Analysis of The Basis for Induction and Maintenance of T Cell Responses in DNA Vaccination

A thesis by:

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To My Parents

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ABSTRACT

This study is aimed at finding the role of dendritic cells in induction and maintenance of immune responses following DNA vaccination. For this purpose, two DNA vaccines were generated, one expressing full C5 cDNA (fifth complement of mice) and the other expressing just 1.6 kb of the 5' end of cDNA. Both vaccines express the epitope for recognition of the A18 T cell hybridoma and both induced C5 specific immune responses following i.m. inoculation of A/J mice. Three routes of entry i.e., (i.m.), abrasion (Scarification) of leg skin and abrasion of ear skin were chosen for inoculation with the DNA vaccine encoding the full C5 protein. Deep cervical lymph nodes were found to be the best targets following DNA inoculation of the ear. Kinetic analysis of these lymph nodes showed the highest T cell immune response 10 to 12 days following DNA vaccination. While all routes of vaccination led to C5 specific T cell responses with time, an early response was detected in cervical lymph nodes following vaccination in the ear.

Isolation of dendritic cells from cervical lymph nodes following DNA vaccination allowed detection of C5 expression. C5 was actively expressed in a DC enriched population while the other cells in lymph node showed no expression. That suggested direct transfection of DCs

following DNA vaccination. However, while DNA vaccination resulted in transfection of a small proportion of dendritic cells only, it led to general activation of all dendritic cells, providing optimal conditions for effective T cell activation. The sites, kinetics and extent of T cell activation following DNA vaccination was investigated in a transgenic model. It was demonstrated that T cell activation is initiated in the cervical draining lymph nodes and persisted for longer than 40 weeks although antigen expression was not demonstrable more than 12 weeks for keratinocytes and 2 weeks for dendritic cells. Crosspriming of dendritic cells by C5 expressed in keratinocytes did not occur unless keratinocyte death was induced by irradiation. About 2% of dendritic cells present in the draining lymph nodes were estimated to be transfected, both by confocal analysis of GFP expression and by functional titration assay.

Notably, the construct used for the series of experiment described so far was shown to result in intra-cellular expression in mammalian cells, but not in secretion. A secretory C5 construct was constructed and it was shown that C5 specific antibodies have been detected only in the case of the secretory construct while the other constructs were unable to generate any antibodies.

CONTENTS

INDEX OF TABLES AND FIGURES

ABBREVIATIONS

Ab	antibody
APC	antigen presenting cell
BCR	B cell receptor
BM	bone marrow
BrdU	bromodesoxyuridine
CFA	complete Freund's adjuvant
C5	fifth component of complement
CD	cluster of differentiation
CTL	cytotoxic T lymphocyte
cDNA	complementary DNA
CFSE	carboxyfluorescein-diacetatesuccinimidyl-ester
cpm	counts per minute
CMV	cytomegalovirus
d	day (s)
DC	dendritic cell(s)
DMSO	dimethylsulfoxide
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immuno sorbent assay

FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FDC	folicular dendritic cell
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony stimulating factor
GFP	green fluorescent protein
gp	glycoprotein
ICAM	intercellular adhesion molecule
i.d.	intradermal
IFN-g	interferon gamma
Ig	immunoglobulin
IL	interleukin
i.m.	intra-muscular
IMDM	Iscove's modified Dulbecco's medium
i.p.	intra peritoneal
kDa	kilodalton
KS	Bluescript
LC	Langerhans cell
LFA	lymphocyte function associated antigen
LPS	lipopolysaccharide

Mab	monoclonal antibody
MACS	magnetic cell sorter
MHC	major histocompatibility complex
NP	nucleoprotein
NK	natural killer cell
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PE	phycoerytherin
Rag	recombination activating gene
RME	receptor mediated endocytosis
RNA	ribonucleic acid
RT	room temperature
SD	standard diviation
SV40	simian virus 40
TCR	T cell receptor
T_h	helper T cell
TNF-a	tumor necrosis factor alpha
UV	ultra violet

1. Introduction

1.1 Function of the Immune System

The survival of a species is dependent upon the ability to respond to and adapt within a dynamic environment. The organism must be capable of withstanding continual pressure from potentially harmful agents (pathogens) such as bacteria, viruses and parasites, many of which find the internal environment of another organism favourable for their own survival (Pincus, *et al.*, 1992). This could prove damaging, if not fatal, to the infected host and therefore many organisms have evolved an inbuilt protection mechanism, known as the immune system. The immune response is made up of a complex sequence of events; it is triggered by the introduction of a stimulus (Immunogen or antigen) and usually culminates in the elimination of the provoking agent. Indeed, the primary function of the immune response is to discriminate between self and non-self and thereby to eliminate the latter, be it a pathogenic micro-organism, a tissue allograft, or an environmental substance such as proteins in pollens, grasses, or food (Kuby, 1994).

1.2 Innate and Adaptive Immunity

In mammals, the immune system comprises a complex network of cellular and molecular elements and is divided into the innate and adaptive (acquired) systems. Innate immunity is the first line of defense against an organism that has managed to penetrate the external barriers of the body, such as skin. The main cell types involved are phagocytic, and include neutrophils and macrophages, which are capable of

ingesting and eliminating various infective micro-organisms. (Edwards and Watson, 1995). Another mechanism of innate immune defense is the activation of complement, a system of several proteins present in the blood, acting against micro-organisms by forming pores in their membranes and by facilitating their uptake by phagocytic cells which bind complement components with specific receptors. Innate immunity does not confer immunological memory to a particular pathogen that has been eliminated. Adaptive immunity on the other hand, which is mediated by lymphocytes, is based on recognition of a specific antigen, derived from a particular organism. Upon recognition of the antigen, lymphocytes mediate the elimination of the pathogen and additionally confer immunological memory towards the antigen, such that subsequent infections will be recognised and eliminated more rapidly than the initial infection (Sprent, 1994). Innate and adaptive immune responses are not mutually exclusive. Innate immunity will limit the initial spread of an infection, if it cannot eliminate the pathogen itself, which allows

time for adaptive immunity to develop. In addition, there is a considerable amount of cross talk between both systems; cells of the innate immune system produce many molecules important in stimulating the production of an adaptive immune response.

1.3 T and B Lymphocytes

A variety of cells play a role in the immune response, but the most prevalent are the lymphocytes, which are located primarily in the secondary lymphoid organs (spleen and lymph nodes), but are capable of circulating throughout the body in the blood and lymph (Parrot and Wilkinson, 1981). There are two types of lymphocyte: T and B lymphocytes, both of which possess on their surface antigen specific receptors. The B cell receptor (BCR) is a cell surface form of the immunoglobulin (Ig) molecule (Pleiman, *et al.*, 1994; Reth, 1994) and interaction of the BCR with antigen stimulates the B cell to differentiate into a plasma cell, which secretes immunoglobulins (antibodies) of the same antigen specificity as the BCR (Reth, 1992). Immunoglobulins bind directly to antigen, which ultimately leads to the elimination or neutralisation of the pathogen bearing this antigen. Immunity mediated by B cells and antibodies are referred to as humoral immunity.

T cells are lymphocytes, which develop and differentiate in the thymus before seeding the lymphoid tissues. The T receptor (TCR)

only recognizes Ag once it has been degraded into peptides and presented in a complex with molecules of major histocompatibility complex (MHC) which is distinct from, but related to, immunoglobulin (Davis and Bjorkman, 1988). MHC molecules are peptide binding glycoproteins and are divided into two classes MHC class one (MHC I) and MHC class two (MHC II). All nucleated cells display MHC class I on their surface, whilst only specialized cells known as antigen presenting cells display MHC class II. The latter group includes macrophages, dendritic cells and B cells.

The TCR consists of an antigen-binding portion formed by two different polymorphic chains, which are associated with CD3, a complex of polypeptides involved in signalling cellular activation. The antigen binding portion may consist of an ab heterodimer or gd heterodimer: the great majority of peripheral T cells have ab T cell receptors. The selection of TCR specificity in the thymus is thought to involve two processes: (1) positive selection of thymocytes bearing receptors capable of binding self MHC molecules, which result in MHC restriction (Benoist and Mathis, 1989), and (2) negative selection by elimination of thymocytes bearing high affinity receptors for self MHC molecules alone or self antigen, which results in self tolerance (Kisielow, et al., 1988). Both processes are necessary to generate mature T cells that are self MHC restricted and self tolerant. Thymic stroma cells, expressing high level of MHC class II, and I are thought to play a role in positive selection, whereas negative selection is primarily carried out by bone marrow derived cells.

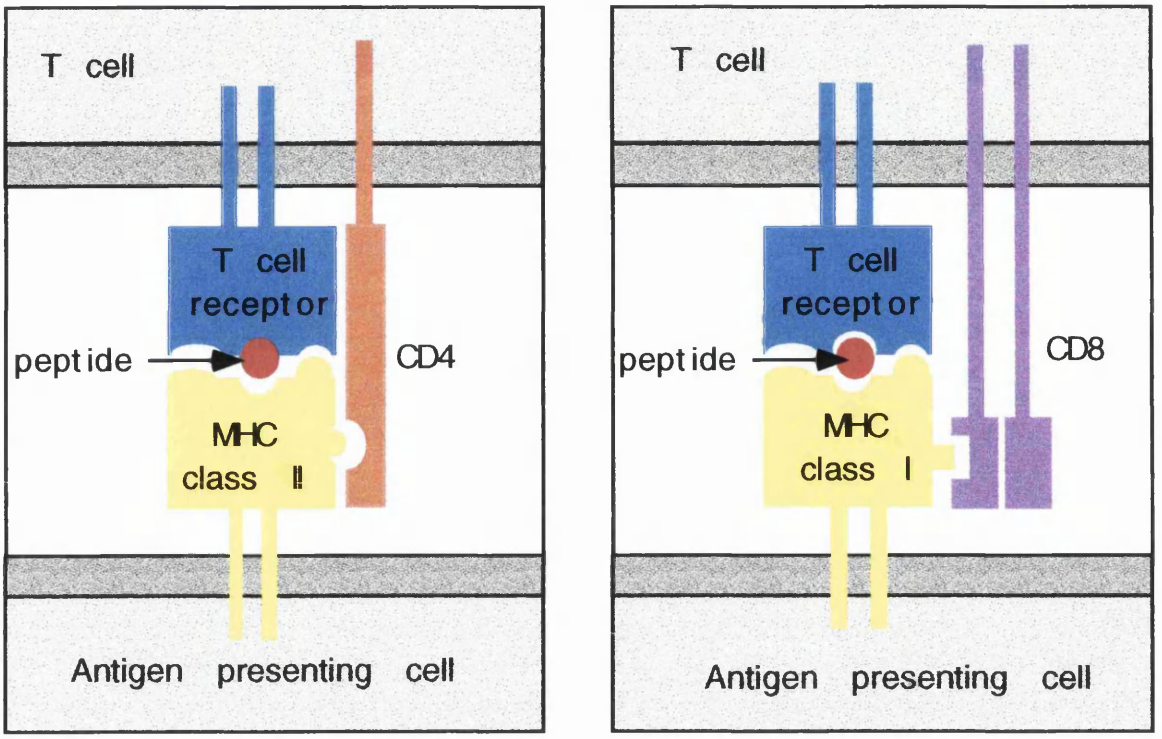


Figure 1.1 The peptide:MHC complex is recognised simultaneously by the T cell antigen receptor and by co-receptor molecules.

The co-receptor molecules CD4 and CD8, on the surface of distinct sets of T lymphocytes, assist the T cell receptor in recognising target cells by binding to MHC molecules on the surface of target cell. When the T cell receptor recognises a peptide:MHC complex on another cell, a co-receptor molecule also binds to the MHC portion of the complex, thus forming part of the receptor and helping to activate T cells. The two types of co-receptor distinguish between the two main classes of MHC molecules. CD4, which is found on the surface of helper and inflammatory T cells, binds only to MHC class II molecules; CD8, which is found on the surface of cytotoxic T cells, binds only to MHC class I molecules.

(*Adapted from Janeway and Travers, 1995*)

There are two main subpopulation of T cells, which can be distinguished according to their expression of the co-receptors, CD4 or CD8. These molecules act as receptors for class II and class I MHC molecules, respectively, and contribute towards both T cell immune recognition and cellular activation. Most CD4 T cells recognize antigen associated with MHC class II molecules and these cells act predominantly as helper T cells (Th) (See T cell activation). Antigens, which cannot induce B cells to produce antibody without T cell help, are called T cell dependent antigens. However a number of antigens directly stimulate B cells, which are called T independent antigens. The majority of protein antigens are T dependent while many T independent antigens are large non-protein polymeric molecules with repeated epitopes. .

CD8 T cells, are primarily responsible for cytotoxic destruction of virally infected cells (Allison, et al.,1982, Haskins, *et al.*, 1983, Meuer, *et al.*, 1983). $CD8^+$ T lymphocytes are stimulated to produce cytotoxic mediators (Such as perforin or granzymes) and are hence known as cytotoxic T lymphocytes (CTL) (Podack, *et al.*, 1985). Although CD8 T cells can act alone when killing target cells, their differentiation from naive CD8 positive T cells is often dependent on help from CD4 positive helper T cells (Keene and Forman, 1982). Furthermore, for effective CD8 CTL priming, this help must be provided in a cognate manner, such that both the CD4 cell and CD8 cell recognize antigen on the same antigen presenting cell (Bennett, *et al.*, 1997). One recent

explanation suggests for this requirement CD4 T cells are needed to convert the antigen presenting cell into a cell that is fully competent to prime CTL. In this "dynamic model" dendritic cell offers co-stimulatory signals to both CD4 and CD8 cells, but it initially stimulates the T helper, which, in turn, stimulates and conditions the dendritic cell to differentiate to a state where it can directly co-stimulate the cytotoxic T cell. (Lanzavecchia, 1988; Ridge, *et al.*, 1988; Bennett, *et al.*, 1988).

1.4 Antigen Processing and Presentation

The generation of antibodies allows the elimination of invading extracellular organisms. However, foreign antigens can also be found within cells. Many viruses and certain bacteria, for example Shigells and Salmonella, reside and replicate within the cytosolic compartment of cells and other pathogens can be located in the vesicular compartment of cells (endosomes and lysosomes). For example the parasite Leishmania and Toxoplasma, and bacterial toxins that have been endocytosed, are found in macrophage vesicles (Mimms, 1982).

The immune recognition system that has evolved to detect antigens is mediated by products of the MHC (Moller,1987; Zinkernagel and Doherty, 1974; Zinkernagel and Doherty, 1979). The MHC encodes a set of highly polymorphic gene products, which are functionally designated to transport small peptide fragments of degraded

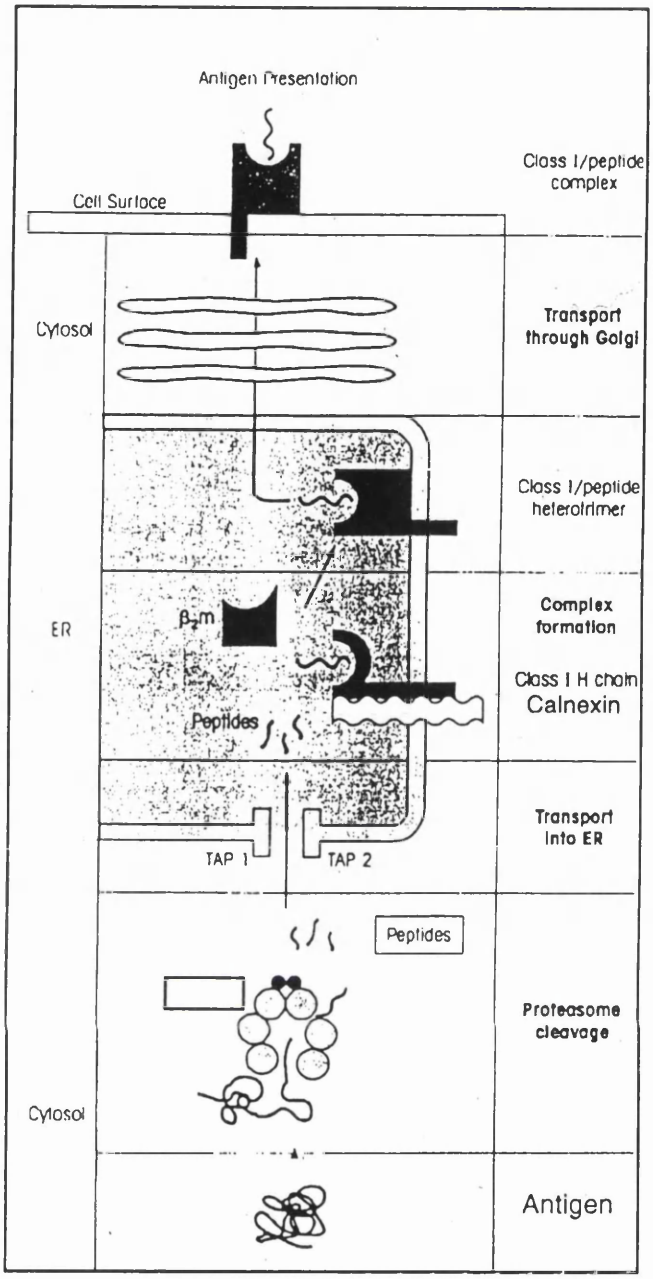


Fig 1.4 Processing and presentation of endogenous antigen by MHC class I. Cytosolic antigens are degraded by the proteasome complex into peptides, which are transported into the endoplasmic reticulum (ER) by transporters associated with antigen processing (TAP)-1 and -2. In the ER, the peptide and β2 microglobulin (β2-m) from a heterotrimer with the MHC class I molecule heavy chain, which becomes released from the molecular chaperone, calnexin. The complex is then transported to the cell surface via the Golgi.

intracellular protein to the cell surface, where the peptide is displayed for TCR recognition (Powis and Geraghty, 1995; Riley and Olerup, 1992). The component chains of MHC class I become associated during synthesis in the endoplasmic reticulum (ER). The association is assisted and stabilised by the membrane bound molecular chaperone calnexin. In this stage MHC class I molecules bind antigenic peptides that are derived from the cytosol (Elliot, 1991; Monaco, 1992). During antigen processing cytosolic proteins are degraded to peptides by cytosolic proteolytic activity which is mainly provided by the 20S and 26S proteasomes (Rock, et al., 1994). The 20S proteasome is a barrel shaped 700kDa complex, consisting of 12-15 different subunits. The 26S proteasome consists of a 20S core particle, plus one or two regulatory complexes that have ATPase and ubiquitin-binding activities. Both proteasomes have at least three different endopeptidase activities and produce peptides of between 3-15 amino acids in length (Williams, et al., 1996). Peptides of this length are transported into the ER by two specialised transporters associated with antigen processing (TAP), known as TAP1 and TAP2 (Shepherd, et al., 1993; Spies, et al., 1992; Van Kaer, et al., 1992). TAP1 and TAP2 are members of ATP-binding-cassette family of transporters that function as a heterodimer in the ER membrane and transport peptide into the ER in an ATP-dependent manner. In the ER, peptides associate with newly synthesised calnexin-bound MHC class I molecules to form a stable antigen-MHC complex. The complex is subsequently transported the cell surface, via

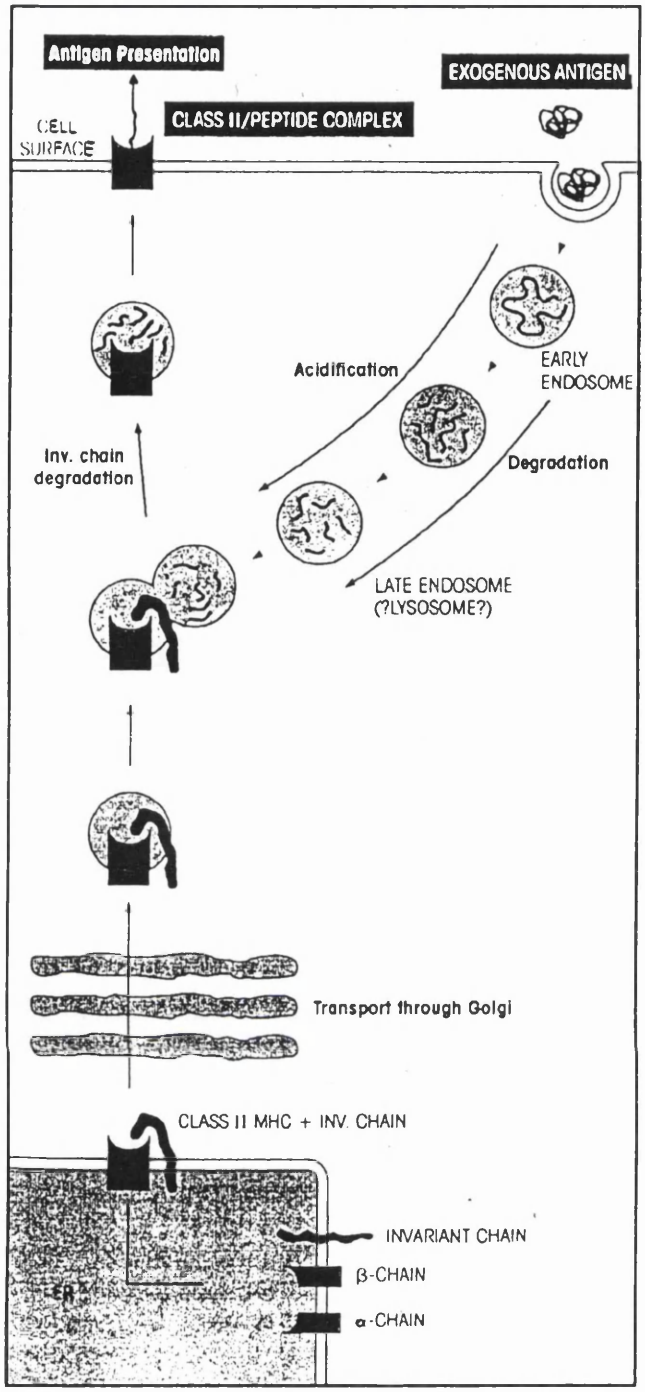


Fig 1.3 Processing and presentation of exogenous antigen by MHC class II. Class II α and β and invariant chains assemble in the ER and are transported through the Golgi and trans Golgi reticulum to endosomes, which contain peptides derived from uptake of exogenous antigen. Degradation of the invariant chain allows peptide binding and the complex is transported to the cell surface.

the Golgi apparatus and the trans-Golgi reticulum. The calnexin association is lost, presumably during transport to the cell surface, although the detail of its dissociation are not clear. At the cell surface the MHC class I-antigen complex is recognised by CD8 T lymphocytes, through engagement through TCR. This interaction increases the sensitivity of T cells to antigen presentation by MHC class I molecules by about 100 fold (Janeway, 1992).

However, MHC class II molecules are present vesicular (endosomal and lysosomal) derived antigens. These antigens are generated from the antigen processing activity of endosomal proteases, such as Cathepsin D and B, on endocytosed proteins (Neefjes and ploegh, 1992; Sadegh and Germain, 1992). Therefore it is necessary that cytosolic derived peptides do not bind to MHC class II during synthesis in ER. This is achieved by the co-assembly of class II molecules in the ER with the third chain known as Invarient (Ii) chain (Cresswell, 1992; Hammerling and Moreno, 1990). The Ii chain is a transmembrane protein that rapidly forms trimers upon synthesis in the ER. Each subunit of the trimer associates with an MHC class II dimer to produce a nonamer complex, which is additionally stabilised by calnexin. The Ii chain is important for efficient ab dimerisation, prevention of premature peptide binding in the ER and for directing the molecules through the biosynthetic pathway to endosomal compartments presumably via its endosomal targeting signal (Bakke and Dobberstein, 1990). In the endosome, the Ii chain is removed mainly

by proteolysis, thus allowing for antigenic peptide loading. Successful peptide loading has been shown to require the class II like molecules HLA-DM in humans (Fling, et al., 1994) or H-2 M in mice (Zechel, et al., 1996). These molecules are thought to induce the dissociation of the peptide fragments of the Ii chain involved in prevention of premature peptide binding, the class II-associated Ii chain (CLIP) peptides (Romagnoli and Germain, 1994; Sloan, et al., 1995). The MHC class II-antigen complex is subsequently transferred to the cell membrane, where it is recognised by CD4 T lymphocytes.

1.5 T Cell Activation

When T cells encounter APCs, the first interactions are transient (Dustin and Springer, 1989) and initiated by cell adhesion molecules on the cell surfaces. This helps the TCR to sample peptide/MHC complexes on the APC. Adhesion molecules mediate the binding of one cell to other cells. Families of these molecules include the integrins and the immunoglobulin superfamily. When TCR recognise peptide/MHC complexes, with additional signals from co-stimulatory molecules, T cells develop into effector cells (Liu and Janeway, 1992). Once activated, these additional signals are no longer required for effector function.

The effector T lymphocytes do not re-circulate through the lymph nodes, but migrate into sites of inflammation, where they encounter

antigen responsible for their expansion. $CD4^+$ effector T lymphocytes are stimulated to produce cytokines upon encounter with antigen. Cytokines are chemical mediators of immune responses and therefore influence the behaviour of all cells of the immune system (Arai, *et al.*, 1990; Paul and Seder, 1994).

Activated and naive CD4 T cells can be identified by the expression of surface markers such as MHC class II, CD25, CD44, CD69 and CD62-L (Male, et al., 1996). As shown in table 1, while naive T cells express MHC class II^{lo}, $CD25^{lo}$, $CD44^{lo}$, $CD69^{lo}$ and $CD62\text{-}L^{hi}$, activated T cells have an MHC class II^{hi}, $CD25^{hi}$, $CD44^{hi}$, $CD69^{hi}$ and $CD62\text{-}L^{lo}$ phenotype.

Characteristic surface markers of $CD4^+$, naive, memory and activated T cells

Phenotype	Naive T cell	Memory T cell	Activated T cell	Function
CD2	++	+++	+++	T cell activation, adhesion receptor for CD48
CD25	–	+	+++	T cell proliferation and differentiation, IL-2 binding
CD44	+	+	+++	Extra and inter cellular adhesion, T cell costimulation
C62-L	+++	++	–	Leukocyte homing, rolling and extravasation
CD69	–	–	+++*	Costimulation, early activation regulation

Clones of mature helper T cells fall into two major groups, which are functionally defined according to the cytokines they secrete. Th1 cells interact preferentially with mononuclear phagocytes, while Th2 cells tend to promote B cell division and differentiation (Mosmann and Coffman, 1989). Th1 lymphocytes produce IFNg and IL-2 and are effective inducers of cell mediate responses (Cher and Mosmann, 1987) and Th2 cells produce, among others, IL-4 and IL-10 and are important in generation of humoral immunity (Boom, *et al.*, 1988). The differentiation of CD4+ T lymphocytes into Th1 or Th2 occurs during priming, although the factors governing differentiation are not clear.

The cytokines produced by the Th1 and Th2 subsets exhibit cross-regulation; that is, the cytokines secreted by one subset can block the production and/or activity of the cytokines secreted by the other subset. For instance, IFN-γ preferentially inhibits proliferation of the Th2 subset, and IL-10 down regulates secretion of IFN-γ and IL-2 by the Th1 subset. Similarly, IFN-γ and IL-2 promote IgG2a production by B cells by inhibit IgG1 and IgE production. On the other hand IL-4 promotes production of IgG1 and IgE and suppresses production of IgG2a. It is possible that a major physiological function of Th2 cells is to control Th1 dependent and macrophages-mediated inflammation because IL-10 is the prototypic anti-macrophages cytokine and IL-4 antagonize many of the activities of IFN-γ.

Macrophages and other antigen presenting cells also produce cytokines that regulate immune effector functions. Interleukin 12 (IL-12) is

secreted by activated macrophages in response to bacterial or protozoan infections. IL-12 induce proliferation of NK cells and Th1 cells resulting in increased IFN-γ production. Since IFN-γ activated macrophages, it has a positive feedback effect inducing even more IL-12. Cross-regulation also influences cytokine production by macrophages; the Th2 derived cytokines IL-4 and IL-10 have both been shown to inhibit IL-12 production.

