The roles of CD40 and OX40 during the induction of T cell tolerance versus T cell immunity

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

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Abstract

Maintenance of T cell tolerance to self-antigens is crucial to prevent immune responses against our own tissue, which can result in autoimmune pathology. Experimentally, the outcome of an immune response can depend on the form in which the antigen is administered. Systemic administration of soluble antigen results in antigen-specific T cell tolerance, while administration of the same antigen in conjunction with an adjuvant leads to T cell immunity. Both tolerance and immunity involve some degree of T cell activation, a process in which costimulatory receptor-ligand pairs on T cells and dendritic cells (DC) are of crucial importance. Tolerance induction is thought to be the result of peptide presentation by resting DC, which are lacking full costimulatory potential. The precise signals that drive a T cell towards tolerance, rather than a productive immune response, are not well defined. This project has addressed this issue by asking three questions:

A. Can exogenous ligation of defined costimulatory receptors convert a tolerogenic signal into an immunogenic one?

B. How does expression of CD154, OX40 and RANKL on T cells, and CD40, OX40L and RANK on DC differ during induction of T cell tolerance versus T cell immunity?

C. Do T cells become tolerant on exposure to antigen-loaded DC lacking CD40?

It was found that agonistic antibodies to CD40 and OX40 overcame a tolerogenic signal, and prevented the induction of tolerance. CD154, OX40 and RANKL were expressed on T cells under conditions leading to either tolerance or immunity. Up-regulation/induction of CD40, OX40L and RANK on DC, however, was only observed during the induction of T cell immunity. The administration of antigen-loaded CD40-deficient DC mimicked tolerance induced by soluble peptide.

Collectively, the results suggest that the CD40-CD154 interaction provides an important checkpoint in the decision between T cell tolerance and immunity. Investigating the process of tolerance induction may provide a rational basis for therapeutic targeting of costimulatory pairs in adverse immune reactions in humans.

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Abbreviations

APC	antigen presenting cell
APC	allophycocyanin
Bm-DC	bone marrow-derived dendritic cell
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CNS	central nervous system
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DLN	draining lymph node
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/macrophage colony stimulating factor
IFN	interferon
IL	interleukin
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
ICOS	inducible costimulator
LN	lymph node
LPS	lipopolysaccharide
MACS	magnetically activated cell sorting
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
pMOG	MOG peptide
pOVA	ovalbumin peptide
PD-1	programmed death 1
PD-L1/PD-L2	programmed death ligand 1/programmed death ligand 2
PE	phycoerythrin
RANK	receptor activator of NFκB
RANKL	RANK Ligand
s.c.	subcutaneous
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
T reg	regulatory T cell

Publications and Presentations

Publications

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1. CHAPTER 1- Introduction

1.1. T cell activation

T cells are a subset of lymphocytes defined by their development in the thymus and by heterodimeric receptors on their surface. T cells bearing the α, β T cell receptor (TCR) can broadly be divided into $CD4^+$ and $CD8^+$ T cells, according to their selective expression of these co-receptors (Davis and Bjorkman, 1988). This introduction concentrates on $CD4^+$, so–called T helper (Th) cells. $CD4^+$ T cells require three signals for effective activation (Kalinski et al., 1999), which are illustrated in Fig. 1.1.

1.1.1. Signals 1, 2 and 3 lead to effective T cell activation

The first signal required for T cell activation consists of TCR binding to peptide-MHC complexes displayed on the surface on antigen presenting cells (APC). The expression of CD4 restricts $CD4^+$ T cells to recognition of processed peptides bound to MHC class II molecules (Guermonprez et al., 2002). Although B cells and macrophages also express MHC class II complexes and are able to activate naïve $CD4^+$ T cells *in vitro* (Askew et al., 1995; Cassell and Schwartz, 1994), dendritic cells (DC) are the only MHC class II expressing cell type in the T cell areas of the lymph node (LN) (Steinman et al., 1997). DC are the initiating APC for primary immune response *in vivo* (Banchereau and Steinman, 1998; Steinman, 1991), and they are thus referred to as professional APC (Lassila et al., 1988). They efficiently take up protein, which is processed to peptides, and these processed peptides are loaded onto MHC class II molecules for recognition by the TCR.

The second signal required for effective T cell activation is delivered through a variety of costimulatory molecules found on DC and T cells. Although costimulation can simply promote more efficient engagement of TCR molecules to enhance the initial activation, it has become clear that it also serves to provide additional signals leading to cell division, augmented cell survival or induction of effector functions. In

the last decade, an increasing number of costimulatory receptor-ligand pairs have been discovered, revealing the complexity of signalling involved in the regulation of T cell expansion, contraction, and inactivation. These can be divided into two main groups, the immunoglobulin (Ig) superfamily, including CD28, PD-1 and ICOS; and the TNF receptor (TNFR) superfamily, including CD40, OX40 and RANK, both of which will be introduced in more detail in section 1.2. These receptor-ligand pairs are shown in Fig. 1.2, although it is important to emphasize that this is only a summary and not an exhaustive list of the molecules with costimulatory activity.

Finally, signal 3 plays a role in directing the effector functions of T cells. The paradigm is that naïve (Th0) cells differentiate predominantly into effector Th1 or Th2 cells, which are defined by the cytokines secreted during the immune response (Mosmann and Coffman, 1989). The Th1-Th2 divergence is influenced by a variety of factors, including the cytokine environment present at the initiation of the immune response, the antigen (Ag) dose, and the presence of costimulatory molecules (Constant and Bottomly, 1997). The presence of interleukin-12 (IL-12) drives development of Th1 cells that secrete the pro-inflammatory cytokines interferon-γ (IFN-γ) and tumour necrosis factor α/β (TNF-α/β), principally in response to intracellular pathogens. Th2 cells secrete IL-4, IL-5 and IL-13 and initiate antibody production, generally against extracellular pathogens. All three signals are regulated through the information DC receive at the site of Ag exposure, via expression of 'pattern-recognition receptors', such as Toll-like receptors (TLR). In this way, DC exert control over the outcome of the immune response. The change from an Ag-capturing to an Ag-presenting, T-cell-priming mode, coupled with the expression of the necessary costimulatory molecules and cytokines, allows DC to initiate the appropriate adaptive immune response.

1.1.2. Experimental Autoimmune Encephalomyelitis as a model of T cell-driven autoimmunity

Experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, is an autoimmune disease that is driven by $CD4^+$ T cells specific for components of the central nervous system (CNS) myelin sheath. These cells traffic to the CNS parenchyma, resulting in focal areas of inflammation and demyelination throughout the CNS (Martin and McFarland, 1995). EAE can be induced in mice by active immunization with complete Freund's adjuvant (CFA) mixed with CNS auto-Ag, notably myelin basic protein (MBP) (Anderton and Wraith, 1998; Zamvil et al., 1985), proteolipid protein (PLP) (Anderton and Wraith, 1998) and myelin oligodendrocyte glycoprotein (MOG) (Mendel et al., 1995), or by the adoptive transfer of MBP-reactive T cells (Lafaille et al., 1997; Zamvil et al.). Central to the aetiology of most experimental autoimmune models is the activation of $CD4^+$ T cells of the Th1 functional phenotype, and the $CD4^+$ T cell lines and clones that transfer EAE invariably produce IFN-γ and/or, TNF-α/β on antigenic challenge *in vitro* (Ando et al., 1989; Powell et al., 1990). Immunodominant T cell epitopes within myelin auto-Ag have been defined, allowing EAE induction with peptide Ag. The 35-55 peptide of MOG (pMOG) has been shown to cause EAE in H-2^b mice (Mendel et al., 1995; Mendel et al., 1996). Since a number of knockout mice are available on the H-2^b background, including CD40- and CD154-deficient mice, EAE provides a good model to study the importance of these costimulatory molecules in a disease setting.

1.2. The molecular basis of costimulation

A large number of costimulatory receptor-ligand pairs have been identified, some of which are shown in Fig. 1.2. The molecules shown are expressed at various stages during the immune response. Although some redundancy exists, the requirements for many of these molecular interactions probably reflects a need for different signals at different times and stages during the immune response. In addition to allowing development of alternative effector functions or creation of long-lasting immunity, this provides a number of checkpoints during T cell activation, which may serve to control the final outcome of an immune response.

1.2.1. Members of the immunoglobulin superfamily

Although a large number of costimulatory molecules are expressed after TCR ligation, some are found constitutively expressed on naïve T cells, and are thus considered especially important during the early stages of T cell activation. One such molecule is CD28, a member of the Ig superfamily, which is constitutively expressed on naïve T cells (Linsley and Ledbetter, 1993). CD28 binds to CD80 or CD86, found at low levels on resting DC, and upregulated on activated, mature DC (Banchereau et al., 2000). CD28 engagement can enhance T cell proliferation (Jenkins et al., 1991), cytokine secretion (Jenkins et al., 1991), and expression of anti-apoptotic molecules such as Bcl-xL (Boise et al., 1995). Experiments using CD28-deficient T cells clearly show that CD28 is required for initial T cell proliferation *in vivo* (Howland et al., 2000). CD28-deficient mice show reduced Th2 responses with defective immunoglobulin class switching, but normal cytotoxic T cell responses, indicating differential requirements in the activation of helper or cytotoxic T cells (Shahinian et al., 1993).

Another receptor for CD80 and CD86 is expressed by both $CD4^+$ and $CD8^+$ T cells after activation, namely cytotoxic T lymphocyte Ag-4 (CTLA-4). CTLA-4 is a close homologue of CD28, and an important negative regulator of T cell responses. It binds CD80 and CD86 with 20-50-fold higher affinity than CD28, and is thought to

deliver a negative signal to the T cell (Oosterwegel et al., 1999), thus downregulating T cell responses after initial activation. CTLA-4 deficient mice develop fatal lymphoproliferative disease with multiorgan tissue destruction, illustrating the critical role of CTLA-4 in moderating T cell activation and maintaining peripheral self-tolerance (Tivol et al., 1995).

Other costimulatory molecules with negative effects on T cell activation have recently been described, including Programmed Death-1 (PD-1), which binds to Programmed Death Ligand 1 and 2 (PD-L1 and PD-L2), also members of the Ig superfamily. PD-1 is expressed on activated T and B cells, as well as myeloid cells (Agata et al., 1996). PD-L1 is constitutively expressed on naïve T cells, B cells, macrophages and DC, is upregulated on T cells, macrophages and DC after activation (Liang et al., 2003), and has also been found in non-lymphoid organs such as heart, placenta, lung and pancreas (Liang et al., 2003). In contrast, PD-L2 was only inducible on macrophages and DC after cytokine stimulation (Yamazaki et al., 2002). Ligation of PD-1 by both PD-L1 and PD-L2 inhibits T cell activation (Khoury and Sayegh, 2004). Blocking PD-1 worsens EAE induced by immunisation with pMOG in CFA (Salama et al., 2003), and PD-1-deficient mice develop multiple autoimmune diseases (Nishimura et al., 1999; Nishimura et al., 2001), suggesting a possible role for PD-1 in controlling the activation of autoreactive T cells.

ICOS, a third member of the Ig superfamily family, is expressed on activated but not naïve T cell (Hutloff et al., 1999). ICOS binds to B7h, which is constitutively expressed on B cells, some macrophages and DC (Sharpe and Freeman, 2002; Yoshinaga et al., 1999). Experiments with ICOS-deficient T cells indicate that the ICOS-B7h interaction plays a role in T cell activation and proliferation *in vitro*. *In vivo*, it is essential for germinal centre formation (Dong and Nurieva, 2003), and a lack of ICOS has an effect on Th2, but not Th1 development (Dong and Nurieva, 2003). ICOS thus appears to be less crucial for *in vivo* T cell activation, but more important for fine-tuning effector T cell differentiation and function.

1.2.2. TNF/TNFR family members

The TNFR-TNF superfamily consists of an increasing number of molecules, many of which have been reported to play a role at various stages in the activation of T cells. The TNFR family members are type I transmembrane proteins, and are believed to exist either as monomers or as self-assembled oligomers, forming trimeric signalling complexes when interacting with their ligand. The ligands are members of the TNF family, which are type II transmembrane proteins, and thought to be expressed as trimers on the cell surface. Although some redundancy exists between members of these families, knockout studies show specific functions for most of the molecules, which cannot be compensated for by other members of the family. Most accounts of TNR and TNFR-family members take the simplistic view that one molecule is expressed by T cells, while its molecular partner is expressed by APC. Interestingly, in some cases, both receptor and ligand can be expressed by T cells (Croft, 2003). Although it is not known whether this occurs generally *in vivo*, it might indicate a potential role for TNFR-TNF-family interactions in communication between T cells, amplifying signals that were initiated by APC. However, this project concentrates on DC-T cell interactions, and expression of these molecules will be discussed with respect to receptor-ligand expression on these two cell types. The three receptor-ligand pairs of particular relevance to this project are described in further detail below.

The CD40-CD154 interaction

The CD40-CD154 interaction has been known to be crucial in the initiation of T cell immunity for some time (Grewal and Flavell, 1998). The gene encoding CD154 is found on the X chromosome in both humans and mice. Mutations in the CD154 gene result in the X-linked hyper-IgM syndrome in humans (Notarangelo and Peitsch, 1996; Villa et al., 1994). CD40-deficient mice have significant defects in thymus-dependent T cell responses, failing to form germinal centres and showing defects in immunoglobulin class switching (Castigli et al., 1994; Kawabe et al., 1994), while CD154-deficient mice similarly fail to form germinal centres and show decreased antibody responses (Grewal et al., 1995; Xu et al., 1994). Early reports suggested

that CD154$^{-/-}$ T cells fail to prime *in vivo* (Grewal et al., 1996), but more recently, it was shown that CD154$^{-/-}$ T cells become activated, but are unable to sustain a response *in vivo*, and are defective in Th1 development *in vitro* (Howland et al., 2000). Mice deficient for CD40 or CD154 fail to develop EAE (Grewal et al., 1996; Grewal et al., 1995), and mice lacking expression of CD40 in the CNS similarly do not develop symptoms of EAE (Becher et al., 2001). Blocking the CD40-CD154 interaction prevents the induction of EAE (Gerritse et al., 1996; Laman et al., 2002; Samoilova et al., 1997), while agonistic anti-CD40 monoclonal antibody (mAb) potentiates EAE (Ichikawa et al., 2002), further illustrating the importance of the CD40-CD154 interaction in T cell priming.

CD40 is found at low levels on resting APC, and is upregulated after APC activation (Cella et al., 1997; Inaba et al., 1994; Vremec and Shortman, 1997), whereas CD154 is transiently induced on naïve T cells after TCR stimulation (Roy et al., 1993). Splenic CD4^{+} T cells show CD154 expression 4 hrs after *in vitro* anti-CD3 stimulation, which peaks between 6 and 8 hrs, and returns to resting levels between 24 and 48 hrs (Roy et al., 1993). The CD40-CD154 interaction primarily signals to the APC, resulting in sustained activation of nuclear factor-κB (NF-κB) transcription factors (O'Sullivan and Thomas, 2002), which regulate DC differentiation and cytokine production. In culture, CD40 signalling results in increased expression of CD80 and CD86 (Caux et al., 1994), enhanced Ag presentation (Delamarre et al., 2003; Machy et al., 2002) and IL-12 production (Cella et al., 1996; Koch et al., 1996). CD40 signalling also enhances DC survival through upregulation of the survival molecule Bcl-2 (Bjorck et al., 1997). Although some evidence for CD154 signalling into the T cell also exists (van Essen et al., 1995), the significance of this remains to be investigated. CD40 ligation on DC is a crucial trigger for IL-12 release and priming of a Th1 type response (Cella et al., 1996; Koch et al., 1996), and the CD40-CD154 interaction synergises with IL-12 in selectively enhancing IFN-γ production by T cells (Peng et al., 1996). In the absence of IL-12 release, a default Th2 type response is observed (Stuber et al., 1996).

While CD28 is required for the initiation of the T cell response, CD154 is required for sustaining Th1 responses (Howland et al., 2000). A soluble isoform of CD154 has been found in T cell supernatants after activation (Graf et al., 1995). Soluble CD154 was also detected in human serum of individuals with systemic lupus erythematosus (Kato et al., 1999) and rheumatoid arthritis (Tamura et al., 2001), but the significance of soluble CD154 during T cell responses remains to be clarified. CD40 ligation delays clonal deletion of Ag-specific T cells and enhanced T cell clonal expansion in response to super-Ag (Maxwell et al., 1999). This may be due to *de novo* OX40L expression on DC, which is induced after CD40 ligation, and which signals to the T cell through OX40 (Fillatreau and Gray, 2003). The importance of this interaction is further explained in the next section.

As described above, CD40 and CD154 are crucial for effective T cell activation in lymphoid organs. The CD40-CD154 interaction is however also important in the migration of DC from the periphery to the draining lymph node (DLN). The paracortical regions in DLN have been shown to be site of Ag-specific DC-T interaction (Ingulli et al., 1997). In $CD154^{-/-}$ mice, Ag-specific DC failed to migrate out of the skin and fewer DC accumulated in the DLN after contact sensitisation (Moodycliffe et al., 2000). This migratory defect was accompanied by a decrease in TNF-α. It appears that CD40 ligation of DC induces their migration out of the periphery, but that an important effect of CD40 ligation is TNF-α release by cells in the peripheral tissue displaying CD154 (Moodycliffe et al., 2000). These CD154-displaying cells have not been identified yet, but possible candidates include mast cells and keratinocytes (Flores-Romo, 2001).

Once in the DLN, the CD40-CD154 interaction is essential for DC longevity (Miga et al., 2001). Administration of blocking anti-CD154 antibodies accelerated disappearance of DC in the DLN, leading to a deficiency in sustaining T cell responses over a longer period of time (Miga et al., 2001), fitting with previous data that CD28 appeared to be the most important costimulatory molecule for initial T cell activation, while CD40 on DC was required for sustained T cell responses (Howland

et al., 2000). The CD40-CD154 interaction is, as a result, crucial for DC migration as well as T cell activation, and also appears to be important during effector T cell activation in the target organs later in the immune response (Becher et al., 2001; Grewal and Flavell, 1998). A model for the role of CD40-CD154 in T cell function is shown in Fig. 1.3.

The OX40-OX40L interaction

OX40 and OX40L-deficient mice have rèduced primary $CD4^+$ T cell responses to common protein Ag, some viruses, and in contact-sensitivity reactions (Kopf et al., 1999; Murata et al., 2000). OX40L-deficient mice also show reduced severity of EAE (Ndhlovu et al., 2001), and administration of a neutralising anti-OX40L Ab ameliorates EAE (Nohara et al., 2001; Weinberg et al., 1999). As with CD154, OX40 is not constitutively expressed on naïve T cells, but peaks in expression 3-4 days after initial activation signals. It is rapidly and highly re-expressed on effector T cells, and can be induced by TCR signals in the absence of CD28 (Akiba et al., 1999; Gramaglia et al., 1998; Nohara et al., 2001). Like CD28, OX40 ligation promotes Bcl-xL and Bcl-2 expression in T cells and thus prolongs T cell survival (Rogers et al., 2001). This effect is illustrated in naïve $CD4^+$ T cell populations that are deficient in OX40. The early proliferation in these T cells is not impaired, but marked apoptotic cell death occurs 4-5 days after T cell activation, resulting in lower frequencies of Ag-specific effector T cells being generated late in the primary response, and fewer T cells entering the memory pool (Gramaglia et al., 2000). Correspondingly, OX40L is expressed on DC many hours or days after DC activation (Ohshima et al., 1997; Rogers et al., 2001), and its induction is dependant on CD40 ligation (Fillatreau and Gray, 2003). These data indicate that the OX40-OX40L interaction is very likely to be important after the CD40-CD154 interaction has taken place.

Overexpression studies further reinforce the view that the OX40-OX40L interaction provides late-acting signals that allow the survival of newly generated T effector cells. Transgenic expression of OX40L by DC (Brocker et al., 1999), or T cells

(Murata et al., 2002), increases the number of Ag-specific T cells and produces autoimmune-like symptoms, normally associated with aberrant T cell activation. Similarly, administration of agonistic antibodies for OX40 after immunisation results in increased numbers of Ag-specific T cells, and an increased memory T cell population (Gramaglia et al., 2000). Although OX40-deficiency had similar reducing effects on both $CD4^+$ and $CD8^+$ T cell responses, the primary effect of OX40-deficiency may be on $CD4^+$ T cells, which need to be "conditioned" in order to activate $CD8^+$ T cells (Bennett et al., 1998; Ridge et al., 1998). Analogous results in $CD8^+$ cells have been obtained in studies looking at the role of a different TNFR superfamily member in $CD8^+$ T cells, namely 4-1BB, suggesting a possible role for OX40 in $CD4^+$ T cells, and 4-1BB in $CD8^+$ T cells in some systems (Croft, 2003). However, some research also reports distinct roles for OX40L, such as a requirement for OX40L on B cells for the induction of primary Th2 responses (Linton et al., 2003), and expression of OX40L on a unique APC population, found at the T cell-B cell interface and in B cell follicles (Kim et al., 2003), which might provide co-stimulatory signals to memory T cells over extended periods of time. A model for the role of OX40-OX40L in T cell function is shown in Fig. 1.4.

The RANK-RANKL interaction

RANKL (receptor activator of NFκB ligand, also known as TRANCE, osteoprotegerin ligand, or osteoclast differentiation factor), is a recently described member of the TNF superfamily, that was discovered during attempts to clone novel genes involved in regulation of apoptosis and function of DC (Anderson et al., 1997; Wong et al., 1997). Its receptor is RANK, and this receptor-ligand pair displays remarkable sequence homology with CD154 and CD40 respectively (Anderson et al., 1997). However, both RANK and RANKL-deficient mice have impaired lymph node organogenesis and increased bone density (Dougall et al., 1999; Kong et al., 1999), indicating these molecules have additional functions in organ development, as well as in the mature immune system. So far, no data exists with respect to RANK- or RANKL-deficient mice and their susceptibility to EAE.

Similar to CD154, RANKL is expressed on T cells in a TCR-dependent manner after *in vitro* activation. It is detected as soon as 4 hours after T cell stimulation, but its expression peaks at 48 hours, and remains high until at least 96 hours (Josien et al., 1999). This expression is strongly enhanced by CD28 stimulation (Josien et al., 1999). RANKL is additionally found on immature DC in the skin, where it may play a role in regulating long-term survival of interstitial DC (Cremer et al., 2002). RANK is also expressed on these cells, but while RANKL is lost during maturation and migration to DLN, mature DC retain expression of RANK, becoming dependent on RANKL expression by other cell types in DLN, e.g. T cells, for survival (Cremer et al., 2002). *In vitro* treatment of these mature DC with soluble RANKL induces Bcl-xL expression in DC and thus promotes DC survival, as well as cytokine production (IL-1, IL-6 and IL-12) by DC (Josien et al., 1999). Treatment of Ag-loaded, mature DC with soluble RANKL before subcutaneous injection also affects immune responses, resulting in prolonged DC survival in the DLN as well as increased primary and secondary T cell responses (Josien et al., 2000). Although T and B express low levels of RANK, soluble RANKL has no effect on these cells (Josien et al., 1999).

The CD40-CD154 and the RANK-RANKL interactions appear to show some redundancy, both leading to IL-12 production and promoting Th1 type responses. This is further evident in that RANK-RANKL can compensate for CD40-deficiency in response to a number of viruses (Bachmann et al., 1999). However, their functions do not completely overlap, exemplified by the fact that RANKL exerts its effects mainly on DC, while the CD40-CD154 is also crucial during T-B cell interactions and subsequent antibody isotype switching (Banchereau et al., 1994). RANKL expression on anti-CD3 activated T cells *in vitro* peaks at 48hrs (Josien et al., 1999). CD154 on the other hand is more rapidly expressed, and expression wanes within 24-48 hrs (Roy et al., 1993). CD40 ligation of cultured human DC *in vitro* also greatly increases RANK expression (Anderson et al., 1997), suggesting that RANKL may act at a later time-point than CD154 during T cell activation to regulate DC function and survival, providing an additional checkpoint during T cell activation by DC.

1.2.3. Dendritic cells as the initiators of $CD4^+$ T cell responses

The DC located in most peripheral non-lymphoid tissues are in an immature, or quiescent, state. Here, they sample their environment for Ag. Upon exposure to inflammatory signals or mediators, DC undergo migratory, phenotypic, and functional changes (Flores-Romo, 2001). They migrate from the periphery via afferent lymphatics to DLN, lose their high endocytic and phagocytic activity, up-regulate their levels of costimulatory molecules, such as CD40, CD80 and CD86, and concentrate peptide-loaded MHC class Π molecules at their cell surface (Flores-Romo, 2001). On arrival in the LN, these mature, activated DC are capable of stimulating naïve Ag-specific T cells.

DC subsets

A number of DC subsets can be identified in humans and mice. The correspondence between these subsets remains unclear, possibly because human DC are generally isolated from peripheral blood, while murine DC are purified from secondary lymphoid organs. CD11c is a surface molecule used as a DC marker, although it is also expressed on other cells of myeloid origin. Expression of CD4 and CD8α is generally used to distinguish different DC subsets, and it is thought that $CD8\alpha^-$ DC are of myeloid origin, while $CD8\alpha^+$ are of lymphoid origin (Cella et al., 1997). Mouse spleen contains three distinct mature DC populations, $CD4^+$, $CD8\alpha^-$; $CD4^-$, $CD8\alpha^-$; and $CD4^-$, $CD8\alpha^+$ (Kamath et al., 2000; Vremec et al., 2000). $CD8\alpha^+$ DC are primarily found in the T cell rich paracortex, and $CD8\alpha^-$ DC ($CD11b^+$) reside in the outer edges of the paracortex, near B cell rich follicles (parafollicular region) (Pooley et al., 2001). DEC-205 is another phenotypic marker for DC, which has been investigated to segregate DC subset function. In the spleen, the expression of CD8α correlates with DEC-205 expression (Kronin et al., 2000). In the LN on the other hand, an additional $CD8\alpha^-$, DEC-205^+subset, can be located (Kronin et al., 2000).

A further subset of DC, which is $CD11c^{int/low}$, has been identified, and is called plasmacytoid DC. These plasmacytoid DC are found in all lymphoid tissue as large, round cells, which change to DC morphology after stimulation with viral products, CpG (further described below) or IL-2 *in vitro*. They then become potent producers of interferon (IFN)-α and IFN-β (O'Keeffe et al., 2002), and are thus thought to be important in the defence against viral pathogens.

Ever since the identification of DC subsets, attempts have been made to link DC ontogeny to DC function. Conflicting data exists with respect to the induction of Th1 versus Th2 type responses by $CD8\alpha^+$ or $CD8\alpha^-$ DC. Both $CD8\alpha^+$ and $CD8\alpha^-$ DC have been shown to efficiently prime Ag-specific T cells *in vivo*, inducing Th1 and Th2 responses respectively when transferred into naïve hosts (Maldonado Lopez et al., 1999b). In this system, it is suggested that $CD8\alpha^+$ DC selectively express IL-12 and prime Th1 type responses (Maldonado Lopez et al., 1999a). However, the maintenance of peripheral tolerance is also attributed to these DC (Belz et al., 2002). Others reported that both DC subtypes can induce either type of immune response, and that the microbial signals, which the DC receive, directs the outcome of the Th response (Manickasingham et al., 2003). Additionally, evidence suggesting that $CD8\alpha^+$ cells mature from $CD8\alpha^-$ precursors clearly exists (Martinez del Hoyo et al., 2002), and thus both DC subtypes can be derived from clonogenic myeloid precursors (Traver et al., 2000). $CD8\alpha^+$ and $CD8\alpha^-$ DC may represent different maturation stages of the same DC population. The most recent research suggests that both DC subsets receive signals from the surrounding environment at the time of Ag exposure through for example TLR, described below. These signals mature the DC in the appropriate way to ensure that the type of immune response, which most effectively deals with the pathogen at hand, will be initiated. Interestingly, different DC subsets express different repertoires of TLR (Boonstra et al., 2003; Reis e Sousa, 2004). At present, the role and significance of DC subsets for the type of T cell response remains to be clarified.

Toll-Like receptor expression on DC

Mammalian TLR are part of a family of 'pattern-recognition receptors', a term first coined over 15 years ago (Janeway, 1989). They recognise conserved microbial structures, including unmethylated CpG sequences on bacterial DNA (TLR9) (Hemmi et al., 2000), lipopolysaccharide (LPS) from Gram-negative bacteria (TLR4) (Hoshino et al., 1999) or double-stranded RNA (TLR3) (Alexopoulou et al., 2001). DC express a range of TLR, and different subtypes of DC express varying levels of the nine murine TLR identified so far (Kapsenberg, 2003). TLR triggering has pleiotropic effects on DC, for example promoting survival, chemokine and cytokine secretion, expression of chemokine receptors, or migration (Akira, 2003). The adaptive immune response generally starts with T cell recognition of peptides on DC, which have matured after previous TLR ligation. TLR are therefore crucial proteins linking the innate and adaptive immunity (Akira et al., 2001), and additionally, the cytokines and chemokines secreted in response to TLR ligation influences the outcome of adaptive immune responses. There follows a brief summary of the main effects of LPS and CpG, which signal through TLR4 and TLR9 respectively, and which are used as adjuvants in this project (also see Fig. 1.5). Both TLR4 and TLR9 signal through MyD88, an adaptor protein resulting in the activation of the transcription factor NF-κB, which controls expression of a number of pro-inflammatory cytokines.

LPS as an adjuvant

LPS is an integral component of the outer membrane of Gram-negative bacteria and can provoke a life threatening condition called endotoxic shock (Ulevitch and Tobias, 1995). LPS is a ligand for TLR4, but the LPS recognition complex may variably contain other components such as CD14, MD2, heat shock proteins 70 and 90, and the chemokine receptor CXCR4 (Triantafilou et al., 2001; Underhill, 2003). Both TLR4 and MD2 are required for responsiveness to LPS, since MD2 is physically associated with the extracellular domain of TLR4 on the cell surface (Underhill, 2003). LPS induces release of inflammatory cytokines as well as IFN-β. The induction of inflammatory cytokines, such as IL-1β or IL-12, is dependent on the intracellular adaptor molecules MyD88 and TIRAP, which lead to NFκB

activation and subsequent induction of cytokine gene transcription in DC (Akira et al., 2001). IFN-β (a type I IFN) release and subsequent upregulation of CD40, CD80 and CD86 through autocrine and paracrine signalling is MyD88 independent, but regulated through IFN regulatory factor 3 (IRF-3) (Kaisho and Akira, 2003). However, additional MyD88-independent pathways of LPS activation of DC also exist, which remain to be investigated.

LPS has been shown to fully activate DC, and its adjuvant effect has been used in numerous systems to enhance the T cell response *in vivo* and *in vitro* (Khoruts et al., 1998; Maxwell et al., 2002). *In vitro* treatment of mouse DC with LPS greatly enhances Ag loading onto MHC class II, and display of these MHC class II:peptide complexes on the cell surface (Inaba et al., 2000). LPS co-administration *in vivo* similarly improves presentation of protein Ag by both $CD8\alpha^+$ and $CD8\alpha^-$ DC (Reis e Sousa and Germain, 1999), which may be an indirect effect of the inflammatory properties of LPS (Manickasingham and Reis e Sousa, 2000). It also induces splenic $CD8\alpha^-$ DC migration, from the marginal zone to the T cell areas of the white pulp (De Smedt et al., 1996; Reis e Sousa et al., 1997). LPS thus enhances both the T cell priming abilities of DC, as well as causing their migration into the T cell areas where that priming can occur. LPS stimulation results in IL-12 release by DC, and the subsequent induction of a Th1 type response under most experimental conditions (Langenkamp et al., 2000). However, Th2 type responses after LPS stimulation have also been described (Boonstra et al., 2003; Pulendran et al., 2001). Interestingly, the IL-12 production after LPS stimulation is a transient effect, and the DC subsequently becomes refractory to further stimulation, an effect described as exhaustion.

