α-DEC205xOva].

6.2.2 Monitoring of effector cell numbers following immunization with the [FabxOva] conjugates

Intrigued by the small difference in tumour protection following immunization with [α-CD11cxOva] and [α-DEC205xOva], blood samples were taken 7 days after tumour challenge, to compare the number of CD8 T cell and NK cell numbers in each group with their clinical outcome. Figure 6.2.2 (A) shows that immunization with [α-CD11cxOva] generated the highest number of CD8 T cells, followed by [α-DEC205xOva], Ova (1 mg) and [α-MHCIIxOva], which agrees with the functional data discussed in previous chapters and suggests that the number of effector CD8 T cells cannot be used to predict the clinical outcome in our tumour model.

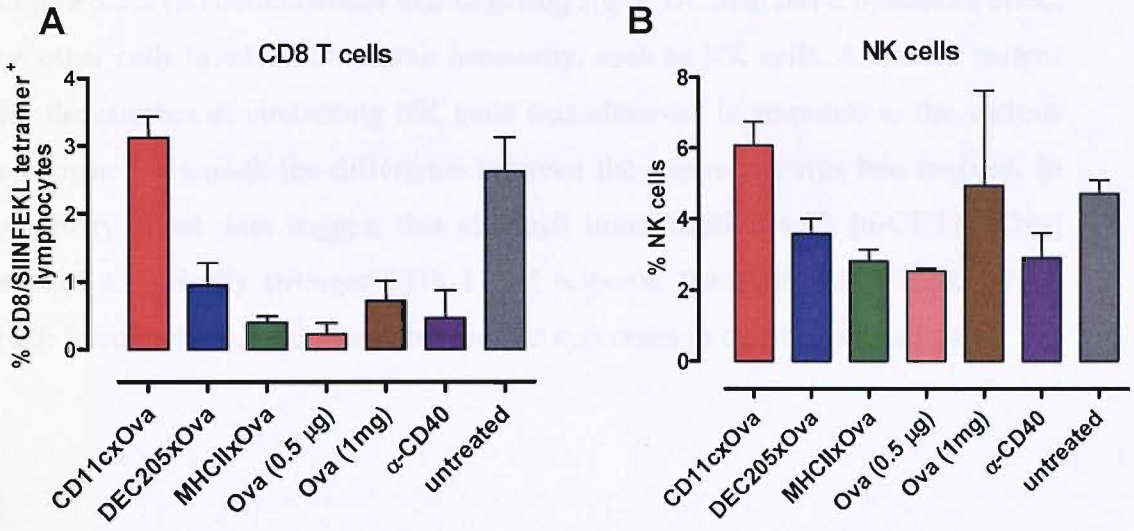


Figure 6.2.2 Generation of effect CD8 T and NK cells following immunization with the different [FabxOva] conjugates.

The mice shown in Figure 6.1 were monitored for tumour development and bled 7 days after challenge with B16-Ova ($4x10^6$) melanoma cells s.c.. (A) The number of circulating Ova-specific CD8 T cells was measured by staining with α-CD8 mAb and SIINFEKL tetramer. (B) Blood samples were also stained with α-NK1.1 mAb and the number of NK cells measured by flow cytometer.

The magnitude of the endogenous CD8 T response here is higher than that previously reported in chapter 3 (Figure 3.2.7 (A)), because in this case we are actually measuring the secondary response to Ova expressed by the tumour cells. Moreover, even the untreated animals, which were not immunized prior to tumour challenge, have high numbers of Ova-specific CD8 T cells, suggesting that the tumour itself has a degree of immunogenicity. However, despite having high numbers of circulating Ova-specific CD8 T cells, these naïve mice were still unable to control the development of the tumours as they only survived for 12 days after tumour challenge. Furthermore, because the tumour cells were administered in the absence of a maturation signal, it seems likely based on our in vivo data discussed in chapter 3 (Figure 3.2.4) that these untreated animals have been tolerized against Ova, which might explain their poor therapeutic outcome.

Figure 6.2.2 (B) demonstrates that targeting Ag to DC also has a bystander effect on other cells involved in tumour immunity, such as NK cells. A similar pattern for the number of circulating NK cells was observed in response to the various conjugates, although the difference between the treatments was less marked. In summary, these data suggest that although immunization with [α-CD11cxOva] evokes a markedly stronger CD8 T cell response than with [α-DEC205xOva], both treatments showed somewhat similar outcomes in our melanoma model.