A variety of cytokines have been shown to act at various stages of B-cell activation, proliferation, and differentiation. Studies with the Th1 and Th2 subsets, which produce distinct profiles of cytokines have helped to demonstrate the effect of these various cytokines. Both subsets, for example, can induce proliferation and differentiation of activated B cells. However, in general Th2 cell lines are more effective helper cells for activating resting B cells than are Th1 cell lines. This finding probably reflects the important role of IL-4, which is produced only by Th2 cells, as a competence and progression signal.

Th1 and Th2-supported humoral responses also exhibit some consistent differences in the isotype of the antibody secreted. Th1 cells induce IgG2a, whereas only Th2 cells induce IgG1 and IgE. This difference is due to the difference pattern of cytokines produced by these subsets. Only Th1 cells produce IFN-γ , which inhibits IgG1 production and enhance IgG2a production. In contrast, only Th2 cells produce IL-4, which induces IgG1 or IgE. Current evidence supports the view that cytokines promote class switching by inducing

rearrengement of the heavy-chain constant region genes. Cytokines also stimulate antibody secretion by plasma cells. For example, secretion of IgM and IgG antibody by cultured plasma cells is inhibited by more than 90% in the presence of monoclonal antibody to IL-6, suggesting that this cytokine stimulates secretion of these isotypes.

In addition to priming of naive T lymphocytes, other outcomes of initial interaction with antigen can occur; the production of immunological memory and the production of anergic T cells. The former manifests itself as increased rapidity of immune response to a pathogen that has been encountered previously, and is thought to be mediated by clonally expanded populations of memory cells (Ahmed and Gray, 1996; Di Rosa and Matzinger, 1996). In kinetics of primary T cell responses, both CD4 and CD8 can be broken down into three distinct phases: I) activation and expansion II) death III) stability or memory. During the initial phase, antigen-driven expansion of the specific T cells and their differentiation into effector cells occur. In several viral systems, between 100 to 5000 fold expansion of virus specific CD8 T cells takes place (Oehen, *et al.*, 1992; Uehara, *et al.*, 1992). Substantial expansion of CD4 T cells has also been reported for several antigenic systems (Kearney, *et al.*, 1994; Kelso, *et al.*, 1991). This decline corresponds to the period of death, previously described as ensuing the initial phase of expansion and during which most of the activated T cells undergo apoptosis and effector activity subsides as the amount of antigen declines (McHeyzer-Williams and Davis, 1995). This phenomenon, termed activation-induced cell death (AICD), serves as

a mechanism for regulating cell numbers and maintaining homeostasis (Kawabe and Ochi, 1991; Web *et al.*, 1990). Both active, through Fas and TNF-R (Zheng *et al.*, 1995), and passive, by lymphokine withdrawal (Lenardo, 1996), mechanisms have been involved in this stage. This contraction of the T cell response can be as dramatic as the expansion, and in most instances more than 95% of the antigen-specific T cells disappear. The remaining specific T cells can referred as a pool of memory cells that can persist for many years.

It is well established that T cell memory, as assessed by accelerated recall responses in vivo, is long lived (Sprent, 1994). However, the nature of T cell memory has remained controversial, with debate centered on two opposing views. One view postulates that memory is due to long-lived memory cells that do not require contact with specific antigen for their survival. The other envisions long term memory as the result of continuous stimulation of T cells by persisting antigen. It is conceivable that memory T cells may be antigen-independent but still cycle because of some non-specific stimulus. It is also plausible that memory cells are long-lived but need antigen stimulation to maintain a state of readiness. So there is still considerable debate regarding the mechanisms by which long-term immunity is maintained (Ahmed and Gray, 1996)

Anergic T cells can be produced as a result of antigen binding to the TCR in the absence of Co-stimulatory signals. The anergic state is one in which the T lymphocytes do not proliferate and differentiate upon

subsequent exposure to antigen, which is mainly due to their inability to produce IL-2 (Garciasanz and Lenig, 1996; Schwartz, 1996). Anergy can be induced in vitro and in vivo with APC, lacking costimulatory molecules (Jenkins and Schwartz, 1987; Lamb, *et al.*, 1983). Whereas the mechanism of anergy induction mentioned above relies on the lack of a signal, another system has been described using an altered peptide ligand for the interaction with the T cell receptor, which can result in T cell anergy in the presence of co-stimulation (Sloan-Lancaster *et al.*, 1993). Here, T cells are stimulated with MHC presenting a synthetic peptide analogue of the cognate antigen that contains an amino acid substitution at a residue critical for TCR binding. This form of anergy induction might have a different application in vivo. Naturally occurring altered peptide ligands designed by pathogens could act as antagonists for viral epitopes, thereby preventing CD8 T cells to respond (Bertoletti, *et al.*, 1994; Klenerman, *et al.*, 1994).

1.6 Dendritic Cells

Dendritic cells (DCs) are key elements in the establishment of T cell immune responses and, as such, have extraordinary antigen presenting capacity. Their potential as antigen-presenting cells relies on their ability to endocytose, process and retains a wide variety of antigens on their surface and to present these to specific T cells. The main function of DC is to present antigen to naive T lymphocytes,

thereby inducing their proliferation and differentiation into effector T cells (Steinman and Inaba, 1989). They have a distinctive dendritic morphology, and capable of cytokine production, express receptors, such as FcgRII (Astier, *et al.*, 1994) and FceRII (Maurer and Stingl, 1995), high level of MHC, co-stimulatory and adhesion molecules, which specialises them for efficient antigen presentation (Steinman and Witmer, 1978). The efficacy of DCs in T cell binding and activation seem to relate to quantitative aspects and their regulation. For example, MHC products and MHC peptide complexes are 10 to 100 times higher on DCs than on other APCs like B cells and monocytes (Banchereau and Steinman, 1998).

Ontogeny

DC are derived from the bone marrow (Thomas, *et al.*,1993) as allogeneic bone marrow transplantation leads to donor-strain specific Langerhans cells in the skin (Katz, *et al.*, 1979; Frelinger, *et al.*, 1979). However, recent investigation indicates that dendritic cells are of diverse origin, with two types of myeloid precursors and a lymphoid precursor implicated in their generation (Inaba, *et al.*, 1992; Young, *et al.*, 1995; Caux and Banchereau, 1996). Most DC are considered to be of myeloid origin: the main evidence for this is from studies of DC development in cultures stimulated by granulocyte macrophage CSF (GM-CSF) (Inaba, *et al.*, 1993). However it was shown that mouse thymic DC are derived from a lymphoid committed, early intrathymic precursor and they are characterised by their expression of CD8 (Wu, *et al.*, 1991, 1995, 1996, Matsuzaki, *et al.*, 1993). Also a proportion of DC

found in murine spleen are lymphoid related and $CD8^+$ (Vremec *et al.*, 1992; Ardavin, *et al.*, 1993; Matsuzaki, *et al.*, 1993).

Performance

Dendritic cells are found in lymphoid organs, where priming of naive T lymphocytes occurres, and are distributed in low numbers in the circulation, both in the blood (Freudenthal and Steinman, 1990; Thomas and Lipsky, 1994) and in lymph (Coughlan, *et al..*, 1996; Liu and Macpherson,1991; Mckeever, *et al.*, 1992) and in virtually all organs of the body. They can be identified in the heart, kidney (Austyn, *et al.*, 1994), oral cavity (Van Wilsem, *et al.*, 1994), lung (Gong, *et al.*, 1992; Nicod and el Habre, 1992; Sertl, *et al.*, 1986), liver (Woo, *et al.*, 1994), gut (Liu and Macpherson, 1995; Maric, *et al.*, 1996; Vidal, *et al.*, 1989), thymus (Crowley, *et al.*, 1989) and skin (Schuler and Steinman, 1985). DCs isolated from non-lymphoid sites are able to capture and process antigen (Austyn, 1996), either by macropinocytosis or receptor mediated endocytosis (RME) (Lanzavecchia, 1996). Macropinocytosis is the cytoskeletal-dependant, non-specific, fluid phase uptake of extracellular particles (up to 0.5 μm diameter) in large vesicles and enables a cell to take up half of its own fluid volume in one hour (Scheicher, *et al.*, 1995). DCs isolated from the skin (Langerhans cells) (Reis e. Sousa, *et al.*, 1993), liver (Matsuno, *et al.*, 1996), bone marrow (Scheicher, *et al.*, 1995) and peripheral blood (Lanzavecchia, 1996) have been shown to possess constitutive macropinocytosis properties. RME can mediate the uptake of particles larger than 0.5 μm and can be facilitated on DC by Fc receptors, the mannose receptor (MR)

(Lanzavecchia, 1996) or DEC-205 (Jiang, *et al.*, 1995). The former is responsible for internalising antibody-antigen complex (Coughlan, *et al.*, 1996), and the latter two are both capable of binding to carbohydrate containing ligands. Additionally, LC (Reis e. Sousa, *et al.*, 1993), liver DC (Matsuno, *et al.*, 1996) heart and kidney DC (Austyn *et al.*, 1994) and bonh forceps, resuspended in c*ulture* medium. The cell suspension was then incubated in a petri dish for 2-3 hrs at 37oC. Non-adherent cells were washed off and adherent cells were scraped off, washed and collected for any further co-culture or lysis for RNA preparation.

Migration and functional heterogeneity

As initiation of an immune response occurs in lymphoid tissues, an essential feature of antigen-loaded DC is the capacity to migrate. Migration from the site of antigen entry has clearly been demonstrated *in vivo* for skin DC (Langerhans cells) (Cumberbatch and Kimber, 1992; Kripke, *et al.*, 1990; Shelley and Juhlin, 1976) and for intestinal DC (MacPherson, *et al.*, 1995). This migration can be experimentally induced by skin grafting, skin contact sensitization with Fluorescein Isothiocyanate (FITC), or *in vitro* culture of skin explants (Larsen and Austyn, 1991; Macatonia *et al.*, 1987). Immature dendritic cells, inparticular Langerhans cells (LC), as sentinel cells resident in non-lymphoid organs, where they continuously internalise and process antigen. Maturation signals induced by injury or infection result in

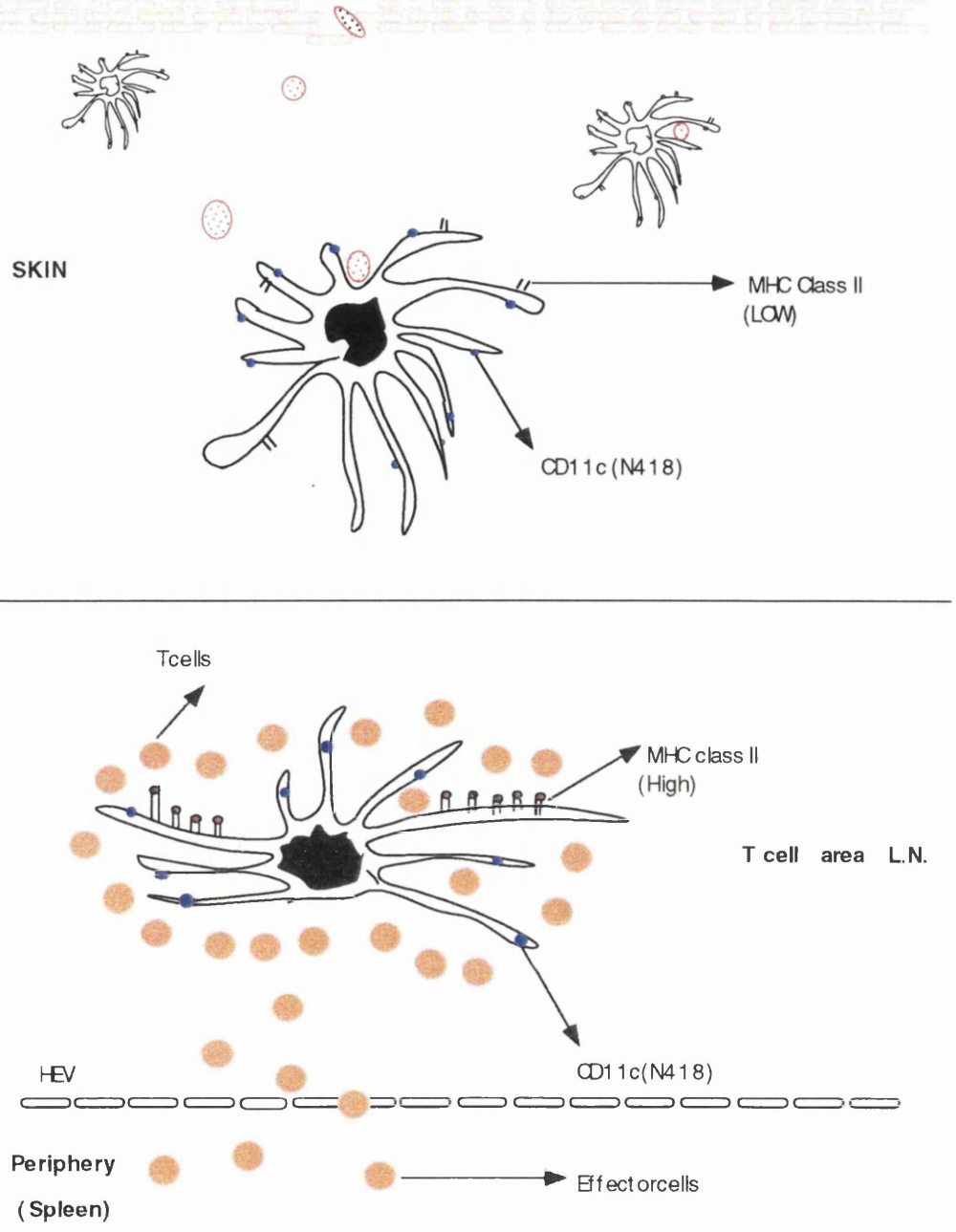


Figure 1.2 Langerhans cells take up antigen in the skin and migrate to lymphoid organs where they present it to T cells. Langerhans cells can ingest antigen but have no co-stimulatory activity. In the presence of antigen (red circles), Langerhans cells (MHC class II low) take up antigen locally in the skin and then migrate to the lymph nodes where they differentiate into dendritic cells (MHC class II high) that can no longer ingest antigen but have gained co-stimulatory activity.

migration of LC via afferent lymph to the T cell areas of the draining lymph nodes, whereby expression of CD44 and b2 integrins might support homing. In mice, GM-CSF and IL-1 have been reported to mediate maturation of LC (Heufler, *et al.*, 1987), whereas TNFa was implied in maintenance of LC in culture without induction of maturation (Koch, *et al.*, 1990). However, another *in vitro* culture system uses GM-CSF and IL-4 to generate immature DC from human peripheral blood mononuclear cells, which can be driven into maturity by TNFa or LPS (Sallusto and Lanzavecchia, 1994).

Distinct migration patterns, as those for LC, have also been shown for blood dendritic cells, which migrate into the T cell area of the spleen, but not into lymph nodes when injected intravenously or when introduced in cardiac allografts, and subcutaneously injected DC traffic into the draining nodes but not further (Austyn *et al.*, 1988; Kupiec-Weglinski, *et al.*, 1988; Larsen, *et al.*, 1990).

Comparison of DC isolated from non-lymphoid and lymphoid organs reveals a functional and phenotypic heterogeneity. DC from skin (Schuler and Steinman, 1985), lungs (Pollard and Lipscomb, 1990), heart and kidney (Austyn, *et al.*, 1994), are superior in their ability to internalise and process antigen both by macropinocytosis and phagocytosis in comparison to DC in lymphoid organs (Reis e. Sousa, *et al.*, 1993). However, DC in non-lymphoid tissue are relatively inefficient antigen presenting cells, as measured by their ability to produce proliferation of antigen-specific T cells where as DC in

lymphoid organs are extremely potent antigen presenting cells (Inaba, *et al.*, 1993b). Phenotypically, DCs in lymphoid organs differ from DCs in non-lymphoid organs in having higher expression level of MHC class II and co-stimulatory molecules (Larsen, *et al.*, 1994). These findings have led to the understanding that DC form an antigen capturing network in a tissue, where they are specialised for effective antigen internalization and processing. These cells are generally considered in the literature as immature processing DCs. Upon antigen uptake, the cells leave the tissue and migrate to regional lymphoid tissue, via the lymphatics or blood. In the lymphoid tissue, the cells lose their antigen capturing and processing capacity, but acquire all the properties necessary for efficient presentation and are hence regarded as mature presenting DC (Austyn, 1996).

The rate of DCs turnover varies significantly depending on the anatomical location of the cells. DCs in the skin have been demonstrated as having a long lifespan of several weeks (Katz, *et al.*, 1979), whereas DC in the spleen (Steinman, *et al.*, 1974) or small intestine (Pugh, *et al.*, 1983) have lifespans of only three to four days.

A model of DC function, exemplified by the Langerhans cells of skin, shown in Figure 1.2. In this model DC collect foreign antigens in peripheral sites, process the antigen, migrate to the T cell area of lymph nodes, and then mature into a form, competent to stimulate an immune response.

1.7 Immunisation and Vaccine Development

Modern immunology grew from Jenner's and Pasteur's original successes with vaccination. Many vaccines have been developed following these initial triumphs over smallpox and rabies. However, while these vaccines have virtually eliminated a number of diseases, their rare harmful side effects have led to a search for better ways to vaccinate against such illnesses. Moreover, great challenges remain in vaccine development against diseases for which no vaccine exist, including malaria and AIDS. Today, infectious disease is still the number one cause of death worldwide. Microbes of one kind or another kill more than 13 million every year, mostly infants. Acute respiratory infections take the biggest toll among the young; in 1992 they killed about 2.8 million children younger than five years. Diarrhoeal diseases took another 2.2 million, and malaria carried off more than a million. Vaccines offer the best hope for reducing the appalling toll. Immunization is simply the best medicine.

The explosion in knowledge about the immune system and its interaction with pathogens has made vaccine development a highly attractive approach to disease control. Recombinant DNA technology has opened new avenues for manipulating microbial genomes and has the potential to improve vaccines tremendously. This, coupled with a growing awareness of the need for vaccines to prevent diseases prevalent in developing countries, newly described diseases and

animal diseases important in agriculture has created a resurgence of interest in this area (Janeway and Travers, 1997).

The goal of immunization is to elicit protective immunity and immunologic memory so that a subsequent exposure to the pathogenic agent will elicit a heightened immune response with successful elimination of the pathogen. Several factors must be kept in mind in developing a successful vaccine. First and foremost, the development of an immune response does not necessarily mean that the state of immunity has been achieved. Often the branch of immune system that is activated is critical, and therefore vaccine design must recognise the important differences between activation of humoral and cell mediated branches. A second important factor is the development of immunologic memory. For example, a vaccine may induce a primary response that is protective but may fail to induce memory-cell formation, leaving the host unprotected after the primary response to the vaccine subsides. Vaccines designated to induce humoral antibody production must display epitopes that are accessible to the immunoglobulin receptor on B cells. B cells generally recognize epitopes that are hydrophilic and many of those epitopes are not sequential and thus require the native structure of the protein to generate their conformation. Inactivated or attenuated bacteria or viral vaccines often display native epitopes and thus induce humoral antibody production. Purified proteins and polysaccharides are also effective inducers of humoral immunity. But, for some infectious agents notably viruses, bacteria, protozoa, and fungi that are

intracellular pathogens, a cell mediated immune response is necessary to confer immunity. A vaccine designated to induce this type of response must activate T cells as strongly as possible. Unlike B cells, which recognize epitopes on native antigen, T cells recognize antigen that has been processed and is presented along with MHC molecules. T cell epitopes tend to be internal, hydrophobic, and linear peptides that are not revealed until the protein is denatured and unfolded in the course of antigen processing. These requirements place certain constraints on potential vaccines: to activate CD4 T cells, a vaccine must be processed by antigen-presenting cells and presented in association with class II MHC molecules and to activate CD8 T cells, a vaccine must be capable of replicating in host cells where its peptides can associate with class I MHC molecules. For this reason, attenuated vaccines that permit some limited viral replication or bacterial growth within host cells are most effective for the induction of cytotoxic activity. In addition to the differences in processing routes for class I and for class II MHC presentation, potential vaccines for inducing a cell mediated response are limited by constraints arising from the preferential interaction of MHC molecules with different peptides. Therefore, an immunodominant T cell epitope for one individual may not serve as an immunodominant T cell epitope for another individual who expresses a different set of MHC on antigen presenting molecules.

Construction of synthetic peptides for use as vaccines to induce either humoral or cell mediated immunity requires an understanding of the

nature of T cell and B cell epitopes. Potential B cell epitopes of a protein antigen can be identified by examining its structure for peptide sequences representing sites that are accessible, hydrophilic, and mobile. Ideally, vaccines for inducing humoral immunity should include peptides composing immunodominant B cell epitopes. Such a peptides can be identified by determining the dominant antibody in the sera of individuals who are recovering from a disease and then testing peptides for their ability to react with that antibody with a high affinity. These experiments highlight the important differences among MHC molecules in peptide presentation. The MHC polymorphism within a species will therefore influence the level of T cell responsiveness by different individuals to different peptides.

Moreover, different subpopulations of T cell probably recognise different epitopes. For example immunisation with the amino terminal residues (1-17) of hen egg lysozyme suppressed the response to native lysozyme. By identifying suppressing peptides and eliminating them from synthetic vaccines, it might be possible to generate enhanced immunity. These suppressive peptides may also be valuable in situations where it is desirable to decrease the immune response, as in treating autoimmune diseases.

In designing synthetic peptide vaccines against viruses, the current approach is to look for invariant regions, whose amino acid sequence is highly conserved. Some regions of hemagglutinin (HA) molecule of influenza virus for example, display high levels of amino acid

variation which generate the type and subtype differences enabling the virus to escape the immune system. But invarient regions, which mediates essential biological functions, also are present in the HA molecule. For example the sialic acid binding site on HA allows the virus to bind to sialic acid residues on cell surfaces. Although this region on the intact viral particle does not normally induce antibody formation, synthetic peptide vaccines of this conserved region were found to neutralise viral infectivity against a number of different influenza types and subtypes.

Synthetic peptide vaccines are being evaluated for hepatitis B virus, diphteria toxin, Heamophilus influenza and various glycoprotein of HIV.

Furthermore, the vaccine itself should produce only limited, if any, undesirable side effects. It should be relatively inexpensive and mass producible for population vaccination.

1.8 DNA Vaccines

The use of DNA and mRNA as vectors for immunisation (variously termed genetic, nucleic acid or DNA immunisation) is a relatively

recent development in the field of vaccine research. These vaccines are composed of an antigen-encoding gene whose expression is regulated by a strong mammalian promoter incorporated into a plasmid backbone of bacterial DNA. The first paper demonstrating the efficacy of a DNA vaccine in an animal model was published in 1993 (Ulmer, *et al.*, 1993). The rationale for using nucleic acids as vaccines came from the initial observations that intramuscular (i.m.) injection of non-replicating plasmid DNA expression vectors or mRNA-encoding reporter genes could result in the *in vivo* expression of proteins in mouse (Wolff, *et al.*, 1990). This ability to express proteins *in vivo* offers the opportunity to generate immune responses against foreign antigens encoded by the nucleic acid. In addition, both humoral and cell-mediated immune (CMI) responses, such as cytotoxic T-lymphocytes (CTL), can be induced. The protective activity of DNA vaccines has been amply demonstrated by immunogenicity and protection studies against several viruses and other intra-cellular pathogens, such as *Leishmania, Plasmodium* and *Mycobacterium*. Moreover, animal species ranging from rodents and fish to non-human primates and humans have been vaccinated with DNA. Protection was first demonstrated in a mouse model of influenza, where a cross-strain protective CTL response was generated by i.m. injection of DNA encoding influenza nucleoprotein (NP) (Ulmer, *et al.*, 1993). Subsequently, the direct immunisation with DNA was shown to elicit protection against homologous challenge with influenza virus in chickens (Robinson, *et al.*, 1993) and mice (Montgomery, *et al.*, 1993), bovine herpes virus in mice and cattle

(Cox, *et al.*, 1993), rabies virus (Xiang, *et al.*, 1994), *malaria* (Sedegah, *et al.*, 1994), *Leishmania* (Xu, *et al.*, 1994), and *tuberculosis* (Tascon, *et al.*, 1996) in mice; DNA vaccines were also shown to generate an immune response against HIV gp 160 and Hepatitis B surface antigen in mice and non human primates (Wang, *et al.*, 1993).

In general, CTL responses require endogenous expression of the antigen, such as during immunisation with live viruses or replicating vectors, whereas subunit proteins, polysaccharide conjugate, or inactivated virus vaccines generate humoral immune responses, but not CTL. However, since somatic cells express only low level of MHC class I molecule and appear to lack co-stimulatory molecules required for efficient antigen presentation, a major paradox is to understand how intramuscular immunisation with plasmid DNA primes for CTL responses. A recent study (Corr, *et al.*,1996) using bone marrow chimeras, demonstrated that the CTL response induce following DNA immunisation was restricted to the haplotype of the bone marrow APC.

Furthermore, reports by Kim *et al.* (1997), Iwasaki *et al.* (1997) and Tsuji *et al.* (1997) demonstrated that co-stimulation with DNA expression vectors, encoding an antigen and the co-stimulatory molecule CD86 (B7-2), significantly augment the CTL response to the encoded antigen. One interpretation of these data is that immune enhancement may be mediated by an increase in the level of CD86

expressed through direct transfection of professional APCs such as interstitial dendritic cells located in muscle.

Several studies have investigated the characteristics of DNA uptake and protein expression by cells *in vivo* (Wolff, *et al.*, 1990, Sato, *et al.*, 1996). However, the basic mechanisms of DNA uptake and antigen presentation remain unclear. For example, although it has been demonstrated that muscle cells express genes after intramuscular (i.m.) injection of DNA, it is unclear whether antigen presentation is mediated by these cells or tissue dendritic cells take up the DNA and express it. However, there is evidence for an exogenous pathway whereby antigens that are not expected to gain access to the cytoplasm are presented on MHC class I molecules (Pfeifer, *et al.*, 1993; Kovacsovics-Bankowski, and Rock, 1995). The most relevant example is in the *in vivo* phenomenon of cross-priming (Bevan, 1976) : antigens from donor cells are acquired by bone-marrow-derived host antigen-presenting cells (APCs) and presented on MHC class I molecules. It was clearly demonstrated that dendritic cells, but not macrophages efficiently present exogenous antigens derived from tumours, transplants or infected cells, stimulating class I restricted $CD8^+$ CTLs (Albert, *et al.*, 1998).

Another prevailing question has been what factors contribute to DNA vaccine immunogenicity? Recent studies suggest that elements in the plasmid backbone may play a role. Sequences consisting of unmethylated CpG dinucleotides flanked by two 5' purines and two 3'

pyrimidines present in the plasmid have been shown to act as adjuvants (Roman, *et al.*, 1997). These CpG motifs appear to mediate direct activation of B cells by stimulation of IL-6 and macrophages by stimulation of IL-12, IFN-a, b, and g (Pisetsky, 1996). They also seem to promote: the development of Th1 dependent CTL responses, activation of DCs via stimulation of IL-12, and finally induction of IFN-g by NK cells (Takunaga, *et al.*, 1992; Krieg, *et al.*, 1995; Stacey, *et al.*, 1996; Klinman, *et al.*, 1996; Sparwasser, *et al.*, 1997, 1998). A perhaps more significant observation is that addition of CpG-containing oligonucleotides can redirect the response induced by a recombinant protein such as HBsAg from Th2 to Th1 and modulate the IgG isotype from IgG1 to IgG2a (Davis, *et al.*, 1998). The immunotherapeutic potential of CpG-containing DNA in the treatment of allergic disease is suggested by a report from Roman et al., 1997. Co-stimulation with a protein antigen and CpG containing DNA was shown to suppress synthesis of antigen-specific IgE upon subsequent boosting with the antigen. Potentially, immunization with an allergen and CpG-containing DNA may elicit blocking IgG antibodies as well as a Th1 response, which could suppress IgE synthesis and eosinophil recruitment upon re-exposure to the allergen.