CpG as an adjuvant

CpG-rich motifs are found in microbial DNA, and they are ligands for TLR9 (Hemmi et al., 2000), which is found on endosomes inside the cells. Three different types of CpG have been identified so far, CpG A, which induces IFN-α release by DC, but is a poor stimulator of B cell proliferation; CpG B, which conversely

stimulates B cell proliferation, but does not induce IFN-α release by DC, and CpG C, which stimulates both B cells and DC (Reis e Sousa, 2004). Similar to LPS, CpG has been used as an adjuvant in a number of experimental systems, and induces a Th1 type response (Jakob et al., 1999). DC activation by CpG at the time of T cell activation enhances immune responses (Boonstra et al., 2003), and enhances T cell responsiveness even after previous tolerance induction (Ichikawa et al., 2002). TLR9 signals exclusively via MyD88, inducing production of pro-inflammatory cytokines, and augmentation of CD40, CD80 and CD86 levels on DC (Hemmi et al., 2000). Release of type I IFN is also stimulated, although the exact pathway leading to IFN-gene induction is unknown (Reis e Sousa, 2004). Additionally, CpG provides a survival signal to DC, inducing upregulation of survival molecules Bcl-2 and Bcl-xL (Park et al., 2002). CpG thus increases DC survival as well as inducing DC maturation, leading to the initiation of effective T cell immunity.

1.3. T cell tolerance

TCR diversity coupled with flexible Ag recognition allows T cells to respond to peptides from virtually all molecules and/or cells, including our own tissue (Mason, 1998). However, the immune system is able to distinguish between harmless self-molecules, and molecules or cells that might be dangerous to us. An immune response should only be initiated in the latter case, whereas a tolerant state to self-molecules is maintained in healthy individuals by a variety of mechanisms.

1.3.1. Central tolerance

Tolerance is usually defined as the failure to respond to Ag, resulting in avoidance of autoimmunity and pathology. Intrathymic deletion of T cells with high affinity for self-Ag plays a crucial role in limiting the autoreactive T cell repertoire (Kappler et al., 1987). The thymus contains a variety of professional APC, which express both MHC class I and II molecules. These APC include DC, macrophages, B cells and thymic epithelial cells (TEC) (Derbinski et al., 2001), which can all express a large array of self-Ag. Immature T cells that bind their specific Ag with high affinity in the thymus, are deleted at the CD4, CD8 double-positive stage. This process is termed central tolerance. In recent years, it has become evident that even "tissue specific" Ag are expressed in the thymus. In particular medullary TEC express a vast range of Ag which are known to be putative targets in autoimmune diseases, for example insulin and MOG (Derbinski et al., 2001). The transcription factor AIRE, which is primarily expressed in the TEC and DC in the thymus, may be responsible for expression of these "tissue specific" Ag (Mathis and Benoist, 2004). Since immature T cells recognising these auto-Ag become deleted or possibly become regulatory T cells (T reg, see below), expression of tissue specific Ag in the thymus greatly limits the number of potentially autoreactive T cells in the periphery.

1.3.2. Peripheral tolerance

Nevertheless, it is unlikely that all self-peptides can be displayed in the thymus. Central tolerance is inevitably an incomplete process, and autoreactive T cells are

consequently found in the periphery, requiring mechanisms of peripheral tolerance to avoid autoimmune pathology (Anderton and Wraith, 2002). Three major mechanisms have been proposed to be relevant for the maintenance of peripheral tolerance to self-Ag, as well as for the induction of tolerance to administered Ag, namely T cell deletion, anergy, and suppression by T reg. T cells go through a number of stages through their life span and during an immune response, and these different mechanisms may all be necessary to avoid detrimental autoimmune responses at these various stages.

Deletion by activation induced cell death

Activation induced cell death (AICD) is a process of apoptosis induced by repeated activation of T lymphocytes by their specific Ag. The principal mechanism of AICD in $CD4^+$ T cells is the co-expression of Fas (CD95) and Fas ligand (FasL), which has been shown to occur after repeated Ag stimulation, and is important in limiting the immune response to foreign Ag to avoid damage to the host (Lenardo et al., 1999). Resting naïve T cells express little surface Fas, while TCR stimulation in the presence of IL-2, a cytokine important for T cell proliferation, enhance Fas expression. The activated T cell becomes sensitive to FasL induced apoptosis, which is triggered through endogenously produced FasL and signalling through Fas (Refaeli et al., 1998). AICD is important to limit the numbers of Ag-specific cells towards the end of a normal immune response. Similarly, if an Ag is presented chronically, inhibitory genes such as FasL are activated, rendering the T cell susceptible to Fas-induced apoptosis (Goodnow, 2001). AICD may thus limit early activation and expansion of self-reactive T cells, which consistently see their specific Ag, and has a supplementary role in maintenance of peripheral tolerance. The significance of Fas and its ligand for peripheral T cell tolerance is underlined by the fact that mice deficient for either of these molecules show lymphoproliferative disease and systemic autoimmunity (Cohen and Eisenberg, 1991).

Anergy

Anergy can be described as a state of lasting T cell unresponsiveness. Two broad categories of anergy are distinguished, namely clonal anergy and adaptive anergy (Schwartz, 2003). The former is mostly observed in previously activated T cells, and is characterised by growth arrest of the T cell, with lack of proliferation and a block in IL-2 production, but little loss of effector function and other cytokine production. The latter is initiated in naïve T cells, and consists of a more generalised inhibition of proliferation and effector functions (Schwartz, 2003). Clonal anergy does not require T cell proliferation or persistence of Ag, in contrast to adaptive anergy, which requires persistency of Ag to maintain the unresponsive state. Also, clonal anergy can be reversed by addition of IL-2, while adaptive anergy is maintained even after addition of IL-2 (Schwartz, 2003).

Both types of anergy can be induced by a number of circumstances *in vitro* and *in vivo*. Clonal anergy has primarily been studies *in vitro*, and induction of clonal anergy in $CD4^+$ T cell clones is induced through strong TCR signalling in the absence of costimulation (i.e. provision of signal 1 without signal 2) (Jenkins and Schwartz, 1987) or by stimulation with a low affinity ligand in the presence of costimulation (i.e. a low signal 1 with signal 2) (Sloan-Lancaster et al., 1993). Clonally anergised T cells remain viable and provision of IL-2 reverses anergy induction (Beverly et al., 1992). The CD28-CD80/CD86 interaction is critical in the prevention of clonal anergy, possibly by having an inhibitory effect on the production or function of anergic factors (Becker et al., 1995), or through stimulation of IL-2 (Beverly et al., 1992; Jenkins, 1992). The involvement of CTLA-4 on the other hand is less clear. Blocking antibodies to CTLA-4 or genetic CTLA-4 deficiency did not influence the induction of clonal anergy *in vitro* in some systems (Frauwirth et al., 2000), but did have a role in others (Wells et al., 2001). All the same, it is debatable how important results from *in vitro* studies are, and although some transfer studies looking at clonal anergy *in vivo* have been carried out

(Schwartz, 2003), it is adaptive anergy which has been studied more extensively *in vivo*.

Adaptive anergy is often initiated in naïve T cells *in vivo* by TCR stimulation without provision of costimulatory molecules, which is thought to be the case during peptide-induced tolerance. T cells go through a significant phase of proliferation before becoming anergic (Pape et al., 1998), see section 1.3.4. The role of the CD28/CTLA-4-CD80/CD86 interaction for adaptive anergy is debatable. The importance of CD28 is difficult to investigate, since $CD28^{-/-}$ T cells fail to proliferate in response to Ag (Howland et al., 2000), while the role for CTLA-4 is controversial. Some groups report that the induction of anergy *in vivo* was only possible through engagement of CTLA-4, illustrated by a lack of anergy induction when CTLA-4 was blocked (Perez et al., 1997), and inability to induce anergy in CTLA-4-deficient T cells (Greenwald et al., 2001). Others suggest anergy of $CD8^{+}$ T cell *in vivo* was possible in the absence of CTLA-4 (Frauwirth et al., 2001). The exact role of CTLA-4 in T cell activation, anergy and tolerance remains open to discussion, and requires further investigation.

In addition to an increasing number of molecules being identified as positive regulators of T cell responses, negative regulators of T cell activation, such as PD-1 and its ligands PD-L1 and PD-L2 (Greenwald et al., 2002) are becoming apparent. It seems plausible, that rather than anergy being the result of a simple lack of costimulation, it is determined by a balance of positive and negative signals that the T cell receives after TCR recognition.

The molecular mechanisms responsible for anergy induction and maintenance are a subject of intense investigation. A number of signalling pathways are activated after ligation of TCR and costimulatory molecules. Recently, it was shown that during the induction of clonal anergy, a specific set of genes became activated (Macian et al., 2002), including genes encoding Cbl-l, Itch and GRAIL, three ubiquitin ligases (E3s)

(Heissmeyer et al., 2004). E3s confer substrate specificity to the ubiquitin system (Schnell and Hicke, 2003), which targets proteins for degradation. It is suggested that degradation of signalling proteins leads to disruption of the signalling synapse between the APC and the T cell, which ultimately results in T cell anergy (Heissmeyer et al., 2004). Other molecules that may influence tolerance induction are signal transducers and activators of transcription (Stats), which are cytoplasmic transcription factors, playing important roles as mediators in cytokine and growth factor signalling (Darnell, 1997). Stat 3 has recently been described as a negative regulator of inflammatory responses, and $CD4^+$ T cells from mice with disrupted Stat 3 function in macrophages cannot be tolerised through a known tolerogenic protocol (i.v. administration of soluble peptide) (Cheng et al., 2003; Kearney et al., 1994). Stat 3 is thus a signalling molecule that has been identified to specifically be required for tolerance induction. Identification of molecules such as Cbl-l, Itch, GRAIL and Stat3 gives new insight into the molecular events during tolerance induction, and allows identification of new potential targets for immunotherapy.

Regulatory T cells

The idea of a specialised subset of T cells, which limits the outcome of an immune response, was first described in the 1970s (Gershon, 1975). After a long period during which this concept was in disrepute, regulatory T cells (T reg) have regained acceptance as a specialised subset of T cells, which have suppressive or regulatory properties, aiding in the prevention of autoaggressive immune responses. Two different T reg subsets can be distinguished through their mode of action. Naturally occurring T reg are thought to arise in the thymus, perhaps via an altered negative selection by self-Ag (Caton, 2003), but they may have to re-encounter Ag in the periphery to become fully mature (Shevach, 2000). They constitute 5-10% of all peripheral $CD4^+$ T cells and display characteristic surface markers such as CD25 (Sakaguchi et al., 1995), CTLA-4 (Read et al., 2000) and GITR (McHugh et al., 2002; Shimizu et al., 2002). Their importance in peripheral tolerance is demonstrated by the development of autoimmune diseases such as gastritis and thyroiditis when T reg are eradicated (Sakaguchi et al., 1995). Although T reg need to be triggered

through their TCR (Shevach, 2002), once activated, they suppress effector T cells ($CD25^-$) in an Ag-nonspecific fashion (Thornton and Shevach, 2000). *In vitro*, this inhibitory action is dependent on cell-cell contact with the $CD25^-$ cells to be suppressed, and can be overcome by addition of exogenous IL-2 and IL-15 (Dieckmann et al., 2001; Jonuleit et al., 2001; Thornton and Shevach, 1998). *In vivo*, roles for IL-10 and TGF-β have been described (Belkaid et al., 2002; Suri-Payer and Cantor, 2001). However, their target Ag and mechanisms of action remain poorly defined, and their exact role in autoimmune diseases continues to be a topic of intense investigation.

The second type of suppressive $CD4^+$ T cells is induced from conventional $CD4^+CD25^-$ T cells in the periphery. In contrast to the naturally occurring $CD4^+CD25^+$ subset, these T cells carry out their *in vitro* suppressive effects via cell contact independent mechanisms, mainly through secretion of immunomodulatory cytokines such as IL-10 and/or TGF-β (Jonuleit and Schmitt, 2003). Two subsets of induced T reg have been described as Tr1 and Th3 cells (Roncarolo et al., 2001a; Weiner, 2001). Tr1 cells produce large amounts of IL-10 and little TGF-β, while Th3 produce preferentially TGF-β. Tr1 cells were first induced by repeated stimulation of T cells in the presence of IL-10 (Groux et al., 1997). The resulting T cell population controls activation of naïve and memory T cells *in vivo* and *in vitro*, and suppresses Th1- and Th2-mediated responses to pathogens, tumours and allo-Ag (Groux, 2003). The inhibitory effect is mainly through production of IL-10, as shown by the reversal of this effect after administration of blocking antibodies against IL-10 (Groux et al., 1997). Th3 cells producing TGF-β were identified in mice after oral tolerance induction using myelin basic protein (MBP) (Chen et al., 1994), and have similarly been described in humans after oral administration of MBP (Fukaura et al., 1996). TGF-β influences the activity of multiple cell types, and Th3 cells may therefore have a major role in many aspects of immune regulation and T cell homeostasis (Weiner, 2001).

1.3.3. Dendritic cells as tolerogenic APC

It is clear from the existing data that Ag presentation by APC, including DC, plays a major role in the induction and maintenance of tolerance. There are two separate ideas about DC and their role in tolerance. Firstly, a subset of regulatory DC may specialise in inducing tolerance in the T cells to which they present, or secondly, the activation status of the DC regulates their ability to induce tolerance or immunity. Some evidence exists for specialised regulatory DC (Fazekas de St Groth, 1998; Suss and Shortman, 1996), in that $CD8\alpha^+$ DC of lymphoid origin (LDC) have been proposed to negatively regulate $CD8^+$ T cell responses (Belz et al., 2002; Kronin et al., 1996; Kronin et al., 2000). These LDC are the predominant DC population found in the spleen and in the T cell zones of non-immunised mice (Fazekas de St Groth, 1998), and have been found to express high levels of self-peptides (Inaba et al., 1997), making them an ideal candidate for tolerance induction (Fazekas de St Groth, 1998). However, since they may be derived from the same precursors as $CD8\alpha^-$ DC, (Roncarolo et al., 2001b; Traver et al., 2000), and taking into account the accumulating evidence that tolerance is a result of the activation status of the DC (described below), it remains to be seen whether there truly is a subtype of DC specialised for tolerance induction.

It has been shown that T cell tolerance to self-Ag is an active choice of the immune system. The (self-) reactive T cell goes through a phase of activation (Kurts et al., 1997), and evidence exists that the T cell acquires significant effector functions on its way to becoming tolerant (Huang et al., 2003). Originally, it was thought that immature, resting DC, which display MHC/self-peptide complexes and low levels of costimulatory molecules in the absence of inflammatory stimuli, induce peripheral T cell tolerance *in vivo* (Dhodapkar et al., 2001; Steinman et al., 2000). This idea has now been revised to suggest that peripheral tolerance in the steady state is induced by mature, but quiescent DC, displaying high levels of self-Ag in their surface (Inaba et al., 1997), while immunity is initiated by DC that are fully activated through the presence of "danger", such as microbial molecules, which activate DC through TLR ligation as described in section 1.2.3 (Albert et al., 2001; Shortman and Heath, 2001).

The activated DC provide the T cells with much stronger versions of the same signals and/or additional signals to induce immunity. One might therefore suggest that the induction of tolerance versus immunity could be determined by the ratio of quiescent DC to activated/mature DC (Roncarolo et al., 2001b). The emerging consensus is that quiescent DC, which display self-Ag and low levels of costimulatory molecules, and which continuously travel from peripheral sites to DLN, are required for continual induction of tolerance to self-Ag. However, if DC become activated in the presence of "danger" or through maturation signals such as agonistic anti-CD40 antibody, they increase expression of costimulatory molecules. This in turn allows initiation of an effective immune response in the DLN. Although the DC still express self-Ag, the prior establishment of self-tolerance will prevent detrimental immune responses against those self-Ag.

Quiescent DC can induce T cell deletion as well as T cell anergy to foreign and self-Ag, and DC also play a role in the induction and modulation of T reg *in vitro* and *in vivo*. Targeting ovalbumin peptide (pOVA), or hen egg lysozyme (HEL) to resting DC via DEC-205 results in deletion of both Ag-specific $CD4^+$ and $CD8^+$ T cells (Bonifaz et al., 2002; Hawiger et al., 2001), while targeting pMOG to resting DC via DEC-205 causes $CD4^+$ T cell anergy (Hawiger et al., 2004). In both cases, T cells go through a phase of proliferation and activation before becoming anergic or deleted. IL-10 producing T reg may be induced *in vitro* by repetitive stimulation through untreated immature DC (Jonuleit et al., 2000). *In vivo*, steady-state DC present soluble protein to $CD4^+$ $CD25^+$ T cells, and induce their expansion (Yamazaki et al., 2003). At least in humans, T reg themselves can also reduce the capacity of DC to induce T cell proliferation, indicating a feedback loop resulting in maintenance of tolerance (Roncarolo et al., 2001b). Similarly, $CD4^+$ $CD25^+$ T reg are able to directly induce the development of other T reg subsets (Dieckmann et al., 2002; Jonuleit et al., 2002), to elicit a form of infectious tolerance. The ability to induce T reg using modified DC has enormous therapeutic potential, but more research needs to be carried out to fully understand the mechanisms involved.

1.3.4. Peptide-induced tolerance

It has been known for a long time that the form in which the Ag is administered plays a crucial role in the outcome of the immune response (Weigle, 1973). Ag in aggregated form, or administered with appropriate "danger" signals, such as adjuvant, leads to T cell activation and expansion and the generation of a productive immune response. The same Ag administered in soluble, monomeric form (in the absence of "danger"), however, leads to T cell tolerance, as illustrated by the absence of T cell activation when rechallenged with Ag in adjuvant. Administration of soluble Ag via the oral/mucosal, intravenous (i.v.), intraperitoneal (i.p.), or intranasal (i.n.) routes have all been shown to successfully induce tolerance (al Sabbagh et al., 1994; Burkhart et al., 1999; Chen et al., 1995; Liblau et al., 1996; Liu and Wraith, 1995). With the identification of immunodominant epitopes within proteins, it has been possible to move from tolerisation using whole proteins to induction of tolerance through administration of soluble peptides. This approach is very effective in preventing autoimmunity, and inhibits the onset of autoimmunity in EAE (Anderton and Wraith, 1998), as well as in experimental models of arthritis and myasthenia gravis (Paas-Rozner et al., 2000). The therapeutic potential for treatments of allergies and human autoimmune diseases is described further at the end of this section.

The exact mechanisms leading to induction of tolerance after administration of soluble peptide remain an issue of debate. It has been suggested that tolerance is simply due to immune deviation from a Th1 phenotype, which causes autoimmune damage, to a less pathological Th2 cytokine profile (Tian et al., 1996). However, it has been shown that in most systems, induction of peptide-induced tolerance involves the same mechanisms responsible for induction of peripheral tolerance; anergy, apoptosis and induction of T reg. Each has been shown to play a role, depending on the exact dose and route of Ag administration, and in a number of systems, a combination of these mechanisms was responsible for the resulting tolerance. High doses of orally administered Ag may cause deletion of specific T cells (probably through Fas-mediated apoptosis after initial T cell activation, i.e.

AICD) (Chen et al., 1995), or anergy (Friedman and Weiner, 1994). Low doses on the other hand appear to induce Ag-specific T reg of the Th3 subtype (Chen et al., 1994; Friedman and Weiner, 1994). Similarly, i.v. administration of high doses of soluble peptide causes apoptosis of Ag-specific T cells (Liblau et al., 1996), and in some systems it was shown that most Ag-specific T cells are deleted, leaving the remaining Ag-specific T cells functionally impaired, i.e. anergic, and resulting in T cell tolerance (Kearney et al., 1994; Pape et al., 1998). In most experimental systems, tolerance is thus likely to result from several mechanisms collectively acting to maintain tolerance.

Induction of T cell tolerance is an active process

Earlier studies showed that systemic T cell tolerance takes 3 days to become established (Liu and Wraith, 1995). Because only a fraction of T cells are specific for a certain Ag, the number of Ag-reactive T cells during an immune response is small, and impossible to trace. By generating TCR transgenic mice, in which all T cells specifically recognise a certain peptide-MHC complex, and transferring T cells from the TCR transgenic mouse into naïve syngeneic recipients, it became possible to trace a definite population of T cells during the induction of tolerance (Kearney et al., 1994). Initial experiments compared T cell expansion after i.v. administration of soluble Ag to sc injections of Ag in CFA. While in both cases, Ag-reactive T cells proliferated in response to the Ag, T cells only entered B cell follicles and remained Ag responsive upon *in vitro* challenge when Ag was administered with an adjuvant. When T cells encountered their Ag without the presence of adjuvant, they proliferated for some time, but never entered B cell follicles, and the cells that remained were hyporesponsive to antigenic challenge (Kearney et al., 1994).

It has since been shown in a number of systems that although tolerant T cells are ultimately unresponsive to their Ag, they go through a significant phase of activation en route to tolerance induction. The kinetics of T cell activation during the induction of tolerance show transient T cell activation with expression of costimulatory molecules, followed by T cell unresponsiveness to Ag (Kearney et al., 1994).

Similarly, expression of a neo-self-Ag results in initial activation and gain of effector functions, before finally resulting in anergy of the adoptively transferred Ag-specific T cells (Huang et al., 2003). In both systems, T cells undergo significant activation and proliferate in response to Ag-encounter, before becoming anergic. In experimental settings where Ag are targeted to quiescent DC, T cells similarly go through a phase of activation and proliferation, before becoming anergic or deleted (Bonifaz et al., 2002; Hawiger et al., 2001; Hawiger et al., 2004). Since costimulatory molecules are crucial for effective T cell activation, their possible importance during the induction of tolerance is evident and discussed further in section 1.3.5.

DC versus B cells in the induction of T cell tolerance

The APC that is responsible for displaying peptide to tolerise T cells, has been debated for some time. Early data shows that not only DC, but also B cells present processed peptides 4 hours after i.v. administration of soluble protein *in vivo* (Zhong et al., 1997). Nevertheless, it has been shown that $CD8\alpha^+$ DC present to $CD8^+$ T cells, while $CD8\alpha^-$ DC present to $CD4^+$ T cells after i.v. administration of Ag (Pooley et al., 2001). Also, T cell-DC interactions, rather than T cell-B cell interactions, were observed in the parafollicular regions of the DLN after s.c. administration of soluble protein (Ingulli et al., 1997). More recently, it was shown that 4 hours after s.c. administration of soluble protein, most protein was found on the $CD8\alpha^-$ DC subset, in the parafollicular regions of the DLN, to which Ag-specific T cells home after administration of Ag (Ingulli et al., 2002). Additionally, it was shown that protein administered s.c. was presented to T cells in the DLN in two temporally distinct waves. Even though processed protein was found on B cells throughout the 24 hours period examined, the peptide-expressing cells in T cell areas were found to be DC (Itano et al., 2003). Finally, DC display peptide for several days longer on their surface compared to B cells after i.v. administration of peptide (S. Anderton, personal communication), and B cell-deficient μMT mice can be tolerised to induction of EAE (Vella et al., 1996). Collectively, these data suggest that it is DC that present processed peptides to T cells and tolerise them, rather than B cells.

Further supporting this argument is the notion that T cells need signals different from TCR stimulation to enter B cell areas. In the presence of "danger", such as of LPS or the proinflammatory cytokines IL-1 and TNF-α, T cells migrate into B cell follicles (Pape et al., 1997). It was similarly shown in an oral tolerance model that T cells do not enter B cell follicles after a tolerogenic stimulus, while they do after administration of an immunogenic one (Smith et al., 2002), and that T cells need to receive costimulatory signals from DC before they can enter B cell areas (Fillatreau and Gray, 2003). It appears that although B cells may present peptide on their surface after administration of soluble Ag, T cells will home to T cell areas and first need to interact with DC before being able to enter B cell areas and possibly re-encounter their Ag.

Tolerance to EAE

All three mechanisms of tolerance described above are thought to play a role in therapeutically-induced tolerance to myelin Ag. FasL-mediated apoptosis is important in some mouse strains in the recovery from EAE, however, it also functions in T cell-mediated killing of oligodendrocytes (Sabelko Downes et al., 1999). Direct induction of T cell apoptosis with high-dose Ag can similarly induce remission of EAE (Critchfield et al., 1994). Administration of soluble peptides from PLP or MBP result in protection from subsequent EAE induction (Anderton and Wraith, 1998). At the population level, tolerant T cells are similar to anergic T cells described above, since this tolerance is characterised by reduced T cell proliferation and cytokine production upon rechallenge with Ag in adjuvant. Immune deviation towards a Th2 phenotype has not proved to be successful in suppressing on-going EAE (Khoruts et al., 1995), but EAE could be prevented if Th2 cells were present during the initiation of the Th1 response (Kuchroo et al., 1995).

Several tolerisation protocols result in the induction of a suppressor T cell population. Th3 cells described in section 1.3.2, producing large amounts of TGF-β, and some IL-10 and IL-4 in an Ag-specific fashion, were first isolated after oral

administration of lower doses of MBP (Chen et al., 1994). These cells successfully suppressed EAE after transfer into naïve recipients. Intranasally-induced peptide tolerance to EAE induces T cell populations that produce IL-10 (Burkhart et al., 1999; Massey et al., 2002). When IL-10 was neutralised, or IL-10-deficient mice were used, mice were no longer protected from EAE after i.n. administration of Ag, further showing the importance of IL-10 in this process (Burkhart et al., 1999; Massey et al., 2002). The search for Ag-specific T reg cells is still ongoing, and their isolation may allow exploitation of novel therapeutic possibilities for the treatment of autoimmune diseases.

Peptide therapy in humans

Over the last years, the findings from peptide-induced tolerance in animal models have been translated into peptide-based therapeutic trials to treat autoimmunity in humans. However, the majority of data has been generated from attempts to treat allergy. A variety of methods, including administration of soluble whole allergenic proteins, or peptides from allergens, have been tested. Results have however been mixed, and most trials showed some adverse side effects of treatment with peptide, such as symptoms of breathlessness and wheeze with onset several hours after peptide administration. Improvements in allergic reactions of patients were found after high dose peptide therapy with Fel d 1 peptides (a cat allergen) (Norman et al., 1996), and promising results indicating induction of T cell hyporesponsiveness after peptide administration have also been reported (Oldfield et al., 2001). Even more encouraging results were found when patients, who previously had severe systemic reactions following bee stings, were treated with a mixture of three peptides from the major bee venom allergen, and all five patients experienced no, or greatly reduced, reactivity to bee stings (Muller et al., 1998). Unfortunately, other trials showed less promising results (Simons et al., 1996). Nevertheless; a number of studies indicate a role for IL-10 in successful treatment using soluble peptide or protein (Akdis et al., 2001; Oldfield et al., 2002), indicating that human peptide therapy may work through induction of enhanced IL-10 production which down-regulates pro-inflammatory responses. Hopefully, ongoing studies will further elucidate the relevant

mechanisms, and will brighten the prospects of developing peptide-based vaccines for human diseases.

1.3.5. The CD40-CD154 interaction in the decision between tolerance and immunity

As described above, T cells go through a significant phase of activation and proliferation on their way to becoming tolerant. The importance of costimulatory molecules such as CD40 and OX40 during the induction of T cell immunity is described above, and is well-documented in the literature. The importance of some costimulatory molecules during the induction of tolerance has been investigated, but this has by no means been extensive. Conflicting evidence exists with respect to the requirements of CD40-CD154 in tolerance. Mucosally induced tolerance to ovalbumin appears to require the presence of CD154 in some systems (Kweon et al., 1999), but not others (Hanninen et al., 2002), while tolerisation via the i.v. route was possible in the absence of CD154 (Howland et al., 2000). The requirements for OX40-OX40L interactions during tolerance induction had not yet been investigated prior to this project.

Exogenous ligation of CD40 has been used extensively to activate APC *in vitro* (Caux et al., 1994; Cella et al., 1996), and more recently, is has been shown that administration of agonistic anti-CD40 mAb *in vivo* boosts T cell immunity in response to a super-Ag (Maxwell et al., 1999). Other experimental systems further support this, in that anti-CD40 administration also prevents tolerance to tumours (Diehl et al., 1999; Grohmann et al., 2001; Sotomayor et al., 1999). Similarly, *ex vivo* blockade of CD40-CD154 has also been reported to result in the induction of potent T reg (Taylor et al., 2002) after transfer of tolerised T cells, and *in vivo* blockade of CD40-CD154 interaction induces T cell tolerance (Tang et al., 1997), and prolongs allograft survival (Honey et al., 1999). CD40-deficient DC have been shown to produce IL-10, but not IL-12, which induced T cell hyporesponsiveness *in vitro* (Gao et al., 1999), again illustrating the importance of CD40 on DC for effective immunity. Similar to the conflicting evidence for the requirements of CD40-CD154

in mucosal tolerance however, there is some evidence that administration of agonistic anti-CD40 does not prevent mucosally induced tolerance (Chung et al., 2004; Sun and Van Houten, 2002), suggesting that T cell tolerance is differentially regulated in different peripheral sites. Nevertheless, the CD40-CD154 interaction is an important candidate as a checkpoint determining between tolerance and immunity.

The interactions of CD28 and CTLA-4 with CD80 and CD86 have been suggested to determine tolerance versus immunity. However, initial T cell activation in T cells lacking CD28 is greatly impaired (Howland et al., 2000). Since T cells undergo a significant phase of activation and proliferation on their way to becoming tolerant, this suggests that the CD28-CD80/CD86 interaction is not disrupted during tolerance induction. In contrast, when T cells lacking CD154 are stimulated by an Ag in adjuvant, they initially become activated, and although they are unable to sustain this activation, they are not rendered tolerant by this initial activation in the absence of CD154 (Howland et al., 2000). Nevertheless, $CD154^{-/-}$ T cells can be tolerised by administration of soluble Ag. CD154 is thus neither required for tolerance induction, nor for initial T cell activation. The main role identified for CD154 is ligation of CD40 on DC, which leads to the induction of OX40L expression on the surface of DC (Fillatreau and Gray, 2003; Ohshima et al., 1997). OX40L is then thought to bind OX40 on activated T cells, resulting in the expression of the survival molecules Bcl-2 and Bcl-xL (Rogers et al., 2001) (also see section 1.1.4). The main effect when the CD40-CD154 interaction is missing, either through a lack of CD154, or by blocking with antibodies, may thus be the subsequent lack of up-regulation of OX40L on DC. This will result in a shortage of survival signals to the activated T cell, and subsequent T cell tolerance. In contrast, provision of anti-CD40 activates the DC and results in OX40L expression, allowing T cell immunity. The CD40-CD154 interaction may therefore be a crucial switch deciding between T cell tolerance and T cell immunity.

1.4. Central Hypothesis

The CD40-CD154 and OX40-OX40L interactions have previously been shown to be of crucial importance during T cell responses, giving a costimulatory signal, i.e. signal 2, resulting in effective T cell activation. The timing of expression of CD154 and OX40 on T cells fits with the timing of tolerance induction. This thesis proposed that the chief defect driving a T cell towards tolerance is the lack of CD40 ligation, subsequently resulting in a lack of the OX40-OX40L interaction. The CD40-CD154 and the OX40-OX40L interactions are thus pivotal in the decision between tolerance and immunity.

1.5. Aims

The above hypothesis was investigated by asking three main questions:

1. Can exogenous ligation of defined costimulatory receptors convert a tolerogenic signal into an immunogenic one?

If the CD40-CD154 and OX40-OX40L interactions are checkpoints determining the outcome of T cell immunity, agonistic antibodies to CD40 or OX40 should prevent the induction of T cell tolerance after administration of a tolerogenic stimulus.

2. How does expression of CD154 and OX40 on T cells, and CD40 and OX40L on DC differ during induction of T cell tolerance versus T cell immunity?

If the presence or absence of costimulatory molecules on DC is responsible for the induction of T cell immunity versus tolerance, DC will upregulate expression of CD40 and OX40L during the induction of T cell immunity, but not T cell tolerance. Since T cells go through a phase of activation during tolerance induction, T cells are likely to express CD154 and OX40 during both immunity and tolerance.

3. Do T cells become tolerant on exposure to Ag-loaded DC lacking CD40?

The lack of CD40 on Ag-loaded DC should not result in T cell priming, but conversely induce T cell tolerance.

These three questions will be addressed in turn in Chapters 3, 4, and 5, respectively.