6.3 Discussion

In this chapter, we investigated the efficiency of the [FabxOva] conjugates as prophylactic vaccines and these preliminary data demonstrated that inoculation with [α-CD11cxOva] and [α-DEC205xOva] together with α-CD40 mAb are the best vaccines for the prevention of tumour development. Although, the functional data for [α-DEC205xOva] showed that this conjugate is not as potent in the generation of T cell immunity as [α-CD11cxOva] (Figure 3.2.4 and 3.2.6) and it is unable to generate Ova-specific mAb (Figure 4.2.1), therapeutically, its protective effects are not significantly different from [α-CD11cxOva] (Figure 6.2.1 (A)). Nevertheless, immunization with these two conjugates was clearly more effective than α-CD40 mAb alone or soluble Ova at equivalent concentration to the conjugates. Although the [α-MHCIIxOva] conjugate showed some efficacy in slowing down the development of the tumours, this did not translate into an increase in survival (Figure 6.2.1(B)).

Furthermore, these results indicate that even though there is a relationship between the trend observed for the treatments evoking the highest numbers of effector cells and tumour regression, the absolute number of CD8 T cells could not be used to predict therapeutic outcome in our model (Figure 6.2.2). However, Bonifaz et al [69] reported that in a similar system to ours, immunization with DEC205 mAb linked to Ova in the presence of α-CD40 mAb, resulted in the protection against B16-Ova melanoma and this protective response was dependent mainly on CD8 T cells and to a much lesser extent, CD4 T cells, which was demonstrated by the decreased resistance to tumour challenge in CD8 T or CD4 T cell knock-out animals.

Although it has been suggested that T cell response to tumour Ag, by way of IFN-γ release, is associated with tumour regression and can be used to predict clinical efficacy [42, 322, 323], often, studies report that the level of functional Ag-specific T cells, detected by assays such as ELISPOT or CTL lysis does not correlate with clinical observations [27, 48, 324]. The significance of a high number of CD8 T cells measured by MHCI-tetramers in relation to tumour rejection is unknown, as in some studies, MHCI-tetramer positive T cells were

found to be functionally anergic in the tumour setting [325] and therefore unable to eradicate tumour cells.

Even though our results show that [α-CD11cxOva] is able to induce bona fide effector cells (Figure 3.2.7), it is possible that in the presence of tumour, many may be rendered anergic and tolerized against Ova, but this remains to be addressed. Nevertheless, as with most animal models and clinical studies using DC-based vaccines, the treatment was well tolerated with minimal local toxicity and no problems associated with autoimmunity, which is of significant relevance to their use as therapeutic agents.

Although the targeting of Ag to DEC205 has been studied by many groups, there are no published data on the effect of Ag delivery via CD11c on T cell immunity. The targeting of mAb harbouring peptide antigens to CD11c in vivo has been reported to induce peptide-specific humoral responses [70, 71], which leads to the presumption that this treatment also provides the T cell help required by B cells, but our studies provide clear evidence that the targeting of this molecules induces potent CD8 and CD4 T cell responses, as well as a robust a humoral response.

The data from a different delivery system, in which Ag-containing liposomes were coated with CD11c or DEC205 mAb, demonstrate that this approach can induce CTL responses and protect mice against subsequent tumour challenge, provided either IFN-γ or LPS was co-administered. Similar to our findings, liposomes coated with α-CD11c or α-DEC205 were equally effective as vaccines [326].

Moreover, we have performed pilot studies assessing the effectiveness of the conjugates in a model where established B16 tumours were treated with the conjugates, in addition to experiments using a different tumour model, where we assessed the growth of the T cell lymphoma EL-4 cells stably transduced with Ova (data not shown). These collective data suggest that [α-CD11cxOva] and [α-DEC205xOva] are indeed the best vaccines for the induction of tumour

protection; however, further experiments need to be done to establish which vaccine is most effective under different conditions.

Nevertheless, these encouraging preliminary data emphasize that vaccines directly delivering Ag to CD11c and DEC205 on DC in vivo have promising therapeutic properties for the treatment of different tumours. Currently, there are no published clinical data on the effectiveness of directly delivering tumour cells or peptides to surface molecules on DC in situ. So far, with the exception of DNA vaccines and viral vectors, all the clinical trials investigating the effectiveness of DC-based vaccines are focused on ex vivo generated DC that require laborious in vitro culturing and Ag loading protocols, which account for most of the costs associated with these trials. This highlights the importance of the strategy reported here as, if successful, this would circumvent these limitations. Until then, the current data on ex vivo DC vaccines will help us to establish the parameters for in vivo targeting, which is the next step in the development of DC-based vaccines.