1.9 Aims of the project:

The main question, which is going to be addressed is: "What is the basis for induction and maintenance of T cell responses following DNA vaccination?" It is well known that dendritic cells play a crucial role in establishing an immune response subsequent to immunisation or infection. What is important to know is, to what extent dendritic cells are involved in genetic immunisation. The extent of involvement may depend on either one or a combination of the following:

I) Dendritic cells become transfected and as a result serve as antigen presenting cells,

II) Professional antigen presenting cells (DCs) pick up the antigen from transfected somatic cells

The model system used to address these questions was previously established in our lab. The model antigen, C5 (fifth component of complement), is synthesized by hepatocytes (Colten, 1992), as a single 200 KDa chain precursor molecule, pro-C5, which is cleaved to a functional heterodimer of 115 kD and 83 kDa. C5 is present in the circulation of normal mice at a concentration of about 50μg/ml depending on age and sex (female mice contain lower levels of C5) (Nilsson and Muller-Eberhard, 1967). A widespread natural genetic mutation which affects 38% of inbred mouse strains results in the absence of secreted C5 protein (Cinader, et al., 1964; Nilsson, et al.,

1975). As a consequence, C5 deficient mice (such as A/J) are not tolerant to C5 (Lin and Stockinger, 1989) and T cell hybrids specific for C5 were generated from these mice. In order to address the questions outlined above, DNA constructs containing cDNA of C5 were established. The C5 cDNA used was previously shown to result in intracellular expression without any secretion, so that the DNA vaccine was expected to result in expression only in cells, which had internalised the vaccine. With this strategy, we wanted to limit uptake of C5 protein from a secreted source of expressed vaccine to allow determination and quantification of APC, which could present C5 following vaccination.

Following immunisation of the C5- mice, these questions were to be addressed:

I) What is the most efficient route of vaccination?
II) What is the earliest time point that T cell activation can be detected?
III) What are the kinetics of the immune responses induced?
IV) What cells are expressing DNA vaccine and which ones are important in establishment of an immune response and generation of T cell memory?

For some of these questions we decided to use transgenic mice which carry MHC class II ($H2\text{-}E^k$) restricted T cell receptors specific for epitope 106-121 of mouse C5. Mice carry the TCR transgene under the control

of the human CD2 promoter. These mice were crossed onto a Rag -/- background to exclude generation of any other TCR by means of endogeous rearrangements.

2. Material and Methods:

2.1 Animals

The mouse strain A/J (H-2^a, C5-/-) was obtained from the SPF (specific pathogen free) unit at National Institute for Medical Research. T cell receptor transgenic mice (A18.A), carrying the H-$2E^k$ restricted TCR (Va11.1^a · Vb 8.3) recognising peptide 106-121 derived from the fifth complement component C5 (Zal, *et al.*, 1994), are bred in conventional animal house facilities.

2.2 Cell Lines and Culture Media

A18 is a class II-restricted C5-specific T cell hybridoma (Lin and Stockinger, 1989), established from a C5-specific T cell line fused with the HAT-sensitive cell line BW a^-b^-according to the method of White et al., (1989). A18 recognises the C5 epitope peptide 106-121, close to the N-terminus of the C5 ß chain, in the context of H-$2E^k$.

CTLL is an Il-2 dependent T cell line, ATCC Cat. No. TIB 214.

COS -7 is a monkey kidney fibroblast cell line with a integrated defective replication origin SV-40 virus genome.

Culture medium was Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL, Paisley, Scotland) supplemented with 5% heat inactivated fetal calf serum (FCS) (Gibco BRL), $2x10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and $5x10^{-5}$ M mercaptoethanol (all Sigma).

Medium for washing cells was air buffered IMDM (AB medium, Gibco BRL) supplemented with 0.21% NaCl, 100 U/ml penicillin, 100 µg/ml streptomycin and 12.5 mM Na OH.

2.3 Bone Marrow Cultures

Bone marrow derived dendritic cells (DCs) were generated as described previously (Stockinger and Hausmann, 1994) with some modifications. Briefly, $5x10^{6}$ bone marrow cells were cultured in 9 cm petri dishes (NUNC) in 10 ml culture medium containing 10% supernatant of Ag8653 myeloma cells transfected with murine granulocyte macrophage colony stimulating factor (GM-CSF) cDNA (about 25 U/ml). On day 4 of culture, non-adherent granulocytes were removed and GM-CSF medium was replaced. Loosely adherent cells were transferred to a fresh dish on day 6 of culture. From day 6 to day 12 these transferred loosely adherent cells were used as a source of

dendritic cells, their purity assessed by MHC class II staining (usually > 80%).

2.4 Magnetic Cell Sorting

Positive selection of lymph node cells was carried out by magnetic cell sorting with the Vario-MACS (Miltenyi, Biotech, Germany). Lymph nodes were digested with a cocktail of 0.1% deoxyribonuclease (Dnase I, Fraction IX, Sigma) and 1.6 mg/ml collagenase (CLS4, Worthington Biochemical Corp., NJ) at 37 °C for one hour followed by washing with 10% FCS IMDM to deactivate the enzymes. For positive selection of DC, cells were stained using biotinylated N418 detecting CD11c (Metlay, *et al.*, 1990) as first labeling step to select dendritic cells (DCs), followed by labeling with streptavidin-magnetic beads in staining buffer (PBS, 5mM EDTA, pH 7.2). Stained cells were then passed over the selection column in a magnetic field twice and washed with running buffer (PBS, 5mM EDTA, 1%FCS, pH 7.2) to increase the purity. Positively selected cells were collected by passing PBS containing 5%FCS in the absence of the magnetic field.

2.5 Determination of Cell Viability and Number

Trypan blue (Sigma) at a final concentration of 0.08% in phosphate buffered saline (PBS, 10.1g NaCl, 0.362g KCl, 0.362g KH_2PO_4, 1.449g Na_2HPO4, in 11 H_2O) was used to determine the viability of cells. Cells

were counted in a 1:1 mixture of Trypan blue using a Neubauer counting chamber (BDH Ltd, UK) under the light microscope. Dead cells (stained blue) were excluded from counting.

Alternatively, Fluorescein diacetate (Sigma) at a final concentration of 10 μg/ml in Dimethylsulphoxide (DMSO, Sigma) was used in a 1:1000 dilution mixture using Neubauer counting chamber (BDH Ltd., UK). Under the UV microscope, the green cells were counted as viable cells.

2.6 Antigen Presentation Assay

Presentation of C5 was assessed by incubating the T cell hybridoma A18 (5×10^4/w) with different numbers of APC in 200 μl in flat bottom 96-well plates (Costar, Cambridge, MA., USA). After 24 hours, 100 μl of culture supernatant was transferred to a fresh plate, and incubated with

5×10^3/ well IL-2-dependent cell line CTLL for a further 24 hours followed by addition of 1μCi of [^{3}H] thymidine per well. 8 hours later plates were harvested (Micro 96 harvester, Skatron Instruments, U.K.) on printed filter mat A paper (Wallac, Finland) and read out was obtained (Wallac 1205, Beta plate counter, Finland) as the mean cpm of incorporated [^{3}H] thymidine.

2.7 Limiting Dilution Assay

For assessment of C5-specific T cell precursors, serial dilutions of spleen cell suspensions (24 replicates for each cell concentration) were cultured in the presence of $2x10^4$/well bone marrow-derived dendritic cells and 10 μg/ml C5 protein in culture medium. Control wells received T cells and dendritic cells, but not C5 protein. After 48 h of culture 50 μl of supernatants were transferred to IL-2 dependent CTLL cells, and CTLL proliferation was assessed by [^{3}H] thymidine incorporation. over 18 h. Reactions were considered positive in wells that exhibited proliferation greater than the mean plus three times the standard deviation value of control wells. The fraction of negative wells (converted to its negative log) is then plotted against cell concentration. Regressional analysis is then used to fit a staight line to this graph, and using the zero term of the Poisson equation "$Fo=e^{-u}$", where "Fo" is the fraction of negative wells, "e" the base of the natural logarithim and "u" the average number of precursors per well. Where u is 1, we have: Fo=0.37. We can predict that when 37% (0.37) of the test wells are negative, there is on average one precursor cell per well. Thus the frequency of precursors specific for a given antigen in a cell population can be extrapolated directly from the graph.

2.8 Keratinocyte Preparation

Keratinocytes were prepared by digesting the dorsal halves of ears with 0.25% trypsin (Sigma) for 25min. at 37°C. Trypsin was washed out with 20%FCS IMDM medium and the epidermal sheets were peeled off with forceps, resuspended in culture medium. The cell suspension was then incubated in a petri dish for 2-3 hrs at 37°C. Non-adherent cells were washed off and adherent cells were scraped off, washed and collected for any further co-culture or lysis for RNA preparation.

2.9 Keratinocyte/Dendritic Cell Co-Cultures

Keratinocytes cell suspension were added to 6 well Costar plates (40mm diameter) and allowed to adhere for 2h. Non-adherent cells were washed off and some of the keratinocyte monolayers were subjected to 1000 Rad irradiation prior to addition of $1x10^6$/well bone marrow derived DC. Keratinocyte/dendritic cell mixtures were incubated overnight. The dendritic cells were then washed off and added in serially titrated numbers to $5x10^4$/well A18 T hybridoma cells. Il-2 production by hybridoma cells was determined 24h later. Keratinocytes were lysed and total RNA was extracted from the lysate in the presence of RNAse inhibitors according to the RNeasy RNA isolation kit manual (Qiagen). Complementary DNA (cDNA) was generated from the mRNA template using a 15-mer poly dT oligonucleotide (Genosys, UK) and the Superscript reverse

transcriptase enzyme according to SuperScript Pre-amplification System protocol (GIBCO BRL). Expression of the C5 construct was detected by polymerase chain reaction using C5 specific primers and general PCR conditions.

2.10 BrdU Labeling and Detection

For continuous BrdU labeling, mice received one intraperitoneal injection of 1mg BrdU (Sigma) in PBS and then were given 0.8 mg/ml BrdU in the drinking water, which was changed every three days. Single cell suspensions of lymph node or spleen were stained with anti CD4 (Pharmingen) and anti Vß8.3 mAb (Förster et al., 1995), resuspended in 200 µl of PBS/1% paraformaldehyde for 20 min. on ice. After washing with PBS, the samples were fixed with cold 70% ethanol for 4 min., followed by another fixation step with 100 µl of PBS/1% paraformaldehyde/ 0.01%Tween for 30 mins at room temperature followed by 30 mins on ice and washed with PBS. Subsequently, the samples were treated with DNase I (Boehringer Mannheim), 200 µl/sample for 30 mins at 37°C. After washing with PBS, the cells were resuspended in 45 µl of PBS/5%FCS/0.5% Tween and stained with anti-BrdU mAb (Becton Dickinson).

2.11 CFSE labeling and adoptive transfer

For labeling of cells before adoptive transfer, spleens of Thy1.1 mice were removed and then gently pressed through a sieve using a syringe plunger. The cells were resuspended in PBS and pelleted by centrifugation. The pellet resuspended in 1ml of 5μm CFSE/10^7 cells (Sigma) for 15 mins at 37°C. Cells were then washed twice once with PBS and once with air buffered medium (AB). CFSE-labeled cells were injected in the Thy1.2 mice tail vein. At different time points after injection spleen cells were stained with either anti-Thy1.1 or anti Thy1.2 (Pharmingen) and anti CD4. Cells were analysed by FACScan as described later.

2.12 Confocal Microscopy

Positively selected dendritic cells from draining lymph nodes of mice vaccinated with GFP-C5 vaccine were stained with biotinylated N418 , followed by Strepavidin Texas RED (Pharmingen). After washing with PBS cells were dropped onto glass coverslips and mounted on glass slides with Histogel (Camon). The confocal images were acquired at the Confocal and Image Analysis Lab (CIAL) at National Institute for Medical Research, London. The confocal system used was an upright Leica TCS-NT with an Argon/Krypton laser source.

2.13 Vaccination

DNA vaccine diluted from the stock (3 mg/ml) to final concentration of 1 mg/ml in sterile, endotoxin free, 0.9% NaCl solution (Sigma). Vaccine has applied under general anaesthetic conditions (Avertin, 300 µl/20g, I.P.). About 150mg of vaccine (150 ml) was scarificated on the ear skin or injected intramuscularly per mouse. At various time points after vaccination lymph nodes and spleen were removed for further analysis.

2.14 Immunization and measurement of antibody response

Mice were injected subcutaneously above the footpads with 10 mg C5 in complete Freund's adjuvant. For determination of antibodies, microtiter ELISA plates were coated with C5 at 10mg/ml (50ml) in carbonate buffer pH9.5 and serial dilutions of serum were tested for the presence of anti-C5 antibodies using secondary alkaline phosphatase coupled anti-IgG1 and IgG2a antibodies.

2.15 FACS Analysis

Analytical flow cytometry was carried out using a FACScan (Becton Dickinson, Mountain View, CA) and the data were processed using

the Cellquest software (Becton Dickinson). Three-colour staining performed with FITC-, PE-, and biotin-conjugated monocolonal antibodies (mAb) followed by strepavidin RED 670 (Gibco BRL, Paisley, and UK). Anti CD4 (H129.19, Pharmingen) was used as PE-conjugate, anti-TCR Vß8.3 (7G8.2, Förster et al., 1995) was conjugated with FITC using standard procedures. Biotinylated anti CD44, CD69, CD62-L (Pharmingen) were used followed by streptavidin RED 670.

2.16 Molecular Biology

2.16.1 Cloning of mC5 cDNA into the Mammalian Expression Vector: pcDNA 3.1

The full length mC5 was cloned into pDNA 3.1 (Invitrogen) under the human cytomegalovirus (hCMV) promoter. The promoter drives constitutive expression in mammalian cells. To optimise and positively regulate the expression, 0.9 kb of 5' untranslated intronic sequence of the immediate early gene of cytomegalovirus (intron A) was cloned in between the CMV promoter and C5 cDNA (Chapman, *et al.*, 1991).

Briefly, KS-mC5 was digested with Sal I restriction enzyme, followed by separation of mC5 from the agarose gel electrophoresis and the 5.4 Kb fragment released from the construct was excised from the gel and purified using the Geneclean protocol (Anachem. U.K.). The pcDNA

3.1 vector was linearised using a unique Xho I site in the multiple cloning region of the plasmid and the terminal phosphates were removed using Shrimp Alkaline Phosphatase (S.A.P.), (Boehringer Mannheim, Germany) treatment for 15 mins at 37°C, followed by purification of the linearised vector by the Geneclean method. The 5.4 Kb. sal I/sal I mC5 fragment and the Xho I linearised/phosphatased vector were ligated together in a 3:1 ratio, respectively, using T4 DNA ligase as per standard protocol (Sambrook, et al, 1989). The ligated plasmid was then transformed into E. Coli bacterial cells as will be described later.

To generate the 1.6 Kb fragment of C5, two primers HH7 and 1.6R were designed for use in a Polymerase Chain Reaction (PCR) (synthesised by Genosys, UK) with a TGA stop codon and a unique XbaI site at the 3' end of the reverse primer. This fragment of the C5 was gel purified (as described before), digested with Sall/XbaI and ligated to pDNA 3.1 already cut with XhoI/XbaI.

2.16.2 Cloning of mC5 cDNA into the Mammalian Secretory Expression Vector

The full length mC5 was cloned into the pSecTag vector (Invitrogen) under the CMV promoter. Briefly, KS-mC5 (Brazil, *et al*, 1997) was digested and gel purified as described before. Separately, pSecTag B was linearised using unique Xhol site in multiple cloning region of

the plasmid and phosphatased/gel purified as described before. The 5.4 Kb. sal I/sal I mC5 fragment (inframe with the secretory signal) was ligated to Xho I linearised/phosphatased vector in a 3:1 ratio, using T4 DNA ligase as per standard protocol (Sambrook, *et al*, 1989).

2.16.3 Cloning of mC5 cDNA into the GFP p-Tracer Vector

The full length mC5 was sequentially digested with the Bsp 120I and NotI restriction enzymes in KS-C5 (Brazil, *et al.*, 1997) and was then gel purified as previously described. The p-Tracer vector (Invitrogen) which expresses GFP under SV40 promotor and the CMV promoter for expression of the gene of interest. p-Tracer was linearised using Not I/SAP. The mC5 gene was then ligated to the linearised vector in a 3:1 molar ratio by standard protocol.

2.17 Preparation of Plasmid DNA

Competent DH5a E coli (dcm^-) were transformed with the ligation reaction, allowed to express Amp-r gene for 1h at 37°C, and subsequently plated out on nutrient agar plates containing 100 ug/ml Ampicillin. Plates were incubated overnight at 37°C, following which colonies were picked from the plates for growth prior to plasmid DNA extraction. This was performed by alkaline lysis method (Sambrook et al, 1989). DNA is eluted from a Qiagen column (Qiagen Ltd, U.K.) as

described in Qiagen plasmid purification hand book (Qiagen Ltd,U.K.). After isopropanol precipitation, the ethanol wash, and vacuum drying, the DNA is taken up in standard sodium chloride solution 0.9% NaCl endotoxin free (Sigma). From the size of Qiagen column used, one can estimate the amount of DNA to be recovered and add the sodium chloride to obtain a solution of about 2-3 mg/ml. Final and accurate concentration and purity were determined by spectrophotometer absorbance reading of DNA at OD 260 and 280.

2.18 Cos-7 Cell Transfection

Cos-7 cells were transfected witnt will elicit a heightened immune response with successful elimination of the pathogen. Several factors must be kept in mind in developing a successful vaccine. First and foremost, the development of an immune response does not necessarily mean that the state of immunity has been achieved. Often the branch of immune system that is activated is critical, and therefore vaccine design must recognise the important differences between activation of humoral and cell mediated branches. A second important detected by RT-PCR as described in 2.19 section.

2.19 cDNA Generation, Amplification, Analysis and Primers

For generation of cDNA, cells were lysed and total RNA was extracted from the lysate in the presence of RNAse inhibitors according to the RNAeasy RNA isolation kit manual (Qiagen). Complementary DNA (cDNA) was generated from the mRNA template using a 15-mer poly dT oligonucleotide (Genosys,UK) and the Superscript reverse transcriptase enzyme according to Superscript Preamplification System protocol (GIBCO BRL). Expression of the C5 construct was detected by PCR using C5 specific primers BamF and BamR under general PCR conditions.

Primer Name	**Sequence**
Bam F	GGG AAG GAT CCA CAT TAA G
Bam R	TAA TGG GAT CCT GTA TGG GA
HH7	CGC TAA ATC CGG ATC CTA AAT T
C5ENI	CTT TAA ATC CGT GTA ACT TCA
1.6 R	GCA ACT TAA ACC ATT TCC CTT ATC

3. RESULTS

3.1 Generation of C5 Expressing DNA Vaccine

The full length C5 construct pcDNA3.1-C5 (5.4) and pcDNA3.1-1.6 (1.6) construct, encoding the 545 amino acids of the N terminus of C5 protein, were generated for use as vaccines. Both expression systems are driven by a Cytomegalovirus (CMV) promoter and to optimise the expression, intron A from early immediate gene of CMV was introduced between promoter and start codon (figures 3.1A & 3.1B). The 1.6 Kb construct encoding the 5' end of C5 sequence was chosen because this part of C5 is known to contain the antigenic epitope recognised by two different T cell hybridomas generated in the lab. One is hybrid A18 specific for epitope 106-121 which was the donor cell line for generation of T cell receptor transgenic strain A18. The other hybrid C8-15 (Stockinger *et al.*, 1992) recognises a not fully characterized epitope within the 1.6 Kb region of C5. The insertion and the correct orientation of the full C5 cDNA and the 1.6 Kb fragment in pcDNA 3.1 vector was confirmed by several restriction enzyme digestions as shown in figures 3.2A and 3.2B respectively.

3.2 Expression of C5 in Mammalian Cells

To investigate the expression of the C5 cDNA in mammalian cells, COS-7 cells were transfected with both vaccines and the expression of C5 was detected by RT-PCR. Figure 3.3 shows the results of the RT-PCR in which b-actin specific primers were used as a positive control to show the optimal reverse transcriptase condition. A PCR of total RNA was carried out in parallel (using C5 specific primers) to rule out any contamination with DNA during the cell lysis. As shown in figure 3.3A and 3.3B, full expression was detected in cells transfected with (5.4) or (1.6) by using different C5 specific primers, along several stretches of C5 sequence, including the far 3' end of the sequence.

3.3 Comparison Between the Immune Response Induced by Full Length C5 (5.4) Vaccine and by 1.6-Kb (1.6) Vaccine.

Since the 1.6 vaccine in contrast to 5.4, only expresses a part of the C5 protein, it was necessary to compare the immunogenicity of both vaccines in $C5^-$ mice *in vivo* . Therefore, 150 μg of each vaccine (5.4 or 1.6) was administered intramuscularly (i.m.) to two groups of A/J $C5^-$ mice. Figure 3.4 shows the IL-2 response of spleen cells re-stimulated with the C5 protein, 5 weeks after inoculation of either vaccine. As a negative control, the empty expression vector (pcDNA 3.1) was inoculated into a third group of mice to control for any unspecific

responses. Similar positive responses were detected in both test groups and no response was detected in the third control group of mice, suggesting recognition of vaccine specific epitopes in both type of vaccines.

3.4 The Immune Response in Relation to the Route of Administration

To determine possible routes of vaccination for an immune response, 5.4 vaccine was administered via three different routes : scarification (abrasion) of ear skin, scarification of leg skin and i.m. injection. 10 days later, lymph nodes draining the vaccination area (cervical lymph nodes in the case of ear vaccination, mesenteric, lumbar and sciatic lymph nodes in the case of leg and i.m. vaccination) were removed and the T-cell response was assayed by re-stimulation of lymphocytes with C5 protein. As shown in figure 3.5, administration of vaccines via ear scarification resulted in a greater immune response in the cervical lymph nodes in comparison with the T cell response from the draining lymph nodes of other routes. The ease of localisation of the ear draining lymph nodes may be responsible for this observation. However, given a longer period (5 weeks after vaccination), all routes of immunisation gave a similar immune response (figure 3.6). In both experiments the empty vector control did not show any C5 specific T cell response.

3.5 Comparison of C5 Specific Immune Responses Following Immunisation with C5 Protein in Adjuvant and DNA Vaccination.

To compare kinetics and magnitude of immune responses following either conventional immunisation with C5 protein in adjuvant or DNA vaccination, two groups of A/J C5-mice were injected either with 150 μg of the full length C5 DNA vaccine (5.4) via the ear skin (scarification) or 10 μg of C5 protein in complete Freund's adjuvant (CFA) subcutaneously. 10 days later cervical lymph nodes and spleen were removed and lymph node cells and splenocytes were re-stimulated with C5 protein. A comparision of the resulting immune response is shown in figure 3.7. In the group immunised with C5 protein (3.7A), no C5 specific immune response was detected 10 days later in re-stimulated lymph node cells, while an IL-2 response was detected in re-stimulated spleen cells. In the group vaccinated with DNA vaccine (3.7B), after 10 days, no response was detected in spleen cells and activated lymphocytes were still restricted to the lymph nodes adjacent to the site of vaccination.

To establish the kinetics of immune response induced by DNA vaccination, cervical lymph nodes and spleen were taken out at four time points (Day 8, 10, 12 and 14) after scarification of ears with the 5.4 DNA vaccine. Following *in vivo* re-stimulation with C5 protein, the highest IL-2 release was observed on days 10 to 12 following

vaccination in lymph nodes (figure 3.8A), while the immune response from the spleen started to rise later around day 14 (Figure 3.8B).

3.6 Isolation and Detection of C5 Expression in Dendritic Cells and Keratinocytes Following Vaccination

Since DCs are the most likely candidate APC responsible for induction of immune responses, it was essential to investigate whether DCs were transfected and expressing C5 protein. A group of A/J $C5^-$ mice was inoculated through ear skin with 5.4 vaccine and detection of C5 expression was carried out in dendritic cells 10 days later on an enriched dendritic cell population from the deep cervical lymph nodes and spleen. The assumption was that Langerhans cells in the ear skin got transfected and migrated to the draining L.N. as a result of local activation. Lymph nodes and spleens were removed, cells were enzyme digested separately, and dendritic cells were positively selected by labelling with N418 antibody and passage through a MACS column. DCs and all other cells (containing B and T cells, called non dendritic cells), were lysed and the total RNA was isolated. In parallel as a negative control a group of mice were inoculated with an empty vector and DC isolation also was performed on lymph nodes and spleen. DC and non-DC were lysed and total RNA were isolated.

After cDNA synthesis from mRNA templates, RT-PCR was performed on both populations of cells from each group, using two C5 specific primers. As a positive control, PCR of cDNA using primers specific for b-actin (a constitutively expressed gene), and as negative control, PCR of total RNA was performed and visualised by agarose gel electrophoresis. As shown in figure 3.9, the 0.7 Kb band (primers BamF/BamR) represents expression of the C5 protein in the lymph node DCs population, while no band was observed with DCs from the empty vector or DCs from spleen of 5.4 vaccinated mice. Also no band was detected from non-dendritic cell populations in both groups, indicating no expression of vaccine in all other cells like B or T cells in the lymph nodes. RNA samples from both groups were also negative for any genomic or plasmid DNA contamination, while both showed a 0.6 Kb band with the b-actin primers (positive control), indicating optimum RT-PCR conditions. This indicates that a population of DCs in lymph nodes draining the vaccination site are transfected and producing C5 protein, but no other cell population in spleen or lymph node was transfected.

The site of vaccination, skin, consists of three main cell populations: Keratinocytes (about 95%), Langerhans cells (about 2-5%) and gd T cells (less than 1%). To address the extent of transfection by DNA in the site of immunisation (skin), 10 days following administration of vaccine to the ears, keratinocytes (from ear's skin) were isolated and lysed as described before and an RT-PCR was performed. As it is shown in

figure 3.9 (D), a C5 specific band (Bam F/BamR) was detected in the keratinocyte population, suggesting expression of C5 in this cell type. Positive ß- actin and negative RNA controls were carried out and run on the agarose gel in parallel.

3.7 Quantification of Transfected Dendritic Cells Following DNA Vaccination

In an attempt to quantify the number of dendritic cells in draining lymph nodes, which express the C5 construct, functional studies were carried out on positively selected dendritic cells from draining lymph nodes of 5.4 vaccinated mice. Isolated DCs were tested for their capacity to activate the C5 specific T cell hybrid A18. In parallel, a standard curve was set up by combining various numbers of bone marrow dendritic cells that had been pulsed with the C5 protein, followed by extensive washing, with non-pulsed dendritic cells and A18 T cells. Comparing the T cell response obtained, it was estimated that about 2% of the dendritic cells in the draining lymph nodes of vaccinated mice processed and presented C5 in the context of MHC class II molecules for recognition by A18 T cells (figure 3.10).

3.8 Analysis of pTracer Expression

Application of DNA vaccine to the ear will affect Langerhans cells and it has been shown (Steinman, et al. 1991) that such cells migrate to draining lymph nodes following a stimulus applied by contact of the vaccine with the skin. Upon stimulation LC dissociate from neighbouring keratinocytes, leave their position in epidermis, pass through basement membrane into the dermis, enter the afferent lymphatics and relocate in the T lymphocyte rich areas of the draining lymph node (Streilein, *et al.*, 1994). It would be of advantage to be able to follow transfected cells by an additional marker independent of C5 protein. A good candidate for such a marker is the green fluorescent protein (GFP). pTracer, a bi-cistronic vector with a multiple cloning site under the CMV promoter, and (GFP) under the control of a SV-40 promoter was chosen and the complete C5 sequence was cloned into the multiple cloning site. The pTracer-C5 construct has the advantage of GFP expression without fusing with the protein of interest (mC5) (figure 3.11A). COS-7 cells were transfected with the pTracer-C5 construct and the fluorescent expression of the GFP was detected under an UV microscope (Figure 3.11C). Since the empty vector itself is capable of expressing GFP in COS-7 cells, restriction enzyme digest analysis and an RT-PCR were performed as described before to confirm integrity of the construct and detect expression of C5 respectively. As shown in figure 3.11B, a 0.7 kb band was amplified in cDNA from pTracer-C5 transfected COS-7 cells lysate, using C5 specific primers (Bam F/BamR). Positive and

negative controls were also run along the sample as described before. The RT-PCR result indicates expression of C5, while the analysis of GFP expression under the UV microscope confirmed the expression of GFP from the same construct.