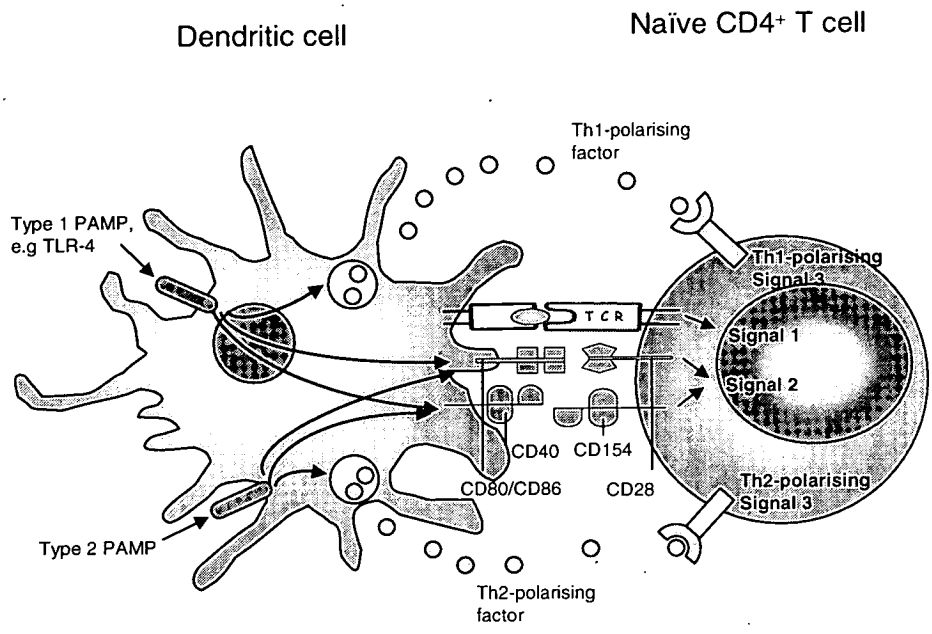

Fig. 1.1:
Signals 1, 2 and 3 lead to effective T cell activation
Signal 1 is the Ag-specific signal that is mediated through the TCR-MHC-peptide complex interaction. Signal 2 can be provided by a variety of costimulatory molecules. Shown here are CD80/CD86-CD28, and CD40-CD154. Other costimulatory molecules shown to be important for effective T cell activation are OX40 and RANKL, binding to their receptors OX40L and RANK, respectively. Signal 3 induces release of the polarising signals that promote the development of Th1 or Th2 cells. Signal 3 also activates DC, resulting in increased expression of costimulatory molecules, i.e signal 2.
(Adapted from Kapsenberg, 2003)

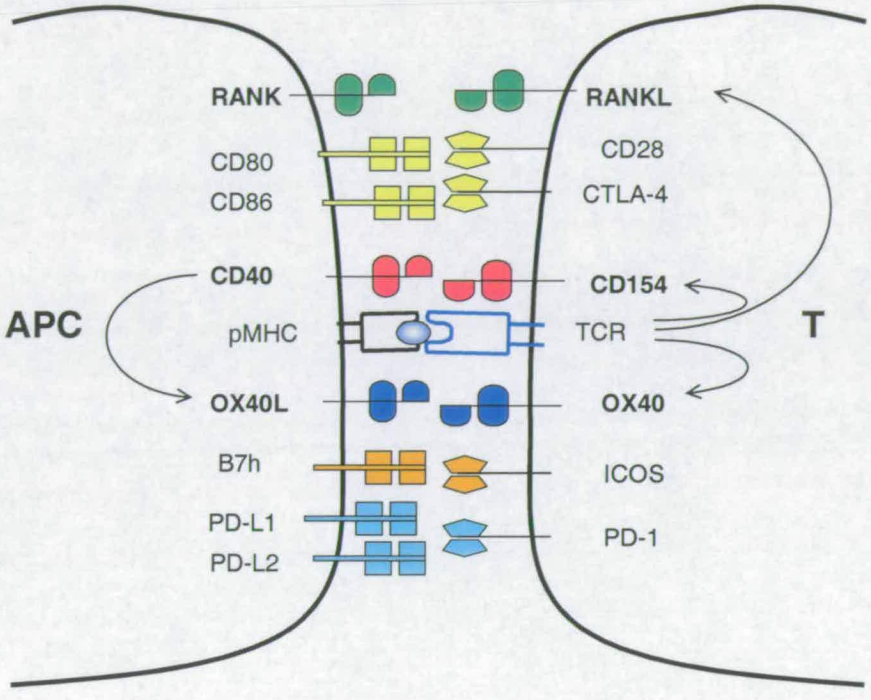

Fig. 1.2:
Summary of some of the costimulatory molecules known to be important in T cell activation
The TCR-MHC-peptide complex interaction induces expression of CD154, OX40, RANKL (as well as CTLA-4, ICOS and PD-1). CD154 expression peaks between 6 and 8 hours, while OX40 and RANKL expression peak 2-3 days after initial T cell activation. CD40 is expressed constitutively at low levels on DC, and becomes upregulated early after DC activation. OX40L is induced (and CD80 and CD86 are upregulated) through CD40 ligation, while RANK has been found to be expressed on mature DC. Expression of these three receptor-ligand pairs are investigated in detail in this thesis.

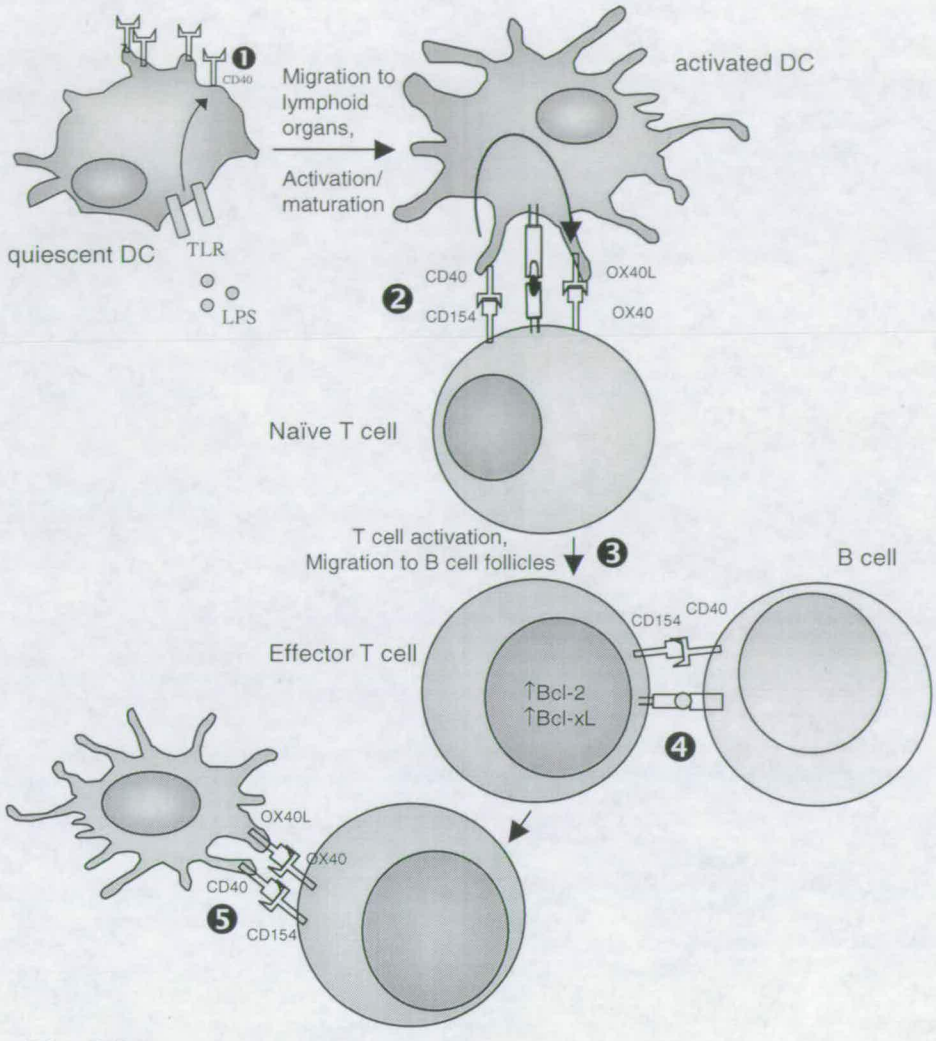

Fig. 1.3:
The role of CD40-CD154 in T cell function
The CD40-CD154 interaction is involved in DC migration to lymphoid organs ❶, and DC maturation, allowing effective T cell activation. CD40 signalling induces up-regulation of a number of costimulatory molecules, such as CD80, CD86, OX40L, enhancing DC survival in the lymph node ❷. Ligation of these molecules by their receptors on T cells results in effective T cell activation and survival ❸, inducing T cell migration into B cell follicles. Once the T cell has become activated, CD40-CD154 is important in B-T cell interactions, allowing isotype class switching ❹. Finally, CD40-CD154 allows entry of effector T cells into peripheral sites, e.g. CD40 expression in the CNS is required for entry of T cells into the CNS ❺. However, it does not appear to play a role in memory formation and is not required for reactivation of memory cells.

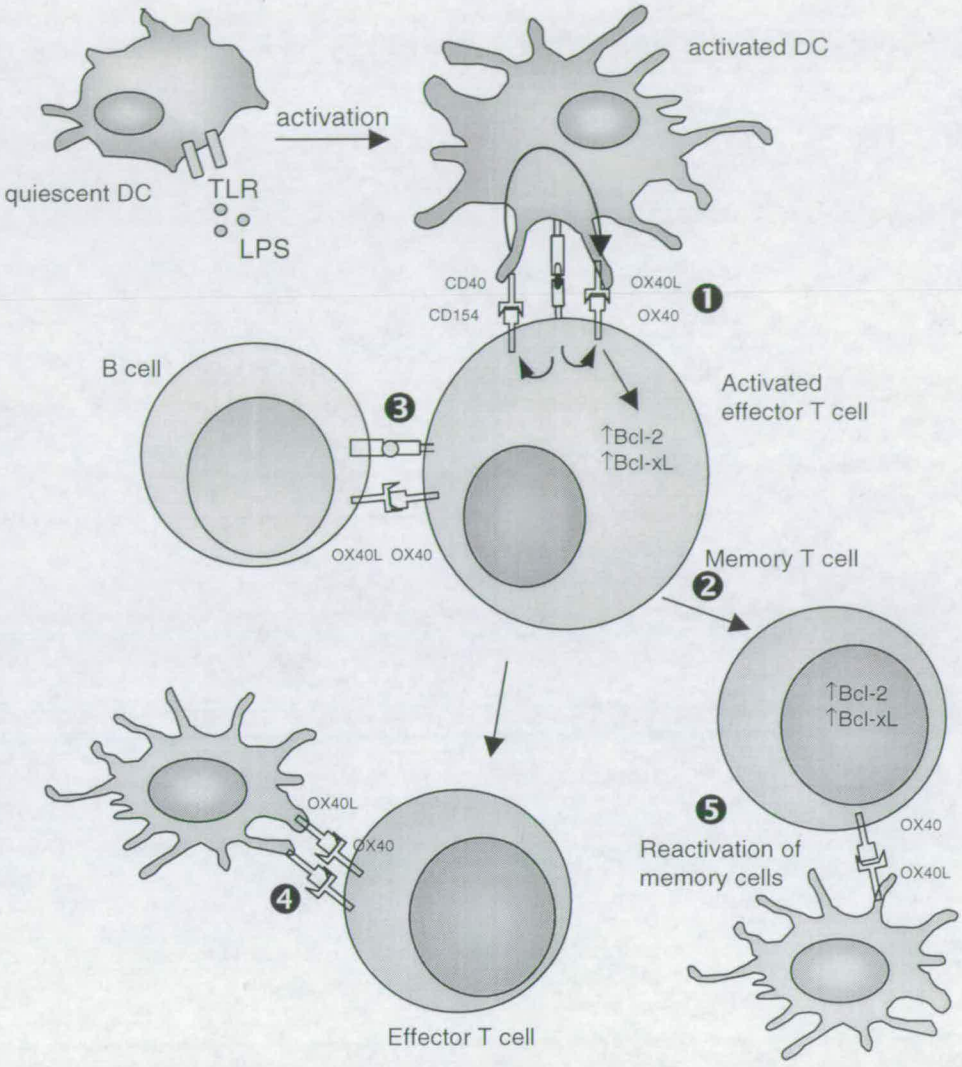

Fig. 1.4:
The role of OX40L-OX40 in T cell function
OX40 is up-regulated after TCR Ag recognition in lymphoid organs. Binding to OX40L on activated DC induces expression of survival molecules in effector T cells ❶. OX40 ligation also allows effective entry into the memory T cell pool ❷. Binding of OX40L on B cells may play a role in induction of Th2 type responses ❸. Finally, OX40L-OX40 is involved in entry of effector T cell into peripheral sites ❹, and reactivation of memory cells ❺.

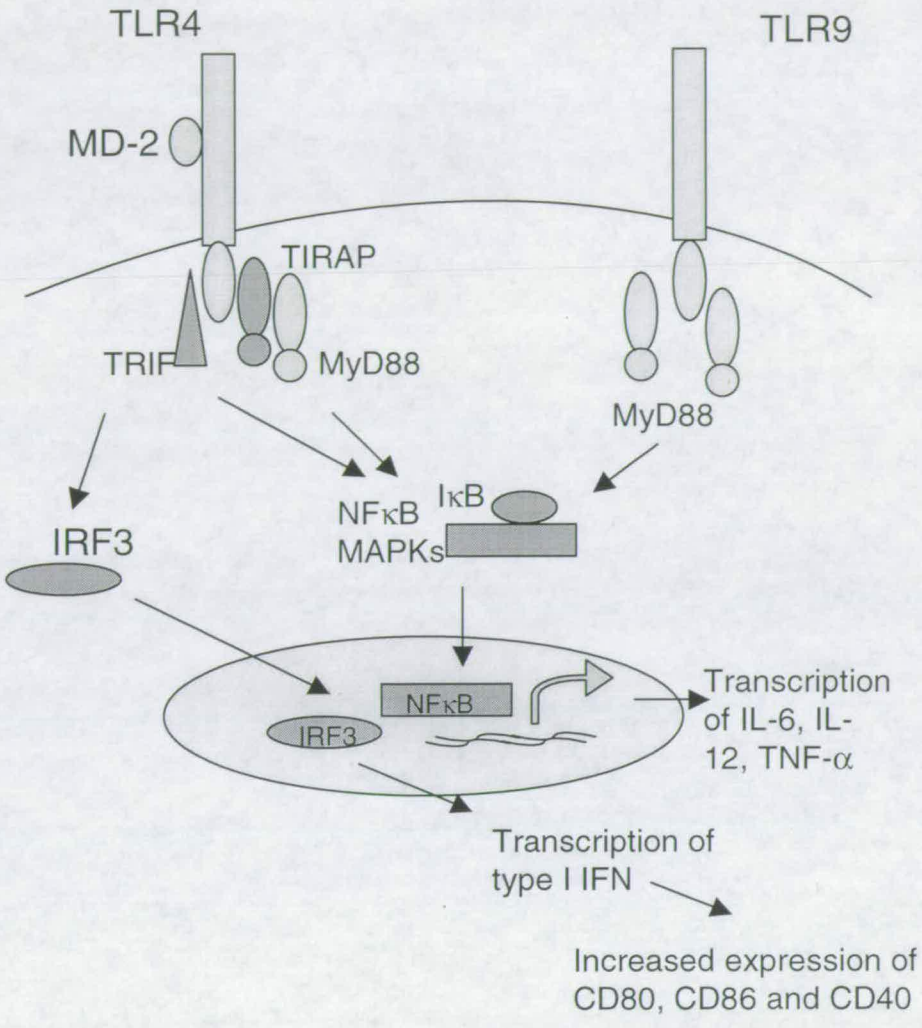

Fig. 1.5:
Possible signalling pathways coupling TLR4 and TLR9 to DC activation
TLR-4 can signal via both MyD88 and TRIF. TIRAP couples MyD88 to TLR4. MyD88 and TRIF promote activation of NFκB and MAPKs, allowing transcription of inflammatory cytokines, such as IL-6, IL-12 and TNF-α. TRIF also activates IRF3, leading to synthesis of type I IFN. TLR9 signals exclusively through MyD88, which does not activate IRF3, but nevertheless results in IFN-β release (a type I IFN). IFN- β then signals in autocrine and paracrine manner, resulting in increased upregulation of CD80, CD86 and CD40.
(Adapted from Reis e Sousa, 2004)

2. CHAPTER 2 - Materials and Methods

2.1. Mice

All mice used were on the C57BL/6 (H-2^b) background and were bred at the Institute of Infection and Immunology Research, University of Edinburgh. C57BL/6 (B6), $CD40^{-/-}$ (Kawabe et al., 1994), $CD154^{-/-}$ (Xu et al., 1994), μMT (Kitamura and Rajewsky, 1992) and B6 Ly5.1 congenic mice were used extensively. OT-II mice expressing an A^b-restricted, transgenic pOVA-reactive TCR (Barnden et al., 1998) were crossed with the respective strains to generate OT-IIxCD40$^{-/-}$ mice, OT-IIxCD154$^{-/-}$ mice, and OT-IIxLy5.1$^+$ mice. Sex-matched, 6-8 week old mice were used for all experiments.

2.2. Reagents

2.2.1. Antigens

Peptides (ovalbumin 323-339, hereafter referred to as pOVA, and myelin oligodendrocyte glycoprotein peptide 35-55, hereafter referred to as pMOG) were synthesized by the Advanced Biotechnology Centre, Imperial College London, using standard F moc chemistry.

2.2.2. Antibodies

The OX-86 hybridoma that produces anti-OX40 and the MAC-49 isotype control hybridoma (anti-phytochrome, rat IgG1 isotype), as well as the MAC-1 control hybridoma (anti-glycoprotein of Chlamydomonas reinhardii, rat IgG2a isotype), were obtained from the European Collection of Cell Culture (ECACC, Wiltshire, GB). The FGK-45 hybridoma that produces anti-CD40 was kindly provided by Prof D. Gray, IIIR, Edinburgh. Antibodies were purified using an AktaPrime (Amersham Biosciences) automated chromatography system using a High Trap protein G HP (Amersham Biosciences).

2.2.3. Adjuvants

Complete Freud's Adjuvant (CFA) and LPS were obtained from Sigma (Poole, GB). CpG_{1668} (TCCATGACGTTCCTGATGCT) was produced by MWG.

Various doses of LPS have previously been shown to induce immunity (Khoruts et al., 1998; Maxwell et al., 2002), and 30μg LPS was tested here, found to work efficiently to prevent tolerance, and thus used in subsequent experiments to induce immunity.

5μg CpG has been used by others to induce immunity (Vicari et al., 2002), and was thus initially tested in the system used here. When it was unsuccessful in inducing immunity, higher doses (10μg and 30μg) were also tested, but similarly did not induce immunity.

2.2.4. Tissue culture media

The following media were used unless indicated otherwise.

Tissue wash medium: RPMI 1640 medium containing 2mM L-Glutamine, $5x10^{-5}$ M 2-ME, 100U/ml penicillin, 100μg/ml streptomycin (all from Gibco, Life Technologies, Paisley, UK Gibco, hereafter referred to as Gibco).

Tissue culture medium: X-VIVO15 serum free medium (BioWhittaker, Maidenhead, GB) supplemented with 2mM L-glutamine and $5x10^{-5}$ M 2-ME.

2.2.5. General reagents

MACS buffer: Hanks medium, supplemented with $5x10^{-5}$ M 2-ME, 100μg/ml penicillin, 100 U/ml streptomycin, and 2% FCS (all from Gibco).

FACS buffer: PBS supplemented with 2% FCS (Gibco) and 0.05% NaN_3.

The PBS used for i.v. administration of cells and antigens was calcium- and magnesium-free sterile PBS (Gibco).

PTX: Pertussis toxin for induction of EAE was obtained from the ECACC, and diluted in PBS (above) for i.p. administration.

2.3. Cell purification

2.3.1. Isolation of naïve $CD4^+$ T cells

Peripheral lymph nodes and spleens were removed, disaggregated, and resuspended in MACS buffer. Cells were depleted of red blood cells (RBC) using RBC lysis buffer (Sigma). $CD4^+$ T cells were isolated by positive selection using CD4-conjugated MACS beads and MS or LS columns (all Miltenyi Biotec), according to the manufacturer's instructions. Cells were incubated at 90µl MACS buffer/10^7 total cells, with 10µl beads/10^7 total cells for 15min at 4°C, before purification on a MACS magnet (Vario MACS, Miltenyi Biotec). A consistent purity of >95% $CD4^+$ was confirmed by flow cytometry.

2.3.2. Isolation of splenic DC for phenotyping

Spleens were removed and experimental groups of mice were pooled (2 mice/group for each time point). Spleens were disaggregated, and resuspended in MACS buffer. Cells were depleted of RBC using RBC lysis buffer. $CD11c^+$ cells were isolated from the spleen by positive selection using anti-CD11c-conjugated MACS beads (Miltenyi Biotec), according to the manufacturer's instructions (with a consistent purity of >80% $CD11c^+$ as confirmed by flow cytometry). Cells were incubated at 400µl MACS buffer/10^8 total cells, with 100µl beads/10^8 total cells, for 15min at 4°C, before purification on a MACS magnet using MS or LS columns. CD11c-purified cells were resuspended in FACS buffer for FACS analysis.

2.3.3. Isolation of splenic DC from naïve mice

Splenic DC were isolated from naive B6 or $CD40^{-/-}$ mice by initial digestion of spleens in 10ml RPMI without serum, supplemented with 2.4mg/ml collagenase D (Worthington Biochemical) and 1mg/ml DNAse (Sigma, final concentrations) for 30 minutes at 37°C. Cells were depleted of RBC using RBC lysis buffer. $CD11c^+$ cells were then isolated by positive selection using anti-CD11c-conjugated MACS beads as described in section 2.3.2. $CD11c^+$ cells were then resuspended in wash medium for further experiments.

Because the simple process of DC purification can induce DC maturation/activation, and upregulation of costimulatory molecules, different purification methods were tested, to ensure the above method was comparable to others with respect to the levels of costimulatory molecules found on DC from naïve mice after purification. Alternatively, DC were isolated using an adaptation of a previously described protocol (Vremec et al., 1992). Spleens were divided into two groups, and one group was digested as above, while the other group was digested as above in the presence of 100U/ml polymyxin B (Sigma). EDTA (1ml, 0.1M, pH 7.2) was added to both groups for the last 5 minutes of incubation, The cells were removed from the digest by centrifugation, prior to depletion of RBC using RBC lysis buffer, and the pellet was resuspended 4 ml of wash medium, and underlayed with 1.077g/cm^3 NycoPrep (Axis-Shield diagnostics), and a low density fraction was collected after centrifugation at 610xg for 15 minutes at 4°C. This fraction was incubated for 30 minutes at 4°C with anti-CD3 (KT-3), anti-Thy.1 (30-H12) and anti-Gr1 (RB68C5). These are all antibodies raised in rats, and DC were purified by depletion of antibody-bound cells using anti-rat Ig-coupled dynabeads (5:1 ratio) (Dynal, Milan Analytica). Dynabead purification was carried out under continuous rotation for 20 minutes at 4°C. The resulting population was >80% CD11c$^+$ as confirmed by flow cytometry. Expression of costimulatory molecules on DC was analysed by FACS analyses as described in section 2.7.1.

2.3.4. Preparation of bone marrow-derived DC

"Myeloid-like" DC were obtained using an adaptation of a previously used protocol (Inaba et al., 1992). Bone marrow cells were flushed from femurs and tibias, depleted of RBC using RBC lysis buffer and cultured in 24-well flat-bottom plates at 3.75x10^5 cells/ml, in 1ml wash medium/well (supplemented with 1% normal mouse serum, Gibco) and 5% GM-CSF supernatant (GM-CSF supernatant from cell cultures of an GMCSF-producing cell line; X-86, kindly provided by Dr A. Knight, IIIR, Edinburgh). The cells are washed with this medium at day 3 and day 6, before collection at day 7 for further experiments. The resulting population was >75% CD11c+ as confirmed by FACS analysis as described in section 2.7.1.

To test the ability of CpG_{1668} to activate DC, DC were incubated with 6μg/ml CpG_{1668} overnight before FACS analysis as described in section 2.7.1.

2.4. *In vivo* antigen administration

2.4.1. Immunisations

Mice received 20μg pOVA or 100μg pMOG emulsified in CFA (Sigma, Poole, GB) s.c. in the hind legs, on day 0. Varying doses of pOVA (ranging from 20-100μg) were originally tested, and 20μg found to give efficient recall responses upon *ex vivo* recall challenge. Similarly, varying doses of pMOG had previously been tested in the lab, and 100μg gave optimal recall responses in the system used. After ten days, spleens and draining inguinal and popliteal LN were removed, disaggregated and used as a source of primed lymphoid populations. Cells were cultured as described in section 2.5.1.

2.4.2. Induction of tolerance with soluble peptides

For experiments investigating the induction of T cell tolerance, mice received a single i.v. dose of 500μg pOVA or pMOG on day -7, or 3 doses of 200μg pOVA or pMOG on days -8, -6 and -4. Some mice then received pOVA/CFA or pMOG/CFA as described above. Three doses of 200μg peptide administered i.p. two days apart had previously been established in the lab to successfully induce tolerance. Varying doses of peptide to induce tolerance i.v. were tested (ranging from 200μg-500μg), and 500μg were required to successfully induce tolerance after adoptive transfer of Ag-specific T cells. In order to keep the dose of peptide consistent, 500μg were used in subsequent experiments to induce tolerance whether or not the adoptive transfer system was used. This dose has also been used by others to induce tolerance after adoptive transfer of Ag-specific T cells (Bansal-Pakala et al., 2001).

2.4.3. Administration of LPS to induce T cell immunity

For experiments using LPS as an adjuvant to prevent tolerance induction, mice received 500μg pOVA and 30μg LPS in PBS i.v.

2.4.4. Administration of antibodies to prevent tolerance induction

Mice received 200μg of anti-CD40, anti-OX40, MAC-1, or MAC49 as indicated on day -7 (at the same time as soluble peptide), and in some cases a second dose on day -5 as indicated. Different doses (ranging from 50μg-200μg) were tested, and 200μg was found to have the most profound effects *in vivo*.

2.4.5. *In vivo* administration of antigen-loaded DC

DC were purified (in the case of splenic DC) or grown (in the case of bm-derived DC) from naïve B6 or $CD40^{-/-}$ mice as described above. DC were resuspended at $2.5x10^6$ cells/ml in wash medium supplemented with 1% normal mouse serum and pulsed with either pOVA or pMOG (53μg/ml, 1:75 of 4mg/ml stock solution; this concentration had been optimised by Georgia Perona Wright) as indicated, in the presence of LPS (0.1 μg/ml, Sigma) for 2 hours. $5x10^5$ DC were injected i.v. into B6 recipients on day 0. Varying numbers of DC ($5x10^5$-$2x10^6$) were originally tested, and $5x10^5$ DC gave efficient recall responses. Some mice also received 200μg anti-OX40 or isotype control antibody MAC 49 on the day of DC injection and 2 days later, as indicated.

2.5. *In vitro* assessment of T cell function

2.5.1. Recall proliferation assays

Lymphoid cell suspensions were cultured in 96-well flat-bottom microtitre plates (Becton Dickinson) at $6x10^5$ LN cells/well or $8x10^5$ spleen cells/well using tissue culture medium. Cultures were stimulated with a dose range of pOVA or pMOG for 48 hours prior to addition of tritiated thymidine (^{3}H-dThd, Amersham) at 0.5

μCi/well. The dose ranges (ranging from pMOG: 0.03-30μM, or ~0.06-60μg/ml, pOVA: 0.001-100μM or ~0.0018-180μg/ml) and cell numbers used were previously optimised in the lab. After a further 18 hours, cultures were harvested and dThd incorporation measured using a liquid scintillation β-counter (Wallac). Results are expressed as mean cpm of triplicate cultures.

2.5.2. Cytokines production assays (ELISA)

Production of some cytokines is difficult to detect by standard ELISA techniques, and cell-based ELISAs were thus adapted from a previously described protocol (Beech et al., 1997) in order to quantify Ag-specific production of cytokines. Cultures were set up with Ag as described above for T cell proliferation assays. After 48 hours, 100μl aliquots of cells were removed from each well, and tested for production of IL-2, IL-4, and IFNγ by ELISA. Microtitre plates (NUNC) were coated with the cytokine-specific capture antibodies JES6-1A12 (anti-IL-2), 11B11 (anti-IL-4) or R4-6A2 (anti-IFNγ) using bicarbonate coating buffer (pH 9.6) at 4°C overnight prior to transfer of cells. After a further 24 hours culture, specifically bound cytokines were quantified using biotinylated secondary antibodies E56-5H4 (anti-IL-2), BVD6-24G2 (anti-IL-4) or XMG1.2 (anti-IFNγ) followed by extravidin peroxidase (Sigma). Cytokines were detected using phosphate citrate buffer, with added hydrogen peroxide and 3,3',5,5'-tetramethyl-benzidine (TMB) (both Sigma). Reactions were stopped using 2M sulphuric acid. All cytokines were quantified with standard curves obtained with known amounts of recombinant mouse cytokines. All monoclonal antibodies and recombinant mouse cytokine standards were purchased from BD PharMingen. The lower and upper limits of detections were as follows: IL-2 and IL-4: 4-1000pg/ml, IL-10 and IFN-γ: 0.4-100ng/ml.

2.5.3. *In vitro* assays of primary T cell activation

Ag-loaded DC were prepared by incubation with 53μg/ml pOVA (1:75 of 4mg/ml stock solution) in wash medium supplemented with 1% normal mouse serum at 37°C for 2 hours in the presence of 0.1 μg/ml LPS (Sigma). Naïve CD4-purified OT-

IIxCD40$^{-/-}$ T cells were labelled with 5μM CFSE (Molecular Probes). Cells were labelled at 10^7/ml in wash medium for 15 minutes at 37°C (continuous shaking). CFSE was quenched with wash medium supplemented with 5% FCS (Gibco), prior to culture with pOVA-loaded DC ($4x10^4$ T cells + $4x10^3$ DC, $8x10^2$ DC, or $4x10^2$ DC/well) in 96-well flat-bottom plates (Becton Dickinson) using wash medium supplemented with 5% FCS. These numbers of T cells with these ranges of DC numbers gave good T cell proliferation in preliminary experiments testing the ability of DC to activate transgenic T cells *in vitro* (see Fig. 5.2). Cells were harvested for FACS analysis at various time-points.

2.6. Adoptive transfer model for T cell tolerance or immunity

In order to phenotype T cells during the induction of tolerance or immunity, CD4$^+$ T cells were purified as described above from OT-II, OT-IIxLy5.1 or OT-IIxCD154$^{-/-}$ mice as indicated. 2-$4x10^6$ cells/mouse were transferred i.v. into syngeneic, naïve recipients on day -1. In some cases, CD4-purified T cells were fluorescently labelled with CFSE (see section 2.5.3) prior to adoptive transfer.

Mice received i.v. injections of 200μl PBS, 500μg pOVA in PBS, 500μg pOVA+30μg LPS in PBS, or 500μg pOVA+30μg CpG one day after adoptive transfer of pOVA-reactive T cells, i.e. day 0. In some experiments, some mice also received 200μg anti-CD40 or 200μg MAC-1 as indicated on the same day.

Mice were sacrificed at various time-points after Ag-administration, and splenic DC were isolated for phenotyping as described in section 2.3.4. The flowthrough of the purification procedure contained the transferred T cells, and their phenotype was assessed as described below.

2.7. Phenotypic analysis of T cell and DC populations after induction of tolerance or immunity

2.7.1. Flow cytometric analysis

Samples were washed with FACS buffer prior to incubations with antibodies. All samples were incubated with the 2.4.G2 anti-Fc receptor antibody to prevent non-specific binding via Fc receptors, prior to incubations with other antibodies. Antibodies were diluted in FACS buffer supplemented with anti-Fc receptor antibody (final concentration of 20μg/ml). FACS buffer used for staining of DC was also supplemented with 2% normal rat serum (Caltag) and 2% normal goat serum (Caltag) to prevent non-specific binding. All samples were collected on a Becton Dickinson FACScan (Mountain View, CA) flow cytometer and analysed using FlowJo Software (TreeStar, USA). All antibodies were obtained from BD Pharmingen (Oxford, GB) unless stated otherwise.