Chapter 7 General discussion

The immunogenicity of tumours has been established in various models but the immune response against tumour associated Ag is generally unable to cope with established disease. Therefore, we developed an Ab-based strategy to target Ag to DC in situ, thereby amplifying Ag-specific T and B cell immunity, which generated encouraging therapeutic results in a mouse melanoma model.

The in vitro targeting of Ova to splenic DC highlighted that the three most effective conjugates in the priming of T responses are [α-CD11cxOva], [α-DEC205xOva] and [α-MHCIIxOva], all of which evoked equally potent responses (Figure 3.2.2). Interestingly, when Ova is delivered to DC in vivo, [α-CD11cxOva] is by far the most efficacious conjugate in inducing CD4 and CD8 T cell expansion (Figure 3.2.3 and 3.2.4). These results suggest that the anatomical structure of lymphoid organs may have a profound effect on the bioavailability of the targeted conjugates, which might affect the overall potency of these vaccines. Nevertheless, throughout this work, both in vitro and in vivo, targeting of DEC205 favoured the priming of CD8 T cells, whereas MHC-II targeting favoured CD4 T cells.

These cumulative data suggest that immunization with [α-CD11cxOva] generates the strongest CD4 and CD8 T cell responses because it targets Ag to the large number of CD11c molecules expressed on the easily accessible and numerous $CD8^-$DC. This DC subset has been demonstrated to migrate from the MZ into the T cell zone of the spleen when activated [89], where they can effectively prime CD4 T cells and transfer Ag to $CD8^+$ DC enabling the priming of CD8 T cells. Therefore, $CD8^-$DC targeted by [α-CD11cxOva] and [α-MHCIIxOva] might be involved in the transfer of Ag to other DC subsets (i.e. $CD8^+$DC) located in less accessible compartments of the spleen. Indeed, DC have been reported to capture and transport Ag from the periphery into the lymphoid organs and recent evidence suggests that migrating DC are capable of transferring intact Ag to the $CD8^+$DC subset [266, 267, 269, 270] which is thought to be most potent, if not the only, subset capable of cross-priming naïve CD8 T cells and generating effector CTL [234].

In contrast, targeting to MHC-II molecules favours the priming of CD4 T cells but fails to prime CD8 T cells, which suggest that the ability of $CD8^-$DC to retain and transfer intact Ag relies on CD11c molecules. Importantly, despite the restricted and lower expression of DEC205 on $CD8^+$DC, it is clear from our results that this subset efficiently cross-prime CD8 T cells even when very low level of Ag is available [265]. These findings emphasize the importance of assessing the effectiveness of a potential therapeutic agent on in vivo models, as the bioavailability of the target molecules can have a profound effect on the overall efficacy of potential therapeutics.

The issue of DC applications in clinical trials has been complicated by reports demonstrating that iDC can induce tolerance. Injections of peptide-pulsed iDC, but not mature DC, in healthy persons can result in Ag-specific inhibition of effector T cell functions [50]. Indeed, immunization with [α-DEC205xOva] [69] or [α-CD11cxOva] in the absence of the maturation signal, provided by α-CD40 mAb, induces the initial proliferation of CD8 T cells but these fail to expand and are rapidly deleted (Figure 3.2.4). Clonal deletion is a major mechanism by which tolerance is established [199] and not unexpectedly, Ag re-challenge of these mice is unable to evoke a secondary response, confirming that targeting of iDC leads to Ag specific tolerance in this model.

The high numbers of T cells generated by the immunization with our conjugates, in particular with [α-CD11cxOva], were demonstrated to be bona fide effector cells when examined using assays routinely used for the monitoring of responses in patients involved in clinical trials. In agreement with the proliferation data, immunization with [α-CD11cxOva] in the presence of α-CD40 mAb, generates the highest number of endogenous IFN-γ secreting effector CD8 and CD4 T cells, followed by [α-DEC205xOva] and [α-MHCIIxOva] (Figure 3.2.7). Many reports have provided strong evidence that signalling of CD40 on DC is the prime means of licensing DC for CTL generation [165, 167] and indeed, it was only when α-CD40 mAb was present that the proliferating CD8 T cells behaved as CTL capable of selectively eradicating splenocytes loaded with Ova (Figure 3.2.7 (C)).