3.9 Confocal Analysis of Dendritic Cells

The pTracer-C5 vaccine used in this experiment allows direct visualization of the transfected cells by fluorescence microscopy. 150 μg of pTracer-C5 DNA was administrated to the ear skin of a group of A/J $C5^-$ mice as described before. 12 days later, DCs were isolated from cervical lymph nodes and GFP expression was analysed. FACS analysis of these DCs, isolated by magnetic cell sorting, did not give a clear signal in the GFP emission range (395 to 410 nm) (although confirmed enriched population of DCs), presumably because of the very low incidence of transfection. However, GFP containing DCs were clearly identifiable by confocal microscopy. As shown in figure 3.12, DCs were stained by a specific surface marker, CD11c (N418-bio), conjugated with Texas Red and transfected DC in addition expressed GFP.

3.10 Quantification of DNA in Lymph Nodes and Spleen Following Ear Scarification of DNA Vaccine

It has been described previously that even in gene gun vaccination, DNA enters the circulation. To test for the presence of DNA in lymph node and spleen 3 days after vaccination, same number of cells from spleen and lymph nodes were collected and directly lysed. A quantitative PCR was performed on the lysates. As shown in figure 3.13, C5 DNA vaccine was detectable in lymph node and spleen, although in a much smaller amount in the latter. This suggests that DNA applied to ear skin gains access to the circulation, but in a very small quantity, which is detectable only by high amplifying cycles of PCR.

3.11 DNA Vaccination Results in Activation of Dendritic Cells

All the DNA plasmids (Vaccines) used so far, were propagated in an E. coli (DH5a, dcm-) prokaryotic system. DNA in prokaryotic systems always has less methylation sites and hence is less protected from restriction enzymes, used mainly by eukaryotic cells as a defense mechanism. Prokaryotic DNA, specifically unmethylated CpG motives were shown to have powerful adjuvant activity on the immune

system (see chapter 1). To test the effect of DNA on the activation status of DC, DCs were isolated by magnetic cell sorting from draining lymph nodes of 5.4 or control vector vaccinated mice and tested for expression of cell surface molecules which are important in T cell stimulation, such as CD40, B7.1, B7.2, and ICAM-1 by FACS analysis. As shown in figure 3.14, both 5.4 vaccine (red line) and empty vector control (green line) resulted in strong upregulation of these molecules compared with DCs from untreated controls (black line), suggesting that prokaryotic DNA, provides the necessary stimuli for activation of dendritic cells in a non-specific manner.

3.12 Persistence of C5 Reactive T Cells

Considering the importance of the persistence of specific T cells in the context of a vaccination, it was important to study the lifespan of the C5-specific CD4 T cells upon DNA vaccination. There has been considerable debate regarding the role of specific antigen in maintaining T cell memory (Beverley, 1990; Swain, *et al.*, 1991; Vitteta, *et al.*,1991; Mackay, 1993; Gray, 1993; Sprent, 1994; Ahmed, 1996; Sprent, *et al.*, 1994). It can be said that periodical reexposure to antigen will enhance the level of T cell memory; experiments showing an increase in the number of memory T cells (or level of protective immunity) after reintroduction of antigen should however not be taken as evidence that memory is strictly antigen dependent. The real question

is whether the pool of memory T cells formed after infection or immunisation can remain relatively stable in the absence of an endogenous depot of specific antigen or is strictly dependent on stimulation by specific antigen. This chronic stimulus could come from low-grade infection (in the case of pathogens) or from antigen persisting on follicular dendritic cells (FDC) or both (Ahmed and Gray, 1996). Resolution of this issue is important for practical reasons (Vaccines) and also because it defines how we view immune memory.

For assessment of the frequency of C5-specific T cells, limiting dilution assays of spleen cells from DNA vaccinated (5.4) A/J mice, were set up at 4 week intervals for 40 weeks. The frequency of antigen specific cells was calculated by regression analysis on three groups of mice in parallel at each time point. These three groups were: I) Splenocytes of 5.4 DNA vaccinated mice, II) as a negative control splenocytes of untreated A/J $C5^-$ mice, and III) as a positive control the splenocytes of TCR transgenic A18 mice. The precursor frequency for C5 specific IL-2 producing T cells, was followed in a cohort of 5.4 vaccinated mice, for 40 weeks. Maximal expansion of C5 specific T cell was detectable around 4 weeks after vaccination (Table 3.1). The frequency of C5 specific cells declines slowly thereafter, but was still above that of the non-treated background controls at 40 weeks after vaccination.

3.13 Kinetics of C5 Expression in Keratinocytes and Dendritic Cells Following DNA Vaccination

To study the persistence of C5 expression in DNA vaccinated mice, eight groups of mice were subjected to 5.4 DNA vaccination. At the end of the every week for a period of four weeks (week 1,2,3 and 4) cervical lymph node dendritic cells were isolated, lysed and cDNA was generated as described before. Keratinocytes isolated on weeks 1 to 4, (in parallel with lymph nodes) and also on weeks 8, 12, 16 and 20 . Keratinocytes were lysed separately and cDNA was generated. A parallel PCR using C5 specific primers (BamF/BamR) was performed on cDNAs from keratinocytes and dendritic cell populations. As demonstrated in figure 3.15, a C5 specific 0.7 Kb band was detected in the dendritic cell population for the first two time points only (days 7 and 14), while expression of C5 was detected until 12 weeks after vaccination in the keratinocyte population.

3.14 Cross-presentation of Protein from Transfected Keratinocytes Occurs Only in a Situation of Excessive Cell Death

A recent study (Casares, *et al.*, 1997) described that DCs from vaccinated mice could stimulate T cells in the absence of added antigen. However, in this case the protein encoded by the DNA construct was secreted so

that it was not possible to define to what extent the stimulatory capacity of DCs was due to direct transfection, in comparison with the uptake of protein synthesized and released by other transfected cells. Although the 5.4 C5 construct does not give rise to secreted C5 protein, there is now substantial evidence that DCs can take up protein from other cells by a mechanism referred to as "cross priming" (Albert, *et al.*, 1998; Huang, *et al.*, 1994; Kurts, *et al.*, 1997). Surprisingly, protein access from other cells to DCs has so far only been shown to result in MHC class I restricted antigen presentation, although there is not a priori reason why this mechanism should not allow access of protein into the class II presentation pathway. We tested this by co-culturing keratinocytes isolated from the 5.4 vaccinated mice with untreated DCs, which were subsequently assayed for their ability to activate the A18 hybrid. This co-culture assay is very sensitive, detecting as little as 6ng/ml of C5 (Brazil *et al.*, 1997), but in the presence of intact keratinocytes which were shown to express C5 by RT-PCR, there was no evidence of any protein transfer to DCs (figure 3.16). In contrast, the exposure of keratinocytes to 1000 Rad irradiation prior to co-culture with DC, resulted in a strong C5 presentation by the DCs. This indicates that cell death induced by this treatment allows cross priming of dendritic cells for presentation in the context of MHC class II.

3.15 Kinetics and Site of T cell Activation Following DNA Vaccination of Transgenic Mice

To investigate the sites, kinetics and extent of T cell activation following DNA vaccination, T cell receptor transgenic (A18 TCRtg) mice were employed, since normal mice have very low precursor frequencies for C5 specific T cells (see Table 3.1) and it is not possible to follow the fate of specific T cells. The A18 TCRtg strain on a Rag-/- background contains CD4 T cells specific for epitope 106-121 of C5 (Zal, *et al.*, 1994). All T cells in the periphery have a naive phenotype as evident by low expression of CD44 and CD69 and high expression of CD62-L. A cohort of A18 TCRtg mice was vaccinated with the 5.4 or vector control construct. At the same time two vaccinated mice per group were injected with BrdU and received BrdU in the drinking water for the next three days. Next the draining lymph nodes and spleens from the BrdU treated mice were analysed for expression of activation markers on CD4 T cells and incorporation of BrdU. In the 5.4 DNA vaccinated mice, all CD4 T cells from lymph nodes draining the site of vaccination had incorporated BrdU, indicating activation and division (figure 3.17A). CD69, an early activation marker, was upregulated and CD62L showed some degree of downregulation whereas no significant changes were observed in CD44 levels yet. CD4 T cells from mice vaccinated with vector control construct or from untreated mice retained their naive phenotype. While CD4 T cells from draining lymph nodes of 5.4 vaccinated mice all appear to be

activated 3 days after vaccination, splenic CD4 T cells had neither incorporated BrdU nor showed any changes in activation markers. This indicates that T cell activation is initiated in the draining lymph nodes. By day 7 after vaccination, some CD4 T cells in lymph node and spleen of 5.4 vaccinated mice expressed an activated phenotype, characterized by downregulation of CD62-L and upregulation of CD44 (figure 3.17B), while T cells in control mice showed no signs of activation. By day 15 after vaccination, all CD4 T cells shown activation phenotype, as CD44 upregulated and CD62-L downregulated both in lymph nodes and spleen (figure 3.17C)

3.16 The Source of Stimulatory Antigen is Short Lived in Vaccinated Mice.

Although RT-PCR is very sensitive and indicated that there is no source of C5 Ag in dendritic cells anymore beyond two weeks after vaccination, we also used a highly sensitive functional test for detection of a persistent source of Ag. This test would also give a positive signal if any Ag had "crossed over" from keratinocytes to DC. 5.4 vaccinated mice, were injected with fresh, naive A18 T cells 21 days after vaccination. The injected T cells carried a different Thy 1 allotype (Thy1.1) and were labelled with the fluorescent dye CFSE to allow monitoring of cell division (Lyons and Parish, 1994). Figure 3.18 shows that 7 days after injection, the Thy1.1 A18 T cells found in the draining lymph nodes of mice vaccinated 20 days before, retained their CFSE

label and naive phenotype. Likewise, injected Thy1.1 A18 T cells present in the spleen had a naive phenotype. Thus, it appears that 20 days after vaccination there is no source of stimulatory antigen present anymore. These results confirm our previous observation of short (two weeks) C5 expression in DCs detected by RT-PCR in section 4.13.

3.17 Expression Analysis of pSec-C5

The C5 cDNA construct used for this series of vaccination was shown to result in intracellular expression in mammalian cells, but not in secretion (Brazil, et al., 1997). This is probably due to an as yet unknown deficiency in the cDNA sequence, probably absence of a full secretory signal sequence at the N- terminal end. Given the exclusive intracellular expression of C5 immune response following vaccination with this construct, this immune response might only be induced by cells that express the DNA and make the protein themselves. In order to allow studies of the effect of secreted antigen in the immune responses, particularly humoral, in comparison with the non-secretory form, a construct was generated which allowed C5 secretion by the transfected cells.

In order to obtain this "secretory C5" construct, the C5 cDNA was ligated in frame to a light chain antibody secretory signal sequence (figure 3.19A). C5 protein secretion can be detected by an extremely sensitive bioassay (Secreted C5 is processed and presented by DCs to

A18 T-cells). To confirm secretion, supernatant of C5 transfected COS-7 cells, was fed to DCs at various time points after transfection. While no response of A18 T cells was detected when DCs were co-cultured with Cos-7 cells transfected with the 5.4 construct, a distinctive response was detected from the supernatant of COS-7 cells transfected with the pSecB-C5 construct (figure 3.19B), confirming expression and secretion of C5.

3.18 Antibody Generated in the Case of only Secretory Construct

To investigate C5 secretion in mice, two groups of $C5^-$ A/J mice were immunised with either secretory C5 construct or 5.4. and re-vaccinated 3 weeks after the first injection. 9 days later serum from each group was tested for any C5 specific antibody responses. As shown in figure 3.20, while no antibody was detectable in the serum of 5.4 vaccinated mice, IgG antibody responses (only IgG2a subclass) were detected in mice vaccinated with the C5 secretory construct. This result confirms the secretion of C5 in vivo and the accessibility of extracellular C5, which is required for B cell activation and thus an antibody response.

3.19 CD4 Precursor Frequency in Mice Vaccinated with the Secretory Construct.

To compare C5-specific T cell precursor frequencies in mice vaccinated with the secretory C5 construct and mice vaccinated with 5.4 DNA vaccine, two groups of mice were chosen and CD4 precursor frequencies were measured in parallel at four time points (weeks 3, 6, 9, 12 and 15). As shown in table 3.2, the precursor frequency in mice vaccinated with secretory C5 is about two fold higher (on weeks 3 and 6) than in mice vaccinated with 5.4. This difference becomes less pronounced over the next 9 weeks, but nevertheless suggests that the presence of secreted C5 may allow activation of a higher number of effector cells. Its effect on the longevity of memory, however, remains to be tested still.

4. Discussion

Vaccine constructs:

DNA vaccination is shown to be a powerful tool for induction of T cell responses to an increasing number of antigens. Inoculation of DNA constructs via the skin results in protective immunity to a variety of pathogens. The underlying basis for induction of a T cell response is still poorly understood. The role of Langerhans cells (dendritic cells) in the skin, which migrate to lymphoid organs, where they play a crucial role in the induction of an immune response following DNA vaccination is investigated in this project. Two vaccine constructs were assembled, one possess full length C5 cDNA (5.4) and the other has 1.6 Kb from the 5′ end of C5 (1.6). There were several reasons that two constructs were made. The C5 cDNA is rather large and it was initially a matter of concern that it might not be fully expressed. Also in several studies it was shown that above a certain size the constructs used as vaccines lose their ability to initiate the immune responses.

Plasmids used for vaccination share the basic attributes of vectors developed for in vitro expression of genes in transfected cell lines. These include a) an origin of replication suitable for producing high yields of plasmid in E.coli; b) an antibiotic resistance (ampicillin) gene to confer antibiotic selected growth in E.coli; c) a strong inhancer/promoter (CMV) and an mRNA transcript termination /

polyadenylation sequences for directing expression in mammalian cells. Both expression systems are driven by a Cytomegalovirus (CMV) promoter which constitutively expresses the fragment. Because expression of many mammalian genes may be dependent on, or may be increased by, the inclusion of interon, intron A from early immediate gene of CMV (Chapman, *et al.*, 1991) was introduced between promoter and start codon of insert (C5). The expression of DNA vaccines in mammalian system (COS-7) was judged by RT-PCR. Full expression of vaccines was confirmed by detecting complete C5 cDNA using various C5 specific primers (see section 4.2). This observation suggests full expression of the large C5 fragment *in vitro*. C5 cDNA used in 5.4 construct encoding a form of C5 protein, which is not secreted by transfected cells with the aim to limit its accessability to antigen presenting cells and investigate the consequences for priming and maintenance of CD4 T cell responses. It is not known what causes the block in secretion of C5, but we recently determined that addition of an optimal leader sequence to the construct results in C5 secretion. The exclusive intracellular expression of the 5.4 construct after transfection into many different cell types, such as fibroblasts, B cells, macrophages and hepatocytes has been extensively tested (Brazil et al., 1997).

Initial approach

The initial approach taken was to test the vaccines *in vivo* . C5 deficient mice ($C5^-$), when immunised with C5 protein, generate a $CD4^+$ T cell response, whereas normal mice ($C5^+$) are fully tolerant to

C5 (Lin and Stockinger, 1989). Inoculation of either DNA vaccine (5.4 or 1.6) intramuscularly on A/J $C5^-$ mice induced C5 specific T cell responses. Both vaccines showed similar ability to induce C5 specific T cell responses, in A/J mice. This may be explained by the fact that the C5 b -chain seems to contain most of the C5 epitopes recognised by T cells. There are 8 independently derived T cell hybrids in the lab, all of which see distinct determinants on the C5 b chain, whereas we have not yet obtained a T cell hybrid recognised the C5 a chain.

Why Vaccination through skin?

DC are highly potent antigen presenting cells distributed throughout the body in low numbers. The evidence is now compelling for the migration of Langerhans cells (LC) from the skin to the draining lymph nodes (Hoefakker *et al.*, 1995; Kripke *et al.*, 1990). It was shown that Langerhans cells migrate from the epidermis of skin explants (Larsen et al., 1990) and this has been shown to occur via the dermal lymphatics (Lukas et al., 1996). This is also the proposed route for LC migration *in vivo* (Austyn and Larsen, 1990).

LC migration is a complicated event. Upon stimulation (for example by scarification) LC dissociate from neighbouring keratinocyte, leave their position in the epidermis, pass through the basement membrane into the dermis, enter the afferent lymphatics and relocate in the T lymphocyte rich areas of the draining lymph node. Each stage represents a different microenvironment, with its own combination

of extracellular matrix proteins, cytokines and cell types. Thus elucidating the mechanisms involved in LC migration in an onerous task but, by analogy to leukocyte migration, it can be hypothesised that migration of LC is mediated by cell adhesion molecules and regulated by chemical mediators. For example TNF-α can stimulate LC migration (Cumberbatch, *et al.*, 1994) *in vivo* and has been shown to produce maturational changes in LC *in vitro* (Chang, *et al.*, 1994).

The outer layer of skin interfaces directly with the external environment and is constantly in contact with innumerable pathogens. As a consequence, immunologic components for the elicitation of both humoral and cytotoxic cellular must be present along the border for counteracting infections. Evidence supporting the immunologic competence of the outer layer of skin includes the observations that: 1) antigens expressed in the epidermis are more immunogenic than those expressed in the dermis, and 2) genetic vaccines inoculated into the epidermis are more potent than those injected intramuscularly (Torres et al., 1997; Raz et al 1994). The large accessible area of the skin and its durability are other advantages of applying genetic vaccines to this tissue. Therefore, the *in vivo* transfection of dermal and epidermal cells, and especially APC, would be expected to provide an efficient route for gene immunisation that mimics the physiological responses to pathogens of the skin, as has recently been demonstrated in viral infections (Condon et al., 1996; Raz et al., 1994).

Following the preliminary observation with the 5.4 construct, the 5.4 plasmid vaccine was inoculated through three main routes; Ear skin, leg skin and muscle (i.m.). The aim of this part was to find a suitable draining lymph node for a particular vaccinated organ. Ease of localisation of the deep cervical lymph node adjacent to the ears and an effective induction of T cell responses following vaccination led to the conclusion that the ear skin was a suitable route of administration. Similar experiment was performed in a large group of mice, to study the kinetics of immune responses in the lymph nodes following DNA vaccination on ears. The highest C5 specific immune responses was observed on days 10 to 12 following vaccination in lymph nodes, while the immune response from the spleen started to rise later around day 14.

The importance of skin but not skeletal muscle cells to DNA-raised responses may reflect the much more highly developed immune surveillance function of skin than muscle (Stingl, 1990). Approximately 2% to 5% of the cells in the epidermis are bone marrow derived dendritic cells. In addition keratinocytes, the predominant transfected cell type in skin, are specialised for enhancing the immune surveillance function of LC. In response to the appearance of microbe or injury, keratinocytes secrete cytokines such as GM-CSF, IL-1 and TNF-a. These cytokines up-regulates the expression on LC of adhesion molecules and MHC class II and stimulate LC migration to lymph nodes (Warfel *et al.*, 1993).

In contrast, lower frequencies of resident dendritic cells in muscle may compromise the efficiency of DNA-based immunisation.

Protein versus DNA vaccination

It was of interest to determine any differences, in T cell activation and migration between two groups of mice, either vaccinated with DNA or with Protein adjuvant. The results of this comparative study showed that immune response from DNA vaccine was delayed by few days in compare to its conventional protein counterpart. Although the amount of protein produced *in vivo* can not be quantified and thus directly comparable with the dose of C5 protein injected, it seems likely that this delay was not due to the amount of antigen but rather to the time needed by the DNA to be internalised and expressed by cells.

The expression of C5 antigen by dendritic cells

Previous studies have shown the presence of gold particles coated with the plasmid of interest in dendritic cells following immunisation with a gene gun (Condon *et al.*, 1996) But whether dendritic cells were expressing and presenting the inoculated plasmid remained unclear. As shown before (Steinman *et al.* 1991) the only migratory cells in skin with the capability of antigen processing and presentation are Langerhans cells. As Langerhans cells co-express strongly co-stimulatory molecules and MHC class II, it is believed that they are the most suitable candidates for stimulation of naive T cells. It has been shown that following conventional protein adjuvant

vaccination via the skin, Langerhans cells actively process the antigen and migrate to adjacent lymph nodes, where antigen presentation is actively carried out (Larsen *et al.*, 1990). In DNA vaccination, like conventional protein adjuvant vaccination, there is no doubt that DCs play a crucial role in establishing a protective immune response but the question is: "Do DCs express the antigen of interest following direct transfection, or do they pick up the antigen from other transfected cells?". Indeed, various routes have been described by which dendritic cells can obtain the protein they present. They can either take up protein secreted by other cell types which were transfected following DNA vaccination, or pick up antigen from transfected cells, an as yet ill defined mechanism referred to as cross-presentation (Corr, *et al.*, 1996; Doe, *et al.*, 1996). Thirdly, they can be transfected themselves (Condon, *et al.*, 1996; Raz, *et al.*, 1994). We performed a series of experiments consisting in isolating dendritic cells from the lymph nodes, adjacent to the site of vaccine administration. RT-PCR analysis showed C5 expression by DCs but not by other cells (including B and T cells) in draining lymph node suggesting direct transfection of DCs following DNA vaccination. The recent studies by Cesares et al., showed the expression of vaccine by PCR in dendritic cells. However, because the exogenous DNA vaccine surrounding the cells may give a positive PCR result, this method is not reliable. In contrast, using the analysis of RNA expression, Dnase I enzyme treatment can be performed to abrogate any DNA contamination of sample before generation of cDNA. As a control we

included the result of PCR performed on RNA in parallel with RT-PCR experiments.

Unfortunately this type of analysis (RT-PCR) does not allow direct quantitation of the number of transfected cells. Further studies with a DNA vaccine encoding GFP as an expression marker (pTracer-C5) were carried out to overcome this problem. The 5.4 construct used contains GFP expressed under a second promoter to allow direct visualization of transfected cells by fluorescence microscopy. The first attempts to construct C5-GFP fusion vector resulted in dramatic decrease if not loss of GFP fluorescent emission. Inaddition, the choice of an adequate filter set is required to detect the fluorescent cells. Unfortunately, using the FITC filter set that was available on FACS analyser, the GFP protein excites in the range of 460 to 490 nm which covers the lower secondary excitation peak (the highest one is at 395 nm).

In an attempt to quantify the number of dendritic cells in draining lymph nodes which express the C5 construct, we set up functional studies and confocal analysis. Indeed, GFP containing dendritic cells were clearly identifiable by confocal microscopy. However, quantification of GFP positive cells among the other dendritic cells by confocal microscopy was almost impossible as laser intensity and microscope conditions need to be adjusted for each cell. Thus, dendritic cells from draining lymph nodes of 5.4 vaccinated mice were tested for their capacity to activate the C5 specific T cell hybrid A18.

Comparing the T cell responses obtained from the lymph nodes dendritic cells with our standard curve, we estimate that about 2% of the dendritic cells in the draining lymph nodes of vaccinated mice processed and presented C5 in the context of MHC class II molecules for recognition by A18 T cells. However, it is worth to mention that this result only shows the proportion of recovered dendritic cells that can both express and present the antigen, thus the number of transfected dendritic cells in lymph nodes is likely to be underestimated.

This observation supports the data obtained in a recent study by Porgador *et al.*, (1998) which showed that 50 to 200 transfected dendritic cells were responsible for the generation of CD8+ T cell responses in each draining lymph node following DNA vaccination.

Cross presentation

While it is possible to estimate the number of directly transfected DC, we cannot formally exclude the possiblity that some of these DC acquired C5 protein expressed in keratinocytes by cross-presentation. Notably, no antigen was transferred to DC from intact keratinocytes, whereas irradiation of keratinocytes provided a strong stimulus for crosspresentation. In addition, its restricted localisation is supported by the absence of antibody responses following vaccination. Thus, C5 message was only detectable in keratinocytes at the site of vaccination

and in a small proportion of DC within the draining lymph nodes.These result also support the restricted endogenous localisation of C5 protein after DNA vaccination.

However, it is still a matter of debate whether normal cell turnover by apoptosis in vivo results in release of antigenic material which could be obtained for presentation by dendritic cells or whether this event only occurs in inflammatory situations encountered during necrosis of cells or tissue (Matzinger, 1994). While there is no obvious sign of inflammation, such as swelling following scarification of the ears, it is nevertheless possible that some keratinocytes die in a manner that results in cross-presentation by skin dendritic cells which then migrate to the draining lymph nodes to activate T cells. However, this event is unlikely to take place at later timepoints after vaccination when the site of scarification has healed.

T cell activation and migration

The vast majority of lymphocytes migrate through the body by passing from the blood to secondary lymphoid tissue by way of specialised endothelium lining the high endothelial venules (HEVs). Lymphocytes are returned to the blood via the efferent lymphatic ducts and the thoracic duct. However, lymphocyte migration to the spleen is completely different, as the spleen is not supported by

lymphatic vessels, and hence migratory cells that extravasate in the spleen migrate directly back into the blood.

Lymphocytes show different migratory behaviour. Moreover within a certain class of lymphocyte, the state of differentiation or activation affects the distribution and migration of such lymphocytes. The migration of effector cells and memory cells is clearly different from that of their naive precursors. This is because the immune system allocates its resources in a rational and economical way, so as to best fight and protect against pathogens and other antigens (Mackay, 1993).

Thus, T cell exported from the thymus (naive T cell) immediately begin to recirculate through lymphoid tissue. Such cells are small and express high level of CD62-L (L-selectin). L-selectin is required for entry of naive CD4 cells into lymph nodes and thus for development of primary CD4 responses in those sites (Bradley, *et al.*, 1994). The molecular profile of naive T cells is consistent with their lymphoid tissue homing. Virtually all naive T cells express CD62-L ^{hi}, CD44 ^{lo} and CD25 ^{lo} phenotype. In contrast upon activation, they lose the CD62-L expression and acquire a high level of CD 44 and CD 25. CD69 is a T cell early activation marker and transiently expressed at a rather short period of time at the begining of activation.

Analysis of the sites and kinetics of T cell activation following DNA vaccination of transgenic A18 mice established that the initial activation step takes place in the draining lymph nodes, in agreement

with our finding of antigen expressing DC in this location. At late time points, spleen T cells are activated as well, but given the fact that the spleen does not contain C5 expressing APC it is most likely that these T cells have migrated into the spleen from lymph nodes.

Persistence of C5 antigen

Although keratinocytes were shown to express C5 up to 12 weeks after vaccination, the presence of antigen in these cells is unlikely to be responsible for the persistence of C5 specific T cell beyond 40 weeks. It is still debatable whether DNA vaccination leads to stable transfection (Kennedy et al. , 1997, Ulmer et al. , 1997). Dendritic cells have been described to have a half life of less than one week in mouse spleen and those DC present in lymphoid organs do not proliferate anymore (Metlay, et al., 1989; Steinman, et al., 1974) . A recent paper by Ingulli *et al.*, (1997) also described that DC which expressed antigen following *in vitro* pulsing, disappeared within few days after adoptive transfer into mice containing transgenic T cells recognising the antigen. However, unpulsed DC survived considerably longer following adoptive transfer. We found no evidence of C5 expressing dendritic cells in the spleen, indicating that the transfected DC found in draining lymph nodes do not migrate to other lymphoid organs. The absence of C5 expression in dendritic cells beyond 2 weeks after vaccination, together with the fact that a fresh cohort of naive transgenic T cells failed to be activated when injected into mice that had been vaccinated 20 days previously, support the conclusion that

there is no long-lived source of antigenic material present. However, the only other conceivable source of long lived antigen could be in the form of antigen/antibody complexes stored on the surface of follicular dendritic cells. Considering the absence of an antibody response, it is also unlikely that antigen was stored in the form of antigen-antibody complexes on follicular dendritic cells which are known to be highly efficient depots of long-lived antigen. It therefore seems that DNA vaccination can induce memory CD4 T cells which can be longlived without repeated antigenic stimulation.

generation of CD4 T cell Memory (Maintenance of immune response)

The development of memory cells is an important issue for vaccination purposes. Indeed, the capacity of the immune system to respond faster and stronger to a reencountered antigen is based on the development of the memory T cell population. These properties are due to both quantitative and qualitative changes in T cells: qualitative, with increase in the frequency of antigen-specific T cells; qualitative because memory T cells seem to develop into effector cells more efficiently than do naive T cells (Sprent and Tough, 1994). This last property may be explained by a higher expression of adhesion molecules than that of naive T cells, allowing them to adhere to antigen presenting cells more efficiently and to respond to lower doses of antigen as compared with naive cells (Pihlgren *et al.*, 1996). This

increase responsiveness might also be due to higher affinity interleukin-2 receptors (Ahmed and Gray, 1996).