DC purity was assessed with FITC-conjugated anti-CD11c. Costimulatory molecules on DC were identified using biotinylated primary antibodies specific for CD80, CD86, CD40, OX40L or RANK (R&D systems), followed by streptavidin-APC conjugate. The following biotinylated isotype control antibodies were used: IgG2a, IgG2b and IgG1.

In experiments involving transfer of OT-II cells, the T cells were identified using biotinylated anti-Vβ5 followed by APC- or FITC-conjugated streptavidin and PE-conjugated anti-Vα2. Transferred OT-IIxLy5.1 cells were identified using APC-conjugated anti-CD4 together with FITC-conjugated anti-Ly5.1. In the case of transfer of previously CFSE-labelled cells, identification used biotinylated anti-Ly5.1 followed by APC-conjugated streptavidin and PE-conjugated CD4. T cell activation was assessed using biotinylated anti-CD69, anti-OX40, or anti-RANKL (R&D Systems), all followed by APC-conjugated streptavidin. Control samples were stained with APC-conjugated streptavidin alone. Once CD154 reaches the cell

surface,. it is rapidly cleaved off. Intracellular staining was thus carried out to investigate CD154 expression (see below).

Intracellular cytokine production was measured *ex vivo* in splenocytes by flow cytrometric staining as described (Openshaw et al., 1995). Briefly, splenocytes (at $5x10^6$/ml) were stimulated with PMA/ionomycin (Sigma, 1μg/ml and 15ng/ml final concentration) in the presence of GolgiStop (diluted 1:1000) (BD Pharmingen) for 4 hours at 37°C, before staining with biotinylated anti-Vβ5 followed by APC-conjugated streptavidin, and FITC-conjugated anti-Vα2, or APC-conjugated anti-CD4 together with FITC-conjugated anti-Ly5.1, as indicated. Cells were then fixed for 20 minutes on ice, washed with cytoperm wash buffer (BD Pharmingen) and stained with PE-conjugated anti-IL-2, anti-IL-4, anti-IL-10 or anti-IFNγ for 30 minutes on ice. To look for CD154 expression, cells were first stained with APC-conjugated anti-CD4 together with FITC-conjugated anti-Ly5.1, before fixing, and stained with biotinylated anti-CD154, followed by APC-conjugated streptavidin, both diluted in Cytoperm wash buffer (BD Pharmingen).

2.7.2. Immunofluorescence

For experiments analysing costimulatory molecules by immunofluorescence, purified $CD4^+$ $Ly5.1^+$ OT-II T cells were fluorescently labelled using Cell Tracker Orange CMTMR (CT orange, Molecular Probes). Since cells stained with CT orange stay fluorescent for up to 3 cell divisions according to the manufacturer, compared to CFSE labelled cells, which loose fluoerescence upon cell division, labelling the cells using CT orange was more likely to allow tracking of T cells using immunofluorescence microscopy. Cells were labelled at 10^7 cells/ml with 25μM CT orange for 15 minutes at 37°C, before adoptive transfer into naïve B6 recipients on day -1. As before, mice received 500μg pOVA in PBS, 500μg pOVA+30μg LPS in PBS, or PBS alone on day 0, Mice were sacrificed 12 and 36 hours after administration of Ag. FACS analysis of spleen cells was carried out as described in section 2.7.1. Spleen tissue sections were also snap-frozen in liquid nitrogen, and 5-

μm cryosections were cut on a cryostat (Meica microsystems). Sections were dried overnight, prior to fixing for 10 minutes in cold acetone, and rehydration for 15 minutes in PBS. Sections were kept in the dark. All washes were carried out for 2 x 3 minutes using TBST buffer (0.05M Tris-Hcl pH 7.6, 0.3M NaCl, .01% Tween 20), and all incubations were carried out at room temperature in a dark, humidified staining chamber.

Sections were blocked with 3% H_2O_2 for 5 minutes, and blocked with avidin-biotin-blocking reagents (Vector) according to the manufacturers instructions for 15 minutes each. Sections were then blocked and stained using the DAKO Catalysed Signal Amplification (CSA) kit. The kit was used according to the manufacturers instructions, but adapted in the final steps for immunofluorescence. Briefly, sections were blocked with protein block (DAKO kit) for 15 minutes, before addition of primary biotinylated antibody for 15 minutes (CD69, CD154, OX40 or OX40L, all from Pharmingen as used above for FACS analysis, used at a 1:100 dilution). Sections were then incubated with streptavidin-biotin-complex from the DAKO kit for 15 minutes, before incubation with amplification reagent for 15minutes. Finally, the sections were incubated with streptavidin-conjugated Alexa Fluoro 647 (Molecular Probes), before two final 3-minute washes in PBS, and mounting with Vectashield mounting medium for fluorescence (Vector). Pictures were taken with 3CCD colour vision camera (controlled by Hamamatsu and Orbit controllers) and evaluated with Openlab version 3.0.9 digital imaging programme (Improvision, Warwick, UK). This work was carried out in collaboration with Dr Paul Garside and Angela Grierson (Department of Immunology, Western Infirmary, University of Glasgow).

2.8. Induction and assessment of EAE

EAE was induced with s.c. injection of 100μg p35-55 in a total of 100μl CFA containing 0.5 mg heat-killed *Mycobacterium tuberculosis* (50 μl into each hind leg). Mice also received 200ng pertussis toxin (ECACC) i.p. in 0.5 ml PBS on the same

day and 2 days later. This protocol has previously been shown to induce EAE in H-2^b mice (Mendel et al., 1995). Clinical signs of EAE were assessed daily using the following scoring index: 0, no signs; 1, flaccid tail: 2, impaired righting reflex and/or impaired gait; 3, partial hind leg paralysis; 4, total hind leg paralysis; 5, hind and fore leg paralysis; 6, moribound or dead. Differences in total disease burden between groups were determined using the Mann-Whitney U test. This statistical test is accepted for comparison of data obtained from EAE experiments, and consistenly used in the scientific community. A different test, namely calculating the area under the curve (of EAE scores plotted against days for each experimental group) and using the Kruskal-Wallis test, another rank sum test similar to the Mann-Whitney U test, can also be used, and may arguably be more accurate. However, the Mann-Whitney U test is the standard test used, and we thus chose to analyse out data using this widely accepted statistical test.

2.9. Generation of bone marrow-chimeric mice

Recipient mice received 1150 cGy of γ-irradiation via a cesium isotope (^{127}Cs) source. One day later, recipients received $5x10^6$ donor bone marrow cells. Bone marrow cells were flushed from femurs and tibias of $CD40^{-/-}$ mice, depleted of RBC using RBC lysis buffer, and depleted of T cells by labelling with a biotinylated anti-Thy1 (clone T24) before incubation with streptavidin-microbeads (Miltenyi Biotec) and negative selection with a MACS CS magnetic column. Chimeras were left to fully reconstitute their peripheral lymphoid system over at least 8 weeks before use in EAE experiments.

2.10. Generation of T cell lines

pOVA and pMOG-specific T cell lines (TCL) were generated from B6, $CD40^{-/-}$ mice and $CD154^{-/-}$ mice. Mice were immunised with 20μg pOVA/CFA or 100μg pMOG/CFA, and DLN were taken 10 days later. TCL were then generated by *in vitro* stimulation of LNC with pOVA or pMOG, and were maintained using a

standard 7-day restimulation/expansion cycle. TCL were restimulated for 3 days with pOVA or pMOG (1^{st} 10μM, then 1 μM peptide) in the presence of irradiated (30 Gy) syngeneic B6 spleen APC. T cell blasts were isolated using a NycoPrep 1.077 animal density gradient (Nycomed Pharma, Oslo, Norway) and expanded in wash medium supplemented with 5% FCS and 5% Con A-activated rat spleen supernatant as a source of T cell growth factors.

For proliferation and cytokine production assays, T cells were cultured in 96-well flat-bottom microtitre plates (Becton Dickinson) at $4x10^4$ T cells/well with $5x10^5$ APC/well using tissue culture medium. Both assays were carried out as described in sections 2.5.1 and 2.5.2. This method had been optimised prior to my arrival in the lab.

3. CHAPTER 3 - Exogenous ligation of either CD40 or OX40 overcomes a tolerogenic signal

3.1. Introduction

Conflicting evidence exists with respect to the requirement of CD154 in the induction of T cell immunity as well as T cell tolerance. Early reports showed that $CD154^{-/-}$ T cells fail to prime *in vivo*, and that CD154 is needed for induction of EAE (Grewal et al., 1996; Grewal et al., 1995). More recently, it has been shown that $CD154^{-/-}$ T cells are unable to sustain a response *in vivo*, and are defective in Th1 development *in vitro* (Howland et al., 2000). Regarding tolerance induction, it has been reported that mucosal tolerance via the oral route requires the CD40-CD154 interaction (Kweon et al., 1999). Other groups however reported that $CD154^{-/-}$ mice could successfully be tolerised via i.v. administration of soluble peptide (Howland et al., 2000).

Exogenous ligation of CD40 on APC through administration of agonistic anti-CD40 monoclonal antibodies has been used extensively to activate APC *in vitro* (Caux et al., 1994; Cella et al., 1996) and *in vivo* (Chung et al., 2004). It has also been shown that administration of agonistic anti-CD40 abrogates tolerogenic presentation to both $CD4^+$ and $CD8^+$ T cells resulting in effective T cell immunity and autoimmune pathology (Bennett et al., 1998; Garza et al., 2000; Hawiger et al., 2001), or prevention of tolerance to tumours (Diehl et al., 1999; Grohmann et al., 2001; Sotomayor et al., 1999). However, others report that administration of agonistic CD40 does not prevent tolerance induction (Chung et al., 2004; Sun and Van Houten, 2002). One effect of CD40 ligation on APC is the up-regulation of OX40L on the surface of the APC (Fillatreau and Gray, 2003). Ligation of OX40 (up-regulated on T cells in response to TCR signalling) has been reported to lead to elevated T cell expression of survival factors such as Bcl-2 and Bcl-xL (Rogers et al., 2001). This chapter describes a series of experiments that tested the requirements for

CD154 expression for tolerance induction, and investigated whether administration of agonistic anti-OX40 and anti-CD40 antibodies prevented peptide-induced tolerance. The relevance of this approach was also tested in the disease setting of EAE.

3.2. Approach

Firstly, the ex vivo recall responses to administration of Ag in CFA (later employed to test the induction or prevention of T cell tolerance) were compared in CD40-deficient, CD154-deficient mice and wild-type (B6) mice. In order to induce systemic T cell tolerance, a high dose (500μg) of Ag (either pMOG or pOVA) was administered i.v. One of the criteria used to define tolerance is unresponsiveness to administration of the Ag in adjuvant, which would normally result in successful T cell priming. Throughout this chapter, mice were tolerised by i.v. administration of Ag. Some groups of mice received the agonistic antibodies anti-OX40 and anti-CD40 at the time of i.v. administration of soluble Ag, and two days later, a protocol which was adapted from other systems (Diehl et al., 1999; Sotomayor et al., 1999). In order to test whether exogenous ligation of CD40 or OX40 prevented tolerance induction, mice were subsequently given Ag in CFA. Tolerance induction would lead to diminished proliferation and cytokine production. These responses would be intact if tolerance had been prevented. EAE was also used to test the relevance of the findings in an autoimmune disease setting.

The experimental outline of this chapter is shown in Figure 3.1.

3.3. Comparison of primary responses in CD40$^{-/-}$, CD154$^{-/-}$ and B6 mice

Previous studies show that CD40$^{-/-}$ and CD154$^{-/-}$ mice are defective in Ag-specific T cell responses (Castigli et al., 1994; Grewal et al., 1995; Kawabe et al., 1994; Xu et al., 1994). Because the extent of T cell priming can be determined by the route of Ag administration and the choice of adjuvant, we compared priming of CD40$^{-/-}$, CD154$^{-/-}$ and B6 mice using the Ag and adjuvant subsequently used throughout this series of experiments, namely pOVA in CFA and pMOG in CFA. CFA contains mycobacterial components that act through TLRs to induce a Th1 type response in most experimental systems (Schnare et al., 2001). The CD40-CD154 interaction is important for the induction of a Th1 type response, since ligation of CD40 increases IL-12 release by DC, which results in IFN-γ production by T cells (Schulz et al., 2000). The lack of CD154 or CD40 could therefore have an effect on T cell priming using Ag in CFA.

B6, CD154$^{-/-}$ and CD40$^{-/-}$ mice were immunised with pMOG/CFA. Ten days after immunisation with pMOG/CFA, cells of the DLN were examined for Ag-specific proliferative capacity and cytokine production. B6 mice showed high Ag-specific proliferation, high levels of IFN-γ, and low levels of IL-4, indicative of a Th1 response (Fig. 3.2). These results were also applicable to immunisation with pOVA/CFA (not shown). While CD40 deficiency led to decreased proliferative capacity, proliferation in CD154$^{-/-}$ mice was comparable to that seen in B6 mice. Cytokine production was also impaired differently in the knockouts compared to B6 control mice (Fig. 3.2). DLN cells from CD40$^{-/-}$ mice failed to produce considerable amounts of IFN-γ, but produced some IL-4. CD154$^{-/-}$ LN cells, on the other hand, produced little IFN-γ, but large amounts of IL-4, corresponding to a Th2 phenotype (Fig. 3.2).

Although CD40$^{-/-}$ mice primed poorly, it was nevertheless possible to make TCL after pOVA/CFA or pMOG/CFA immunisation using DLN from these mice. These TCL were assessed for Ag-specific cytokine production after several rounds of restimulation with syngeneic B6 APC. Similar to DLN cells from mice lacking CD154, TCL cells from CD154$^{-/-}$ mice produced little IFN-γ, but considerable IL-4 production, indicative of a Th2 phenotype (Fig. 3.3, even though the background levels for IL-4 production were high in both groups, there nevertheless was considerable Ag-specific IL-4 production in TCL from CD154$^{-/-}$ mice). TCL cells from CD40$^{-/-}$ mice, on the other hand, showed considerable IFN-γ production after several rounds of stimulation, with little IL-4 production (Fig 3.3). This suggests that the lack of CD154 on T cells produced a stable Th2 phenotype. The deficits from initial priming in the absence of CD40, however, were overcome through restimulation in the presence of CD40 on B6 APC, allowing development of a Th1 phenotype.

B6 mice thus showed optimal T cell expansion, and displayed a Th1 type profile, while CD154$^{-/-}$ mice showed almost optimal T cell expansion, displaying a stable Th2 type profile after Ag/CFA immunisation. CD40$^{-/-}$ mice had reduced T cell expansion, with a Th2 type profile, which can be converted to a Th1 phenotype through restimulation with syngeneic B6 APC.

3.4. Exogenous ligation of CD40 or OX40 influences the cytokine response in CD154$^{-/-}$ mice

The lack of the CD40-CD154 interaction thus shifts the cytokine response towards a Th2 profile. The OX40-OX40L interaction has been implicated to be important for the induction of Th2 type responses (Flynn et al., 1998; Linton et al., 2003). Agonistic antibodies to CD40 and OX40 were used to test if exogenous ligation of these two molecules would influence the cytokine profile in CD154$^{-/-}$ mice. Mice were immunised with pMOG/CFA on day 0, and some groups received anti-CD40 or anti-OX40 antibodies at the same time, and two days later. Ligation of CD40 greatly

reduced IL-4 levels relative to those seen in CD154$^{-/-}$ control mice, but had no significant effect on IFN-γ levels. Ligation of OX40 resulted in increased levels of both IL-4 and IFN-γ (Fig. 3.4). Isotype control antibodies for anti-CD40 and anti-OX40 were not available at the time of these experiments. However, administration of isotype controls in later experiments did not have the effects seen with administration of anti-CD40 or anti-OX40. Exogenous ligation of CD40 thus appeared to shut off IL-4 production in CD154$^{-/-}$ mice without an increase in IFN-γ production, while exogenous ligation of OX40 greatly enhanced both IL-4 and IFN-γ production. This experiments remains to be repeated, but indicated a differential effect of anti-CD40 and anti-OX40 on cytokine production.

3.5. CD154 is not required for peptide-induced tolerance

Previous reports give conflicting results with respect to the importance of CD154 during peptide-induced tolerance induction (Kweon et al., 1999), (Howland et al., 2000). In order to address this question in the experimental system used here, mice received one dose of pOVA i.v. or 3 doses of pMOG i.p., two tolerisation protocols, which have both been used extensively by our laboratory and others to effectively induce T cell tolerance. 7 days after administration of soluble peptide, mice received the same peptide in CFA. Unresponsiveness to this rechallenge with Ag in adjuvant would indicate successful tolerance induction. Since profound effects on proliferative capacity were observed after i.v. or i.p. administration of both pOVA and pMOG (Fig. 3.5), tolerance was induced successfully in the absence of CD154. CD154 was thus not required for peptide-induced tolerance to pOVA or pMOG.

3.6. Peptide-induced tolerance diminishes cytokine responses in both B6 and CD154$^{-/-}$ mice

To further investigate the effects of i.v. administration of pOVA on T cell effector function, pOVA-specific cytokine ELISAs were carried out 10 days after *in vivo* rechallenge with pOVA in CFA. Both the Th1 type response in B6 mice, and the Th2

type response in CD154$^{-/-}$ mice were ablated after administration of soluble pOVA (Fig. 3.6).

3.7. Investigating the roles of CD40 and OX40 in peptide-induced tolerance

Exogenous ligation of CD40 activates APC *in vivo*, and has previously been reported to result in effective immunity against tumours (Diehl et al., 1999; Grohmann et al., 2001; Sotomayor et al., 1999). Based on the central hypothesis, administration of agonistic anti-CD40 should also prevent tolerance induction by administration of soluble peptide. Similarly, if agonistic anti-CD40 prevents tolerance through up-regulation of OX40L on DC, which then binds to OX40 on activated T cells, exogenous ligation of OX40 should prevent peptide-induced tolerance. In order to elucidate the importance of the CD40-CD154 and OX40L-OX40 interactions in the experimental system used here, the ability of agonistic anti-CD40 and anti-OX40 to prevent induction of tolerance was investigated.

Since both i.v. and i.p. administration of Ag resulted in tolerance induction, the protocol chosen to use in subsequent experiment was i.v. administration of Ag, which only required a single dose of 500μg pOVA, rather than three doses of 200μg pOVA.

3.7.1. Exogenous ligation of CD40 or OX40 does not convert a tolerogenic signal into an immunogenic one

To test whether administration of agonistic anti-OX40 and anti-CD40 could convert tolerance into immunity in the experimental system used, B6 or CD154$^{-/-}$ mice received 200μg of anti-CD40, anti-OX40 or isotype control antibody on the same day as i.v. administration of 500μg pOVA and a second dose of antibody two days later. Initially, pOVA was not given in CFA at day 7. Instead, *ex vivo* recall responses to a dose range of pOVA were examined 7 days after Ag administration, to

test whether administration of these antibodies could result in effective T cell priming. Administration of pOVA alone did not result in effective recall responses, and neither anti-CD40, nor anti-OX40 administration resulted in effective T cell priming in B6 or CD154$^{-/-}$ mice (Fig. 3.7).

3.7.2. Exogenous ligation of CD40 or OX40 prevents tolerance induction in B6 and CD154$^{-/-}$ mice

Although administration of anti-CD40 or anti-OX40 did not result in T cell priming, it was possible that administration of these antibodies nevertheless allowed the T cells to maintain responsiveness to subsequent *in vivo* challenge of Ag in adjuvant. B6 or CD154$^{-/-}$ mice therefore received 500μg pOVA i.v. at day -7, with or without anti-CD40, anti-OX40 or isotype control antibodies on the same day and two days later, followed by administration of 20μg pOVA/CFA on day 0 and examination of ex vivo recall responses to a dose range of pOVA on day 10. Ligation of CD40 and OX40 in B6 and CD40 in CD154$^{-/-}$ mice increased proliferative responses to levels comparable to non-tolerised controls, while in CD154$^{-/-}$ mice, ligation of OX40 further increased the proliferative response (Fig. 3.8). Administration of isotype control antibodies did not have this effect. This shows that although ligation of CD40 or OX40 did not result in effective T cell priming, it did prevent tolerisation of the T cells in both B6 and CD154$^{-/-}$ mice.

3.7.3. Exogenous ligation of CD40 or OX40 partially prevents tolerance induction in the absence of B cells

Although DC are responsible for the initiation of primary T cell responses *in vivo* (Banchereau et al., 2000), B cells are important in the induction of effective T cell immunity. CD40 and OX40 are both expressed on activated B cells (Banchereau et al., 1994; Croft, 2003), but the importance of this expression for successful T cell priming remains to be determined (A. Crawford, personal communication). If CD40 and OX40 on B cells do not play a role in the prevention of T cell tolerance, ligation of CD40 and OX40 at the time of administration of soluble peptide in μMT mice, which lack B cells, should still result in restoration of effective proliferative

responses. μMT mice thus received 500μg pOVA i.v. at day -7, with or without anti-CD40, anti-OX40 or isotype control antibodies on the same day and two days later, followed by administration of 20μg pOVA/CFA on day 0 and examination of *ex vivo* recall responses to a dose range of pOVA on day 10. Peptide-induced tolerance to pOVA was successfully induced in μMT mice, indicating that B cells do not play a significant role in the induction of tolerance to soluble peptide. Administration of both anti-CD40 and anti-OX40 partially prevented tolerance induction as evident after immunisation with pOVA/CFA (Fig. 3.9), an effect not seen after administration of isotype control antibodies. Anti-CD40 and anti-OX40 are therefore most likely to act on DC and T cells respectively to prevent tolerance induction, but a role for B cells in restoring responsiveness cannot be totally excluded.

3.8. Induction and prevention of tolerance to EAE

EAE is a mouse model for multiple sclerosis, and can be induced experimentally in mice in various ways (Bhardwaj et al., 1994; Sakai et al., 1988; Zamvil et al.). Here, EAE was induced in H-2^b mice by immunisation with pMOG in CFA. The activation of autoreactive $CD4^+$ T cells of the Th1 functional phenotype after immunisation results in induction of EAE, characterised by a paralysis, which typically peaks around 2 weeks after immunisation with Ag. By four weeks after Ag administration the mice have recovered from the disease.

To investigate whether i.v. administration of Ag could induce T cell tolerance and prevent the induction of EAE, mice received a single dose of 500μg pMOG 7 days prior to the induction of EAE. This approach resulted in tolerance and protection from EAE (Fig. 3.10).

3.8.1. Exogenous ligation of OX40 prevents tolerance induction to EAE in B6 mice

The importance of the CD40-CD154 interaction in EAE is well-documented (Gerritse et al., 1996; Grewal et al., 1996; Samoilova et al., 1997), while the importance of the OX40-OX40L interaction has more recently been described to be relevant for development of clinical symptoms in EAE (Ndhlovu et al., 2001; Nohara et al., 2001; Weinberg et al., 1999). Since the present study showed that administration of anti-OX40 could prevent tolerance induction to a model Ag (pOVA), this approach was extended into the EAE setting. 200μg of anti-OX40 or isotype control antibody was administered i.p. at the time of i.v. administration of pMOG (day –7) and two days later. On day 0, EAE was induced by administration of pMOG in CFA, and i.p. injections of PTX on days 0 and 2. This experiment was carried out once. These preliminary results showed prevention of tolerance induction by administration of anti-OX40, with mice developing clinical signs of EAE similar to those seen in non-tolerised control mice (Fig. 3.10).

3.8.2. Exogenous ligation of OX40 in CD40-deficient mice does not lead to induction of EAE

$CD40^{-/-}$ mice do not develop clinical signs of EAE (Becher et al., 2001). Since ligation of OX40 results in increased number of Ag-specific T cells, and an increased memory T cell population (Gramaglia et al., 2000), we wanted to test whether administration of anti-OX40 could overcome the lack of CD154 signalling, and lead to priming of self-reactive T cells in $CD40^{-/-}$ mice, resulting in induction of EAE. 200μg anti-OX40 or isotype control antibody was given to CD40-deficient mice at the time of EAE induction, and two days later. However, administration of anti-OX40 did not restore the induction of EAE (Fig. 3.11).

3.8.3. CD40 expression on bone marrow-derived cells is required for typical EAE disease progression

Previous reports suggested that CD40 expression in the CNS is of critical importance for development of clinical signs of EAE (Becher et al., 2001). Administration of

anti-OX40 in $CD40^{-/-}$ mice did not restore the induction of EAE, which was potentially due to the lack of CD40 expression in the CNS. Bone marrow chimeras, which lacked expression of CD40 on all bone marrow-derived cells, but not on any other cells in the body, were produced, and allowed to reconstitute for 8 weeks, before induction of EAE. The absence of CD40 on bm-derived cells was confirmed by FACS analysis. The lack of CD40 expression on bone marrow-derived cells resulted in greatly decreased severity of disease compared to B6 controls. Nevertheless, these mice did develop some signs of disease, in contrast to $CD40^{-/-}$ mice, where the global lack of CD40 totally prevented EAE (Fig. 3.12).

3.9. Summary

This chapter investigated the requirement of CD154 on T cells for the induction of tolerance, and the potential to prevent tolerance induction through the administration of agonistic antibodies to CD40 and OX40. It was found that CD154-deficient mice gave Ag-specific *ex vivo* recall responses, suggesting successful T cell priming upon immunisation with Ag. $CD40^{-/-}$ mice however failed to prime efficiently, shown by a considerable decrease in *ex vivo* recall responses. Immunisation of $CD154^{-/-}$ mice with Ag in an adjuvant known to induce a Th1 type cytokine profile resulted in a Th2 type cytokine profile. This Th2 profile could only be partially shifted to a Th1 profile by exogenous ligation of CD40 at the time of Ag challenge (decreased IL-4 production, but no increased IFN-γ production). Exogenous ligation of OX40 at the time of immunisation greatly increased the production of both type 1 and type 2 cytokines in $CD154^{-/-}$ mice. The results indicate a possible quantitative effect of OX40 ligation on the immune response, while CD40 ligation has a qualitative effect with respect to the cytokine profile observed.

Intravenous administration of soluble peptide produced Ag-specific T cell unresponsiveness in the absence of CD154 signalling. This T cell tolerance could be prevented by exogenous ligation of CD40 or OX40, in $CD154^{-/-}$ mice as well as B6 mice. B6 mice were successfully tolerised against the induction of EAE by i.v. administration of pMOG, which could similarly be prevented through administration of OX40 at the time of Ag administration. Expression of CD40 in the CNS was required for optimal induction of EAE, and exogenous ligation of OX40 did not induce EAE in CD40-deficient mice. It is concluded that although the CD40-CD154 interaction is important in T cell responses, CD154 on T cells is not absolutely required for either initiation of T cell immunity or induction of T cell tolerance. Nevertheless, the results presented in this chapter indicate that the CD40-CD154 and OX40-OX40L interactions are indeed important checkpoints determining firstly the outcome of T cell immunity with respect to tolerance versus immunity, and secondly the cytokines produced in response to antigenic challenge.

3.10. Discussion

Although it was originally reported that CD154$^{-/-}$ mice do not prime effectively (Grewal et al., 1996), it has since then been shown that CD154$^{-/-}$ T cells initially expand normally, but cannot sustain a Th1 type response (Howland et al., 2000). It was shown here that CD154$^{-/-}$ mice prime normally compared to B6 mice. In the experimental system employed, the Ag is emulsified in CFA, which is believed to provide a lasting supply of Ag (it remains to be investigated for how long). Although it has been shown that in CD154$^{-/-}$ mice, Ag-loaded DC failed to migrate out of the skin and fewer DC accumulated in the DLN after contact sensitisation (Moodycliffe et al., 2000), an Ag depot may explain the effective recall responses seen here after immunisation of CD154-deficient mice with Ag in CFA, since such a depot may have allowed sufficient numbers of Ag-loaded DC to reach DLN. This Ag depot could thus have rescued proliferative responses, but would not rescue CD154 signalling to DC. A lack of CD154 signalling to DC would cause decreased IL-12 production by DC, and result in a Th2 type profile, which was indeed observed in this system.

This same explanation should however hold true for CD40-deficient mice. Instead, mice lacking CD40 showed greatly reduced proliferative and cytokine responses. CD40 ligation is required for DC migration from the site of Ag administration to DLN, as well as during DC-T cell interactions in the DLN (Miga et al., 2001; Moodycliffe et al., 2000). A better explanation for the recall responses, which were observed in CD154$^{-/-}$ mice, but not CD40$^{-/-}$ mice, may thus be the existence of a different ligand for CD40, which compensates for the lack of CD154 at the site of CFA injection. This would again allow DC migration to DLN, where they could interact with T cells, and in the absence of CD154, induce a Th2 type response. In CD40$^{-/-}$ mice however, the lack of CD40 would result in ineffective DC migration, and thus result in greatly decreased proliferative recall responses. The 70 kDa mycobacterial heat shock protein (HSP 70) has been described to bind CD40 and result in release of chemokines (Wang et al., 2001). The presence of HSP 70 in CFA

might have substituted for the lack of CD154 at the site of CFA injection and allowed efficient T cell priming in $CD154^{-/-}$ mice, but not in $CD40^{-/-}$ mice.

Although conflicting evidence exists with respect to a CD154 requirement for tolerance induction (Howland et al., 2000; Kweon et al., 1999), it was shown here that CD154 was not required for peptide-induced tolerance. The results obtained agree with previous data, that showed that systemic i.v. administration of soluble peptide did not require the presence of CD154 (Howland et al., 2000). The contrasting results were obtained using a different experimental system, and investigated the requirements for mucosal tolerance after orally administered Ag (Kweon et al., 1999). Further evidence suggests that tolerance is differentially regulated at these different peripheral sites. On the one hand, ligation of CD40 has been used extensively to promote anti-tumour immunity (Diehl et al., 1999; Grohmann et al., 2001; Sotomayor et al., 1999), and to activate quiescent DC, breaking peripheral tolerance *in vivo* (Hawiger et al., 2001). On the other hand, in systems investigating mucosal tolerance, ligation of CD40 was not sufficient to prevent tolerance induction, even though it led to upregulation of CD80 and CD86 on DC, and potentiated the proliferation of Ag-specific T cells in lymphoid organs (Chung et al., 2004). Similarly, activation of resident DC through administration of Flt3 Ligand, a growth factor that expands DC *in vivo*, prevented systemic tolerance (Pulendran et al., 1998), while it enhanced mucosal tolerance (Viney et al., 1998). It may well be that tolerance is differentially regulated at different peripheral sites, probably through DC biology.

Exogenous ligation of CD40 successfully prevented the induction of tolerance through i.v. administration of soluble peptide, fitting with data obtained from other experimental systems (Diehl et al., 1999; Hawiger et al., 2001). Since one effect of CD40 ligation on is the upregulation of OX40L, it appears that the agonistic CD40 antibody may be exerting its effects through induction of OX40L, allowing signalling to T cells and subsequent induction of survival molecules, resulting in effective T cell immunity. This is further investigated in the following chapter.

Exogenous ligation of OX40 also prevented the induction of T cell tolerance, probably by directly ligating OX40 on T cells and promoting T cell survival. This was similarly suggested in a recent paper investigating the effects of exogenous ligation of OX40 (Bansal-Pakala et al., 2001). Using a T cell adoptive transfer system, this group reported successful tolerance induction through a single i.v. dose of soluble peptide, illustrated by reduced proliferative capacity upon *in vitro* rechallenge, and lack on IFN-γ or IL-4 production, comparable to the data presented here. They prevented this tolerance through exogenous ligation of OX40 at the time of administration of soluble peptide. Finally, they also report reversal of tolerance through OX40 ligation at the time of giving peptide in CFA (i.e. at the time of *in vivo* rechallenge with Ag in adjuvant, rather than at the time of administration of soluble peptide). This raises the question of the exact timing of the immunogenic effect of the anti-OX40 antibody. While anti-OX40 may indeed have acted at the time of administration of soluble peptide, promoting T cell survival, without actually allowing T cell proliferation (Fig. 3.7), it cannot be excluded that anti-OX40 exerted its effects at the time of rechallenge with Ag in adjuvant. Identification of ways to reverse peptide-induced tolerance may lead to insights into preventing unwanted tolerance against tumours, holding great therapeutic potential, and further investigation needs to be carried out.