Interestingly, DC were observed to present Ova-derived peptides on both MHC-I and -II complexes for long periods of time, and in addition, the conjugate used for the delivery of the Ag had an impact on the length of peptide presentation and to which T cell subset it was favourably presented to. Here, we demonstrated that delivery of Ag via CD11c or DEC205 resulted in a prolonged presentation of peptide complexed with MHC-I molecules, which lasted at least 7 days (Figure 3.2.8). Furthermore, targeting of CD11c favourably presents Ag to CD4 T cells in contrast to DEC205, as substantial CD4 T cell proliferation was still induced after four days from immunization with [α-CD11cxOva], whereas the response to [α-DEC205xOva] was abolished after one day. This prolonged presentation of Ag to CD4 T cell following immunization with [α-CD11cxOva] led us to further investigate its importance in the initiation of humoral responses.

Cellular immunity is generally thought to be responsible for the immunologic rejection of tumours, based on studies showing that the transfer of T cells but not Ab, protects mice from tumour challenge; elimination of endogenous CD8 T cells abrogates both protective and therapeutic antitumour effects; and extensive T cell infiltrates are commonly seen in tumours and allografts undergoing rejection. However, since the mid-1990s, mAb have emerged as important new anti-cancer therapeutics emphasising the role of mAb in the eradication of tumours. Importantly, of all our conjugates, only immunization with [α-CD11cxOva] rapidly generates high titres of Ova-specific IgG, which lasts for longer than a month (Figure 4.2.1). In accordance with our data, a few studies have reported that delivery of Ag via CD11c is characterised by a striking ability to evoke a rapid and strong Ab response [70, 71]. Furthermore, this potent humoral response is not dependent on the presence of an adjuvant, as immunization with α-CD40 mAb or various TLR-L has no impact on the IgG titres (Figure 4.2.1 and 4.2.4).

To stimulate an Ab response, the native Ag must be recognised not only by DC but also by B cells, which indicates that Ag targeted to CD11c can be made available for B cells, supporting studies demonstrating that DC are capable of transferring intact Ag to B cells [198, 298]. Furthermore, the fact that a maturation signal was not essential for the generation of this strong humoral

response unlike for the priming of a robust CTL response, also indicates that the main role of CD11c played in the initiation of an Ab response is to transport and transfer intact Ag to B cells, and not necessarily to provide co-stimulatory signals to B cells, unless these signals are provided regardless of the DC maturation status. Nevertheless, the fact that immunization with [α-CD11cxOva] is also accompanied by robust CD8 and CD4 T cell responses indicates that a proportion of the bound Ag must be also internalized and processed.

These data could be interpreted as being consistent with observations that targeting of CD11c delivers Ag to the $CD8^-$DC subset, which has been reported to prime T cells and induce strong humoral responses, in contrast to the $CD8^+$DC subset which is mainly associated with efficient cross-priming abilities [288, 289] as previously discussed. Moreover, these findings also suggest that independent of the DC subset being targeted, the internalization of the vast number of CD11c molecules on DC is either very slow or it recycles back to the surface, thereby enabling the transfer of native Ag to B cell and possibly the $CD8^+$DC subset, as proposed earlier. In parallel, the proportion of Ag that is internalized, is processed and presented to CD4 T cells which provide the required help for B and CD8 T cell maintenance and differentiation.

Conversely, DEC205 has been reported to efficiently internalize Ag and deliver it to the lysosomal compartment where it is degraded for T cell presentation, in particular to CD8 T cells. Indeed, Steinman et al [305] proposed that Ag delivered via DEC205 is transported to the late endosomal compartment as a result of its EDE cytoplasmic tail sequence, which facilitates the loading of peptide into MHC complexes. Thus, it is possible that its inability to generate an Ab response is not totally dependent on the specialization of the $CD8^+DEC205^+$ DC subset but more a consequence of its inability to retain intact Ag. Moreover, although [α-MHCIIxOva] targets the same DC subsets as [α-CD11cxOva], it is unable to evoke a potent mAb response in our model, which reinforces the notion that CD11c has the crucial ability to retain intact Ag independent of the DC subset being targeted. Alternatively, a proportion of the [α-MHCIIxOva] conjugate may be delivered directly to B cells, which is presumably not able to generate an Ab response, perhaps because it prevents Ova from being presented

by DC to CD4 T cells, which are also critically important for the mounting of a robust anti-Ova humoral response.