Another question is the life-span of memory T cells. It has long been argued that memory cells have a prolonged lifespan and can often survive without the need for secondary contact with the infectious organism concerned (Gray, 1993; Zinkernagel *et al.*, 1996). This notion is supported by the observation that immunisation against small pox in childhood using vaccinia virus leads to an elevated precursor frequency of virus specific CD4 and CD8 cells for up to 50 years (Demkowics *et al.*, 1996). However, it is still uncertain whether the prolonged life span of memory cells is an intrinsic property of these cells or whether it reflects their intermittent stimulation either by residual deposits of specific antigen or through cross-reactive contact with environmental antigens. Resolution of this issue is important for practical reasons (Vaccines) and also because it defines how we view immune memory. FDC can trap antigen in the form of antigen-antibody complexes and retain it on their cell surface for long periods of time. It has been postulated that B cells or other APCs pick up this trapped antigen and present it to T cells (Celada, 1971; Feldbush, 1973; Mandel, *et al.*, 1980; MacLennan, 1994). Thus, antigen depots on FDCs could potentially play a role in sustaining CD4 T cell memory. However, as no antibody response was detected following DNA vaccination with 5.4 (See section 3.18), it seems that FDCs could not play a serious role in sustaining CD4 T cell memory. We also can not

ignore stable expression and continous supply of antigen by stable transfected cells subjected to DNA vaccination.

Turnover studies on cells with a memory phenotype in mice (Tough and Sprent, 1995; Bruno, *et al.*, 1996; Tanchot, *et al.*, 1997), have indicated that at least some of these cells divide with regularity without expressing the features of effector cells.

The notion that memory cell survival requires persistent contact with antigen stems from the observation that T and B cell memory can decline rapidly following adoptive transfer unless the primed cells are co-transferred with specific antigen (Feldbush, 1973; Gray and Skarvall, 1988; Gray and Matzinger, 1991; Gray *et al.*, 1996; Kundig *et al.*, 1996). However, we cannot exclude a stimulation by cross-reactive environmental antigens or via cytokines (Beverly, 1990; Selin *et al.*, 1994, Unutmaz *et al.*, 1994). Indeed it would seem almost inevitable that some memory cells are subject to intermittent stimulation via mechanisms that are not antigen specific. Nevertheless, it has to be borne in mind that late memory cells can remain in interphase for prolonged periods and display a resting phenotype. Hence to sustain the view that memory cells require chronic or intermittent stimulation, one has to argue that such stimulation leads to a very weak form of signaling which is sufficient merely to maintain cell survival without inducing over activation or entry into the cell cycle.

To assess the potential role of antibody responses in the maintenance of memory cells, the precursor frequency of C5 specific T cell was compared between two groups of A/J mice, one vaccinated with secretory C5 construct and thought to generate antibodies, and the other vaccinated with the non-secretory 5.4 construct and thus unable to generate antibodies. Although some differences were observed in the level of precursor frequencies in the first few weeks of priming between two groups of mice as reported before in another system (Gray et al., 1996), the frequencies of specific T cells after 12 weeks were almost comparable in presence and absence of antibody. Thus, these results suggest that DNA vaccination can induce long-lived CD4 T cells, in the absence of antibody response.

Previous studies carried out in μMT mice (Brundler *et al.*, 1996; Di Rosa and Matzinger, 1996; Asano and Ahmed, 1996; Topham *et al.*, 1996) support our observations. In these mice, the lack of antibody production due to the targeted disruption of the Igμ heavy chain transmembrane region, prevents the formation of immune complexes on FDCs. The general finding was that the generation and long term survival of memory $CD8^+$ cells is much the same in μMT mice as in normal mice.

The adjuvanticity of prokaryotic DNA

One of the most intriguing questions that is frequently asked is " Why does gene vaccination which generates only picograms or nanograms of gene product (antigen) induces a strong immune response (usually Th1 response), whereas immunisation of animals with the same dose of antigen (picograms or a few nanograms) does not elicit any response at all? " In animal models potent adjuvants magnify the immune response to low concentrations of the injected antigen. However, since gene vaccination is usually employed without any adjuvant, as "naked" plasmid DNA (dissolved in normal saline), it seems difficult to explain such an efficacity of DNA vaccination. However, the discovery that certain DNA sequences in plasmid DNA backbone act as adjuvants (Klinman *et al.*, 1997; Sato *et al.*, 1996), and that more specifically several types of polynucleotides are mitogenic for lymphocytes (Brown *et al.*, 1998), may in part resolve that question. For example polymers composed of guanosine and cytosine (poly G:C), or inosine and cytosine (poly I:C), are potent inducers of IFN-a and activate the lytic potential of macrophages and natural killer (NK) cells. By synthesising oligodioxynucleotides (ODN) from different regions of the mycobacterium genome, single stranded 45mer ODN where identified which also activate macrophages and NK cells (ref). This cell activation was attributed to DNA sequences containing a CpG motif within a palindromic hexamer. Thus, DNA vaccines seems to contain two conceptually distinct units: i) a transcription unit that

directs antigen synthesis and ii) an adjuvant unit in the DNA backbone (CpG motifs).

What are the potential mediators of the DNA adjuvant effect? First, the access of DNA to the circulation observed upon DNA vaccination, even by gene gun (Barry and Johnston, 1997; Klinman, et al., 1998) plays obviously an important role in this adjuvant effect. Indeed, it was shown that the immediate removal of the ear after vaccination does not prevent the immune response (Torres, *et al.*, 1997). The passage of DNA in the circulation seems also to happen after scarification, since we showed by PCR analysis that the vaccine was higly present in lymph node while was weaker in spleen. Second, it has been reported that DNA had potent immunostimulatory activity leading to polyclonal B cell activation and macrophage activation (Stacey et al., 1996). The data presented in the present work, confirm that DNA provides activation signals to dendritic cells. Considering that all DC in the draining lymph nodes are activated, whereas only a very low number of DC was directly transfected, it seems that small amounts of DNA reaching the circulation can exert stimulatory activity, but fail to be internalised and transcribed by cells.

In view of recent data showing the crucial importance of DC activation (following DNA vaccination) for stimulation of CD8 T cell responses (Porgador *et al.*, 1998), the finding that DNA vaccination non-specifically activates a large number of dendritic cells demonstrates that this type of genetic immunisation provides ideal conditions for

the initiation of T cell responses. The success in achieving long-lived memory responses by DNA vaccination may therefore lie in optimal activation of most antigen specific T cell precursors, rather than in long-term storage of antigen leading to periodic re stimulation.

Recent similar studies

A number of recent studies point to the importance of DCs in inducing immunity following DNA vaccination. Our observation provide mechanisms underlying few recent observation. Ulmer *et al.* (1996) showed that F1 (H-2^d X H-2^K) mice injected with myoblast cell line (H-2^k) transfected with a foreign gene developed H-2^{d-} and H-2^{k-} restricted immunity. Corr *et al* (1996) generated bone marrow chimers in which the haplotypes of bone marrow was mismatched with the haplotype of somatic cells at the site of injection and demonstrated that CTL responses induced following DNA immunisation was restricted to the MHC haplotype of the bone-marrow APC. In recent study Porgador *et al.*, (1998) showed 50 to 200 transfected dendritic cells responsible for generation of CD8+ T cell responses in each draining lymph node following DNA vaccination.

These result offer new insights into enhancing the immune response to weak plasmid DNA-encoded immunogens. Other strategies that have been reported to augment the immune response include; the co-delivery of expression vectors encoding IL-2 and GM-CSF with antigen. Further studies (Manicken et al. 1997) also reporting

enhancement of the immune response to naked DNA vaccine by immunisation with transfected dendritic cells, support the major role of dendritic cells in genetic immunisation.

5. Future studies

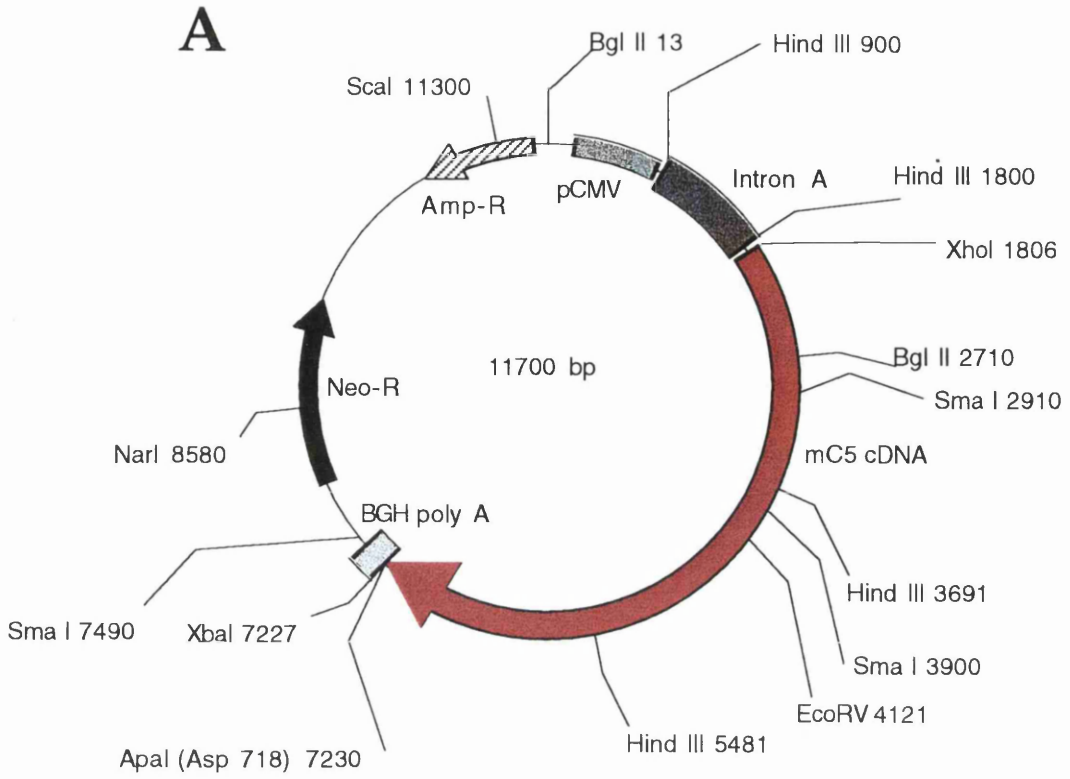
Experiments are already in progress to generate a dendritic cell specific expression construct with a promoter from CD11c gene. Although it was established that the CMV promoter used generally in DNA vaccination vectors is a powerful promoter, it would be of interest to generate a DNA vaccine that only targeted dendritic cells. To assess whether this will allow generation of $CD4^+$ and $CD8^+$ immune responses.

It would also be of interest to study in more details the role of antibodies in maintenance of $CD4^+$ T cell memory. In general the antibody responses induced by DNA vaccination is significantly lower than that induced by protein vaccination (Raz, 1997). The C5 antibody response generated as a result of vaccination with the secretory C5 construct is very low and variable. One option is to generate C5 cDNA again with new primers and study the 5' end of template by primer extension method. The other option is to generate new constructs with other secretory tags in order to optimise the C5 secretion.

Finally, I would like to focus on CD8+ T cell responses following DNA vaccination. While immunisation with C5 protein were induces CD8 T cell responses, the situation could be different in DNA vaccination, where the antigen is expressed intracellularly and may therefore gain access to the class I presentation pathway. I will attempt to generate CD8+ T cell clones from A/J mice vaccinated with the C5 construct with the aim to finally clone the TCR of a C5 specific CD8+ T cell clone to generate TCR transgenic mice. This would allow us to study activation and regulation of CD4 and CD8 T cell responses specific for the same antigen.

6. FIGURES

Figure 3.1: pcDNA 3.1-C5 (5.4) and pcDNA 3.1-1.6 (1.6) constructs.
pcDNA 3.1-C5 (5.4) is a mammalian expression vector (A). The expression is driven by a CMV promoter and enhanced by intron A. Referred to in the text as 5.4 vaccine.
pcDNA3.1-1.6 expresses 1.6 Kb of N-terminus from C5 generated by PCR with an XbaI site designed in reverse primer for the purpose of cloning (B).


A
Bgl II 13
Hind III 900
ScaI 11300
Amp-R
pCMV
Intron A
Hind III 1800
XhoI 1806
Bgl II 2710
Sma I 2910
11700 bp
Neo-R
NarI 8580
mC5 cDNA
BGH poly A
Hind III 3691
Sma I 7490
XbaI 7227
Sma I 3900
EcoRV 4121
ApaI (Asp 718) 7230
Hind III 5481

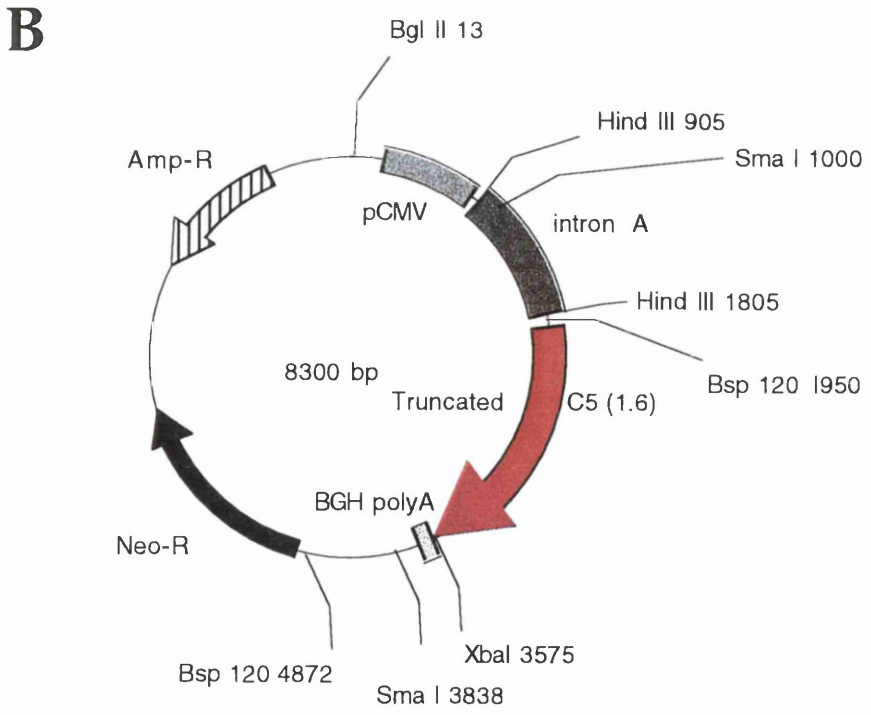

B
Bgl II 13
Hind III 905
Amp-R
Sma I 1000
pCMV
intron A
Hind III 1805
8300 bp
Truncated
C5 (1.6)
Bsp 120 I950
BGH polyA
Neo-R
XbaI 3575
Bsp 120 4872
Sma I 3838

Figure 3.2:Restriction enzyme analysis of pcDNA3.1-C5 (5.4) and pcDNA3.1-1.6 (1.6) A) The restriction pattern of pcDNA3.1-C5(5.4) encoding the full length C5 protein, visualised on an ethidium bromide gel. Lines 2 to 6 correspond to the restriction digests with ApaI (11700), Bgl II (9000 and 2697 Kb fragments), Hind III (7200,1891,1790 and 900 Kb fragments), Asp 718 (11700 Kb fragment) and SmaI (7120, 3590 and 990 Kb fragments) enzymes respectively. Line 7 shows uncut plasmid and line 1 and 8 show molecular weight markers.
B) Restriction enzyme analysis of pcDNA3.1-1.6 (1.6) encoding 545 N-terminal a.a. of C5 protein. Lines 1 to 4 correspond to the restriction digests with XbaI (8300 Kb fragment), Bgl II (8300 Kb fragment), HindIII (7400 and 900 Kb fragments) and Sma I (2828 and 5472 Kb fragments) enzymes respectively. Line 5 shows molecular weight marker.
These experiments confirmed the insertion and right orientation of both cDNAs.

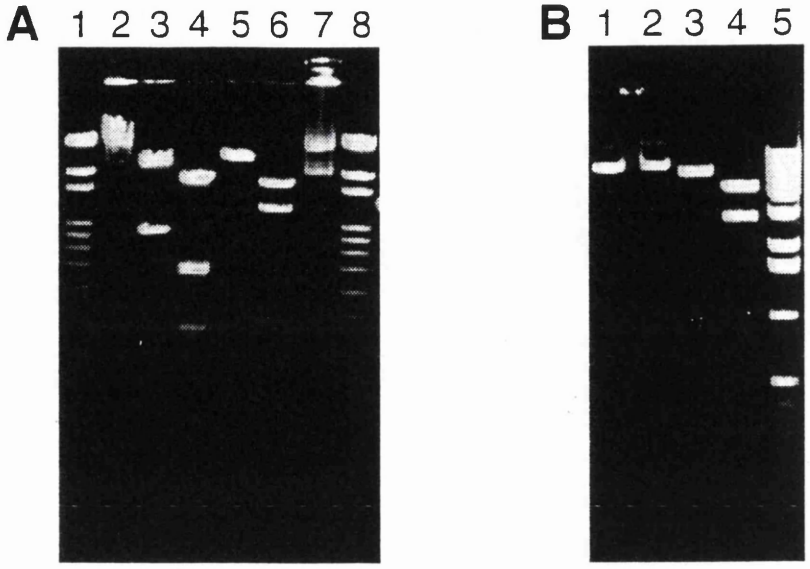


Figure 3.3: Analysis of the transient C5 mRNA expression in 5.4 / 1.6 transfected COS-7 cells. RT-PCR on transfected COS-7 cells with 5.4 was performed, using C5 specific primers. The order of primers and size of DNA fragments expected from PCR are shown on a schematic representation of the C5 sequence. Line 1 corresponds to primers HH7/BR, line 2 to β-actin, line 3 to PCR of total RNA, line 4 to marker, line 5 to HH7/C5 ENI and line 6 to BF/BR primers (A). These amplifications stretching all along the C5 sequence confirm full expression of C5 message in a mammalian system.

RT-PCR on COS-7 cells transfected with the 1.6 construct was performed, using a set of primers HH7/1.6R (shown on schematic linear 1.6) (B). The 1.6 band indicated full expression of vaccine.

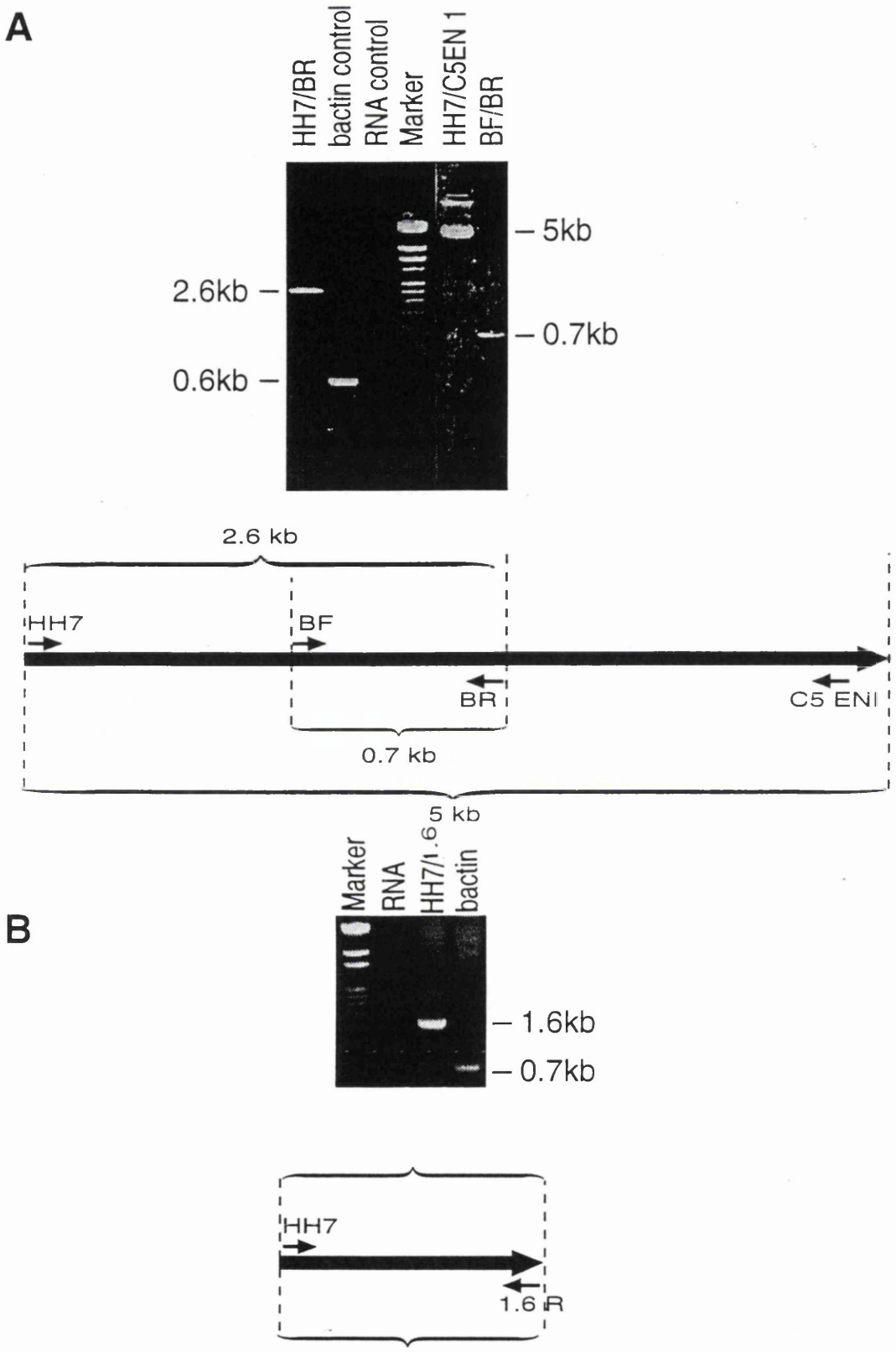

A
HH7/BR
bactin control
RNA control
Marker
HH7/C5EN 1
BF/BR
– 5kb
2.6kb –
– 0.7kb
0.6kb –
2.6 kb
HH7
BF
BR
C5 EN1
0.7 kb
5 kb
B
Marker
RNA
HH7/1.6
bactin
– 1.6kb
– 0.7kb
HH7
1.6 R

Figure 3.4: Comparison between the immune response induced by full length C5 (5.4) vaccine and by 1.6 vaccine. Three groups of A/J mice (each group 3 mice) were chosen for vaccination. The first group was vaccinated with 150 μg of 5.4 vaccine intramuscularly (yellow) and the second group received the same quantity of 1.6 DNA vaccine intramuscularly (blue). As a negative control, the third group was vaccinated with 150 μg of the empty vector (Black). The IL-2 response of pooled spleen cells from each group stimulated with C5 protein *in vivo* was determined 5 weeks after vaccination. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.

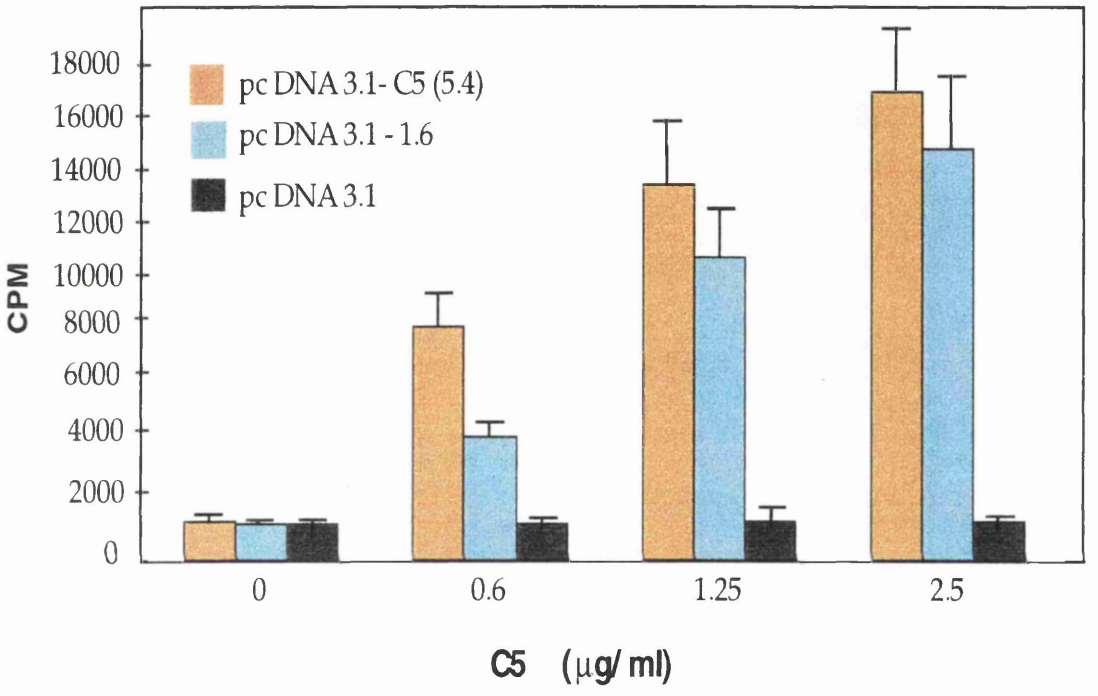
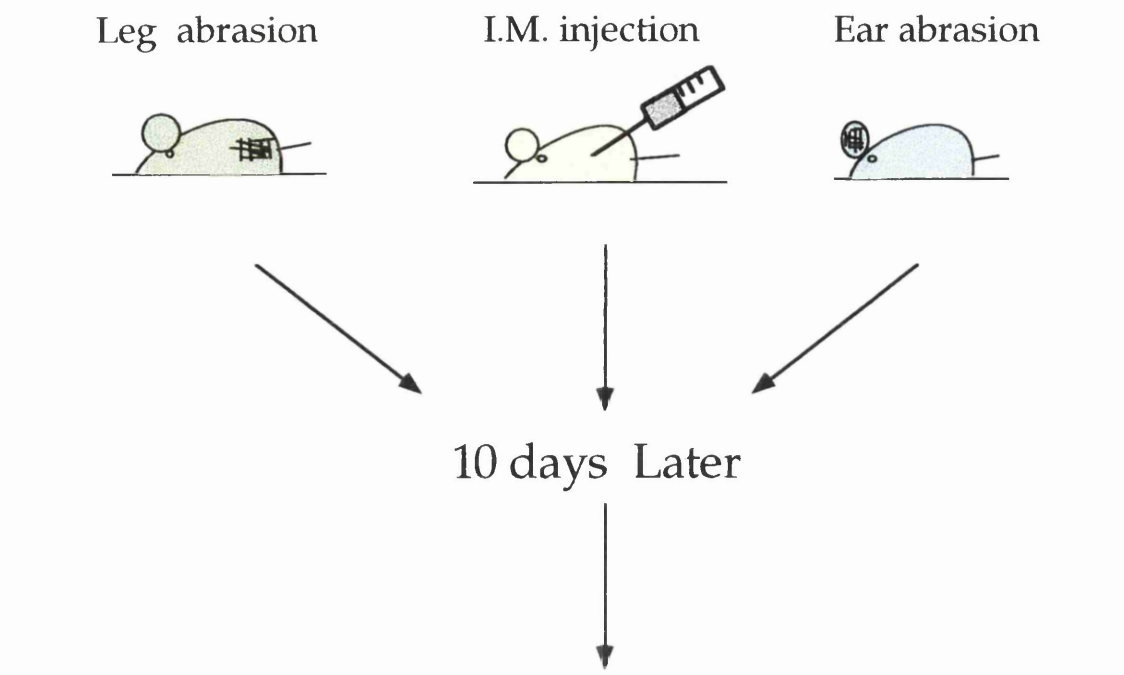

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CPM
pc DNA 3.1- C5 (5.4)
pc DNA 3.1 - 1.6
pc DNA 3.1
0
0.6
1.25
2.5
C5 (µg/ ml)

Figure 3.5: Early lymph node immune response in relation to the route of DNA vaccine administration. IL-2 production by draining lymph node cells, adjacent to the site of immunisation (leg skin, green area; muscle, yellow area; ear skin, blue area) in response to different concentrations of C5 protein, was determined in 3 groups of mice (each group consisted of four mice) 10 days after vaccination with 5.4 vaccine. As negative control mice vaccinated with the empty vector (two mice in each group) were shown in parallel. Ear DNA vaccination gave the highest early immune responses. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.