The importance of the CD40-CD154 interaction for the induction of EAE has previously been investigated in some detail (Gerritse et al., 1996; Grewal et al., 1996; Samoilova et al., 1997). CD40 deficient mice did not develop clinical signs of EAE, which correlates with more recently obtained data from a different group (Becher et al., 2001). Similarly, this group reported that the lack of CD40 on bone marrow-derived cells resulted in decreased disease severity, which is also shown in this chapter. Exogenous ligation of OX40 could not induce disease in CD40-deficient mice. Unfortunately, the ability of agonistic anti-OX40 to induce disease in mice lacking expression of CD40 on bone marrow-derived cells could not be investigated, due to time constraints. This antibody greatly enhanced cytokine responses in CD154-deficient mice, inducing large amounts of IFN-γ, and also prevented peptide-

induced tolerance to pOVA and pMOG. It seems likely therefore, that exogenous ligation of OX40 in CD40-deficient bone marrow chimeras would greatly enhance disease scores seen in these mice. This possibility remains to be investigated.

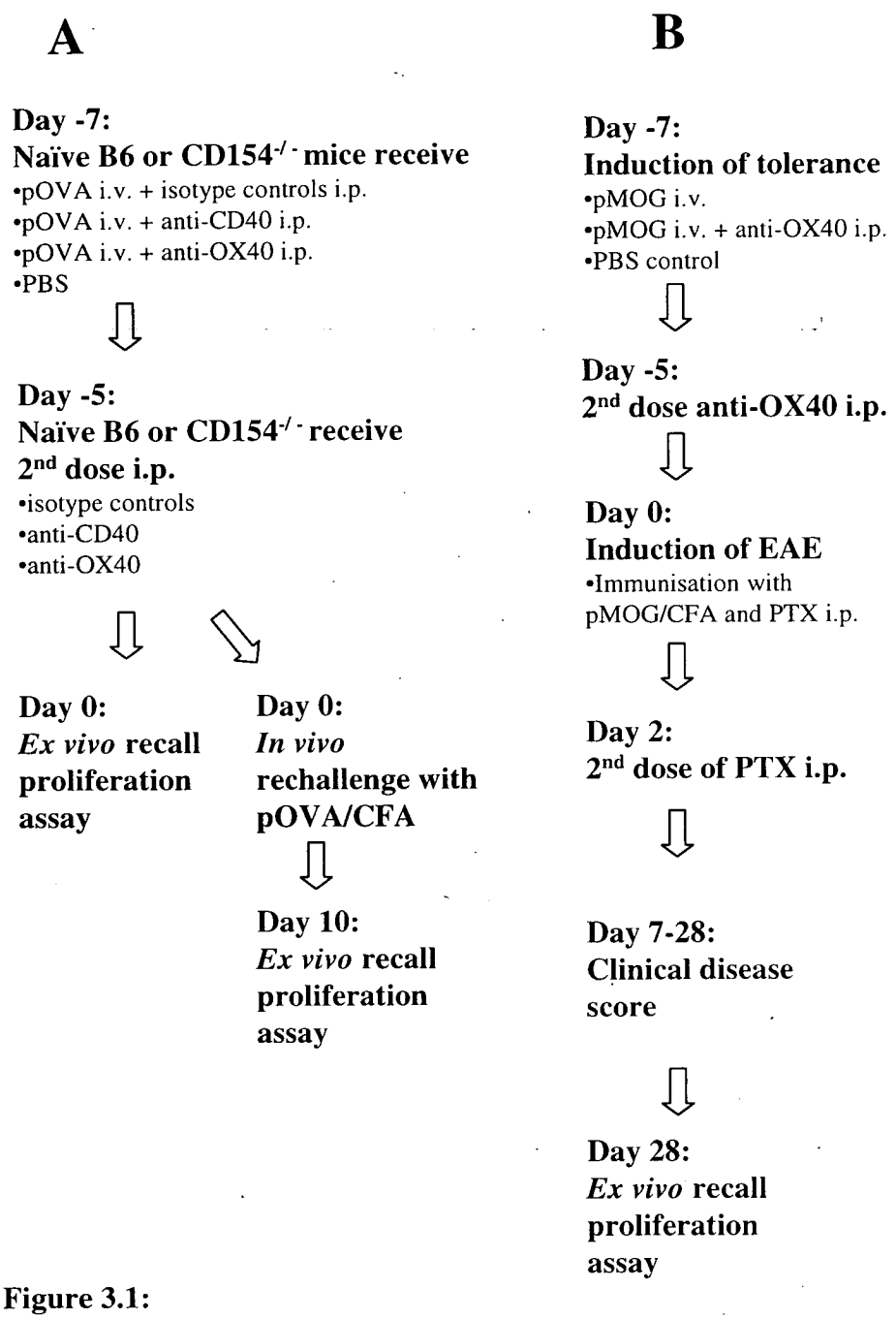

Figure 3.1:
Experimental outline of Chapter 3
A: Does exogenous ligation of CD40 or OX40 prevent the induction of tolerance? 200µg anti-CD40, anti-OX40 or isotype control antibody was co-administered with 500µg pOVA on day –6, and mice received a second dose of antibody on day –4.
B: The EAE model. Tolerance induction and induction of EAE.

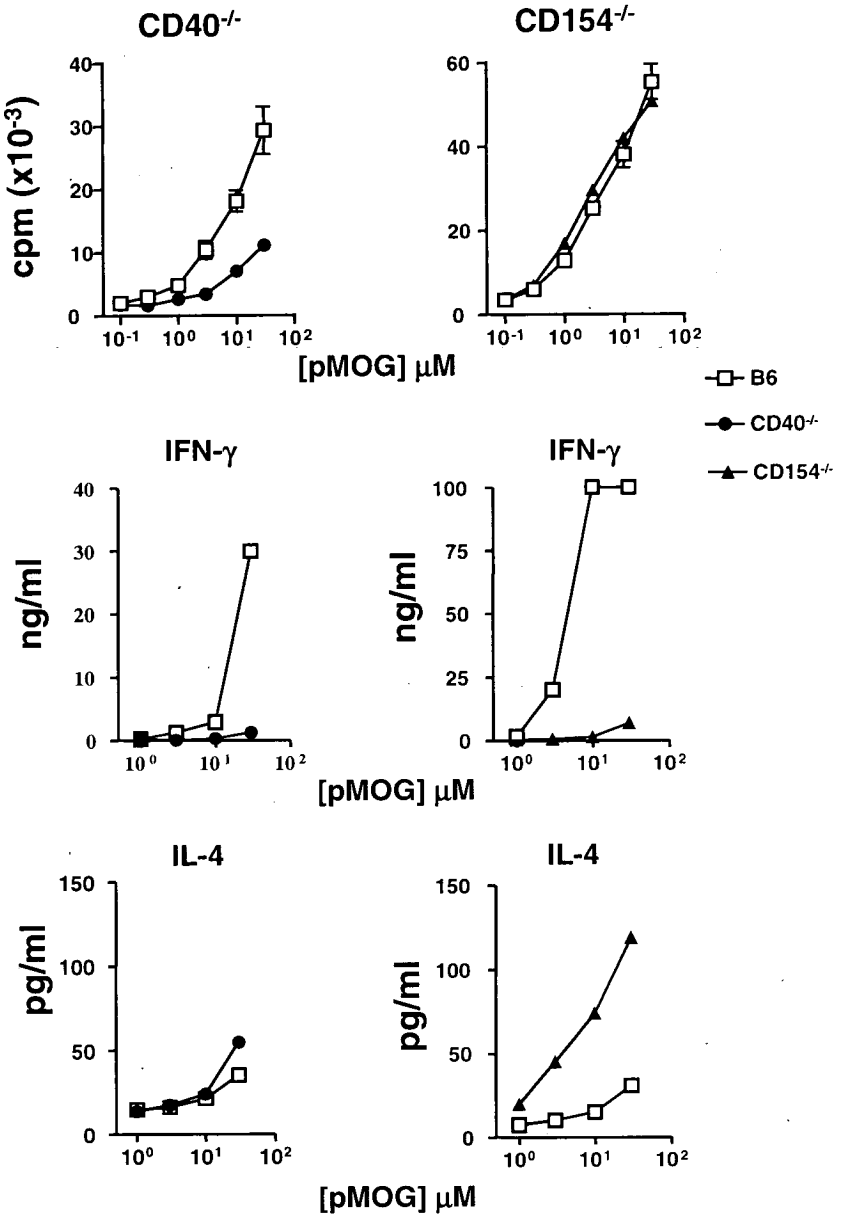

Figure 3.2:
Comparison of T cell priming in CD40$^{-/-}$, CD154$^{-/-}$ and B6 mice after immunisation with pMOG in CFA
Mice received 100μg pMOG in CFA on day 0. Draining lymph nodes were assessed 10 days later for pMOG-specific proliferative responses or by ELISA for pMOG-specific IFN-γ or IL-4 production. Background levels were as levels seen at lowest concentration of Ag in each group. For each cytokine datapoint, the SEM was <20%. These data are from two of six experiments giving consistent results.

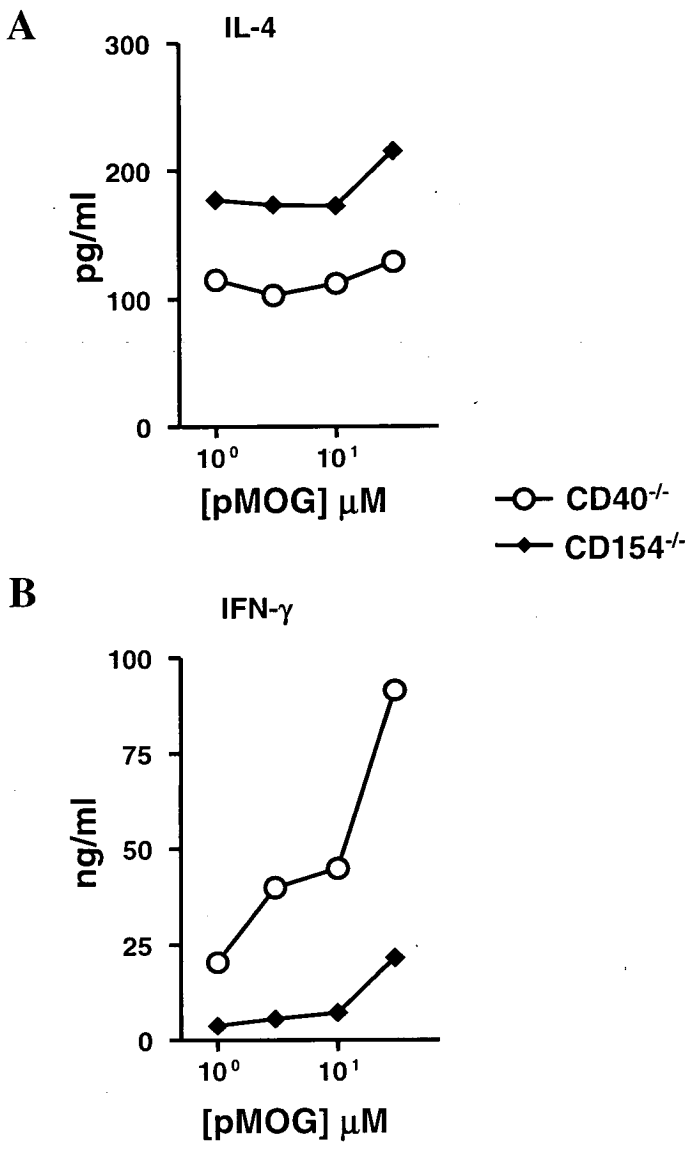

Figure 3.3:
Cytokine production by TCL derived from CD40$^{-/-}$ or CD154$^{-/-}$ mice

Mice received pMOG in CFA. DLN were taken 10 days later, and TLC established as described in the materials in methods. pMOG-specific IL-4 and IFN-γ production (A and B respectively) was assessed by ELISA. Background levels were as follows: IL-4(pg/ml): CD154$^{-/-}$ mice 98, CD40$^{-/-}$ mice 116; IFN-γ (ng/ml): CD154$^{-/-}$ mice 0.7, CD40$^{-/-}$ mice 2). For each datapoint, the SEM was <20%. These data are from one of two experiments giving consistent results.

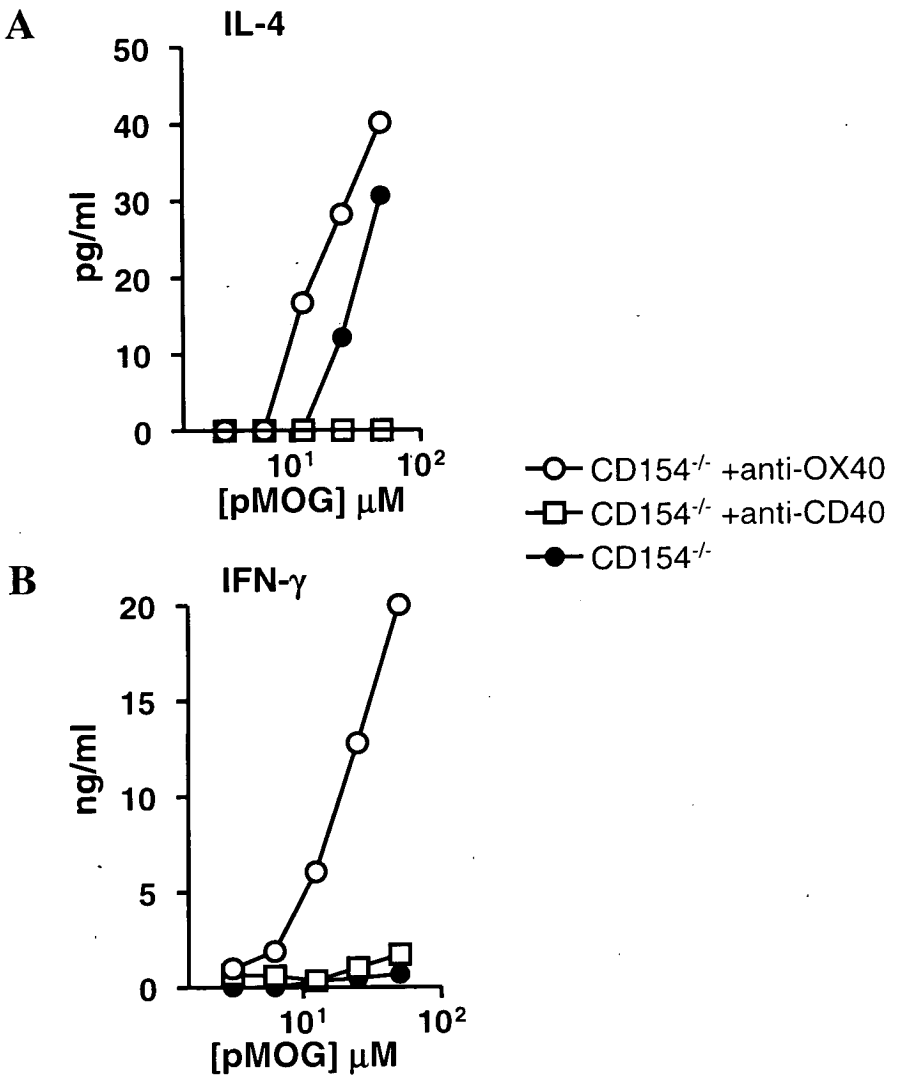

Fig. 3.4:
Exogenous ligation of CD40 or OX40 influences the cytokine response in CD154$^{-/-}$ mice
CD154$^{-/-}$ mice received 100μg pMOG in CFA at day 0. As indicated, some groups also received 200μg anti-OX40 or anti-CD40 at the time of pMOG in CFA administration and 2 days later. Draining lymph nodes were assessed 10 days later by ELISA for pMOG-specific IL-4 and IFN-γ production to a dose range of pMOG (A: IL-4, B: IFN-γ). The effects seen with anti-CD40 and anti-OX40 were not found using isotype control antibodies. Background levels in each group were below detectable levels. For each datapoint, the SEM was <20%. These data are from one preliminary experiment.

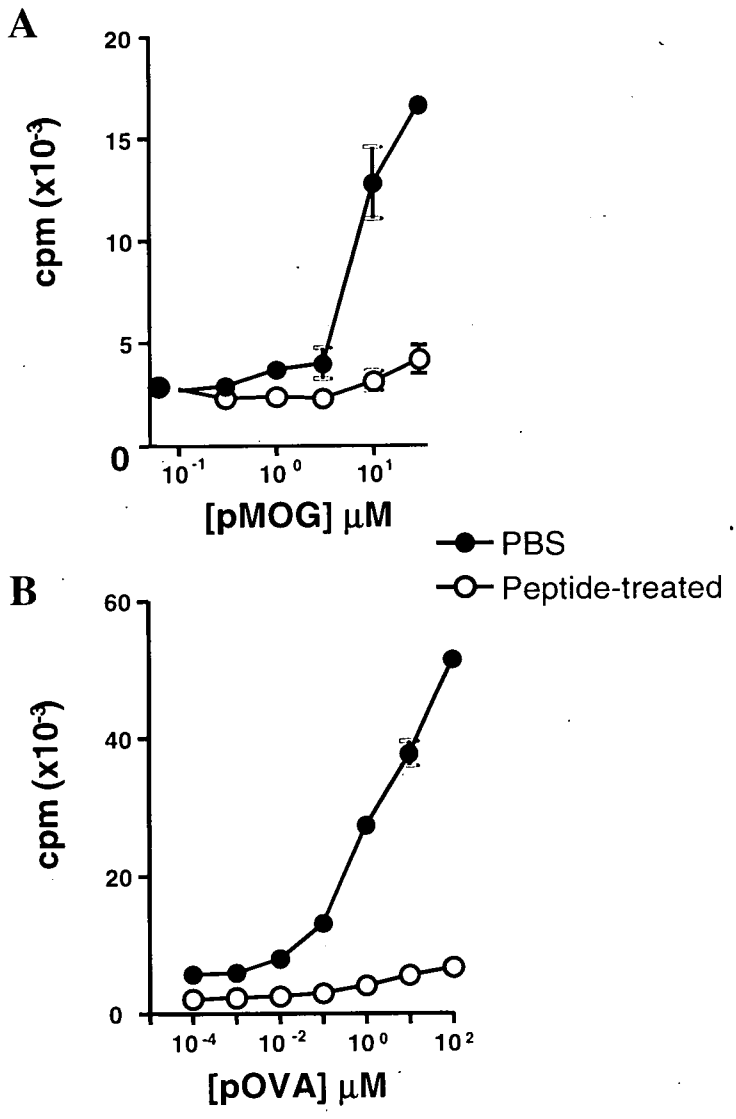

Fig. 3.5:
CD154 is not required for peptide-induced tolerance
Mice received 3 doses of 500µg pMOG or PBS i.p., on days -8, -6 and –4 (A) or a single dose of 500µg pOVA i.v. on day –7 (B), before immunisation with pMOG or pOVA in CFA on day 0. Draining lymph nodes were assessed 10 days later for pMOG or pOVA-specific proliferative responses respectively. These data are from one of five giving consistent results. Background cpm in each group were as follows: pMOG: PBS 3291, peptide-treated CD154$^{-/-}$ mice 2834; pOVA: PBS 2375, peptide-treated CD154$^{-/-}$ mice 6224.

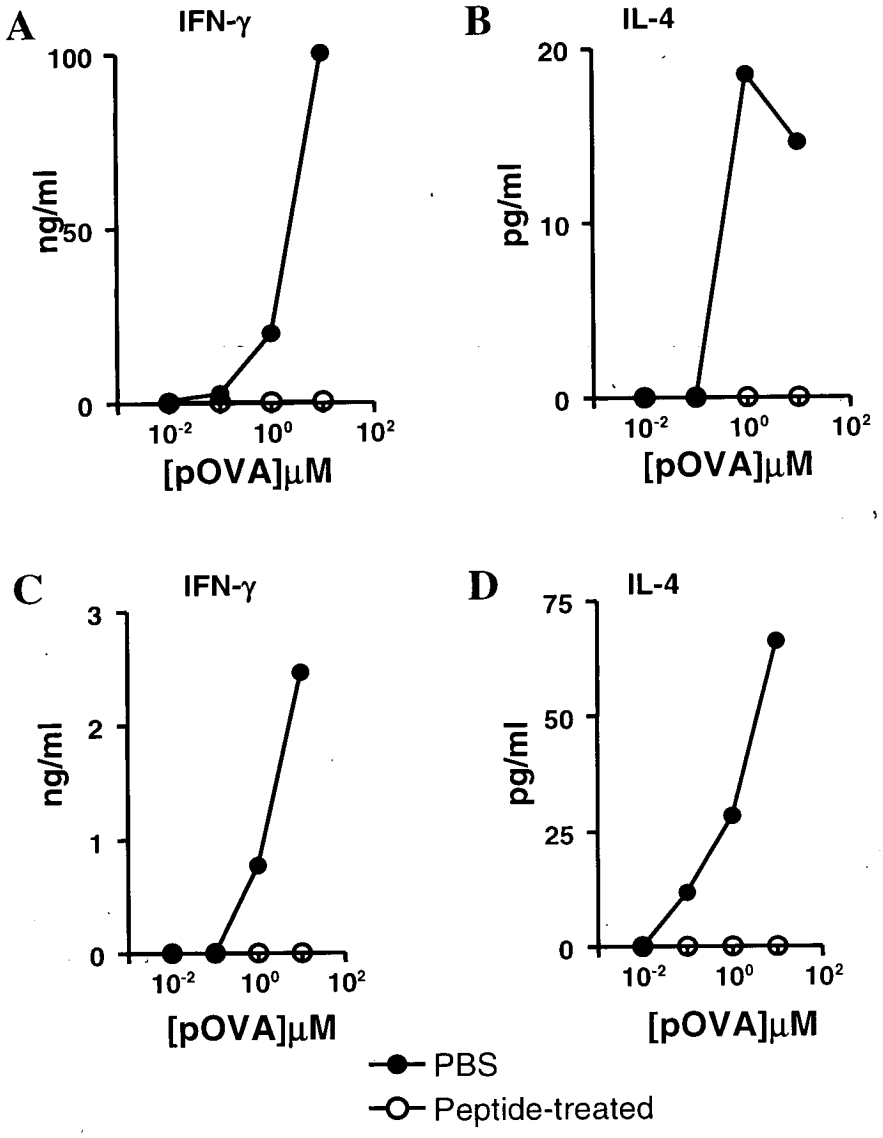

Fig. 3.6:
Peptide-induced tolerance diminishes cytokine responses in B6 and CD154$^{-/-}$ mice
Mice received 500μg of pOVA i.v. on day –7, before administration of pOVA in CFA on day 0. Draining lymph nodes were assessed 10 days later by ELISA for pOVA-specific cytokine production. Shown are IFN-γ and IL-4 production by B6 mice (A and B respectively) and CD154$^{-/-}$ mice (C and D). These data are from one of three experiments giving consistent results. The background cpm for all cytokines was below detectable levels in all groups. For each datapoint, the SEM was <20%.

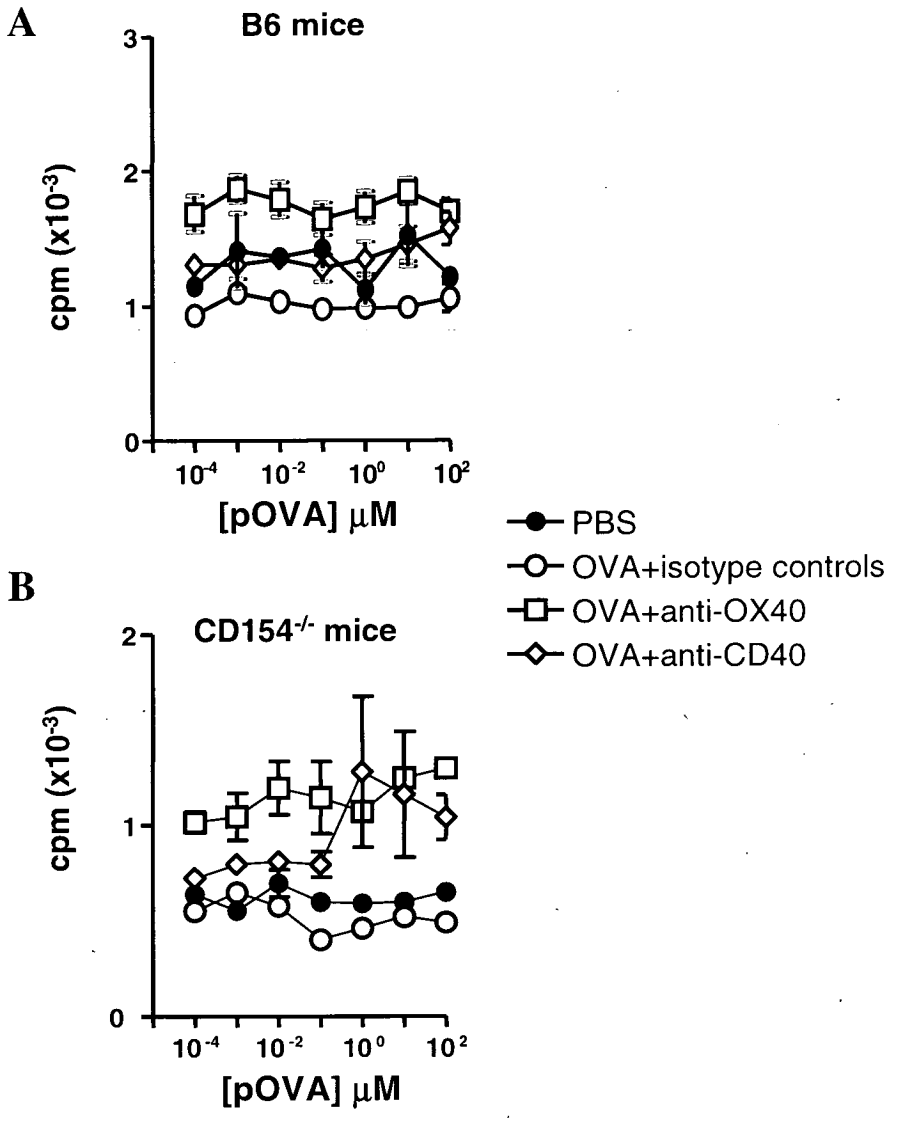

Fig. 3.7:
Exogenous ligation of CD40 or OX40 does not convert a tolerogenic signal into an immunogenic one
B6 or CD154$^{-/-}$ mice received a single dose of 500µg pOVA i.v. As indicated, some groups also received 200µg anti-OX40 or anti-CD40 at the time of pOVA administration and 2 days later. Draining lymph nodes were assessed 7 days later for proliferative responses to a dose range of pOVA (A: B6 mice, B: CD154$^{-/-}$ mice). Background proliferation was as proliferation observed at 10^{-4} for each group. These data are from one of three experiments giving consistent results.

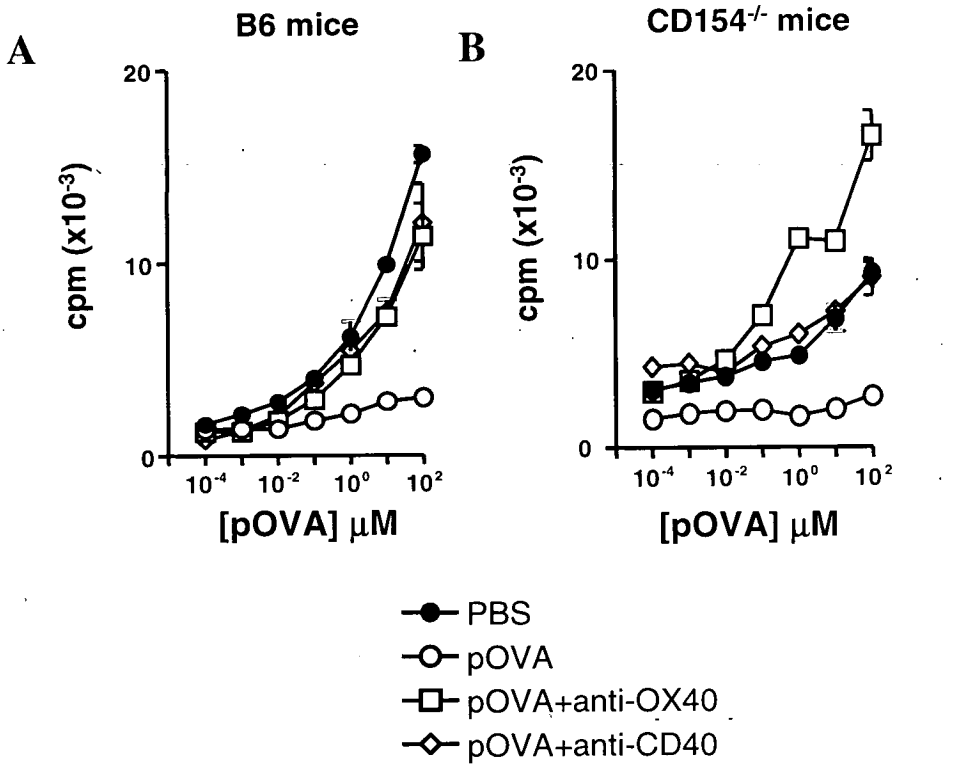

Fig. 3.8:
Exogenous ligation of CD40 or OX40 prevents tolerance induction in B6 or CD154$^{-/-}$ mice

B6 or CD154$^{-/-}$ mice received a single dose of 500µg pOVA i.v. on day –7. As indicated, some groups also received 200µg anti-OX40 or anti-CD40 at the time of pOVA administration and 2 days later. Mice were then immunised with pOVA/CFA on day 0, and draining lymph nodes assessed 10 days later for proliferative responses to a dose range of pOVA (A: B6 mice, B: CD154$^{-/-}$ mice). The effects seen with anti-CD40 and anti-OX40 were not found using isotype control antibodies. These data are from one of three experiments giving consistent results. Background cpm were as follows: B6: PBS 2038, pOVA 1530, pOVA+anti-CD40 1214, pOVA+anti-OX40 1483; CD154$^{-/-}$ mice: PBS 3489, pOVA 2341, pOVA+anti-CD40 6205, pOVA+anti-OX40 3328.

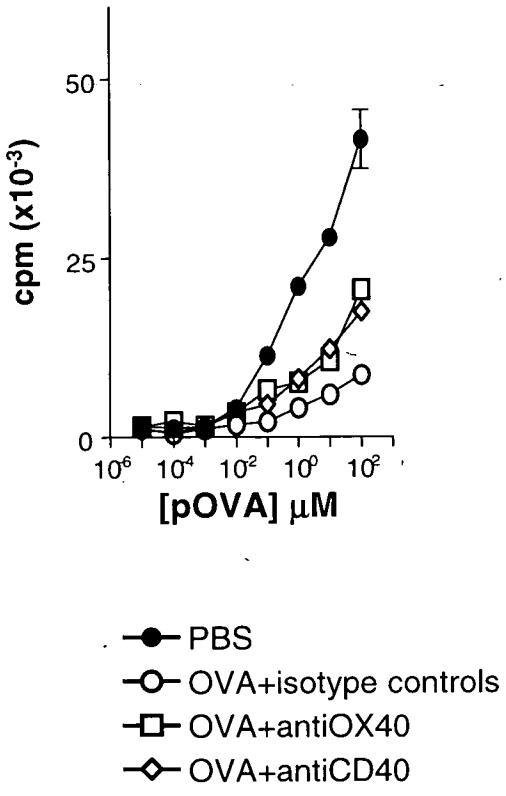

Fig. 3.9:
Exogenous ligation of CD40 or OX40 partially prevents tolerance induction in B cell-deficient mice
µMT mice received a single dose of 500µg pOVA i.v. on day –7. As indicated, some groups also received 200µg anti-OX40 or anti-CD40 at the time of pOVA administration and 2 days later. Mice were then immunised with pOVA/CFA on day 0, and draining lymph nodes assessed 10 days later for proliferative responses to a dose range of pOVA. These data are from one of two experiments giving consistent results. Background levels were as follows: PBS 1656, pOVA 1234, pOVA+anti-CD40 902, pOVA+anti-OX40 1473.