The therapeutic benefits associated with mAb treatment is a result of the ability of circulating mAb to bind to the target and either induce its lysis by complement- or antibody-dependent cellular cytotoxicity (CDC or ADCC, respectively) [327], or phagocytosis of the complex. Antibodies can be particularly effective at removing metastatic tumour cells, thus, provided an appropriate target can by identified, the unique ability of [α-CD11cxOva] to evoke a potent a humoral response may have very beneficial therapeutic effects.

The interaction between B and CD4 T cells via CD40:CD40-L signalling is known to be required for the formation of an effective humoral response to T dependent-Ag, as patients with a genetic mutation in the CD40-L gene have severely impaired Ab responses, lacking B cell memory and circulating IgG, IgA and IgE [328]. Indeed, for Ova, depletion of CD4 T cells in immunized mice completely abrogates the secretion of mAb in response to [α-CD11cxOva] (Figure 4.2.2). In contrast to the generation of CTL responses observed here (Figure 3.2.4), administration of α-CD40 mAb was unable to even partially restore the secretion of Ab by these depleted animals, which suggests that CD4 T cells are providing help directly to B cells via cytokines or additional costimulatory molecules (Figure 4.2.2). Other molecules that may be involved in this process include OX40/OX40L [300], BAFF and APRIL and their receptors (BAFFR, TACI, BCMA) [301], CD95/CD95L [302], ICOS/ICOSL, which has been suggested to act upstream of CD40-CD40L interactions [303].

Studies investigating the biodistribution of the targeting mAb over a period of time support our proposed hypothesis that the anatomical structure of the lymphoid organs has an impact on the availability of our targets. These studies demonstrated that α-CD11c and α-MHCII mAb, unlike α-DEC205 mAb, quickly accumulate in the lymphoid organs, in particular to the spleen (Figure 5.2.1 and 5.2.2). Furthermore, the immuno-histochemical and -fluorescent data (Figure 5.2.4 and 5.2.5), indicate that part of the effectiveness associated with [α-CD11cxOva] in the priming of T cells is a result of the easy accessibility to large

numbers of DC expressing high levels of CD11c molecules when the mAb is drained into the MZ of the spleen.

MZ resident cells include MZ- macrophages, MZ-B cells and MZ-DC, which are strategically located to capture blood borne pathogens. Thus, in this manner, the system ensures that Ag in the soluble form or complexed with circulating DC is transported to the edges of the white pulp in the spleen, where DC have been reported to initiate their migration into the T cell zone, where they can prime CD4 T cells thereby providing help for a humoral response [134]; and as proposed earlier, transfer Ag to $CD8^{+}$DC for the cross-priming of CTL. Hence, immunization of [α-CD11cxOva] delivers Ag to all subsets of DC and these are then able to migrate towards the T cell zone where they can interact with T and B cells.

In contrast to α-CD11c mAb, α-DEC205 mAb failed to accumulate in the spleen, which is intriguing because this conjugate can mediate strong CD8 T cell responses and therefore, must have access to the $CD8^{+}$DC subset located in the lymphoid organs. Difficulty visualizing the $DEC205^{+}CD8^{+}$ DC within the lymphoid organs mainly due to the small number of these intergitating cells found only in the deep regions of the T cell zone has been reported by many groups [91, 329]. However, the staining of these cells has been previously reported to be augmented by inducing their maturation with LPS [91, 330], as shown in our phenotypic profiles (Figure 3.2.1 (C)). Together these findings suggest that the reason why α-DEC205 mAb is not retained in the spleen is due to its inability to reach this small number of DC that in the absence of a strong danger signal or maturation signal is expressed at very low levels.

However, when mice were pre-treated with FLT-3L (Figure 5.2.3), which increases the number of DC in all subsets, or with LPS that induced their maturation (data not shown), there was a clear increase in the accumulation of α-DEC205 mAb to the spleen, which suggests this mAb can actually reach a proportion of the target cells and prime CTL (Figure 3.2.5). Possible ways in which this may occur include: (1) the leakage of soluble mAb through the conduit system, which has been reported to be responsible for the exclusion of

the large soluble molecules into the T cell zone [245, 315]; (2) a very small number of circulating $CD8^+$DC may be targeted by the mAb before reaching the T cell zone. In both cases, the increase in DEC205 expression or number of $DEC205^+$ DC would facilitate the accumulation of α-DEC205 mAb to the spleen, which is not normally detectable in our assays.