IL-2 response from draining lymph node cells

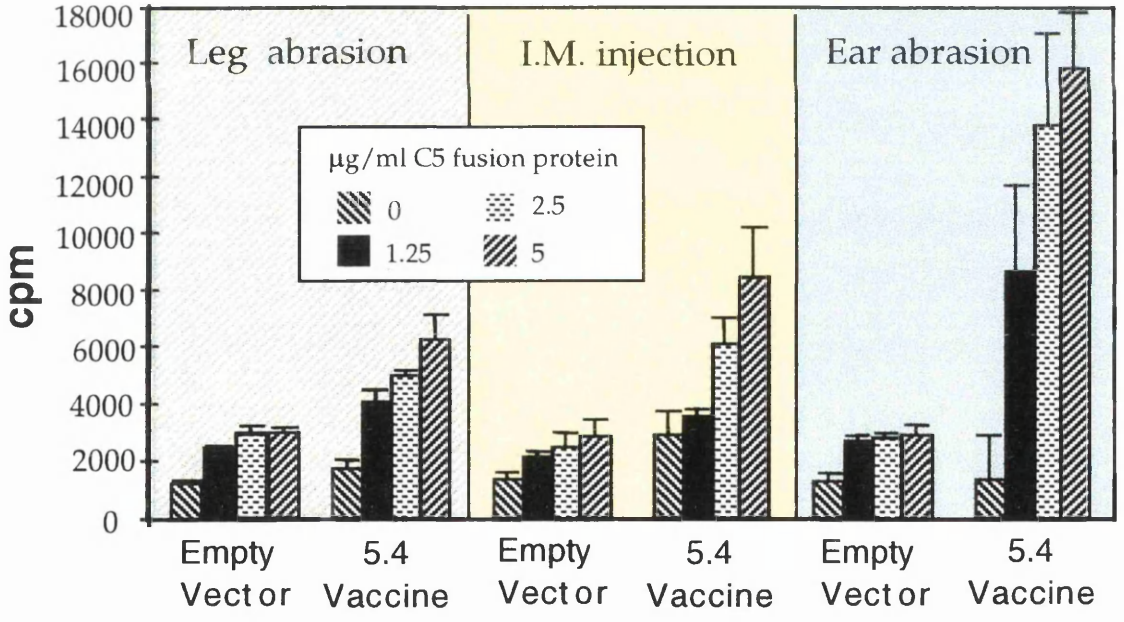


Figure 3.6: Late spleen cells immune response to DNA vaccination. IL-2 production by splenocytes from mice vaccinated 5 weeks earlier with 150 μg of 5.4 vaccine either through leg abrasion (green area), i.m. injection (yellow area) or by ear skin scarification (blue area) was determined *in vitro* in response to different concentrations of C5 protein. The splenocyte from 4 mice were pooled in each group. As negative control, IL-2 response of pooled splenocytes from empty vector vaccinated mice (each group consist of 2 mice) was determined. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.. These results indicate that C5 specific immune responses induced by DNA vaccination through 3 routes of administration can be detected later in the spleen of mice.

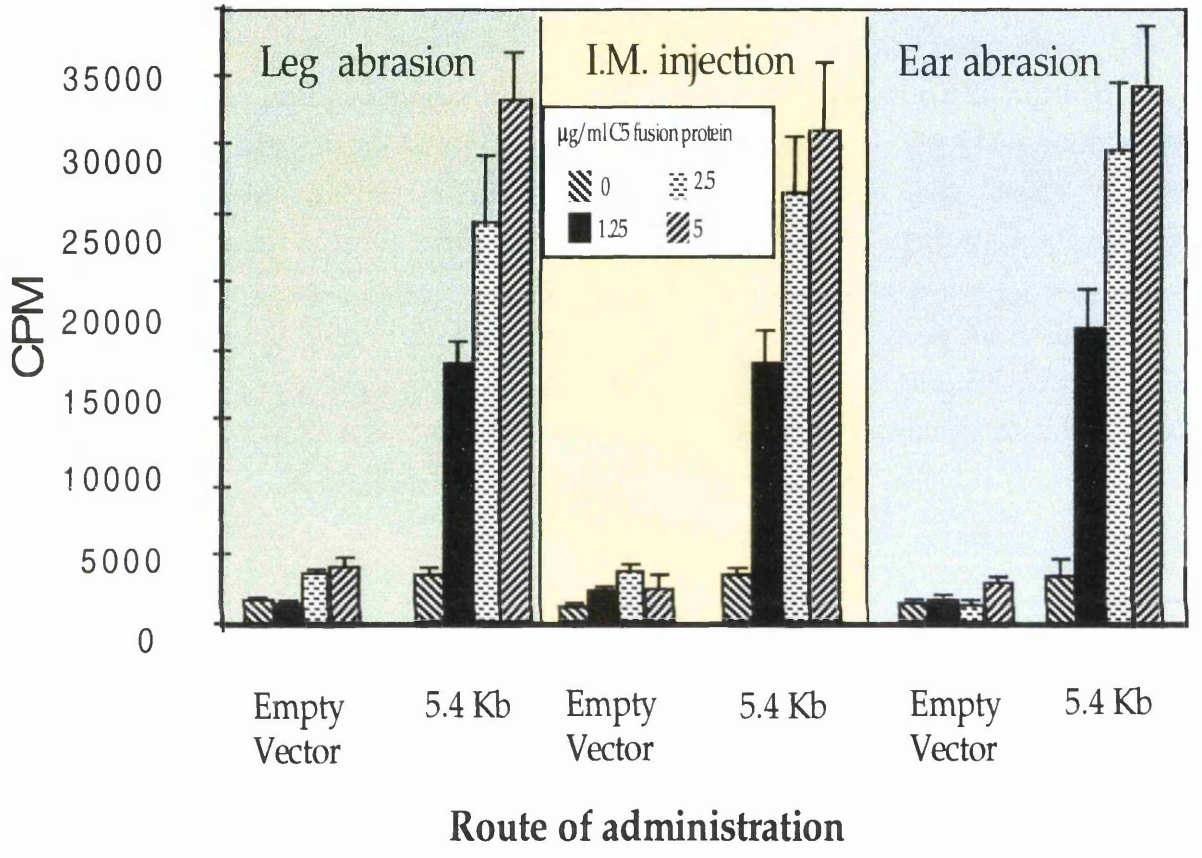
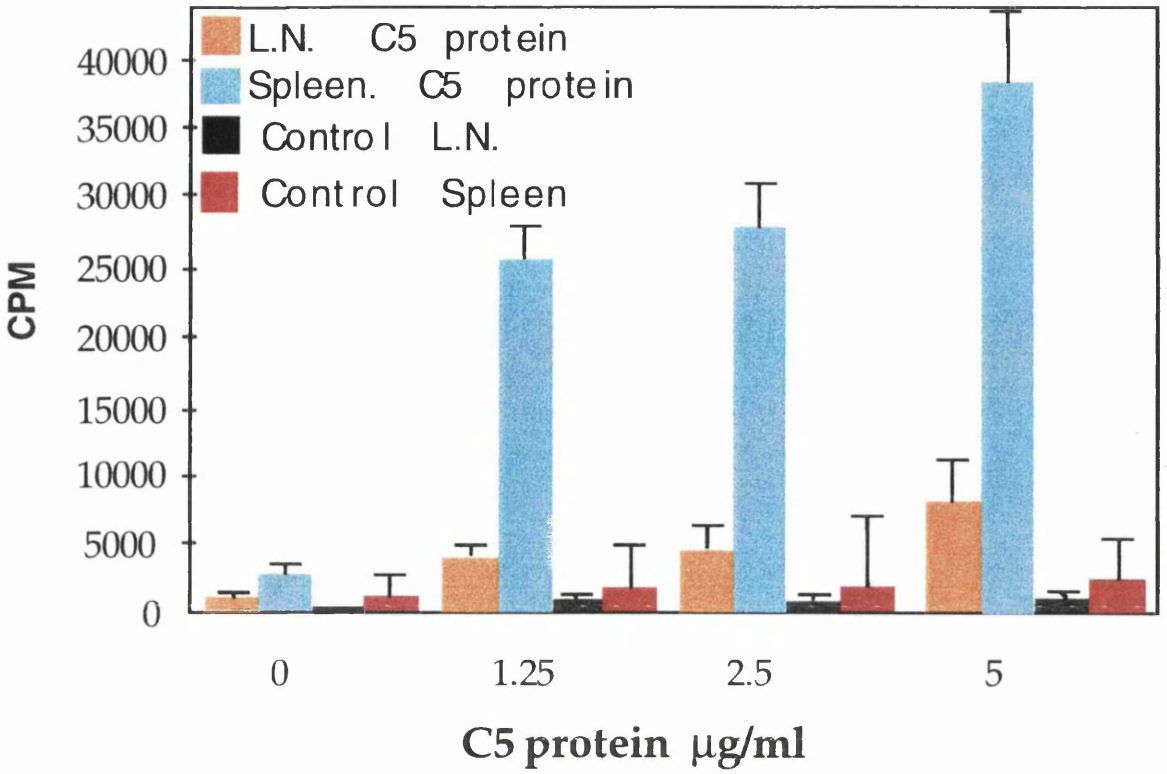

Leg abrasion
I.M. injection
Ear abrasion
µg/ml C5 fusion protein
0
2.5
1.25
5
CPM
35000
30000
25000
20000
15000
10000
5000
0
Empty Vector
5.4 Kb
Empty Vector
5.4 Kb
Empty Vector
5.4 Kb
Route of administration

Figure 3.7: Different localisation of C5 specific T cell responses to DNA or protein vaccines. Two groups of A/J mice (each group consisted of 4 mice) were vaccinated either with 150 μg of 5.4 DNA vaccine by ear skin scarification (B) or with 10 μg of C5 protein in complete Freud's adjuvant subcutaneously (A). IL-2 production by pooled lymph node cells (yellow) and pooled spleen cells (blue) in response to different concentrations of C5 was determined. As negative control, mice (2 mice in each group) were injected with saline (A) or empty vector (B) and IL-2 responses from pooled lymph node cells (black) and pooled spleen cells (red) were shown in parallel. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.

A


L.N. C5 protein
Spleen. C5 protein
Control L.N.
Control Spleen
CPM
40000
35000
30000
25000
20000
15000
10000
5000
0
0
1.25
2.5
5
C5 protein μg/ml

B

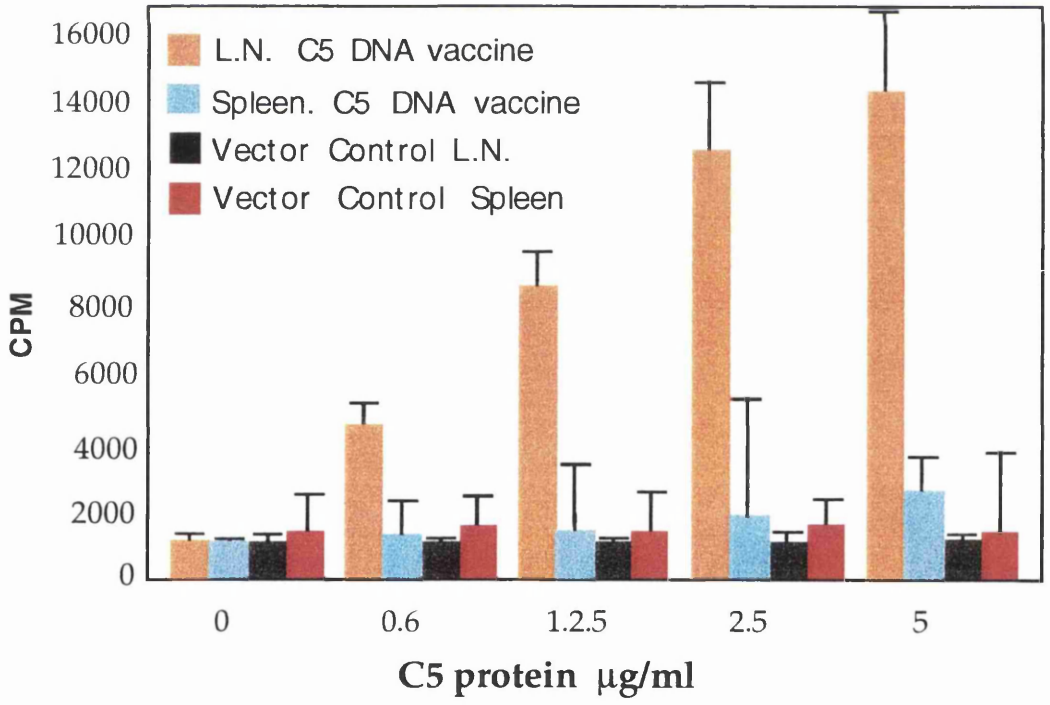
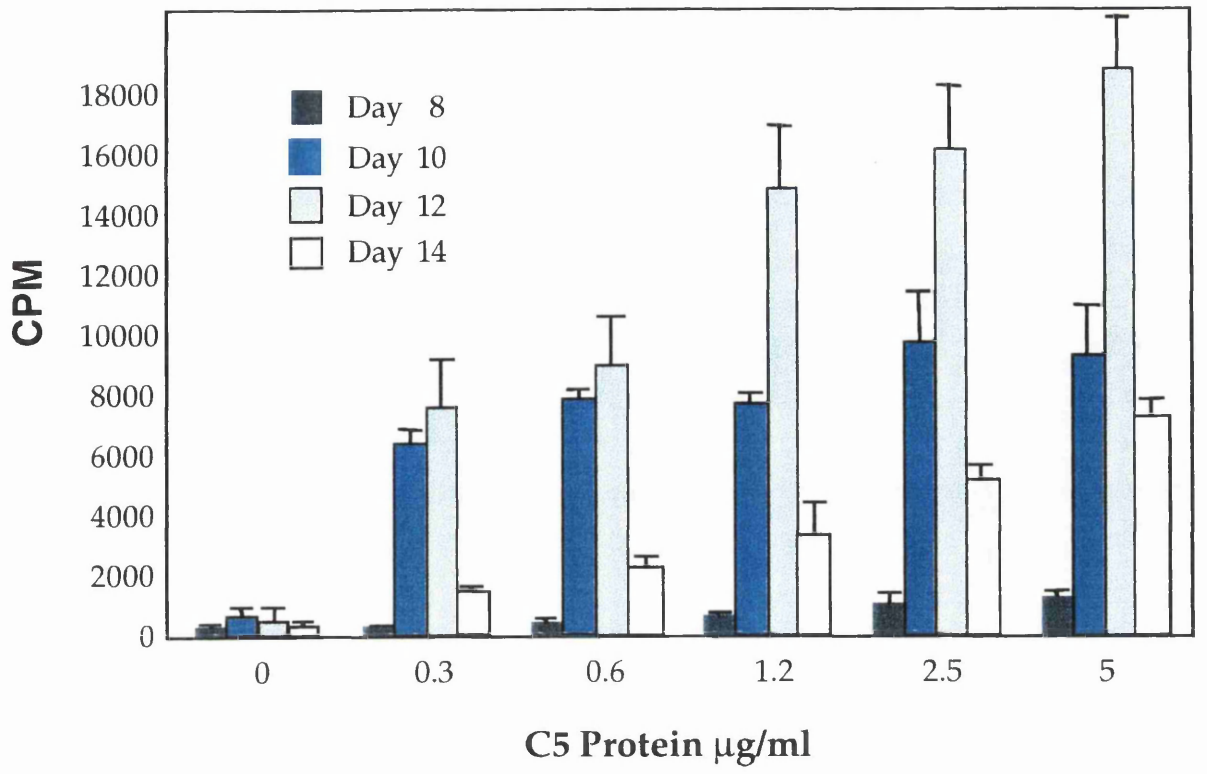

L.N. C5 DNA vaccine
Spleen. C5 DNA vaccine
Vector Control L.N.
Vector Control Spleen
CPM
16000
14000
12000
10000
8000
6000
4000
2000
0
0
0.6
1.2.5
2.5
5
C5 protein μg/ml

Figure 3.8: Kinetic analysis of lymph node immune responses following DNA vaccination. 20 A/J mice were vaccinated with 150 μg of 5.4 DNA by ear scarification. At different time points after vaccination cervical lymph nodes cells and splenocytes from 5 mice per time point were recovered, pooled and re-stimulated by C5. The IL-2 production was then determined. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells. While the highest C5 specific immune response was detected on days 10/12 after DNA vaccination in cervical lymph nodes (A), the immune response can be detected later in spleen around day 14(B).

A


Lymph node
CPM
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Day 8
Day 10
Day 12
Day 14
0
0.3
0.6
1.2
2.5
5
C5 Protein μg/ml

B

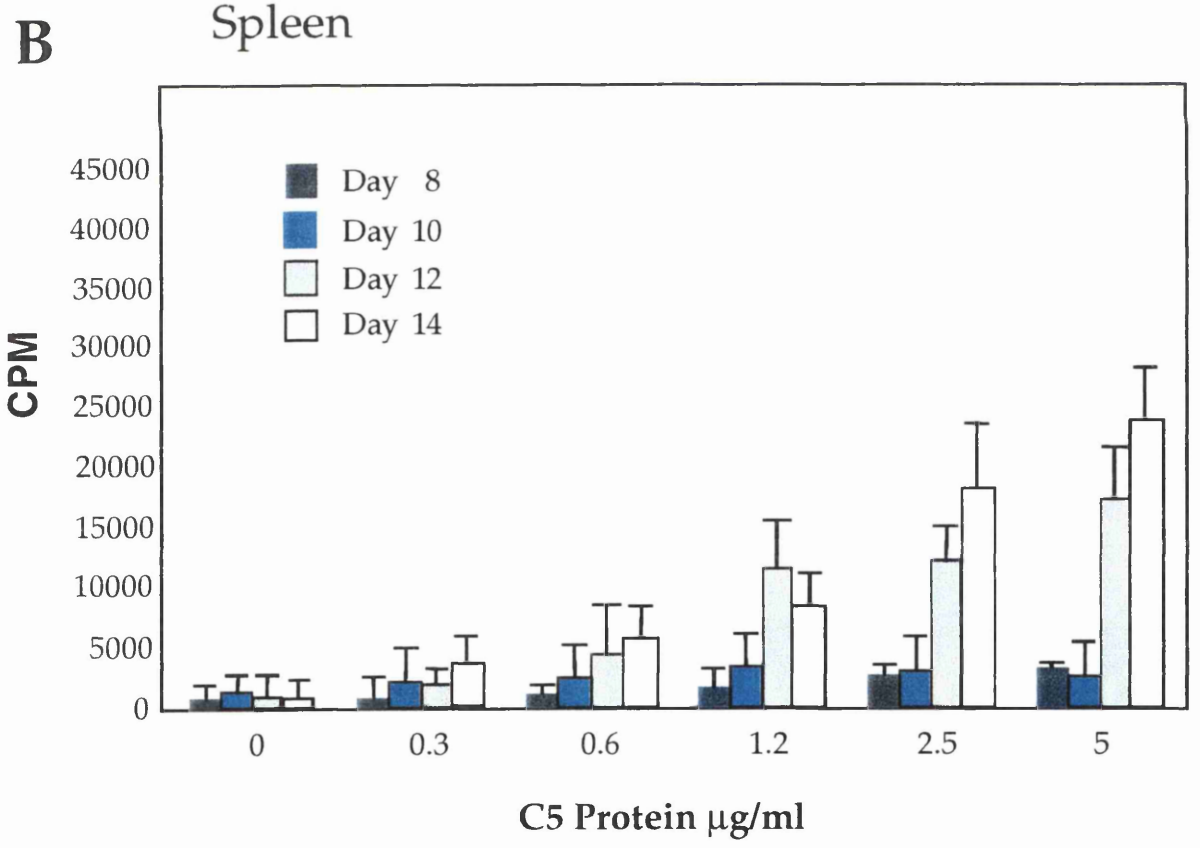

Spleen
CPM
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Day 8
Day 10
Day 12
Day 14
0
0.3
0.6
1.2
2.5
5
C5 Protein μg/ml

Figure 3.9: Expression analysis of C5 protein in lymph node cells and keratinocytes 12 days following DNA vaccination.

RT-PCR was performed for C5 and b-actin messages from spleen and lymph node DC or DC depleted cells (NDC) from 5.4 vaccinated mice (A). As controls expression of C5 message by spleen (B) or lymph node (C) dendritic cells from empty vector vaccinated mice were analysed. In addition, the C5 expression was assessed in keratinocytes from 5.4 or empty vector vaccinated mice.

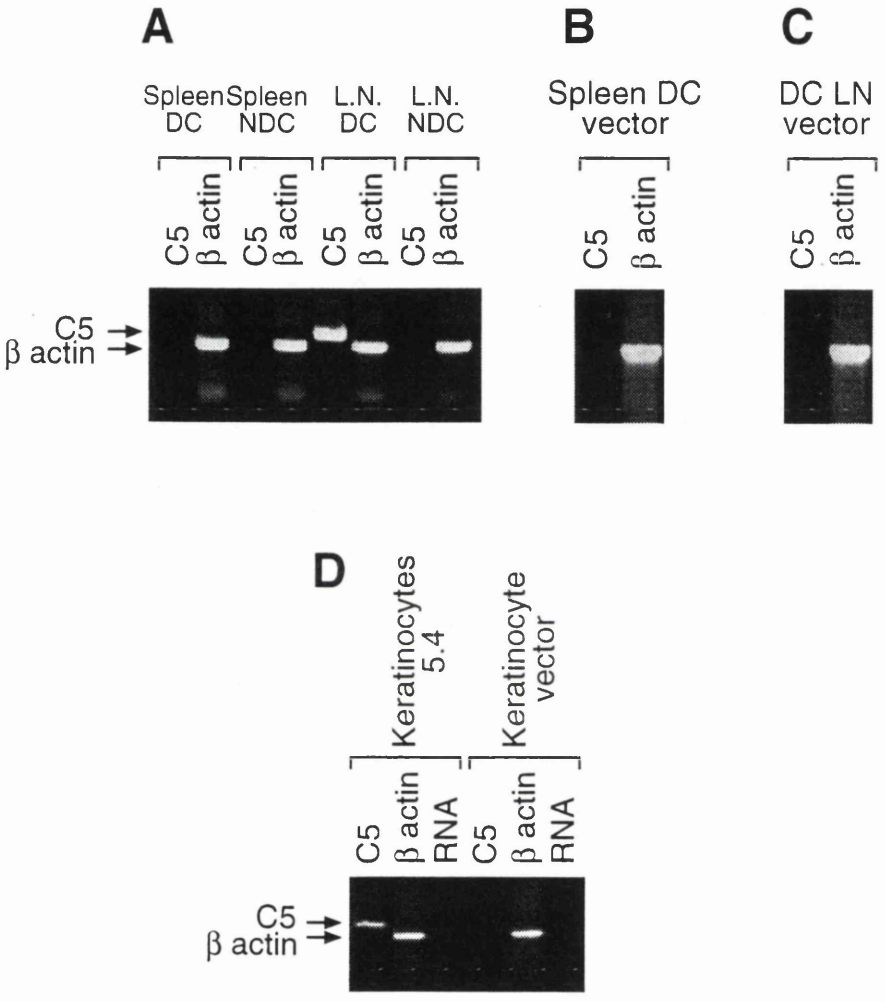

A
Spleen DC
Spleen NDC
L.N. DC
L.N. NDC
C5
β actin
C5
β actin
C5
β actin
C5
β actin
C5
β actin
B
Spleen DC vector
C5
β actin
C
DC LN vector
C5
β actin
D
Keratinocytes 5.4
Keratinocyte vector
C5
β actin RNA
C5
β actin RNA
C5
β actin

Figure 3.10: Quantification of C5 presenting dendritic cells from draining lymph nodes following 5.4 DNA vaccination.

IL-2 production by the C5 specific T cell hybridoma (A18) co-cultured with $2x10^4$/well DC isolated from draining lymph nodes of vaccinated mice was analysed. The percent of C5 presenting DC was then determined from a standard curve set up by co-culturing different ratios of untreated and C5 pulsed bone marrow derived DC with A18 hybridoma cells in vitro. The ratio of unpulsed:pulsed DC is shown on the abscissa, and the counts obtained by co-culture of A18 hybridoma cells and DC from vaccinated mice intersects the standard curve at a ratio of 98 unpulsed to 2 pulsed DC. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.