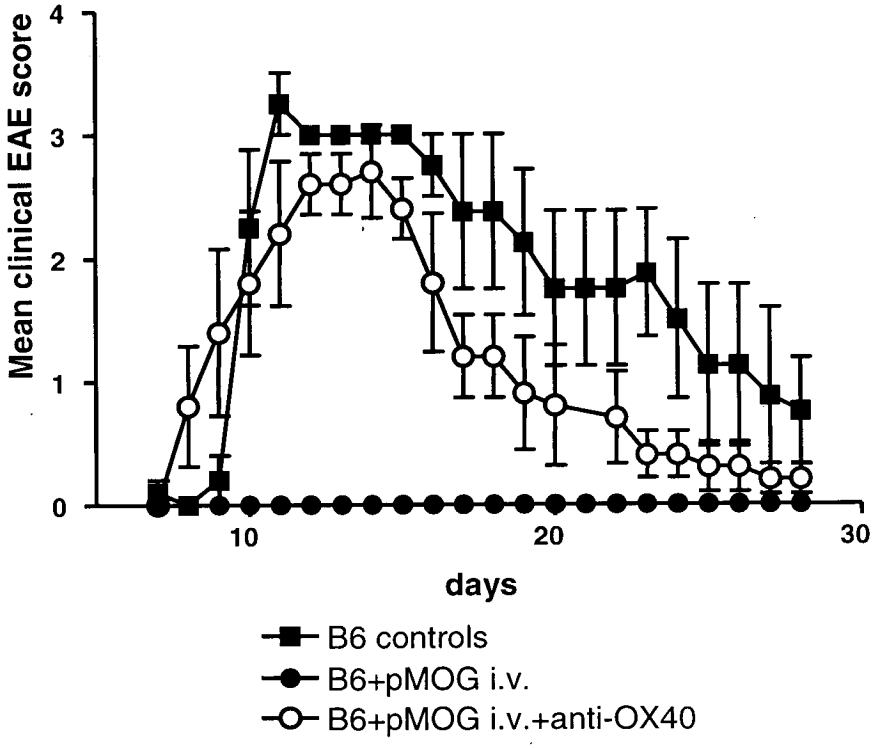

Fig. 3.10:
Exogenous ligation of OX40 prevents tolerance induction to EAE in B6 mice
Mice received a single dose of 500µg pMOG i.v. on day –7. As indicated, some groups also received 200µg anti-OX40 at the time of pMOG administration and 2 days later. EAE was then induced through immunisation with pMOG/CFA on day 0 and PTX was given at the same day and 2 days later. Clinical EAE scores were assessed daily from day 7-28. The effects seen with anti-OX40 were not found using an isotype control antibody. These results are from one preliminary experiment, and remain to be repeated. (p values determined by Mann-Whitney U test were: B6 controls vs. B6+pMOG i.v.: <0.0001, B6 controls vs. B6+pMOG i.v. +anti-OX40: <0.0001)

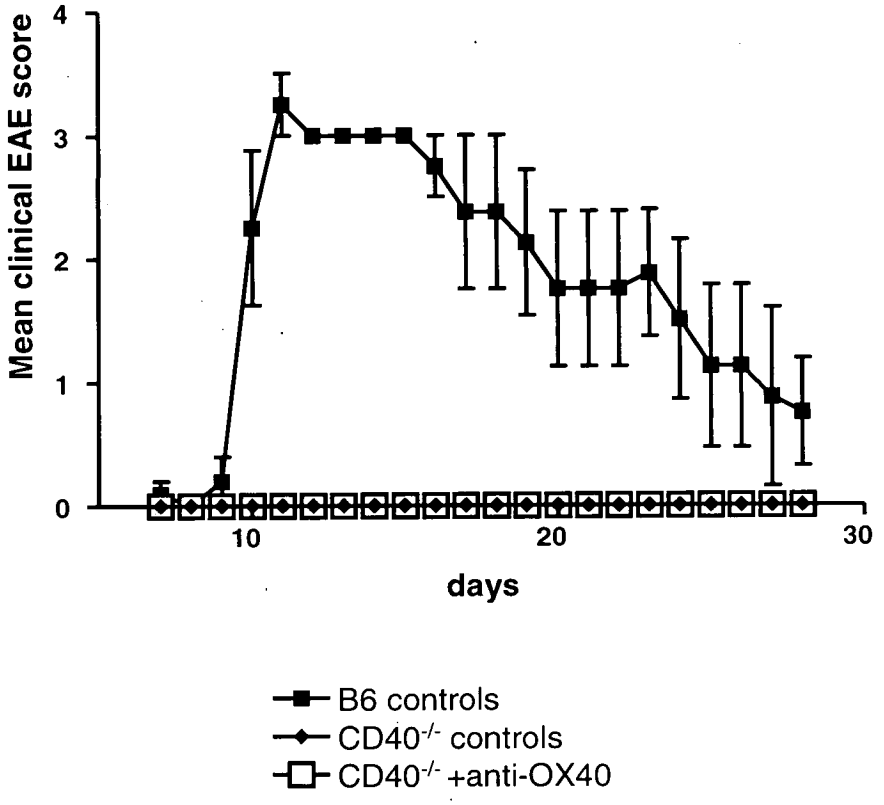

Fig. 3.11:
Exogenous ligation of OX40 does not restore induction of EAE in CD40$^{-/-}$ mice
EAE was induced through immunisation with pMOG/CFA on day 0. PTX was given at the same day and 2 days later. One group also received anti-OX40 i.p. at the time of immunisation and 2 days later. Clinical EAE scores were assessed daily from day 7-28. (p values determined by Mann-Whitney U test were: B6 controls vs. CD40$^{-/-}$ controls: <0.0001, B6 controls vs CD40$^{-/-}$+anti-OX40: <0.0001) These results are from one preliminary experiment.

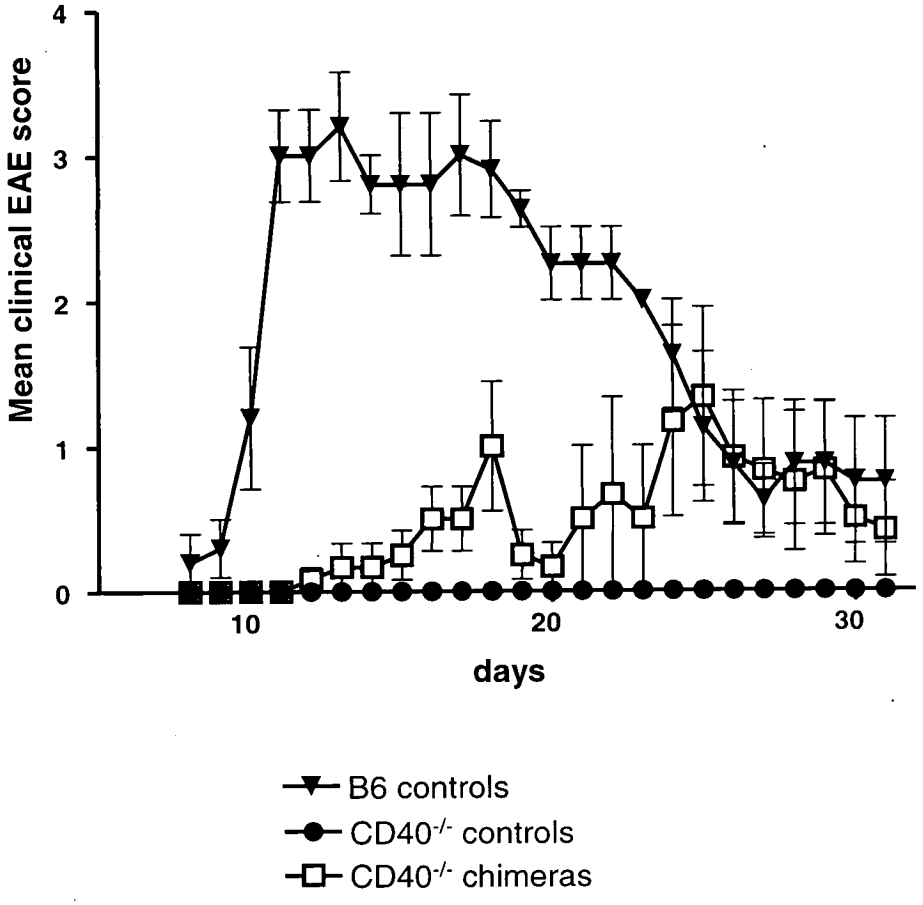

Fig. 3.12:
CD40 expression on bone marrow-derived cells is required for typical EAE disease progression
Chimeras lacking CD40 expression on all bone marrow-derived cells were generated. EAE was induced through immunisation with pMOG/CFA on day 0. PTX was given at the same day and 2 days later. Clinical EAE scores were assessed daily from day 7-28. (p values determined by Mann-Whitney U test were: B6 controls vs. $CD40^{-/-}$ controls: <0.0001, B6 controls vs. $CD40^{-/-}$ chimeras: <0.0001), $CD40^{-/-}$ controls vs. $CD40^{-/-}$ chimeras: <0.0001) These results are from one preliminary experiment.

4. CHAPTER 4 – Kinetics of costimulation during the induction of T cell tolerance versus immunity

4.1. Introduction

The previous chapter illustrated that exogenous ligation of either CD40 or OX40 could prevent the induction of T cell tolerance. This suggests that upregulation of one or both these costimulatory molecules occurred during tolerance induction, and indicates that molecules known to be important for T cell activation may also play a role in T cell tolerance. This chapter investigated at which time-points during this process these receptors and their ligands were expressed. Expression of a third receptor-ligand pair, namely RANK-RANKL, was also examined. The RANK-RANKL interaction was reported to be important for DC survival (Cremer et al., 2002), and treatment of DC with RANKL before transfer into mice enhances their survival in the DLN, resulting in enhanced T cell responses (Josien et al., 2000). This pair could therefore play a role in the decision between tolerance and immunity. All three pairs are thus known to play an important role during T cell activation, but to date their expression during the induction of T cell tolerance has not been investigated.

LPS has known effects on DC migration and activation (Reis e Sousa and Germain, 1999), but most experimental data available stems from histological evaluation with regards to DC location, and from *in vitro* activation of DC. Although it is assumed that tolerance induction is the result of peptide presentation by quiescent DC, which lack full costimulatory potential (Steinman et al., 2003), few experiments actually look at the DC phenotype during the induction of T cell tolerance. Similarly, little *ex vivo* data exists, showing the state of DC activation after LPS administration. The experiments in this chapter were designed to test the view that T cell immunity is the result of DC activation through microbial signals, whilst tolerance induction is a result of peptide presentation by immature DC, and sets out to reveal possible

differences in costimulatory molecule expression on T cells during the induction of T cell tolerance or immunity.

4.2. Approach

Since T cell responses in naïve mice involve a very small number of Ag-specific T cells, it was necessary to use an adoptive transfer system to look at the T cell phenotype during the induction of tolerance. The use of TCR transgenic T cells from OT-II mice (Barnden et al., 1998) allowed the transfer of a specific number of pOVA-reactive T cells into naive B6 hosts, enabling tracking of a defined number of Ag-reactive T cells. OT-II cells express the Vα2 and Vβ5 TCR chains, and initially, the transferred cells were identified using monoclonal antibodies to these TCR chains. However, naïve B6 mice have endogenous $V\alpha2^+$, $V\beta5^+$ T cells, which are not necessarily pOVA-reactive. OT-II mice were therefore crossed with mice expressing the congenic marker Ly5.1 (CD45.1), and were identified by expression of this marker during subsequent FACS analysis (host B6 mice express Ly5.2). OT-II cells were adoptively transferred into naïve hosts, before pOVA was administered in tolerogenic, i.e. soluble form, or immunogenic form, i.e. with LPS as adjuvant. At various time points after Ag administration, mice were sacrificed, and the transferred T cell population in the spleen was analysed by FACS.

DC constitute about 1% of spleen cells, and gating on splenic DC during FACS analysis is difficult due to the heterogeneous level of expression of the CD11c marker. DC were therefore purified before FACS analyses. Although purification may result in DC activation, it was necessary in order to examine the phenotype of the splenic DC population. A group, which received PBS, rather than pOVA in any form, was used as a control for possible DC activation and change in surface phenotype due to the purification process employed. Any reported changes in costimulatory molecule expression are with respect to this control group.

The experimental outline is shown in Figure 4.1.

4.3. Administration of soluble pOVA induces Ag-specific tolerance in TCR transgenic T cells

The previous chapter shows that i.v. administration of soluble pOVA results in pOVA-specific T cell tolerance. In order to look at the phenotype of a defined population of T cells, the OT-II adoptive transfer system was employed in most of the subsequent experiments. It was therefore important to test that systemic administration of soluble pOVA could similarly induce tolerance after the transfer of a large number of Ag-specific T cells. Mice received OT-II cells on day −1, followed by 500μg pOVA on day 0, and *in vivo* rechallenge with 20μg pOVA in CFA on day 7. Greatly reduced T cell *ex vivo* recall proliferative responses were observed on day 17, even after transfer of OT-II cells (Fig. 4.2). Administration of a single dose of soluble pOVA could therefore be used in subsequent experiments to investigate the phenotype of DC and pOVA-reactive T cells during the induction of T cell tolerance.

4.4. Co-administration of LPS (but not CpG) leads to T cell immunity

With the aim to compare T cell phenotype during the induction of T cell tolerance compared to the induction of T cell immunity, LPS and CpG were tested as adjuvants to prevent the induction of T cell tolerance, since both can be administered i.v. at the same time as soluble pOVA. OT-II cells were adoptively transferred into naïve B6 hosts 1 day prior to the hosts receiving either 500μg pOVA, 500μg pOVA with 5μg, 10μg or 30μg CpG (5μg has been used by others to induce immunity (Vicari et al., 2002)), or 500μg pOVA with 30μg LPS (various doses of LPS have previously been shown to induce immunity (Khoruts et al., 1998; Maxwell et al., 2002), and 30μg was tested, and found to work efficiently to prevent tolerance). Splenic populations were assessed 7 days later for proliferative responses to a dose range of pOVA (Fig. 4.3). Co-administration of pOVA and LPS did not result in tolerance induction, but allowed efficient recall responses, while co-administration of pOVA and CpG did not result in effective T cell immunity, since responses were similar to those seen

with pOVA alone, (Fig. 4.3, shown is pOVA co-administration with 30μg CpG, which was the highest dose used. Other doses included 5μg and 10μg CpG, which also did not prevent the induction of tolerance in this system, results not shown). CpG did however have an effect on DC *in vitro*, as shown through upregulation of CD40, CD80 and CD86 on bone marrow-derived DC after incubation with 6μg/ml (1μM) CpG overnight (Fig. 4.4). Other groups have administered CpG intratumorally to prevent tolerance against tumours (Vicari et al., 2002). This very localised delivery of CpG may explain the ability of CpG to prevent tolerance in that system, compared to systemic administration of CpG in the system used here. Since administration of pOVA with LPS resulted in effective T cell immunity, this protocol was used in subsequent experiments to compare the phenotype of DC and pOVA-reactive T cells during induction of T cell immunity versus T cell tolerance.

4.5. Phenotypic analysis of DC and T cells during the induction of T cell immunity versus T cell tolerance

Having established that administration of pOVA alone resulted in T cell tolerance, while co-administration of pOVA and LPS induced T cell immunity, experiments were carried out to look at the phenotype of T cells and DC during these two processes. In all of the subsequent experiments in this chapter, $CD4^+$ OT-II cells were purified by positive selection using anti-CD4-conjugated MACS beads, prior to transfer into naïve recipients on day-1. On day 0, mice received the indicated administration protocol of pOVA alone or pOVA and LPS, and mice were sacrificed at various time points following Ag administration. Spleens were disaggregated, before DC were purified by positive selection using anti-CD11c beads. The flowthrough of this purification was kept to analyse the transferred T cell population.

By day 2 after Ag administration, transferred OT-II cells started to expand in numbers. T cells in groups that received pOVA alone consistently showed greater expansion at day 2 after Ag administration compared to pOVA+LPS groups. In the particular experiment shown (Fig. 4.5), $CD4^+$, $Ly5.1^+$ OT-II cells made up 1.65%

(pOVA alone) and 0.31% (pOVA+LPS) of total lymphocytes on day 2. By day 3, both groups showed significant expansion (4.52% and 3.79% respectively). By day 4, the pOVA-specific T cell population in mice that had received pOVA alone started to decrease in number (3.19), indicating the induction of tolerance, while in mice that had received pOVA+LPS, the T cells continued to expand in numbers (7.18%) (Fig. 4.5).

The greater expansion seen at day 2 after administration of pOVA alone could have been due to earlier entry into proliferation phase in this group, or due to greater expansion of T cells at these early time points. In order to distinguish between these two possibilities, CD4-purified Ly5.1$^+$ OT-II cells were CFSE-labelled before adoptive transfer into naïve recipients on day-1. The CFSE profile of OT-II cells was examined at days 1, 2, 3 and 4 after pOVA administration with or without LPS. Again, mice that had received pOVA alone showed a greater number of pOVA-reactive T cells on day 2 than those that had received pOVA+LPS. Overlaying the CFSE profiles of groups that had received pOVA or pOVA+LPS allowed comparison of the number of cell divisions undergone in each group (Fig. 4.6b). This showed that the number of cell divisions in each group was similar at day 2, and the difference in T cell numbers are thus not due to earlier entry into cell division after administration of pOVA alone. By day 3 post Ag administration, both groups showed significant expansion as before (compare Fig. 4.5 and 4.6), but the CFSE profiles revealed that the cells had undergone greater numbers of cell division after administration of pOVA+LPS, compared to administration of pOVA alone (Fig. 4.6b). By day 4, pOVA-reactive T cell numbers continued to increase during the induction of immunity, while numbers in mice that had been given pOVA alone had greatly reduced numbers of pOVA-reactive T cells (Fig. 4.6).

Fig. 4.5 and Fig. 4.6 illustrate the variation in percentages of pOVA-reactive cells seen between different experiments. These differences are probably due to the variations in transferred numbers, cell death after transfer (CFSE-labelled cells are more fragile, and prone to dying due to the way they have been prepared), and

differences in effectiveness of Ag administration. Nevertheless, the same trend was consistently observed in all experiments, and variation between experiments was therefore acceptable.

4.6. Expression of costimulatory molecules on T cells during the induction of tolerance or immunity

The establishment of systemic T cell tolerance takes at least three days (Liu and Wraith, 1995), and it was therefore decided to carry out a time course over 4 days, looking at the phenotype of T cells and DC during the induction of T cell tolerance and immunity. The experimental data obtained from days 3 and 4 were the same in all experimental groups unless indicated otherwise, and the results for days 1, 2 and 3 are therefore shown.

The transferred T cells were identified during FACS analysis by staining for the congenic marker Ly5.1, and gating onto $CD4^+$, $Ly5.1^+$ cells. Expression of CD69, CD154, OX40 and RANKL was analysed on this population of cells. CD69 is a marker accepted to be expressed very early after T cell stimulation. Since there is no evidence that this molecule has costimulatory capacity, it was solely used as a marker of T cell activation in these experiments. Although T cells expressed higher levels of CD69 after administration of pOVA with LPS (Appendix Fig. 1), the marker was expressed after administration of either pOVA alone, or pOVA with LPS, indicating successful initial T cell activation in both cases.

4.6.1. Tolerance is not due to the absence of CD154 expression on T cells

Mice were sacrificed on days 1, 2 and 3 after Ag administration, and the expression profile of CD154 on transferred T cells was investigated at these time-points. CD154 was expressed on days 1 and 2 after administration of pOVA and pOVA+LPS (Fig. 4.7). By day 3, only a small percentage of transferred cells still expressed CD154.

CD154 was expressed on the T cell during the induction of both T cell tolerance and immunity, although it appeared to be expressed for longer on T cells during the induction of T cell immunity.

4.6.2. Tolerance is not due to the absence of OX40 expression on T cells

OX40 was found on day 1 during induction of both T cell tolerance and T cell immunity (Fig. 4.8). Interestingly, OX40 was expressed for longer and at higher levels on T cells during the induction of T cell immunity, with some OX40 expression still present on day 2 after Ag administration (34.6%). By day 3, neither of the two experimental groups showed OX40 expression on transferred T cells compared to the PBS control group. Therefore, both CD154 and OX40 were consistently seen to be expressed for longer during the induction of immunity compared to induction of tolerance.

4.6.3. Tolerance induction results in rapid expression of RANKL on T cells

Similar to CD154 and OX40, RANKL was expressed on T cells during induction of tolerance and immunity (Fig. 4.9). In contrast to CD154 and OX40 however, RANKL was expressed earlier and at higher levels during induction of tolerance compared to induction of immunity. This was clearly noticeable when a more detailed time course analysis was carried out (Fig. 4.10), with the pOVA group beginning to express RANKL by 12 hours, whereas the pOVA+LPS group did not express RANKL until 48 hours. Both groups still expressed some RANKL on day 3 after Ag administration (Fig. 4.9).

Although the level of RANKL appeared to vary between experiments (compare Fig. 4.9 and Fig. 4.10), there was definite up-regulation of RANKL in both experimental groups, which was not seen in the PBS control group, and this upregulation was consistently observed earlier, and on a greater percentage of T cells after administration of pOVA compared to administration of pOVA+LPS.

4.7. Expression of costimulatory molecules on DC during the induction of tolerance or immunity

DC purity after isolation was assessed by staining for CD11c, and was consistently above 80%. The $CD11c^+$ population was stained for expression of CD40, OX40L, RANK, CD80 and CD86 in conjunction with CD8α. Even though the percentages of $CD8\alpha^+$ DC appeared to vary between days 1, 2 and 3, this is most likely due to variation in staining (Fig. 4.11). There was nevertheless a small, but consistent increase in the percentage of $CD8\alpha^+$ DC in the spleen after administration of pOVA alone, and a decrease of $CD8\alpha^+$ DC after administration of pOVA+LPS compared to the PBS control group on each day (Fig. 4.11).

Purification of DC in the presence of polymyxin B (which binds LPS and prevents its DC-activating properties) (Montoya et al., 2002), and purifying DC by depletion, using a cocktail of antibodies against thymocytes, B cells, and granulocytes (Kamath et al., 2000), did not change the level of costimulatory molecules displayed on the surface of DC compared to the described method of positive selection (Appendix Fig. 2). It was therefore chosen to purify DC for phenotyping using positive selection.

Immature DC constitutively express low levels of CD40, CD80 and CD86 (Vremec and Shortman, 1997). In order to control for possible activation and upregulation of these molecules during the purification procedure, levels of these molecules after Ag administration were compared to PBS controls, which were purified in the same way as the other experimental groups. Any upregulation of CD40, CD80 and CD86 must therefore have been due to events that had happened *in vivo*, prior to DC isolation.

4.7.1. CD40 is upregulated on DC during the induction of T cell immunity

The level of CD40 on DC was not increased in mice that had received pOVA compared to PBS controls, but DC from mice that had received pOVA+LPS showed increased levels of CD40 on days 1 and 2 after Ag administration, as indicated by median fluorescence values (MF) in each group (Fig. 4.12). No difference in up-regulation of CD40 could be observed between $CD8\alpha^+$ and $CD8\alpha^-$ DC, although in all three groups (in the steady state as well as after DC activation) MF values indicated slightly higher levels of CD40 on $CD8\alpha^+$ DC compared to $CD8\alpha^-$ DC (Fig. 4.13). By day 3, CD40 levels had returned to normal compared to PBS controls. The $CD11c^+$, $CD40^{low}$ population, which was consistently seen on days 1 and 2 in the group that had received pOVA+LPS, may have consisted of immature DC recruited due to the inflammation occurring after administration of LPS, but this was not investigated in greater detail. Nevertheless, the administration of adjuvant therefore led to increased levels of CD40 on DC during induction of immunity, while during the tolerance induction, CD40 levels on DC did not change compared to PBS controls.

4.7.2. OX40L is not detectable on DC during induction of T cell tolerance or T cell immunity

No significant expression of OX40L on DC compared to isotype controls was observed during the induction of T cell tolerance or T cell immunity over the 3 days time course (Fig. 4.14). The MF values suggested expression of OX40L on DC after administration of pOVA+LPS at the day 1 time-point. However, although this increase was consistently observed, it is very small, and it is therefore difficult to draw conclusions with respect to OX40L expression from the FACS data obtained.

4.7.3. RANK is upregulated on DC during the induction of T cell immunity

DC constitutively expressed low levels of RANK. The level of RANK on DC was not increased in mice that had received pOVA compared to PBS controls, but DC

from mice that had received pOVA+LPS showed increased levels of RANK on day 1 after Ag administration (Fig. 4.15). No difference in upregulation of RANK could be observed between $CD8\alpha^+$ and $CD8\alpha^-$ DC (Fig. 4.16), although similar to CD40 expression, MF values indicated slightly elevated levels of RANK on $CD8\alpha^+$ DC compared to $CD8\alpha^-$ DC, in all three experimental groups. By day 2, RANK levels in this group were returning to normal, and by day 3, RANK levels had returned to PBS controls levels. Interestingly, there was hardly any RANK expression above background levels at day 2 after pOVA+LPS administration, while RANKL was expressed on the T cells at that time. Fig. 4.10 shows that RANKL expression is induced between 24 and 48 hours after pOVA+LPS administration. Although RANK expression had decreased to background levels by day 2 (i.e. 48 hours), there nevertheless is a time window between day 1 and day 2 where both RANK and RANKL could have been expressed.

4.7.4. CD80 and CD86 are upregulated on DC during the induction of T cell immunity

DC constitutively expressed CD80 and CD86, but similar to data investigating CD40 expression, CD80 and CD86 were upregulated after administration of pOVA+LPS, but stayed at levels seen in PBS controls groups after administration of pOVA alone (Figs. 4.17 and 4.18). By day 2, a population of $CD80^{low}$ cells could be identified, which was still present at day 3, again similar to the CD40 expression data described above. Similarly, a large proportions of cells were present at day 2 and 3 that showed intermediate expression of CD86. Nevertheless, it appeared that CD86 stayed up-regulated for longer, since it was still seen at high levels on a proportion of cells on day 3 after Ag administration.

4.8. Summary of costimulatory molecule expression on T cells and DC during the induction of T cell tolerance or immunity

The time course investigating the phenotype of DC and T cells after administration of a tolerogenic or immunogenic protocol was repeated many times. Although slight variations in the duration and level of costimulatory molecule expression were observed, the same trend, summarised in Fig. 4.19, was observed consistently. CD154 (Fig. 4.7), OX40 (Fig. 4.8) and RANKL (Fig. 4.9) were all found to be expressed on T cells after administration of pOVA alone or pOVA+LPS. However, CD154 and OX40 were expressed at higher levels and/or for longer during the induction of immunity than tolerance, while RANKL was expressed earlier and at greater levels during tolerance induction.

Nevertheless, the data obtained suggests that the expression of costimulatory molecules on DC is the deciding factor between tolerance and immunity, since increased expression of CD40 (Fig. 4.12), RANK (Fig. 4.15), CD80 and CD86 (Fig. 4.17 and 4.18) was only ever seen during the induction of T cell immunity. During the induction of T cell tolerance, the expression of these molecules was indistinguishable from the PBS controls.

The CD40, CD80 and CD86 profiles after administration of pOVA+LPS showed two DC populations at days 2 and 3, expressing different levels of CD40, CD80 and CD86 (Fig. 4.12, 4.17 and 4.18). The $CD40^{low}$ population may suggest recruitment of immature DC from the circulation. Interestingly, Fig. 4.13 shows that the $CD40^{low}$ population of $CD11c^{+}$ cells was mostly $CD8\alpha^{-}$. The significance of these observations, if indeed significant at all, remains open for discussion.

4.9. Co-administration of pOVA and CpG does not alter the phenotype of DC, and does not prevent tolerance induction

Even though co-administration of pOVA+CpG did not prevent tolerance induction, it did result in upregulation of co-stimulatory molecules *in vitro*. The phenotype of DC was therefore investigated *ex vivo* after co-administration of pOVA+CpG. Expression of CD40, OX40L and RANK did not change on DC in groups that had received pOVA+CpG compared to pOVA alone or PBS control groups (Fig 4.20, and Appendix Fig. 3-5). Similarly, administration of pOVA+CpG did not change expression of OX40 and RANKL on T cells compared to groups that received pOVA alone (Appendix Fig. 6 and 7). Expression of CD154 on T cells was not investigated in these experiments.

4.10. Administration of agonistic anti-CD40 antibody results in induction of OX40L expression on DC

Administration of agonistic anti-CD40 antibody prevented the induction of tolerance (Chapter 3). Successful T cell priming is normally used as a read-out for DC activation by the antibody. Here, T cell and DC phenotype were also analysed over a 4-day time course. At these early time-points, T cell expansion was comparable in groups of mice that had received pOVA alone, or pOVA and anti-CD40. Analysis of DC phenotype however showed considerable DC activation after CD40 administration, as illustrated by upregulation of CD80 and CD86 on days 2 and 3 after Ag administration (Fig. 4.21). Administration of CD40 also induced expression of OX40L on days 2 and 3 (Fig. 4.21). Increased expression of CD80 and CD86, and expression of OX40L was found at comparable levels in $CD8\alpha^-$ and $CD8\alpha^+$ DC (not shown). The isotype control shown in Fig. 4.21 for day 2 was taken from the group of mice that had received pOVA+anti-CD40. This was because this group had the highest background staining at that day. Nevertheless, an upregulation of OX40L could be observed in that group on day 2 compared to the control staining. By day 4,

expression of OX40L, CD80 and CD86 had returned to levels comparable to those in PBS controls (not shown).

4.11. Expression of costimulatory molecules on CD154-deficient T cells during the induction of T cell tolerance or immunity

It was shown in Chapter 3 that CD154 on T cells is not essential for induction of T cell tolerance. In order to look at the expression of costimulatory molecules on $CD154^{-/-}$ T cells, OT-II mice were crossed with $CD154^{-/-}$ mice. These mice did not express the congenic marker Ly5.1. Purified $CD4^+$ cells from OT II x $CD154^{-/-}$ mice were transferred into naïve $CD154^{-/-}$ recipients, and identified by gating on $V\alpha2$ and $V\beta5$, the TCR chains expressed by OT-II mice. This allowed phenotyping of $CD154^{-/-}$ T cells during the induction of T cell tolerance or immunity. However, $CD154^{-/-}$ mice have endogenous $V\alpha2^+$, $V\beta5^+$ T cells (approximately 0.5% of the $CD4^+$ T cell population in the spleen), which may not be pOVA-reactive. The number of $V\alpha2$, $V\beta5^+$ increases to 1-1.8% after transfer. By gating on $V\alpha2$ and $V\beta5$, the FACS data obtained therefore included endogenous as well as transferred T cells. The percentage of transferred cells positive for the costimulatory molecule being analysed may in reality have been higher than seen on the FACS plots, since endogenous $V\alpha^+$, $V\beta5^+$, pOVA-non-specific T cells will not have been activated by administration of pOVA, and will thereby have increased the number of $V\alpha2^+$, $V\beta5^+$ T cells, which are costimulatory molecule^{-ve}.

As with B6 mice, administration of pOVA+LPS induced immunity in $CD154^{-/-}$ mice (Appendix Fig. 8). In essence, the pattern of costimulatory molecule expression on T cells and DC during the induction of tolerance and immunity was the same in $CD154^{-/-}$ mice as seen in B6 mice (summarised in Fig. 4.22). In the absence of CD154, OX40 was expressed on T cells during the induction of tolerance and immunity (Appendix Fig.9). Similar to results obtained with CD154-sufficient T

cells, OX40 was expressed on a greater percentage of T cells and for longer after administration of pOVA+LPS. OX40L was not seen on any of the experimental groups, similar to results obtained in B6 mice (Appendix Fig.12).