Most importantly, what these results highlight is the efficiency with which DEC205 on $CD8^+$ DC internalizes the Ag and primes the CD8 T cells, bearing in bind that only a very small number of cells are thought to be targeted by [α-DEC205xOva]. Unfortunately, due to the low expression of DEC205 on BMDC we have been unable to determine the kinetics of the internalization process (on-going research), but published data using cells transfected with DEC205 suggest that ligated DEC205 is endocytosed very rapidly; approximately 70 % of initially bound mAb was internalized within 15 minutes [305]. Although the internalization of CD11c has not been studied in detail, research by Rabb et al [331] on the endocytosis of its close relative, CR3 (CD11b/CD18), which is expressed on macrophages and NK cells and also binds iC3b, has demonstrated that in CR3 transfected cells ligated with α-CR3 mAb, approximately 18 % and 40 % of ligated CR3 receptors were internalized within 10 and 40 minutes, respectively.

More recently, Skoberne M et al [332] reported that CR3 is a poor phagocytic receptor on human DC, which ingested less than 10 % of initially bound complexes of red blood cell conjugated α-CD11b mAb in 2 hours, in contrast to 50 % by the integrin $\alpha_V\beta_5$. Therefore, it is possible that CD11c acts in a similar manner and some of its specific features, in particular, the strong Ab production in response to its targeting and proposed ability to transport and transfer Ag to DC, may be a consequence of a slow uptake of Ag or recycling of native Ag to the surface, making it available for MZ and follicular B cells, in addition to $CD8^+$DC.

Finally, we report here that the potent T cell responses observed in our functional assays with [α-CD11cxOva] were translated to a therapy model, where immunization with [α-CD11cxOva] clearly protected the animals from the

growth of B16 melanoma tumours (Figure 6.2.1). Despite the weaker CD8 T cell response evoked by [DEC205xOva] (Figure 6.2.2), animals treated with this conjugate were also shown to be protected after immunization (Figure 6.2.1). Although the difference in the therapeutic outcome between these two conjugates was not significant in this model, it clearly demonstrated that Ag targeting to DC in situ can be a very effective approach to the enhancement of immunity. Moreover, immunization with [α-CD11cxOva] is the only treatment to rapidly generate high titres of Ag-specific mAb, thereby increasing its therapeutic potential in the treatment of tumours that have been shown to be eradicated by mAb therapy.

In summary, we report here that the in situ targeting of Ag to DC via CD11c and DEC205 has a promising therapeutic value, which needs to be further investigated and validated in a clinical setting. Furthermore, the presented data advances our understanding of the possible mechanistic differences associated with the targeting of different molecules on distinct subsets of DC in vivo, and suggests for the first time, a novel role for CD11c molecules in the transfer of intact Ag to B cells and $CD8^{+}$DC. Together these may have an impact on the development of new DC vaccines aiming to amplify and modulate immunity to induce a positive outcome in the treatment of cancer and autoimmunity.

Further Work

The following studies could be performed to confirm our proposed hypotheses:

- To assess the contribution of each DC subset on the priming of CD4 and CD8 T cells following targeting with the different conjugates; DC subsets could be isolated using FACS sorting or MACS separation columns and loaded with the conjugates in vitro and their abilities to prime T cells assessed.

- The administration of α-CD40 mAb was unable to replace the help provided by CD4 T cell to B cells, therefore by using blocking mAb against other co-stimulatory molecules (e.g. OX40/OX40-L or BAFF/BAFFR) and by analysing the expression pattern of these molecules, their contribution towards the production of Ab could be determined.

- Experiments addressing the internalization rate of the distinct molecules being targeted by the conjugates:
  -DC from primary tissues or DC- lines or fibroblasts transfected with the target molecules at comparable levels could be targeted by fluorescently labelled soluble Ag, latex beads or mAb and analysed by FACS or confocal microscopy.
  -The internalization of ^{125}I-mAb could be determined by measuring the radioactivity of these cells following acid stripping of surface bound mAb.

- To visualise the transfer of intact Ag to other lymphocytes, CD11c molecules on DC could be targeted with a fluorescently labelled Ag and the transfer to other DC subsets or B cells assessed by confocal microscopy.

- Other tumour models should be used to investigate the efficacy of the conjugates further and the co-administration of mAb to deplete Treg (e.g.

α-CD25 mAb) could be used to amplify the efficacy of the conjugates even further. Monitoring of the levels and effector capabilities of CD8 T cells generated following treatment would also indicate any suppressive activity by the tumour on these cells.

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