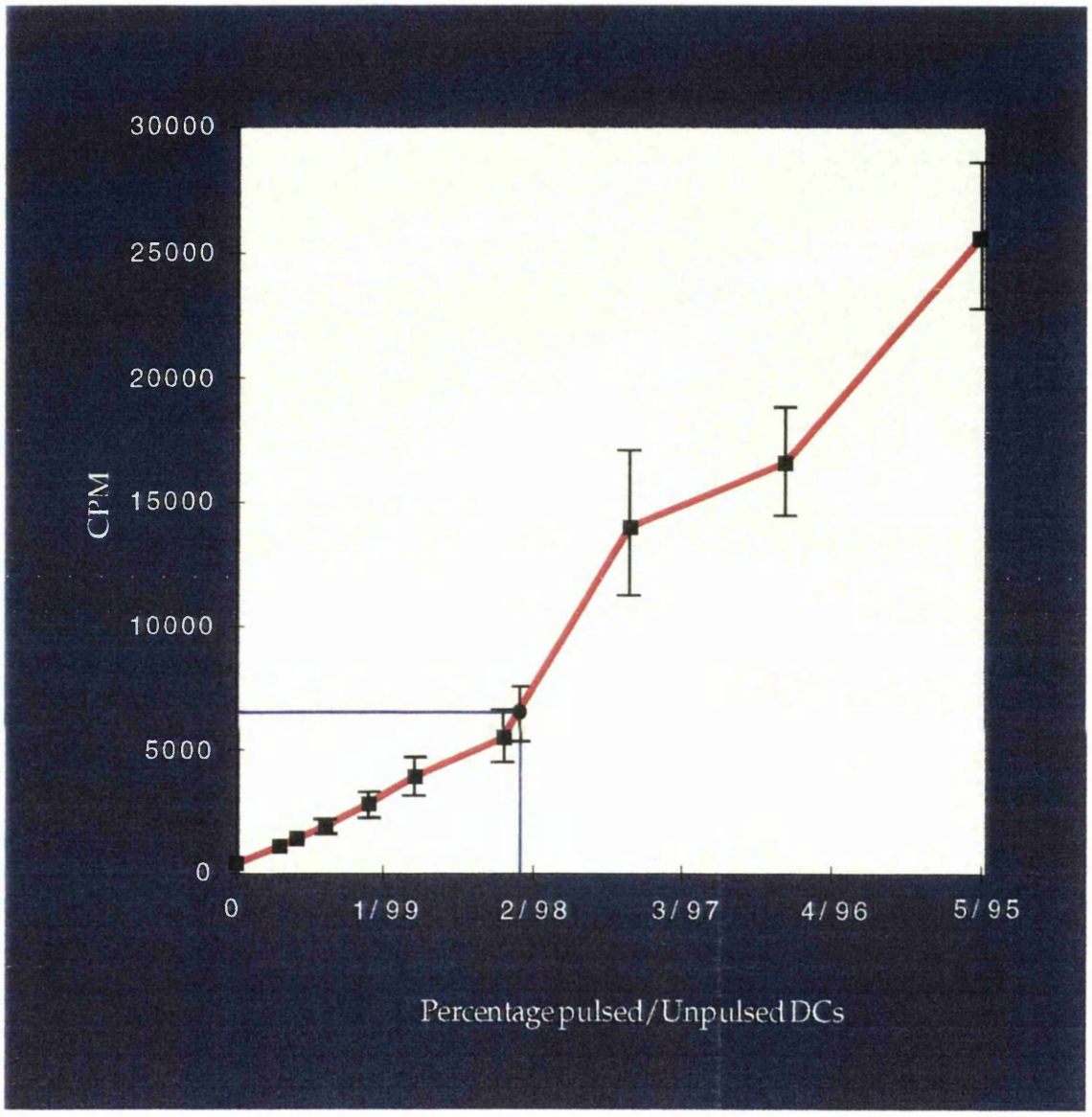
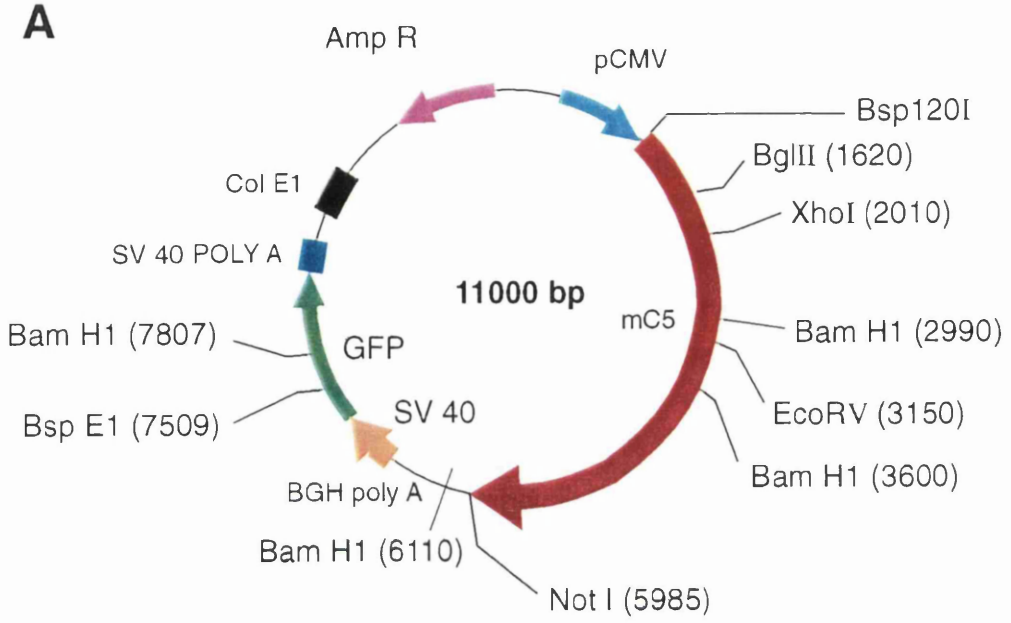
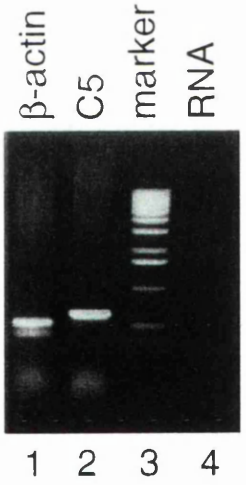

30000
25000
20000
15000
10000
5000
0
CPM
0
1/99
2/98
3/97
4/96
5/95
Percentage pulsed/Unpulsed DCs

Figure 3.11: The pTracer construct and analysis of expression
Full C5 cDNA was inserted between Not I and Asp 718 in the pTracer-C5 construct containing GFP under a separate promoter (A). Assessment of C5 expression in pTracer-C5 transfected COS-7 cells was analysed by RT-PCR using C5 specific primers (BamF/BamR=0.7 Kb). Lane 2 corresponds to C5 expression, lane 1 to the ß-actin positive control and Lane 4 to the PCR product on total RNA (negative control) (B) The expression of GFP in COS-7 cells transfected with pTracer-C5 was confirmed under the fluorescent microscope (C).

A


Amp R
pCMV
Bsp120I
BglII (1620)
XhoI (2010)
Col E1
SV 40 POLY A
11000 bp
mC5
Bam H1 (2990)
Bam H1 (7807)
GFP
EcoRV (3150)
Bsp E1 (7509)
SV 40
Bam H1 (3600)
BGH poly A
Bam H1 (6110)
Not I (5985)

B


β-actin
C5
marker
RNA
1 2 3 4

C

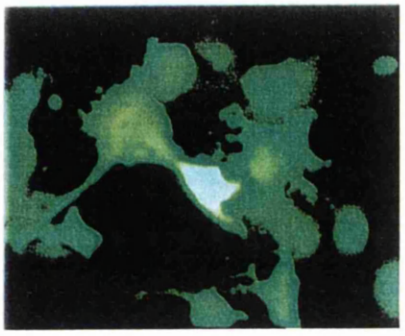
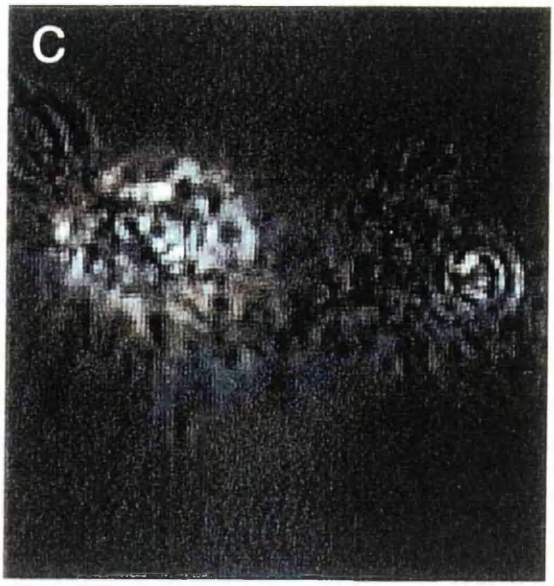
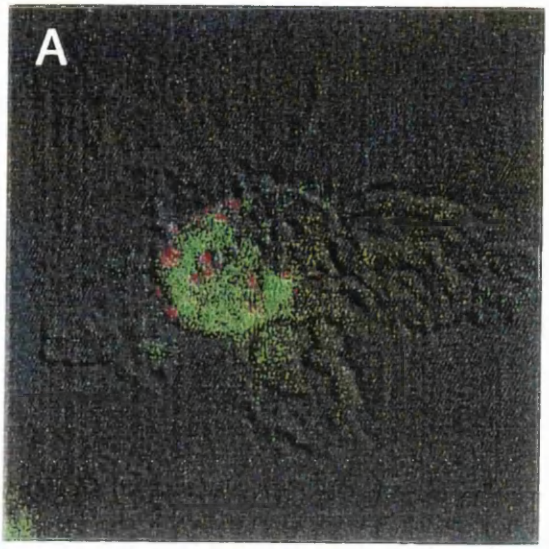
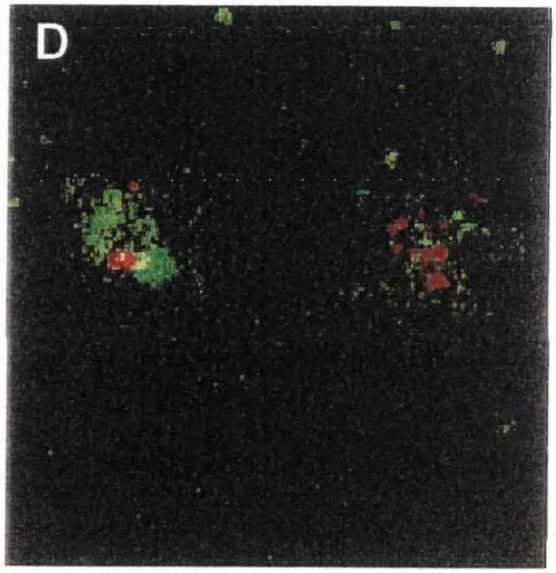


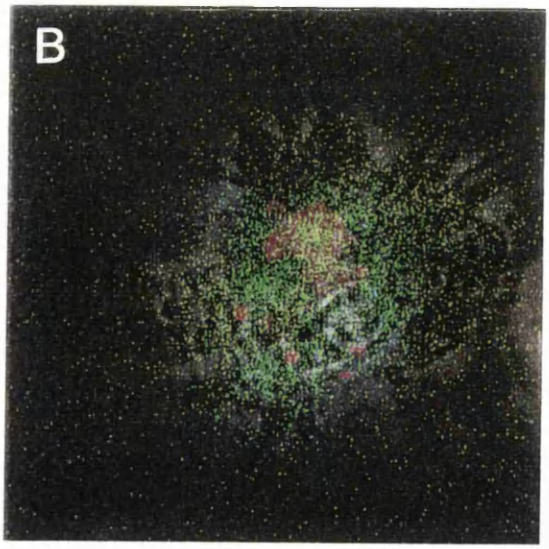
Figure 3.12: Confocal analysis of dendritic cells from draining lymph node of p-Tracer-C5 vaccinated mice.

DC from draining lymph node of pTracer-C5 vaccinated A/J mice were isolated and assessed for the co-expression of GFP (green fluorescence) and CD11c (red fluorescence). The CD11c expression was detected using biotinylated N418 mAb and streptavidin Texas-Red. The concurrently acquired bright field image (shown separately in C) is composited with the fluorescence image (D) to generate overlay (E) which illustrates the dendritic shape of the cells as well as co-expression of dendritic cell specific marker and vaccine construct-expressed GFP. Two other such overlays are depicted in A & B.


C


A


D


B

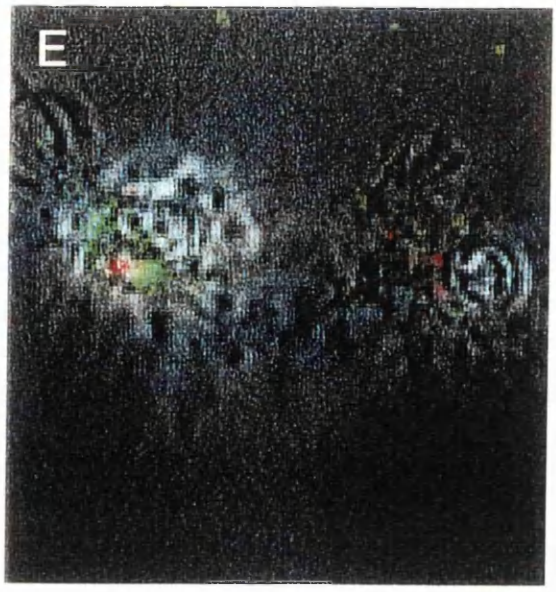
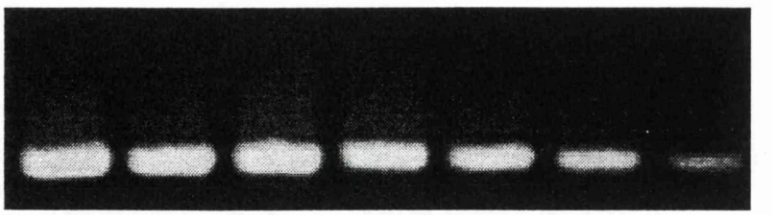

E

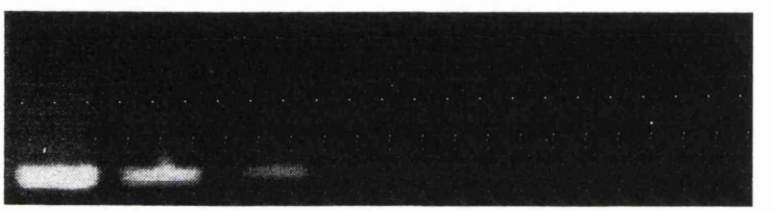
Figure 3.13: Quantification of DNA in lymph nodes and spleen.
Semi-quantitative PCR was performed to compare the amount of C5 DNA between lymph nodes (A) and spleen (B) cells from mice 3 days after vaccination with 5.4. DNA was extracted from 10^6 cells and subjected to different number of PCR cycles using C5 specific primers. The number of PCR cycles is indicated for each lane.

A



40 35 30 25 20 15 10

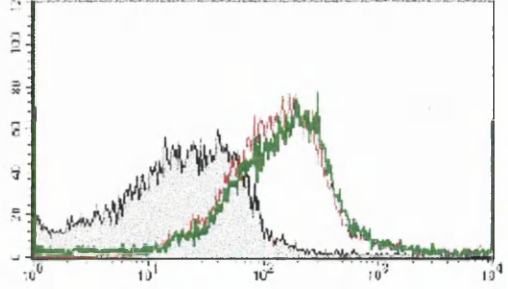
B



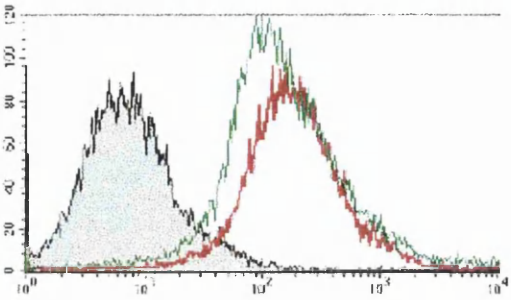
40 35 30 25 20 15 10

Figure 3.14: Activation marker analysis on lymph node dendritic cells
DC from draining lymph nodes of 5.4 vaccinated (red), vector (green) and untreated (shaded gray) were isolated by magnetic cell sorting. Expression of activation markers B7.1, B7.2, CD40 and ICAM-1 was analysed by FACS on gated N418 positive dendritic cells.

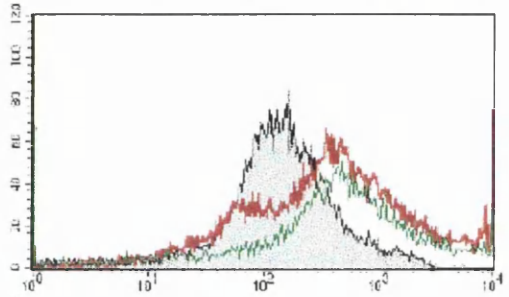
B7-1



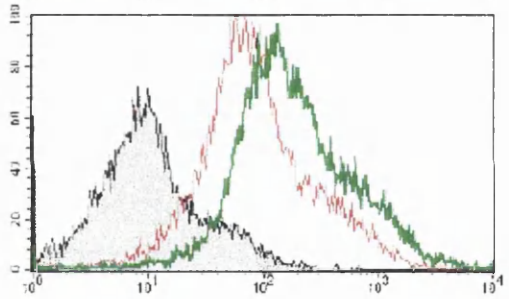
B7-2



ICAM-1



CD40



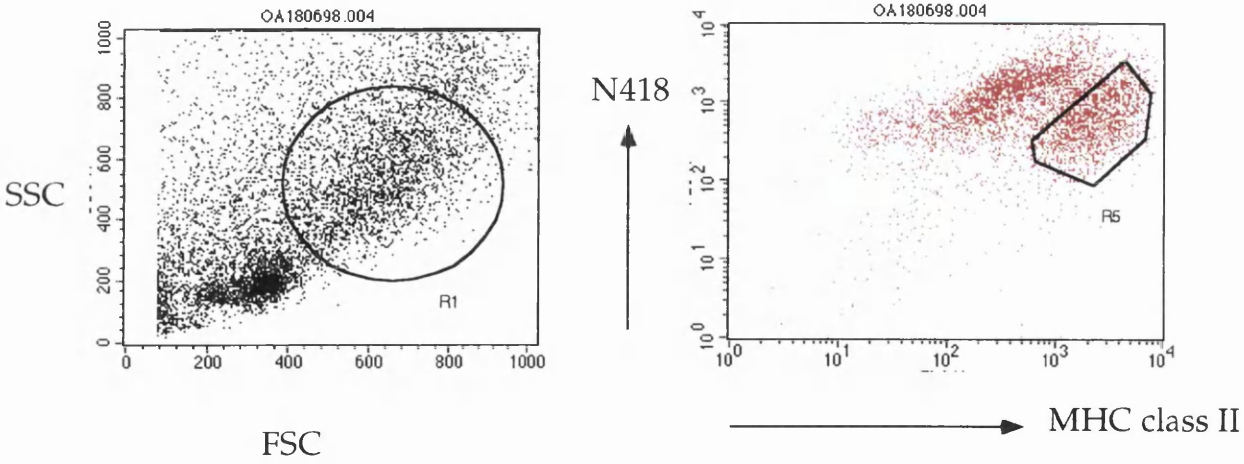


Table 3.1: Frequencies of C5 specific T cell responses following DNA vaccination. 20 A/J mice were vaccinated with 5.4 vaccine and precursor frequencies of C5 specific T cells was determined by limiting dilution analysis on spleen cells every 4 weeks (2 mice for each time point). In parallel, precursor frequencies of A18 transgenic mice (positive control) and untreated A/J mice (negative control) were determined. Results are expressed as the number of C5 specific T cells per million spleen cells after regressional analysis.

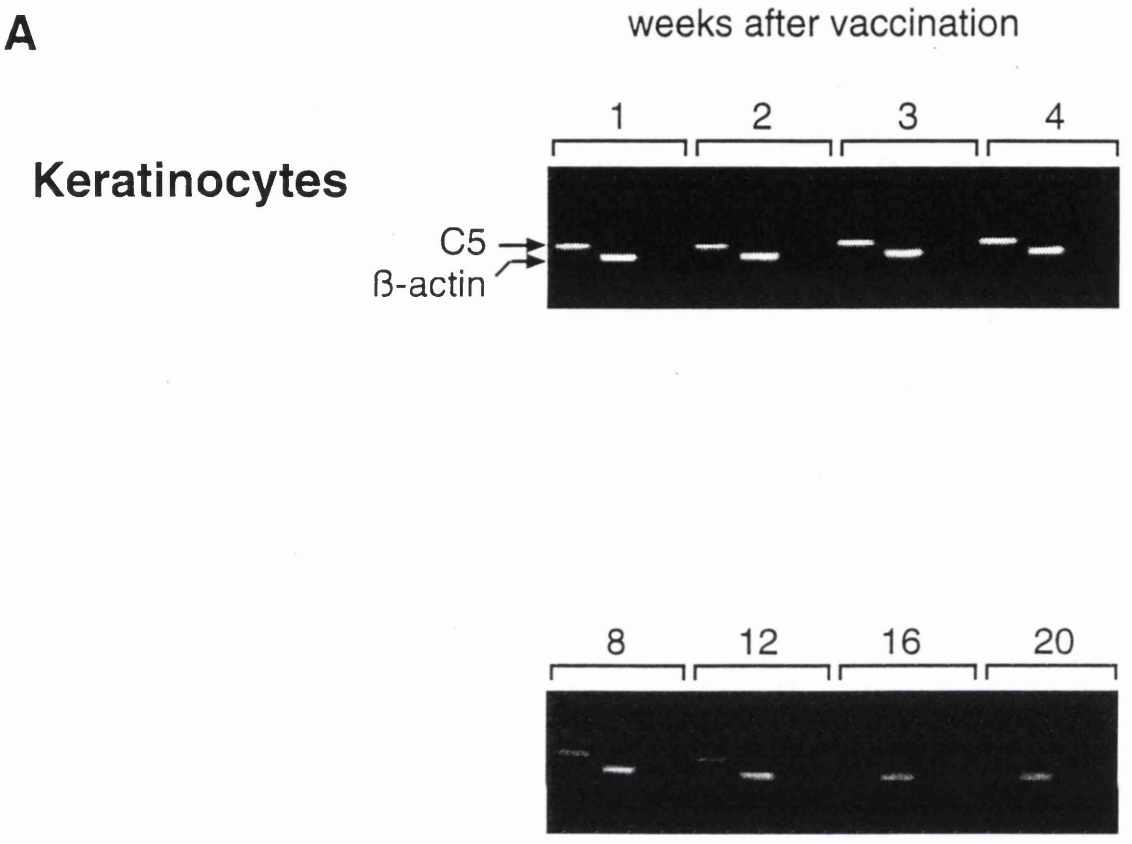
C5 specific precursor T cells per 10^6 spleen cells

weeks after vacc.	untreated	DNA Vaccine	A18 TCRtg untreated
0	5	5	10000
4	5.7	110	13333
8	5	66.6	10000
12	5	50	8000
16	5.7	30	13333
20	5	25	10000
24	5.7	25	10000
28	5	25	8000
32	5	25	10000
36	5	25	13333
40	5	25	10000

Figure 3.15: Kinetic analysis of C5 expression in keratinocytes and lymph node dendritic cells following DNA vaccination.

At different time points after 5.4 vaccination C5 expression was analysed by RT-PCR on keratinocytes and dendritic cells isolated from draining lymph nodes of 5.4 vaccinated mice by magnetic cell sorting (lane 1 of each time point). The second lane of each time point represents β-actin (positive control). The third lane for each time point is a control showing the absence of DNA contamination in the RNA samples (omission of reverse transcriptase before the PCR reaction).

A


weeks after vaccination
1
2
3
4
Keratinocytes
C5
ß-actin
8
12
16
20

B

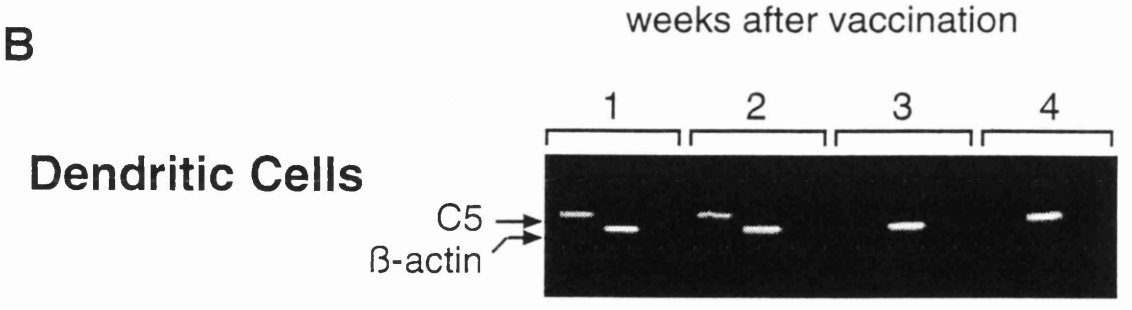

weeks after vaccination
1
2
3
4
Dendritic Cells
C5
ß-actin

Figure 3.16: Cross-presentation analysis of C5 protein from keratinocytes to dendritic cells.

Bone marrow derived dendritic cells were pre-incubated either with C5 protein or keratinocytes (untreated or 1000 Rad irradiated) from vaccinated mice for 12 hrs. Serially titrated numbers of such treated DC were then co-cultured with the A18 hybridoma for 24 hrs and IL-2 production was determined. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.

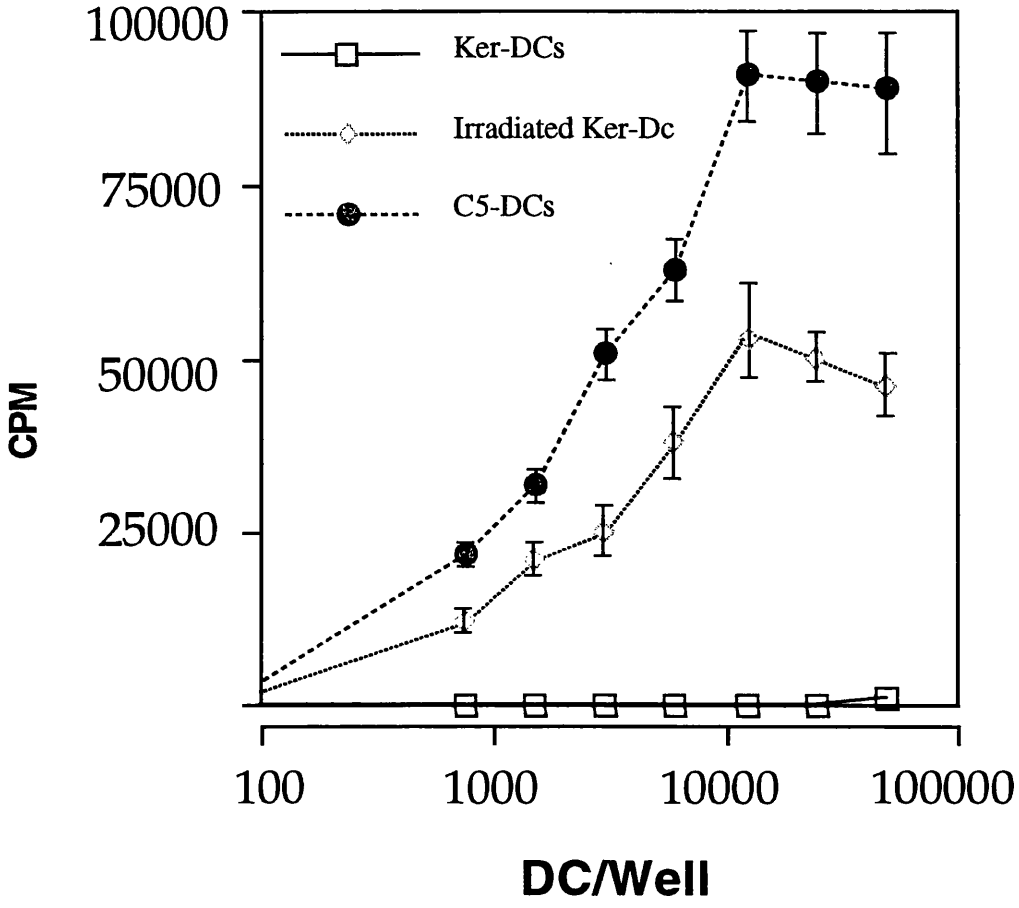
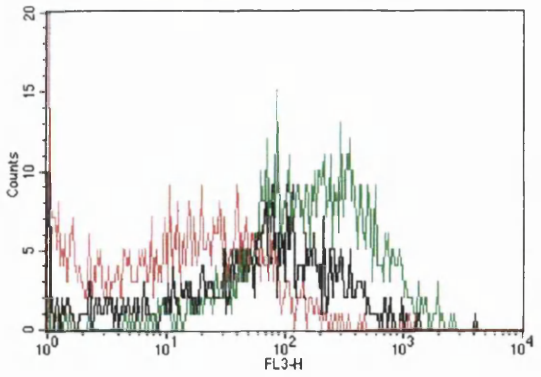

100000
75000
50000
25000
CPM
Ker-DCs
Irradiated Ker-Dc
C5-DCs
100
1000
10000
100000
DC/Well

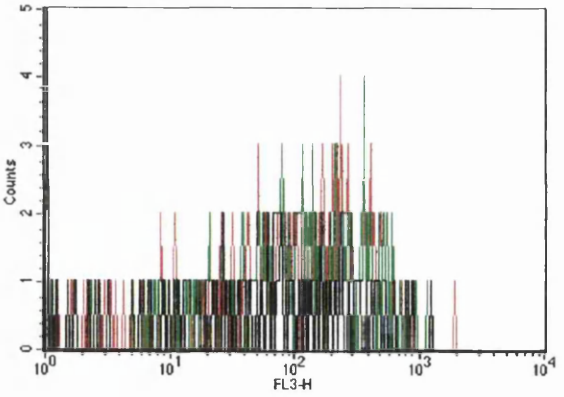
Figure 3.17: Kinetic analysis of T cell activation in transgenic mice.
Lymph node and spleen cells from A18TCRtg mice vaccinated with the 5.4 construct (red lines), the control vector (Green lines) or untreated (shaded gray) were analysed 3 days (A), 7 days (B) and 15 days (C) after vaccination for expression of different activation markers by three colour FACS analysis. The figure shows expression of CD44, CD62-L and CD69 (and BrdU labeling for day 3 after vaccination) on gated CD4 and Vb8.3 positive T cells.