RANKL was expressed on $CD154^{-/-}$ T cells after administration of pOVA and after pOVA+LPS (Appendix Fig.10). RANKL expression again appeared at lower levels after administration of pOVA+LPS, similar to results obtained with CD154-sufficient T cells. CD154 signalling to DC via CD40 has previously been shown to induce RANK expression (Anderson et al., 1997). However, RANK expression in $CD154^{-/-}$ was comparable to that observed in B6 mice (compare Fig. 4.15 and Appendix Fig.13), suggesting an LPS-dependent, CD40-CD154-independent pathway of RANK upregulation. The expression of the three receptor-ligand pairs during induction of tolerance or immunity in $CD154^{-/-}$ mice is summarised in Fig. 4.22. Although the level of RANK on day 2 was only slightly increased compared to the other groups (Appendix Fig. 13), this increase was seen consistently on day 2 after administration of pOVA+LPS, and the summary therefore shows DC to display RANK on their surface at that time-point.

4.12. Expression of costimulatory molecules in B cell-deficient mice during the induction of T cell tolerance or immunity

B cells have been shown to display MHC-peptide complexes on their surface after administration of soluble protein (Zhong et al., 1997). Chapter 3 showed that T cell tolerance could be established in the absence of B cells. Expression of the costimulatory molecules was investigated by transferring OT-II cells into B cell deficient μMT mice. The expression of the costimulatory molecules during induction of tolerance or immunity in B cell-deficient mice is summarised in Fig. 4.23 (the expression of CD154 was not investigated).

Expression of OX40 on day 1 on pOVA-reactive T cells was comparable to data obtained from B6 mice (compare Fig. 4.8 and Appendix Fig. 14). However, OX40 was still found at increased levels on day 2, and thus appeared to stay upregulated longer in the absence of B cells. Interestingly, the expression of RANKL on T cells after administration of pOVA+LPS was negligible, similar to data obtained in $CD154^{-/-}$ mice (Appendix Fig. 15). However, the absence of B cells did not influence the levels of expression of CD40, OX40L and RANK on DC during induction of tolerance or immunity (Appendix Fig. 16 and 17, OX40L is not shown, since no expression was observed). Comparable to results obtained from experiments with B6 mice, the levels of CD40 and RANK were increased on DC during the induction of immunity (Appendix Fig. 16 and 17), indicating that these cells are responsible for effective T cell immunity.

4.13. Histological approach to investigating the expression of costimulatory molecules during the induction of tolerance of immunity

Chapter 3 provided evidence for the lack of OX40-OX40L signalling during tolerance, since exogenous ligation of OX40 can prevent tolerance induction, which has also been observed by another group (Bansal-Pakala et al., 2001). During the induction of either tolerance or immunity, T cells became activated (Figs. 4.7-4.9), indicating definite contact with cells displaying peptide on their surface. These cells were likely to be DC, since similar data was obtained from experiments in B6 compared to B cell-deficient mice (Fig. 4.23). OX40L is induced through CD40 ligation (Fig. 4.21). Interestingly, CD154 was expressed on T cells during both the induction of tolerance and immunity (Fig. 4.7), which should have been sufficient to allow signaling through CD40, resulting in OX40L expression on DC. Nevertheless, only a very small increase of OX40L expression after administration of pOVA+LPS was seen, and the expression of OX40L on DC was therefore minimal under conditions leading to either tolerance or immunity (Fig. 4.14). Since OX40L expression via CD40 signaling requires DC-T cell contact, it was possible that the

actual number of DC in contact with T cells and expressing OX40L was too low to be detected through FACS analysis.

In order to investigate expression of OX40L on DC in contact with T cells, and to validate the data obtained through FACS analysis, immunofluorescent histology was carried out on spleen tissue sections. To identify adoptively transferred T cells, CD4-purified, Ly5.1$^+$ OT-II cells were fluorescently labelled with CT orange before transfer into naïve B6 recipients, and pOVA was administered in tolerogenic (pOVA alone) or immunogenic form (pOVA+LPS), as before. Because the intensity of the fluorescent dye decreased with cell division, two early time-points, 12 hours and 36 hours, were initially chosen to look at expression of costimulatory molecules, in particular CD154, OX40 and OX40L. Mice were sacrificed at these two time-points, and spleen tissue sections were snap-frozen in liquid nitrogen. These sections were firstly stained for Ly5.1, to validate that the fluorescent cells, which could be seen in the tissue sections, actually were the Ly5.1$^+$ OT-II cells, that had been transferred. Staining for Ly5.1 revealed that at 12 hours after administration of Ag, cells identified as CT$^+$ were indeed Ly5.1$^+$ (Fig. 4.24a). However, by 36 hours after Ag administration, a large proportion of the Ly5.1$^+$ cells could no longer be distinguished from background fluorescence (Fig. 4.24b), since the number of Ly5.1$^+$ cells identified through staining was larger than the number of CT$^+$ cells observed. Fluorescently labelling OT-II cells before transfer was thus an effective technique to look at expression of costimulatory molecules at very early time-points, but could not be used to look at later time-points. A double-staining protocol, staining for expression of Ly5.1 and the chosen costimulatory marker, must be developed in order to look at these later time-points. Although a number of different approaches were tested, development of a double-staining protocol proved difficult, and requires further optimisation before it can be used effectively.

Nevertheless, sections were stained for expression of CD69, CD154, OX40 and OX40L, 12 hours after administration of Ag. Correlating with the FACS data obtained from this 12 hours time-point, CD69, CD154 and OX40 were found to be

expressed on CT^+ cells (i.e. on $CD4^+$ OT-II cells) during both induction of tolerance and immunity, while OX40L staining was hardly observed (Fig. 4.25 and Table 4.26). The percentages of $CD69^+$ adoptively transferred cells after administration of pOVA was higher than data obtained through FACS analysis (47.1 vs. 37.6%). However, after administration of pOVA+LPS, FACS analysis revealed a large percentage of pOVA-reactive T cells to be $CD69^+$ (81.2%), while immunofluorescence shows 66.6% of cells to be $CD69^+$. The percentages of CT^+ cells showing expression of CD154 was higher than the results obtained through FACS analysis (pOVA: 25.7 vs. 15%, pOVA+LPS 27.7 vs. 7.43). This could be due to the method of staining. Cells need to be fixed, and intracellularly stained for CD154 expression. Immunofluorescence staining on tissue sections may arguably be a more sensitive method of detection, and intracellular staining may not show a large enough shift during FACS analysis to accurately show all the $CD154^+$ cells.

Immunofluorescence staining for OX40 similarly showed a higher percentage of cells to be $OX40^+$ compared to FACS analysis (pOVA: 27.8 vs. 6.15, pOVA+LPS: 29.3 vs. 19.3%). Very little immunofluorescent staining for OX40L was observed at this early time-point, and was rarely in proximity to CT^+ cells. Unfortunately, it was not possible to investigate whether or not OX40L was expressed close to pOVA-reactive T cells at later time-points.

The use of a laser scanning cytometer (LSC) would allow quantification of the data obtained from OX40L staining, since using the LSC, one can scan tissue sections, and the software available allows analysis of the fluorescence observed not only on the cells of interest, but also in close proximity to those cells. A collaboration with Dr Paul Garside and Angela Grierson in Glasgow was set up, aiming to analyse expression of OX40L in close proximity to CT-labelled T cells. However, since very little OX40L staining was observed at the 12 hour time-point, and the CT-labelled cells could not be identified at later time-points, this approach again required development of a double-staining protocol. In an attempt to quantify the data

obtained from staining for OX40L, I counted OX40L staining in close proximity to transferred T cells. A summary of the histological data is given in Table 4.26.

4.14. Induction of OX40L on CD8α$^+$ DC by an unidentified compound

The initial time courses that were performed to investigate DC and T cell phenotype used LPS from an unknown source, kindly provided by the lab of Prof. D. Gray, IIIR. Interestingly, these first experiments showed upregulation of OX40L specifically on the CD8α$^+$ subset of CD11c$^+$ DC on day 1 (Fig. 4.27), which later appeared to become CD8α^{int} and/or disappeared from the spleen. This upregulation was also observed after administration of LPS alone in CD40$^{-/-}$ mice and CD154$^{-/-}$ mice (not shown), pointing towards a pathway of CD40-CD154-independent induction of OX40L. It was later realised that the LPS was contaminated, possibly with a fungus. Unfortunately, the LPS has since been discarded, and the identity of this contaminant, remains a mystery. Nevertheless, the possibility that CD8α$^+$ DC can specifically upregulate OX40L expression, in a CD40-CD154-independent fashion, in response to an unknown (possibly fungally-derived) agent, is intriguing.

4.15. Summary

The expression of three costimulatory receptor-ligand pairs, namely CD40-CD154, OX40-OX40L and RANK-RANKL, was characterised during the tolerisation or activation of T cells over a time course of 4 days. T cells expressed CD154, OX40 and RANKL during the induction of either tolerance or immunity. CD154 and OX40 were expressed for longer, and on a greater percentage of pOVA-reactive T cells during induction of immunity, while RANKL was expressed earlier and on a greater percentage of T cells during tolerance induction. DC increased their expression of CD40 and RANK only after administration of Ag with LPS, while FACS analysis could not detect OX40L during induction of either immunity or tolerance. OX40L expression was however observed specifically on $CD8\alpha^+$ DC in response to an unknown, possibly fungally derived compound.

Induction of tolerance or immunity in $CD154^{-/-}$ mice was comparable to B6 mice, with similar expression of OX40 on $CD154^{-/-}$ T cells, and CD40, OX40L and RANK on DC during induction of either tolerance or immunity. In contrast to data from B6 mice, little RANKL expression was found in $CD154^{-/-}$ mice after administration of pOVA and LPS. The absence of B cells had little influence on T cell or DC phenotype after Ag administration. Again, no RANKL was detected in response to pOVA+LPS.

Chapter 3 showed that exogenous ligation of anti-CD40 prevented T cell tolerance, and it was here further shown that exogenous ligation of CD40 resulted in up-regulation of CD80, CD86, and most importantly OX40L on DC, re-enforcing the idea that the CD40-CD154 and the OX40-OX40L interactions are pivotal in the induction and prevention of T cell tolerance.

Collectively, it is concluded that presentation of soluble peptide by quiescent or immature DC brings about T cell tolerance, while administration of LPS results in

activation of DC as shown by increased expression of costimulatory molecules, resulting in their ability to successfully initiate T cell immunity.

4.16. Discussion

In recent years, evidence has accumulated suggesting that the activation state of the DC determines the outcome of antigenic challenge. Ligation of TLRs is coupled to DC activation, leading to increased expression of costimulatory molecules on the DC surface, migration into T cell areas, and the induction of effective T cell immunity (Reis e Sousa, 2004). The current consensus view suggests that in the absence of inflammatory stimuli *in vivo*, DC play an important role in the maintenance of peripheral tolerance (Belz et al., 2002; Hernandez et al., 2001; Scheinecker et al., 2002; Steinman and Nussenzweig, 2002; Steinman et al., 2000). The ability of DC to induce tolerance or immunity appears to depend on their maturation stage (Dhodapkar et al., 2001; Jonuleit et al., 2000). Unmanipulated DC *in vivo* express little CD40 (Inaba et al., 1994; Schulz et al., 2000), and DC constitutively take up antigens in the periphery and present them to T cells in lymphoid organs in a tolerogenic fashion, which can be converted into immunogenic presentation after DC activation through CD40 (Hawiger et al., 2001; Maxwell et al., 1999).

This chapter provided direct evidence for the importance of activation state of DC in the decision between tolerance and immunity. Specifically, it showed that administration of pOVA and LPS resulted in significant DC activation, illustrated by increased expression of CD40, RANK, CD80 and CD86, which resulted in T cell immunity. Expression levels of these molecules did not change during the induction of tolerance with respect to steady state controls. It is worth noting that the data presented did not investigate peptide expression on DC. It has been suggested that $CD8\alpha^-$ DC present peptide to $CD4^+$ T cells after s.c. administration of soluble peptide (Ingulli et al., 2002; Itano et al., 2003), and that $CD8\alpha^+$ DC also efficiently present processed peptide to $CD8^+$ T cells after i.v. administration of protein (Pooley et al., 2001). Co-administration of Ag and LPS has been shown to induce DC migration from the splenic marginal zone into T cell areas, enhancing expression of processed peptides on both $CD8\alpha^+$ and $CD8\alpha^-$ DC (Reis e Sousa and Germain, 1999), which express TLR-4 at similar levels (Boonstra et al., 2003). It remains to be

investigated which subset of DC presents peptide after i.v. administration, although unpublished data from our laboratory suggests that both $CD8\alpha^+$ and $CD8\alpha^-$ ($CD4^+$) DC do so (S. Anderton, personal communication).

Administration of Ag and LPS consistently resulted in a small decrease of $CD8\alpha^+$ DC in the spleen, while administration of Ag alone resulted in an increase of $CD8\alpha^+$ DC in the spleen. The $CD8\alpha^+$ subset of DC has previously been implicated in down-regulating $CD8^+$ T cell responses (Belz et al., 2002; Kronin et al., 1996). The turnover of all subsets in the spleen is between 1.5 and 2.9 days, with the $CD8\alpha^+$ DC showing the fastest turnover rate after administration of LPS (Kamath et al., 2000), which agrees with data shown here. Although one may suggest that the decrease in $CD8\alpha^+$ DC after administration of pOVA and LPS is due to preferential $CD8\alpha^+$ DC death and subsequent loss from the spleen, a mechanism by which the number of $CD8\alpha^+$ DC would increase after administration of soluble Ag alone is more difficult to explain. The changes in percentages of $CD8\alpha^+$ versus $CD8\alpha^-$ DC observed here are very small, and the significance remains to be investigated.

Whereas the differences in expression of CD40 and RANK on DC during the induction of immunity versus tolerance were very striking, less obvious differences were evident in costimulatory molecule expression by T cells during the two processes. The general timing of expansion and subsequent loss of Ag-reactive cells after i.v. administration of soluble Ag correlates with previous reports (Kearney et al., 1994). These earlier studies compared T cell expansion after i.v. administration of Ag without adjuvant to administration of Ag in CFA, while this chapter compared administration of soluble peptide alone with co-administration of peptide and LPS. Greater T cell numbers were consistently observed at early time-points after administration of pOVA alone, compared to co-administration of pOVA and LPS. Using a TCR transgenic transfer system, it has been reported that for up to 42 hours after administration of Ag, T cells were difficult to detect by flow cytometry, although histology data showed that the cells were present in the organs examined

(Maxwell et al., 2004). This "loss" of T cells could be overcome by collagenase digesting the samples before flow cytometry. This decrease in T cell numbers was evident after both administration of Ag alone, and Ag and adjuvant, while in the system used here, numbers were lower after administration of Ag and adjuvant. Nevertheless, it cannot be excluded that the lower percentage of pOVA-reactive T cells found here at early time-points after pOVA+LPS administration compared to pOVA alone was due to the isolation procedure used, which did not include a collagenase digest.

Previous data suggests that CD40 ligation acts to greatly enhance RANK expression on DC (Anderson et al., 1997). Nevertheless, RANK was found to be expressed at similar levels after administration of pOVA+LPS in B6 as well as $CD154^{-/-}$ mice. The levels of RANK expression observed in the steady state of B6 and $CD154^{-/-}$ mice were comparable to those described previously (Williamson et al., 2002). While CD154 and OX40 were found for longer and at higher levels during the induction of immunity, RANKL expression was found at higher levels and at earlier time-points during the induction of tolerance compared to induction of immunity. RANKL expression was barely detectable in $CD154^{-/-}$ and B cell-deficient mice during the induction of immunity, while it was clearly present during the induction of tolerance. B cells and CD154 are thus not absolutely required for RANKL expression. Identification of a costimulatory molecule with positive correlation with tolerance induction was surprising, and it is intriguing to speculate about what significance the early RANKL expression during tolerance may have. This is further discussed in Chapter 6.

Chapter 3 highlighted the importance of CD40 ligation as a checkpoint in the decision between immunity and tolerance through the successful prevention of tolerance induction with anti-CD40, comparable to data obtained in other recent studies investigating $CD4^+$ T cell tolerance (Hawiger et al., 2001). This chapter further shows that exogenous CD40 ligation increased expression of CD80, CD86 and OX40L on DC. A similar approach (administration of anti-CD40 i.v.), has

previously been reported to result in upregulation of CD80 and CD86 (Chung et al., 2004). However, this upregulation was already seen by 24 hours after administration of antibody, compared to 48 hours in the results shown here, and also employed a model of mucosal tolerance, in which administration of anti-CD40 did not prevent tolerance induction. The discrepancy in timing may have been due to i.v. versus i.p. administration of the antibody, while inability to prevent tolerance may be due to differential regulation of tolerance in different peripheral sites.

Although CD40 ligation resulted in CD80 and CD86 upregulation, its effect on OX40L expression was not investigated previously (Chung et al., 2004). OX40L was here found to be expressed after exogenous ligation of CD40, but not after administration of Ag with LPS. One explanation may be that only DC receiving a CD40 signal would upregulate OX40L. After administration of Ag in LPS, only DC in contact with the appropriate Ag-reactive (i.e. $CD154^{+ve}$) T cells should upregulate OX40L, and the number of those DC was too small to be traceable during FACS analysis. After administration of anti-CD40 however, a larger number of DC will have been given a signal through CD40 and upregulation of OX40L was seen at days 2 and 3 after ligation. If OX40L is first upregulated at these later time-points after CD40 ligation, this would provide an explanation for the lack of OX40L staining on histology sections, which was carried out at 12 hours, i.e. at an earlier time-point, after Ag administration.

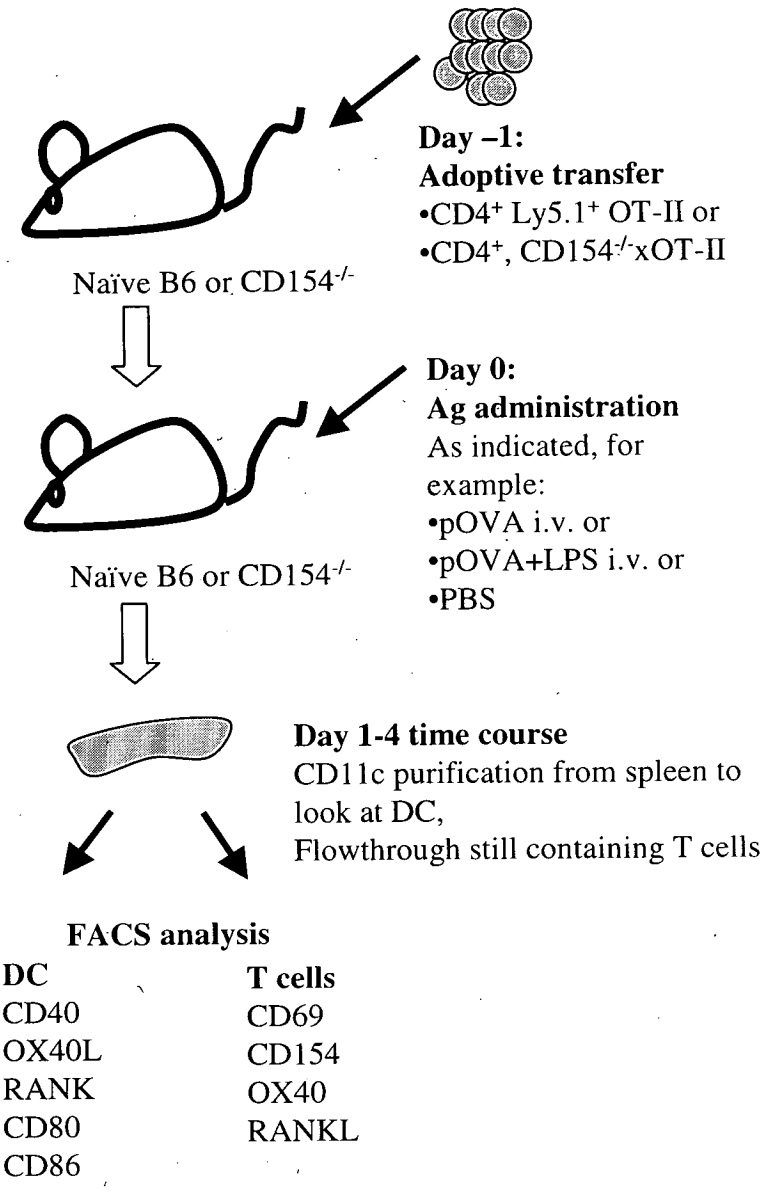

Fig. 4.1:
Experimental outline of experiments investigating T cell and DC phenotypes during the induction of T cell tolerance versus the induction of T cell immunity
Naïve mice received a transfer of pOVA-reactive T cells. One day later, mice were tolerised or immunised with pOVA, and the phenotype of T cells and DC was assessed 1, 2, 3 and 4 days after pOVA administration.

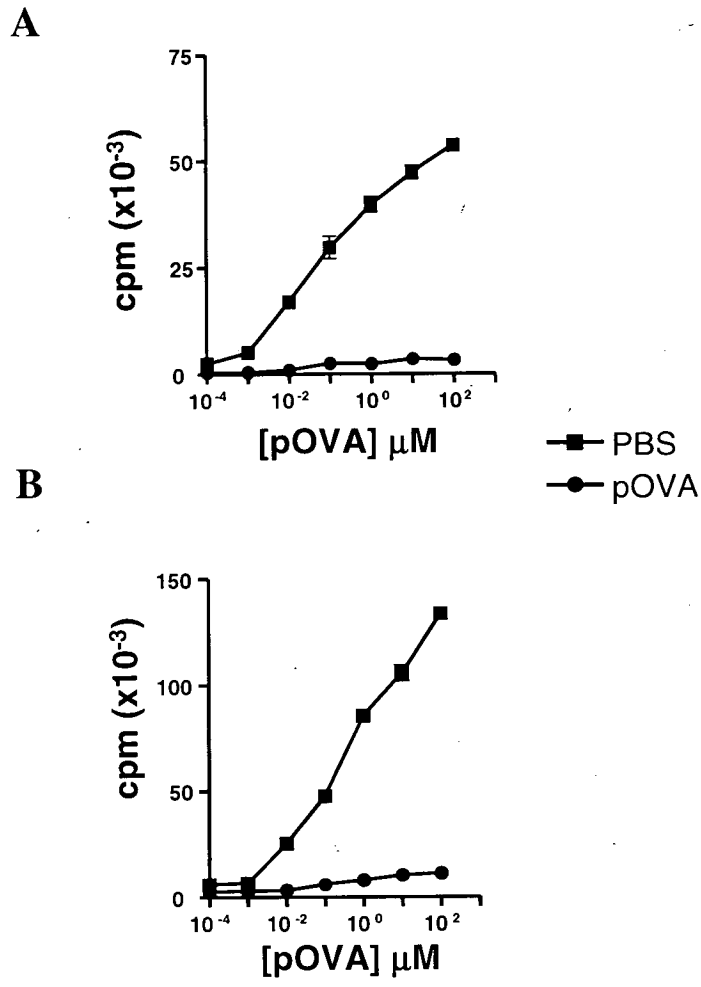

Fig. 4.2:
Tolerance induction in naïve B6 mice versus B6 mice after adoptive transfer of pOVA-reactive cells
A: Naïve B6 mice received a single dose of 500µg pOVA or PBS i.v. on day 0, followed by 20µg pOVA/CFA on day 7, and lymphoid populations (draining lymph nodes shown here) assessed 10 days later for proliferative responses to a dose range of pOVA.
B: B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA i.v. Mice received a single dose of 500µg pOVA or PBS i.v. on day 0, followed by 20µg pOVA/CFA on day 7, and lymphoid populations (draining lymph nodes shown here) were assessed on day 17 for proliferative responses to a dose range of pOVA. Background cpm were as those seen with 10^{-4} µM pOVA.
These data are from one of three experiments giving consistent results.

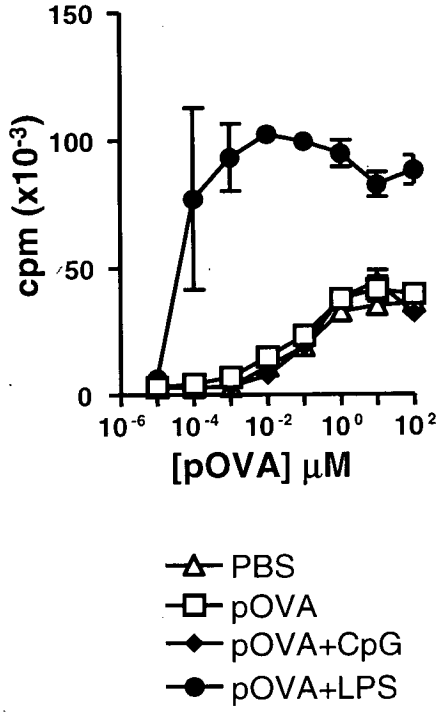

Fig. 4.3:
Co-administration of LPS (but not CpG) leads to T cell immunity
B6 mice received Ly5.1$^+$ OT-II cells on day –1, one day prior to administration of pOVA. Mice received either 500µg pOVA, PBS, 500µg pOVA with 30µg CpG, or 500µg pOVA with 30µg LPS (all i.v.) day 0, and splenic populations were assessed 7 days later for proliferative responses to a dose range of pOVA. Background cpm were as follows: PBS 2304, pOVA 2932, pOVA+CpG 2818, pOVA+LPS 6245. These data are from one of two experiments giving consistent results.

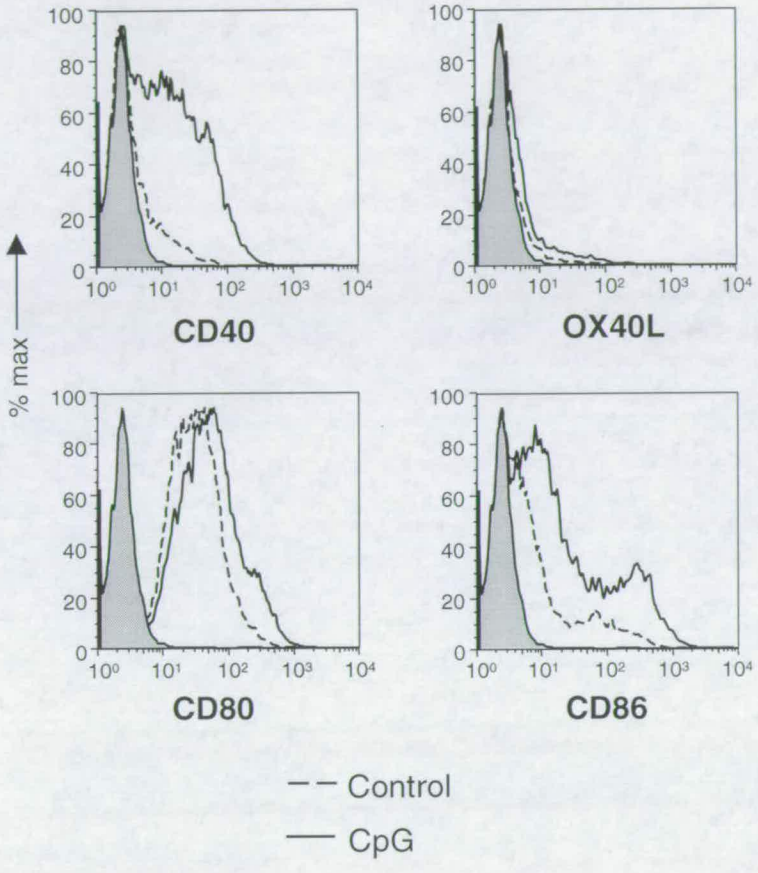

Fig. 4.4:
Phenotype of DC after overnight culture with CpG
B6 bone marrow cells were grown for 7 days in the presence of GM-CSF, cultured with 6µg/ml CpG overnight at 37°C, and stained for CD40, OX40L, CD80 and CD86. Filled histograms are $CD11c^+$ cells stained with an isotype control antibody. These data are from one of two experiments giving consistent results.

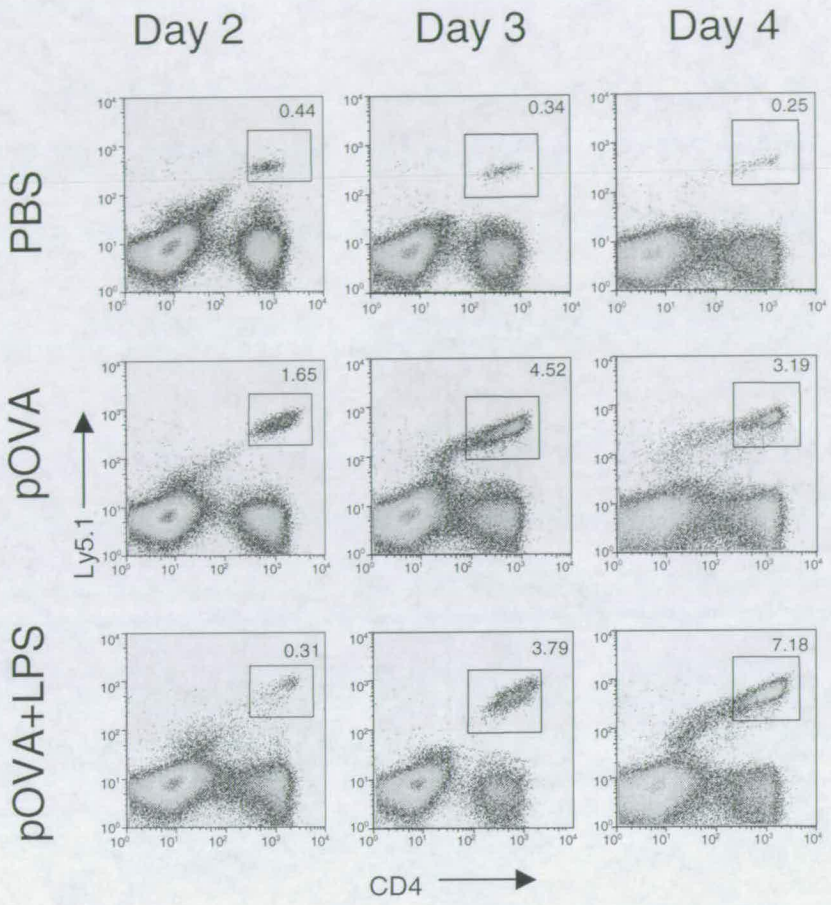

Fig. 4.5:
Expansion of pOVA-reactive CD4$^+$ T cells after administration of pOVA or pOVA+LPS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 2, 3 and 4 post Ag administration, spleen cells were analysed by FACS for Ly5.1 and CD4 expression. Percentages of total cells that were CD4$^+$, Ly5.1$^+$, are shown. These data are from one of eight experiments giving consistent results.