A

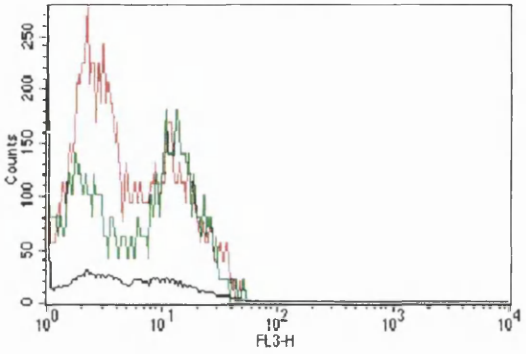
CD62-L L.N. Day 3



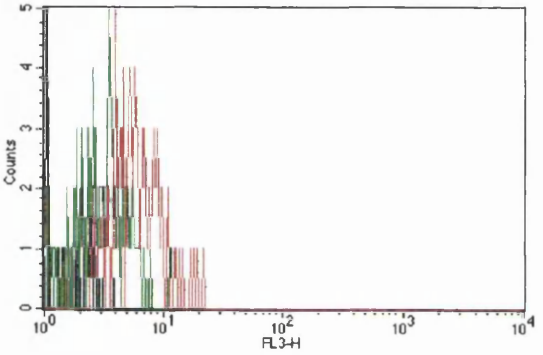
CD62-L Spleen Day 3



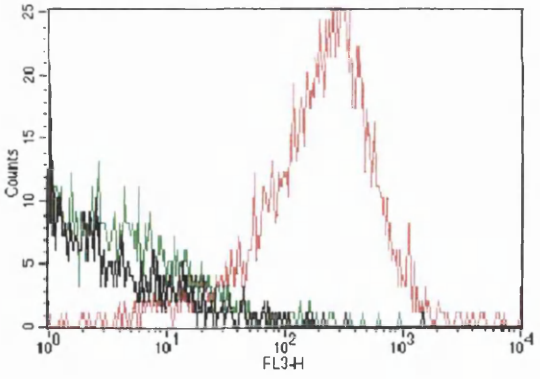
CD44 L.N. Day 3



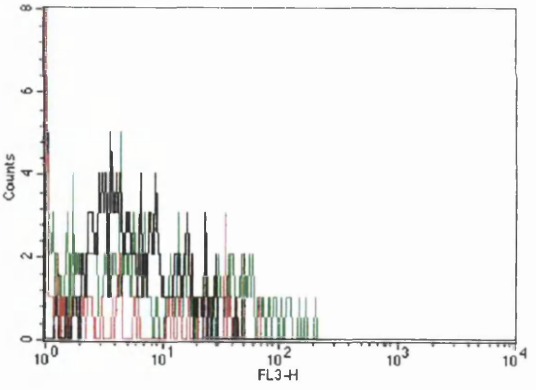
CD44 Spleen Day 3



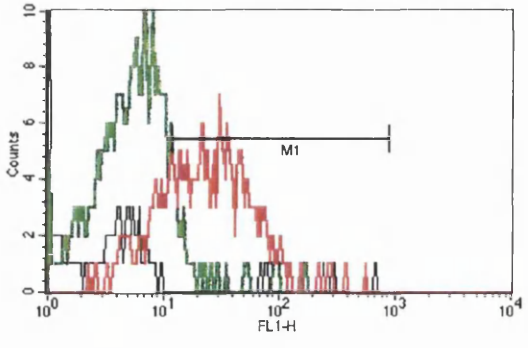
CD69 L.N. Day 3



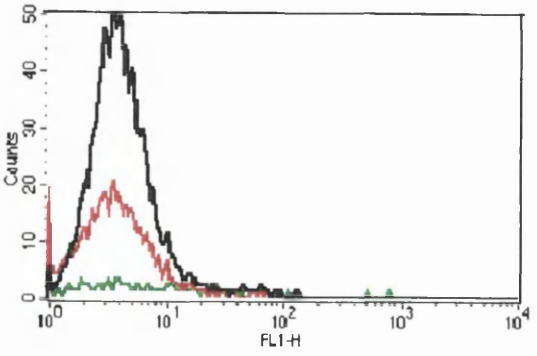
CD69 Spleen Day 3



BrdU L.N. Day 3

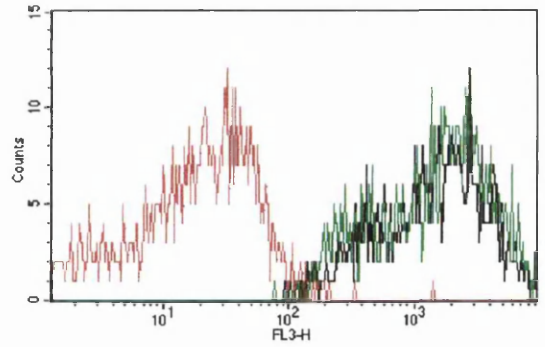


BrdU spleen Day 3

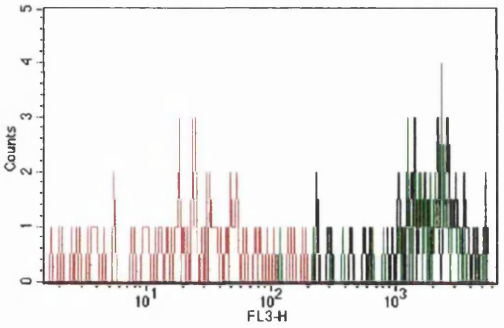


B

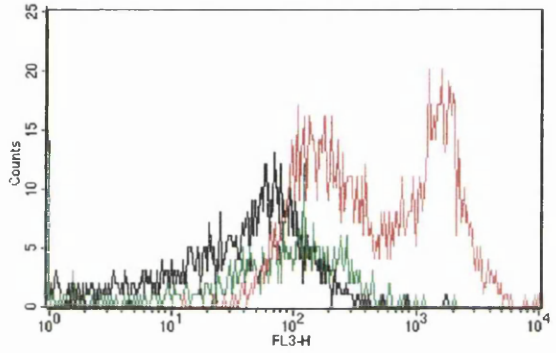
CD62-L L.N. Day 7



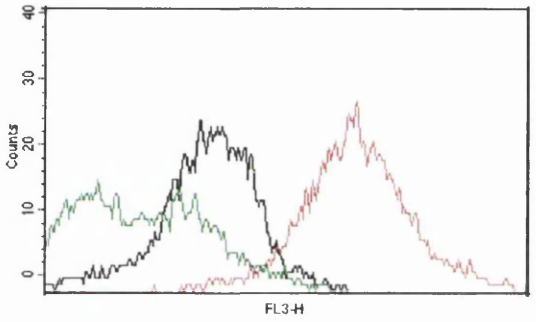
CD62-L Spleen Day 7



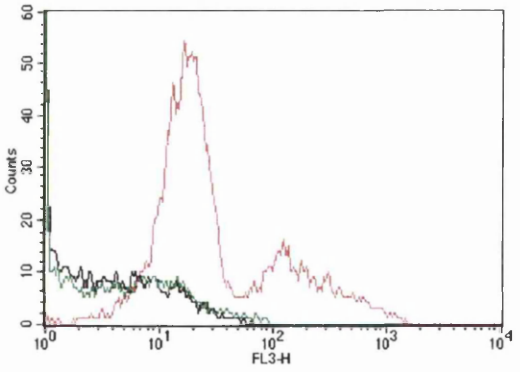
CD44 L.N. Day 7



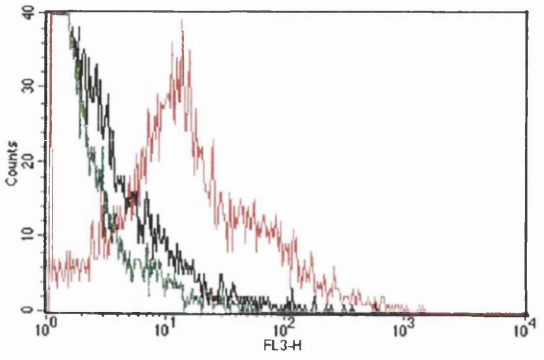
CD44 Spleen Day 7



CD69 L.N. Day 7

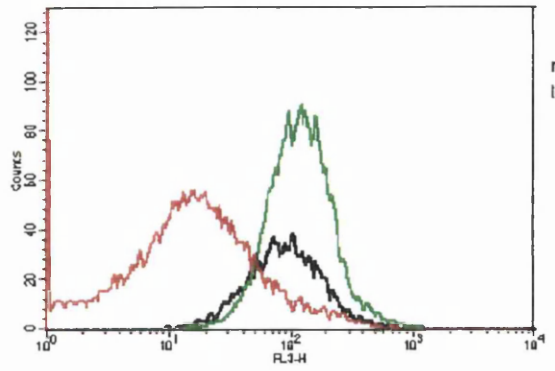


CD69 Spleen Day 7

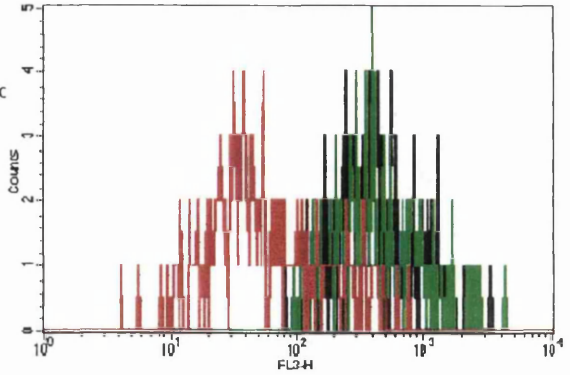


C

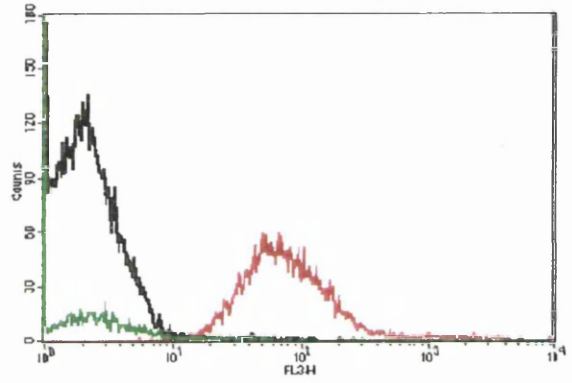
CD62-L L.N. Day 15



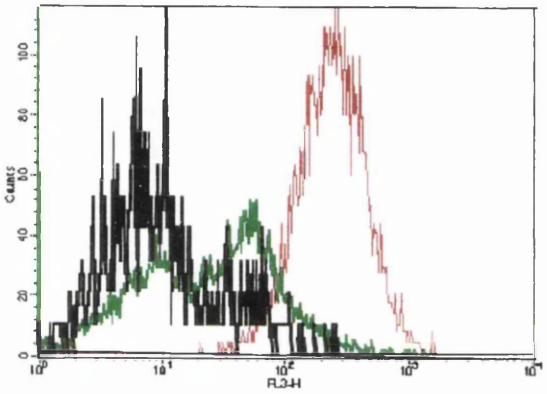
CD62-L Spleen Day 15



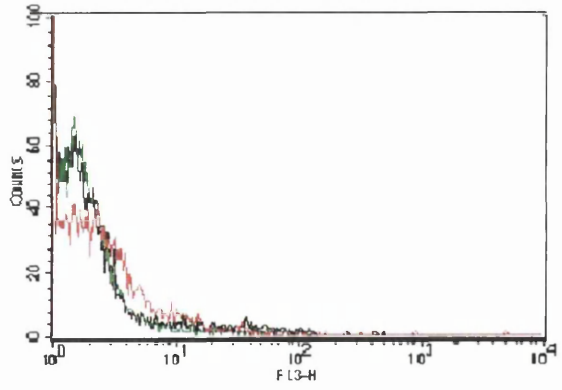
CD44 L.N. Day 15



CD44 Spleen Day 15



CD69 L.N. Day 15



CD69 Spleen Day 15

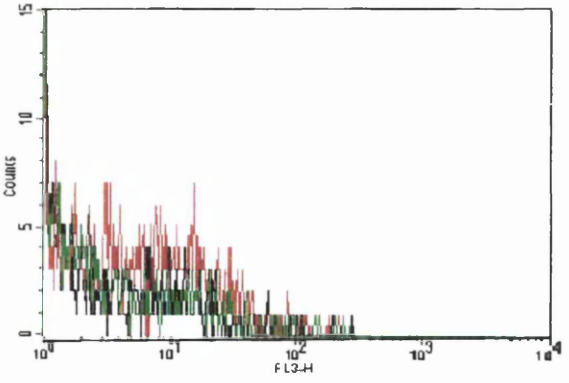


Figure 3.18: *In vivo* T cell proliferation analysis in transgenic mice vaccinated with 5.4 DNA vaccine.

Two A/J Thy1.2$^+$ mice were injected i.v. with 10^6 CFSE labeled naive Thy1.1$^+$ A18TCRtg T cells, 20 days after 5.4 vaccination. 7 days after injection draining lymph nodes were pooled and expression of CFSE (A) CD62-L (B) And CD44 (C) gated on Thy1.1$^+$ was analysed by three colour FACS analysis. The dot plot shows CFSE levels on Thy1.2 negative (Thy1.1 positive) injected T cells. Histograms for the activation markers CD44 and CD62-L show cells gated for expression of Thy1.1 (black lines) or Thy1.2 (endogenous T cells, shaded grey).

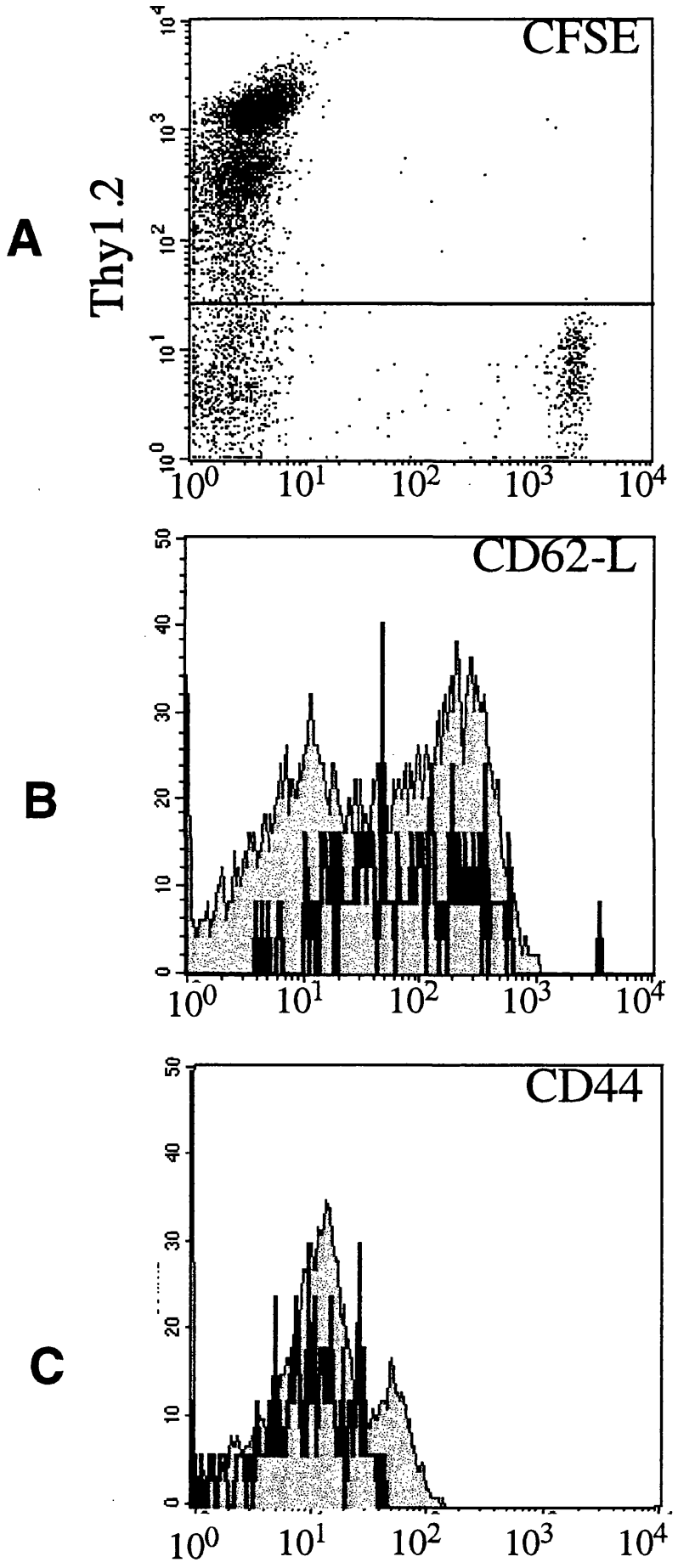
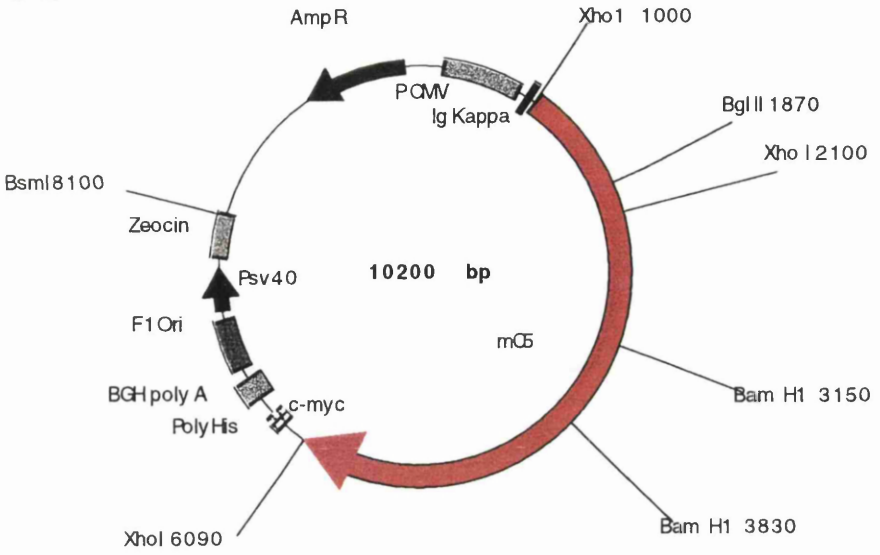

A
Thy1.2
CFSE
B
CD62-L
C
CD44

Figure 3.19: Construct and expression analysis of pSec-C5 *in vitro:*
The Ig kappa light chain secretory signal sequence was fused in frame with full C5 cDNA to generate the pSec-C5 construct. COS-7 cells transfected either with pSec-C5 construct (red and green lines), or with 5.4 construct (yellow and blue). After 5 days (red and blue) and 7 days (green and yellow) of transfection supernatants were incubated 4 hrs with bone marrow derived dendritic cells. Different number of dendritic cells were co-cultured with A18 T cell hybridoma to test their capacity to present C5. IL-2 was measured 24 hrs later. Results are expressed as the mean of triplicate ± SD of 3H thymidine incorporated into CTLL cells.

A


AmpR
Xho1 1000
PCMV
Ig Kappa
BglII 1870
Xho I 2100
BsmI 8100
Zeocin
Psv40
10200 bp
F1 Ori
mC5
BGH poly A
c-myc
PolyHis
Bam H1 3150
XhoI 6090
Bam H1 3830

B

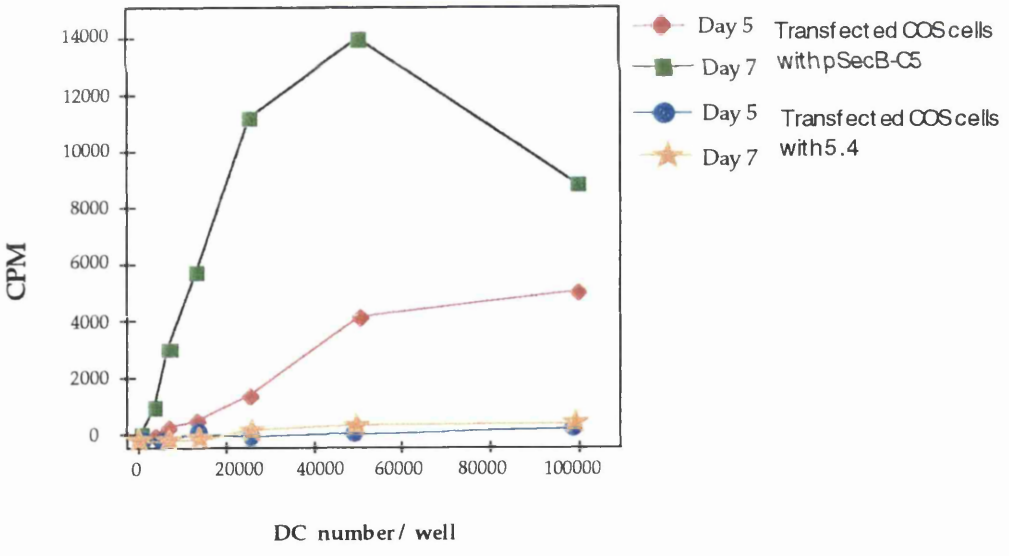
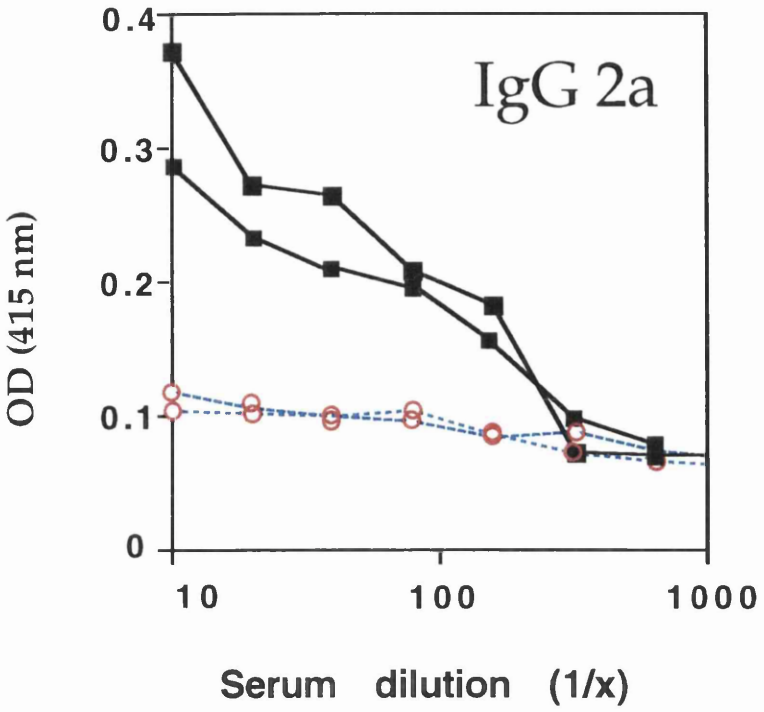

CPM
14000
12000
10000
8000
6000
4000
2000
0
0
20000
40000
60000
80000
100000
DC number / well
Day 5
Day 7
Transfected COS cells with pSecB-C5
Day 5
Day 7
Transfected COS cells with 5.4

Figure 3.20: Generation of antibody in mice vaccinated with pSec-C5
Two 5.4 vaccinated (red circles) and two Sec-C5 vaccinated (black squares) A/J mice were re-vaccinated 3 weeks after the first injection. 9 days later their sera were tested for the presence of IgG2a (A) and IgG1 (B) anti-C5 antibodies by ELISA.

A


IgG 2a
0.4
0.3
0.2
0.1
0
OD (415 nm)
10
100
1000
Serum dilution (1/x)

B

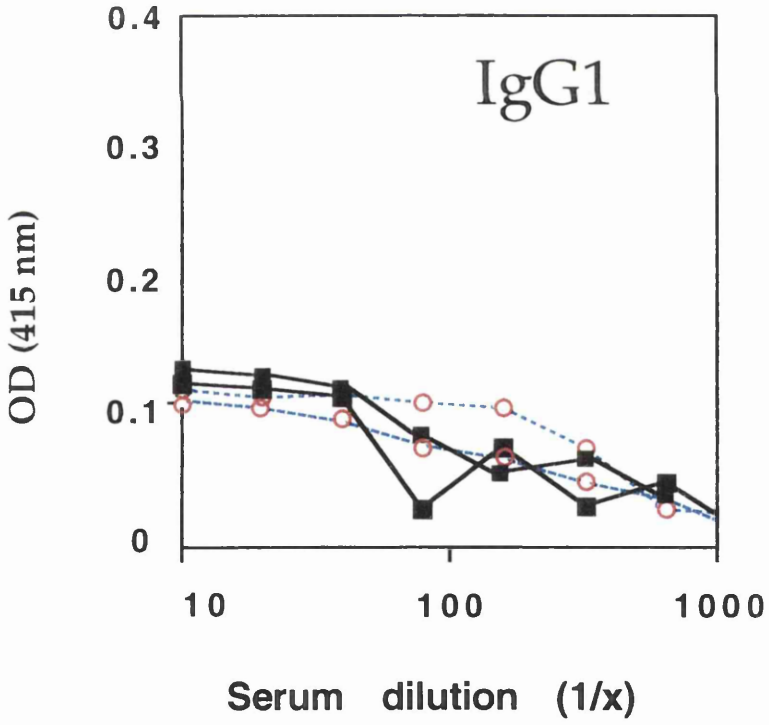

IgG1
0.4
0.3
0.2
0.1
0
OD (415 nm)
10
100
1000
Serum dilution (1/x)

Table 3.2: C5 specific T cell precursor frequency in mice vaccinated with 5.4 in compared with mice vaccinated with pSec-C5.

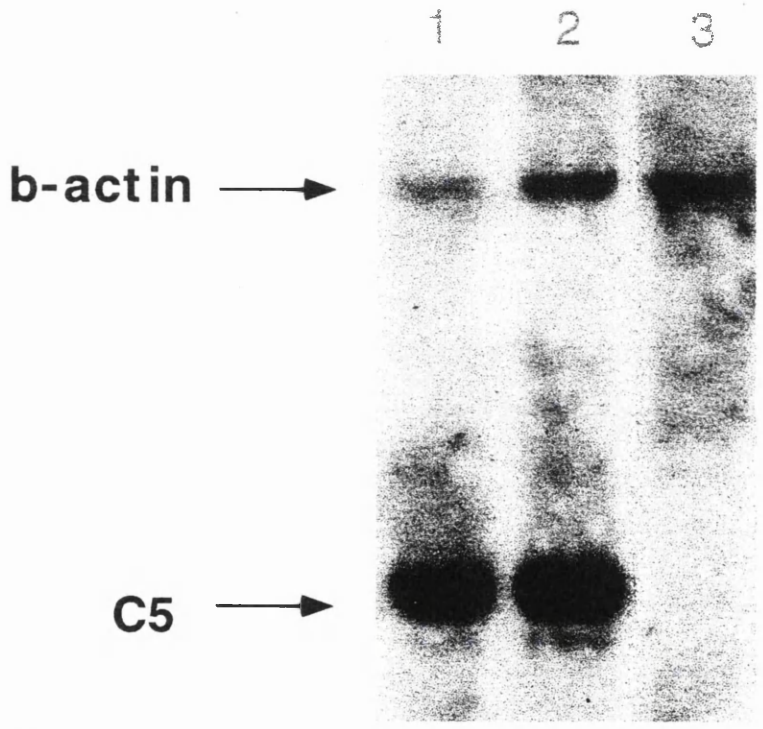
Two groups of A/J mice were vaccinated either with 5.4 or with psec-C5 vaccine and the frequency of C5 specific T cells was determined by limiting dilution analysis on pooled spleen cells every 3 weeks (3 mice each time point). In parallel precursor frequencies of A18 transgenic mice (positive control) and untreated A/J mice (negative control) were determined. Results are expressed as the number of C5 specific T cells per million spleen cells after regressional analysis.

C5 specific precursor T cells per 10^6 spleen cells

weeks after vacc.	untreated	5.4	Sec-C5	A18 TCRtg untreated
0	5	5	5	10000
3	5.7	120	200	10000
6	5	75	133	13333
9	5	50	50	8000
12	5.7	30	33.3	13333
15	5	25	33.3	10000

Figure 3.21: Expression of C5 in COS cells transfected with 5.4 construct.

Independent 5.4 transfectants or vector-only transfected control cells were lysed at 5x106 cells/ml in 1% Brij-96 containing lysis buffer. 25µl of lysate was loaded per lane as shown, and separated on an 8% SDS-PAGE gel, followed by transfer and immunoblotting with H9C4 mAb (anti-C5). While expression of C5 was seen in two independant transfectants (Lane 1 and 2), no C5 expression was detected in vector transfected cells (Lane 3)


1
2
3
b-actin
C5

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