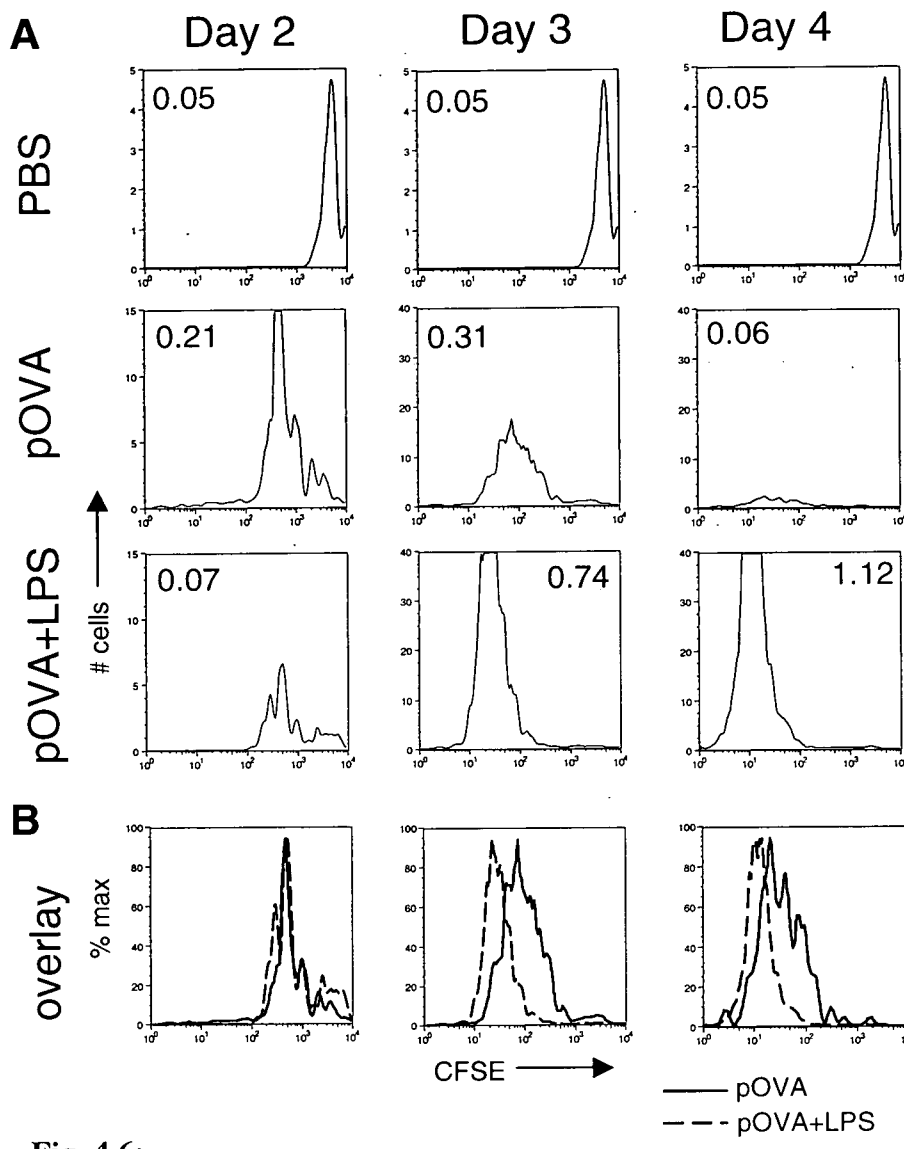

Fig. 4.6:
CFSE profile of pOVA-reactive CD4⁺ T cells after administration of pOVA or pOVA+LPS
B6 mice received CFSE-labelled Ly5.1⁺ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 2, 3 and 4 post Ag administration, spleen cells were analysed by FACS for Ly5.1 and CD4 expression. A: CFSE profiles and percentages of total cells that are CD4⁺, Ly5.1⁺, are shown. B: The overlay allows comparison of number of cell divisions undergone after administration of pOVA or pOVA+LPS. These data are from one of three experiments giving consistent trends for division of pOVA-reactive cells.

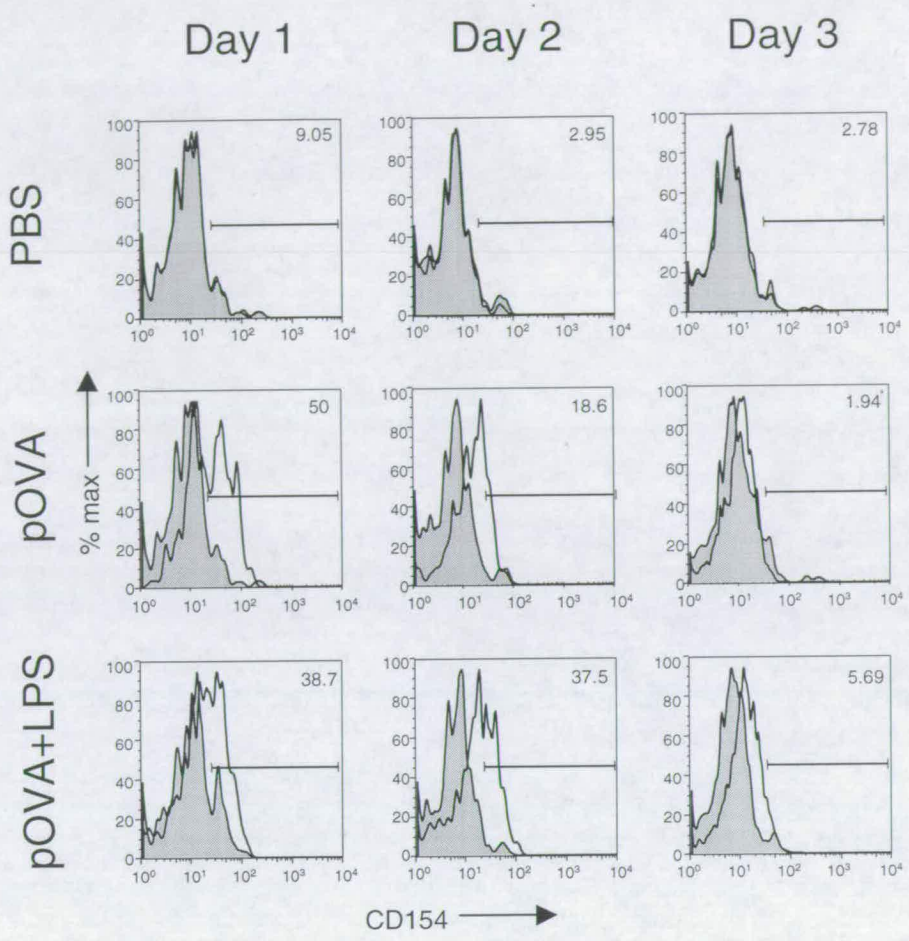

Fig. 4.7:
Tolerance is not due to the absence of CD154 on T cells
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for CD154 expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for CD154 are shown on each graph. These data are from one of two experiments giving consistent results.

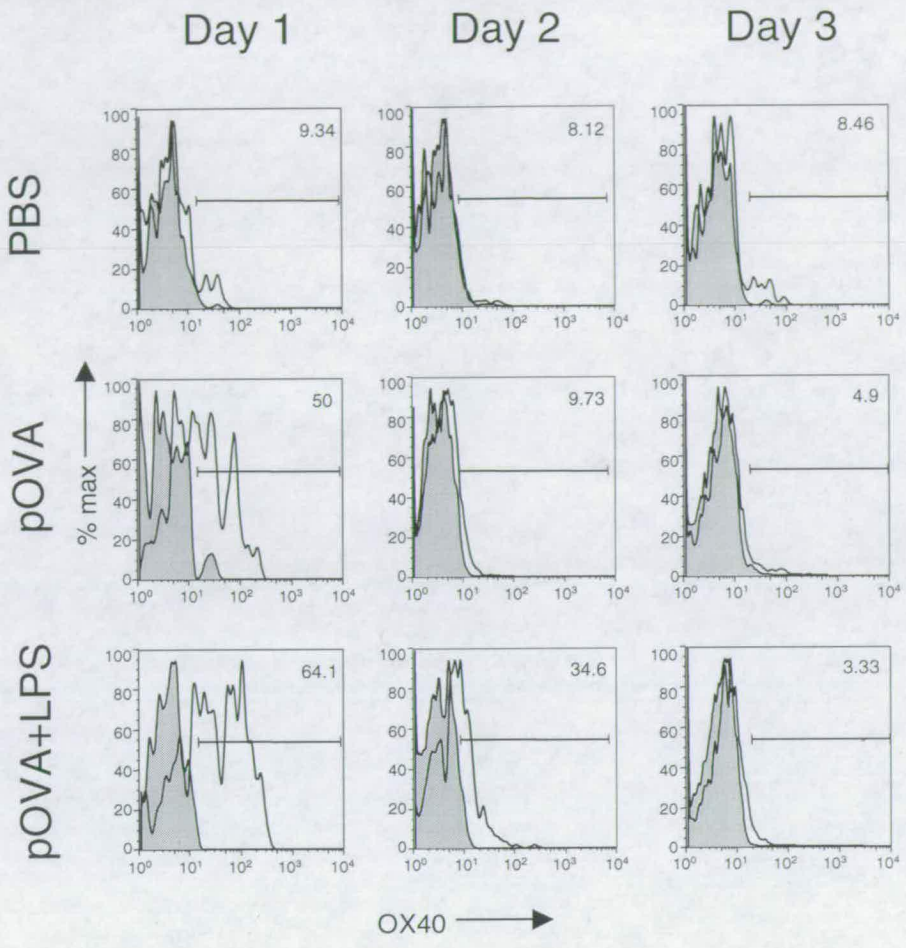

Fig. 4.8:
Tolerance is not due to the absence of OX40 on T cells
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for OX40 expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for OX40 are shown on each graph. These data are from one of eight experiments giving consistent results.

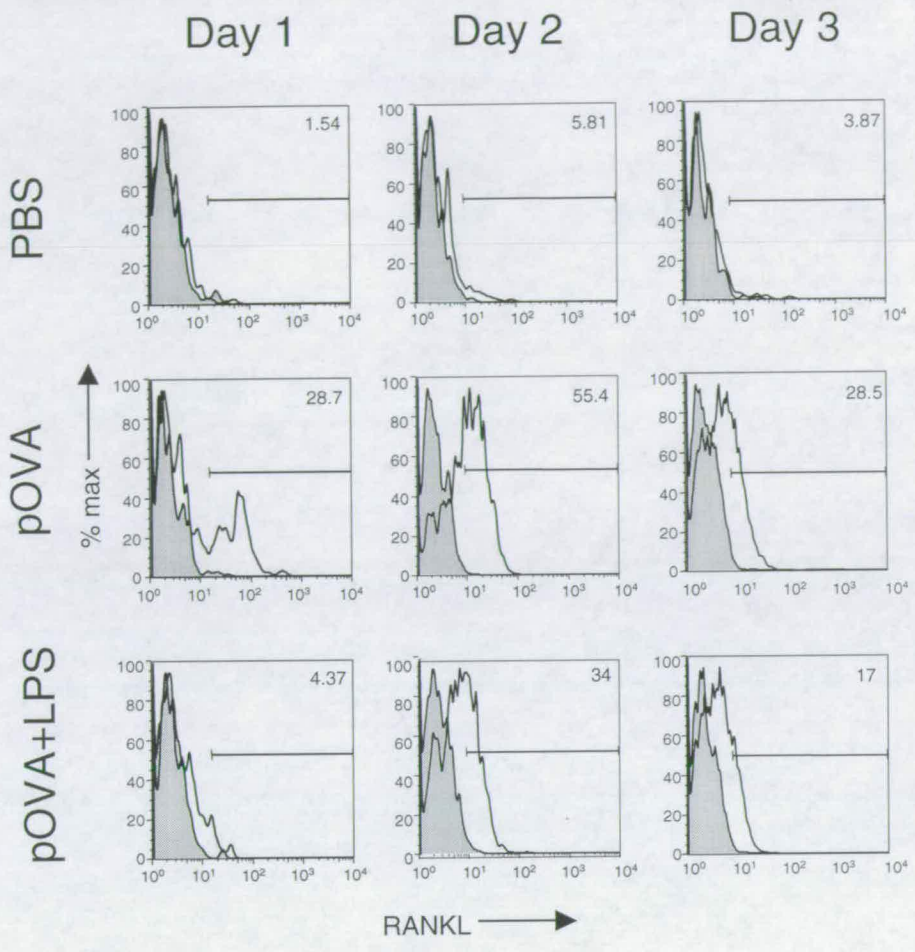

Fig. 4.9:
Tolerance induction results in rapid expression of RANKL on T cells

B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for RANKL expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for RANKL are shown on each graph. These data are from one of four experiments giving consistent results.

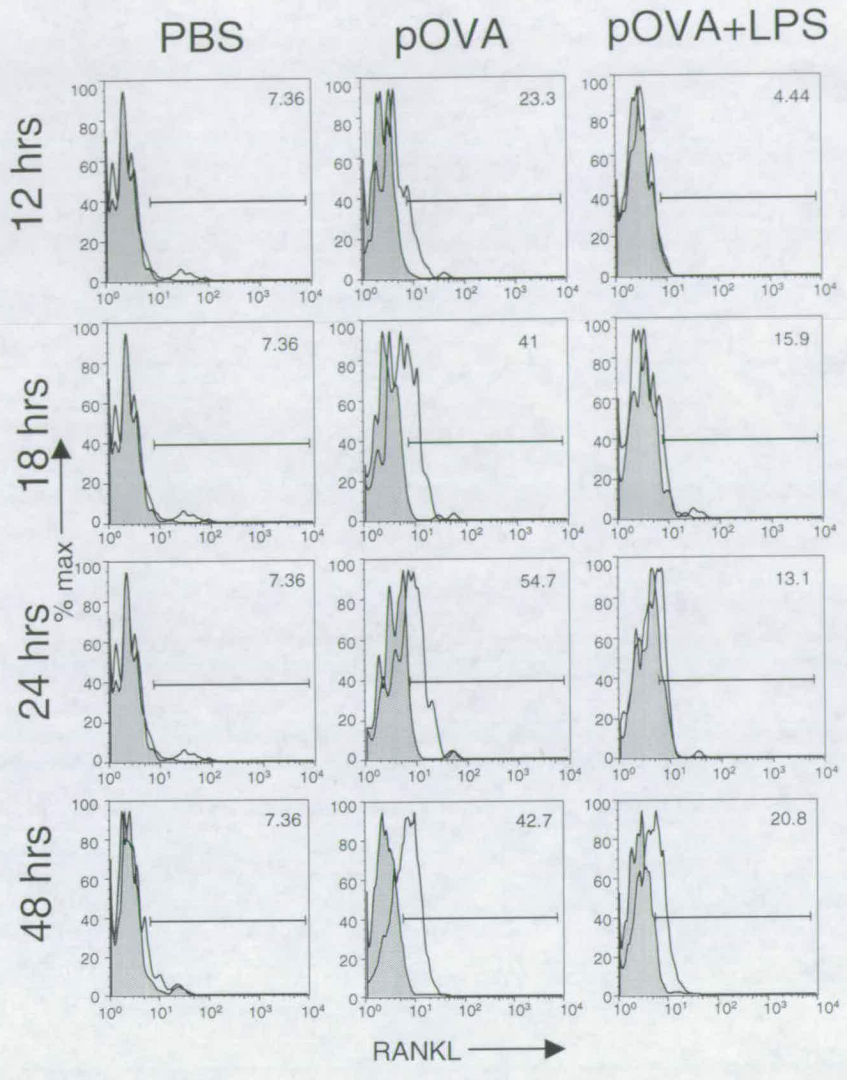

Fig. 4.10:
Detailed time course analysis of RANKL expression on T cells during induction of tolerance versus immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
12, 18, 24 and 48 hours post Ag administration, spleen cells were analysed by FACS for RANKL expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for RANKL are shown on each graph. These data are from one of two experiments giving consistent results.

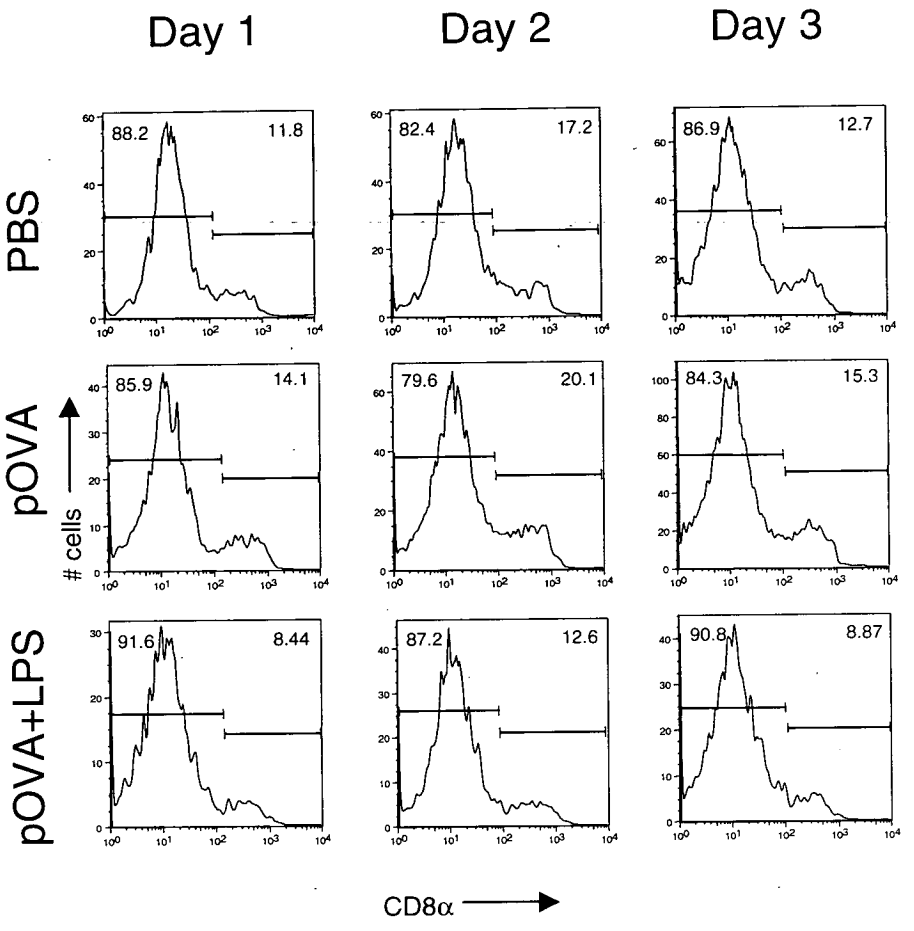

Fig. 4.11:
CD8α expression on DC after administration of pOVA, pOVA+LPS or PBS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS. On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD8α expression on days 1, 2 and 3. Percentages shown are CD8α$^+$ DC vs. CD8α$^-$ DC.These results are from one of eight experiments giving consistent results.

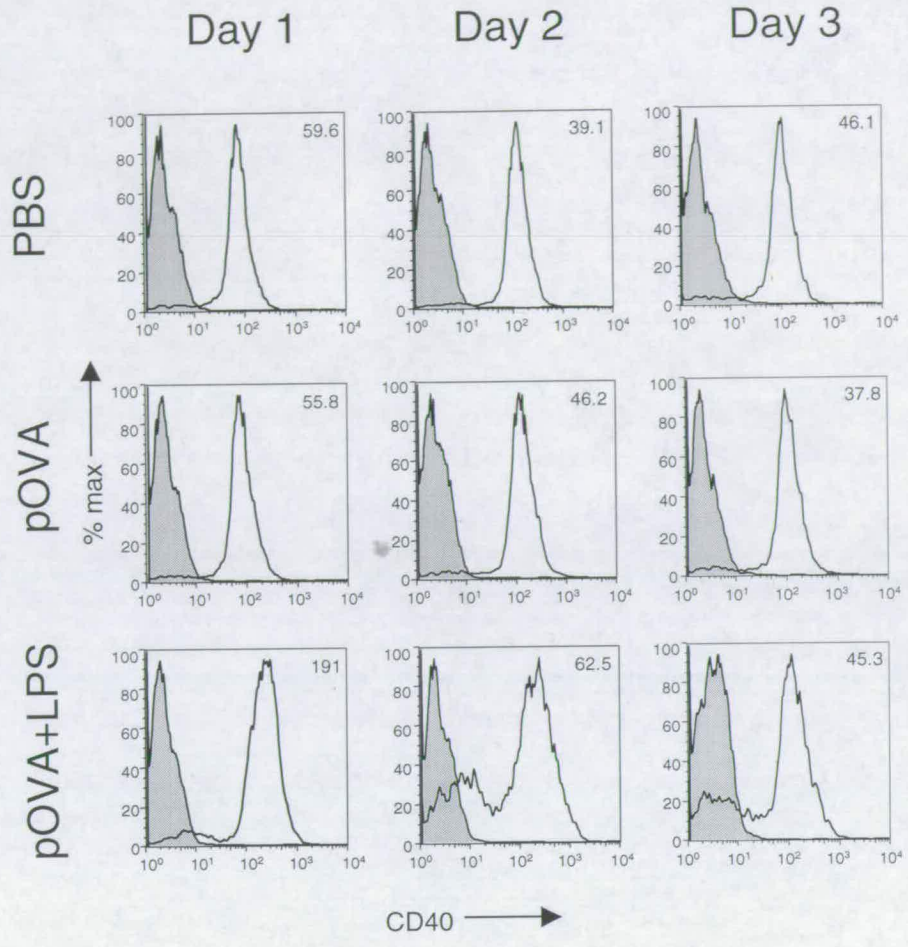

Fig. 4.12:
CD40 expression is upregulated on DC during the induction of immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD40 expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of CD40 stained DC are shown on each graph. These data are from one of six experiments giving consistent results.

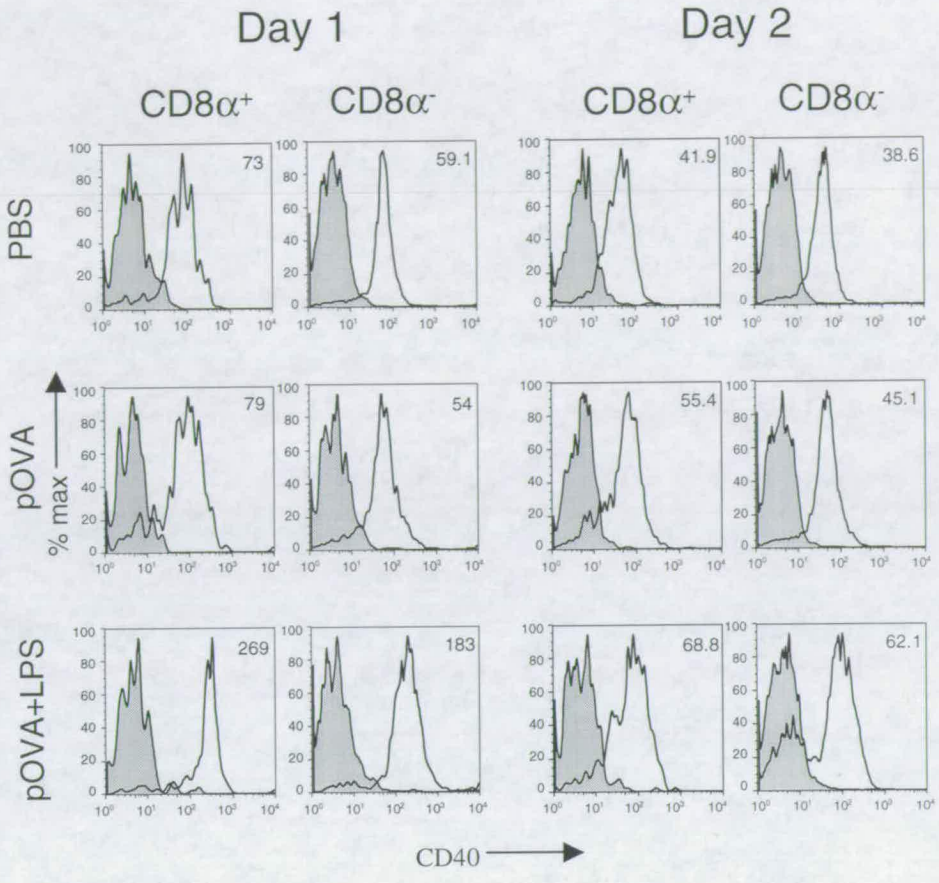

Fig. 4.13:
CD40 expression on CD8α$^+$ versus CD8α$^-$ DC during the induction of tolerance or immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1 and 2 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD8α and CD40 expression on days 1 and 2 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of CD40 stained DC are shown on each graph. These data are from one of six experiments giving consistent results.

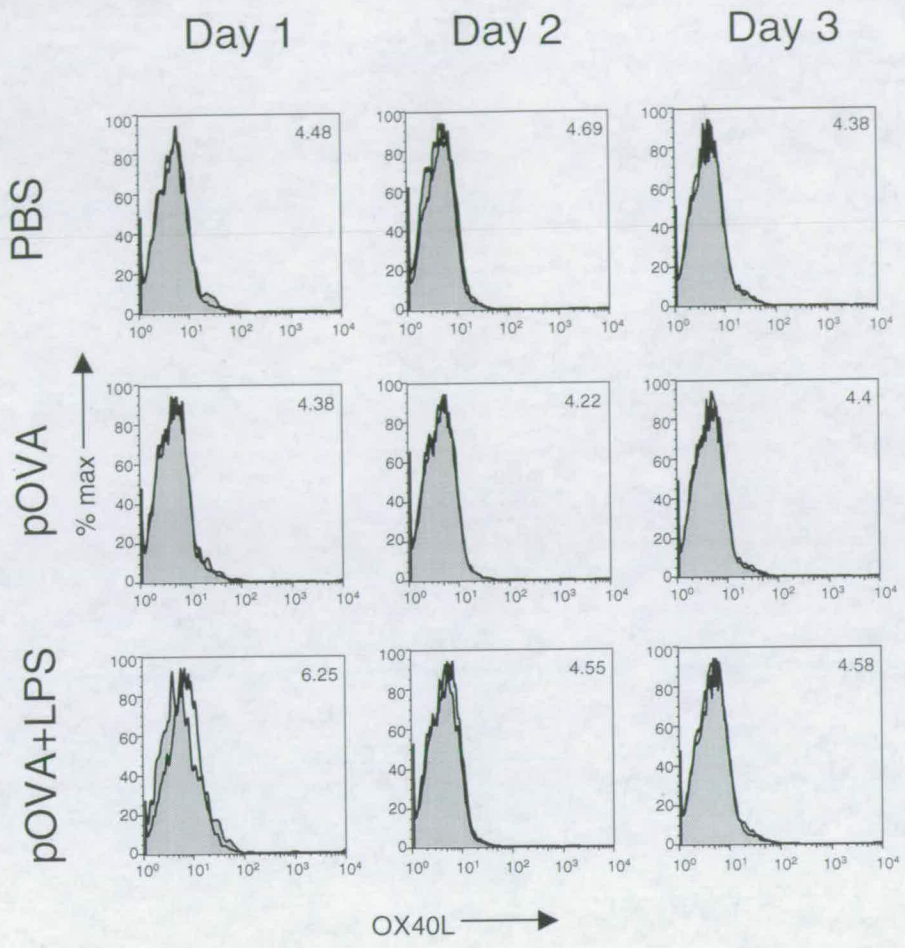

Fig. 4.14:
OX40L expression is not detectable on DC during the induction of tolerance or immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA or pOVA+LPS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for OX40L expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of OX40L stained DC are shown on each graph. These data are from one of several experiments giving consistent results.

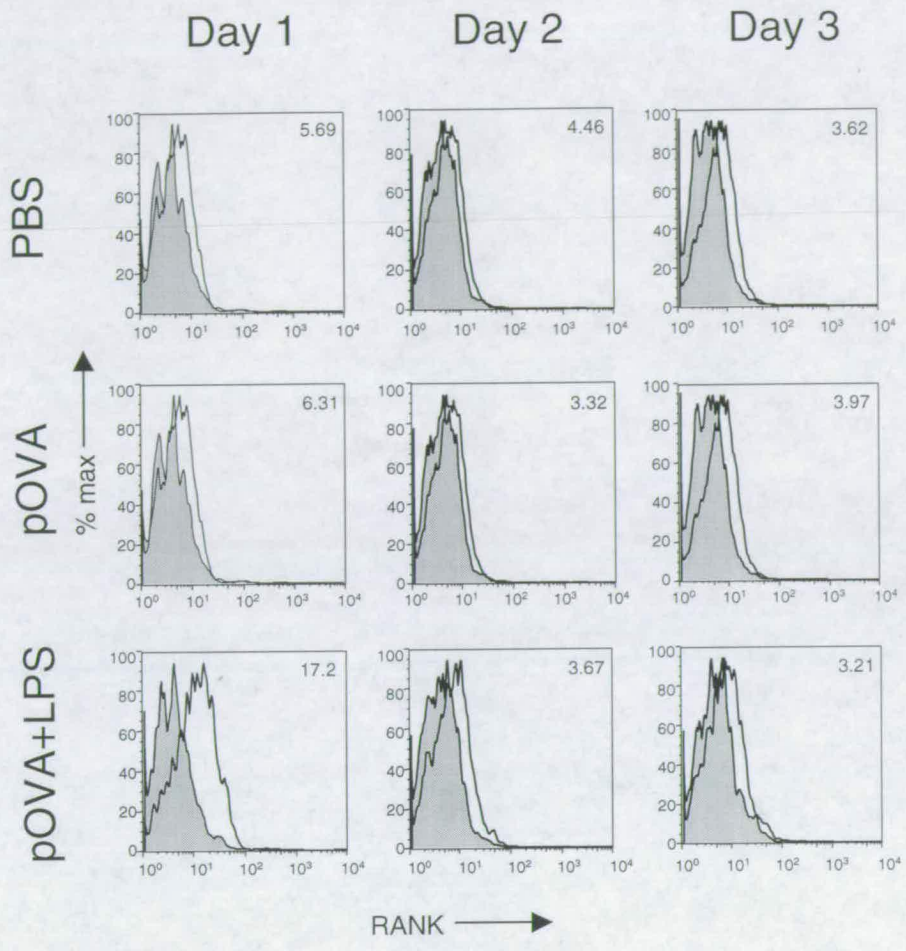

Fig. 4.15:
RANK expression is upregulated on DC during the induction of immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA or pOVA+LPS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen.CD11c$^+$ cells were analysed by FACS for RANK expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of RANK stained DC are shown on each graph. These data are from one of four experiments giving consistent results.

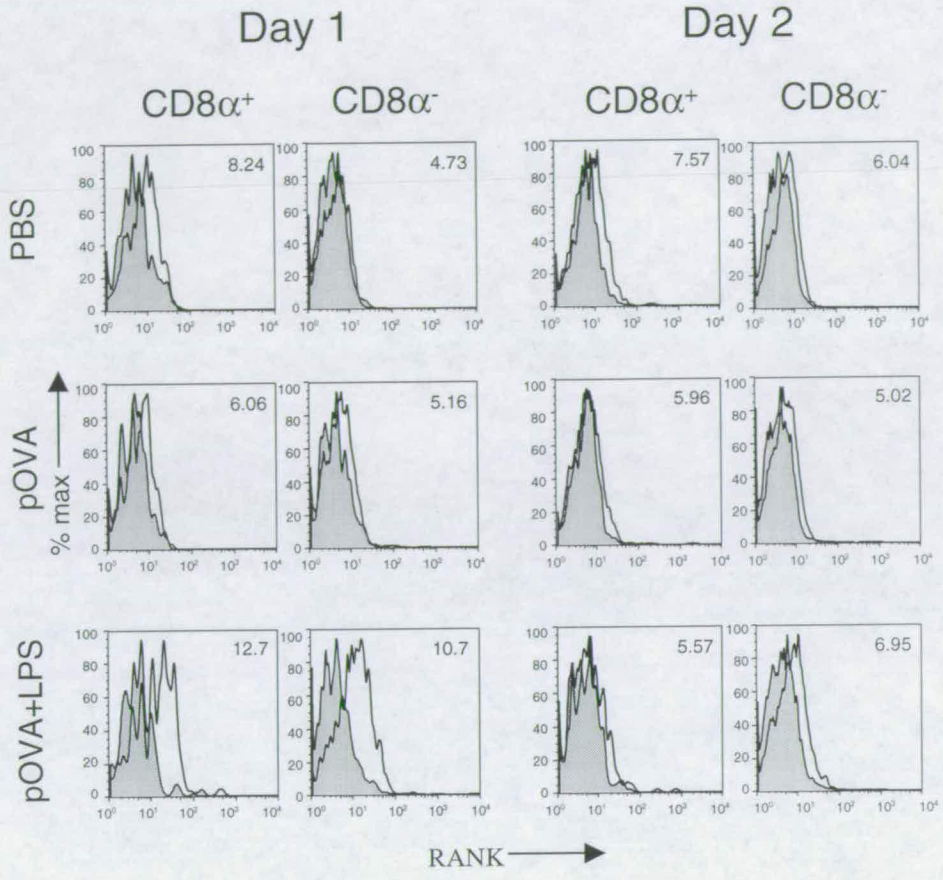

Fig. 4.16:
RANK expression on CD8α+ versus CD8α- DC during the induction of tolerance or immunity
B6 mice received Ly5.1+ OT-II cells one day prior to administration of pOVA or pOVA+LPS.
On days 1 and 2 post Ag administration, CD11c+ DC were purified from the spleen.CD11c+ cells were analysed by FACS for CD8α and RANK expression on days 1 and 2 (filled histograms are CD11c+ cells stained with an isotype control antibody). Median fluorescence values of RANK stained DC are shown on each graph. These data are from one of four experiments giving consistent results.

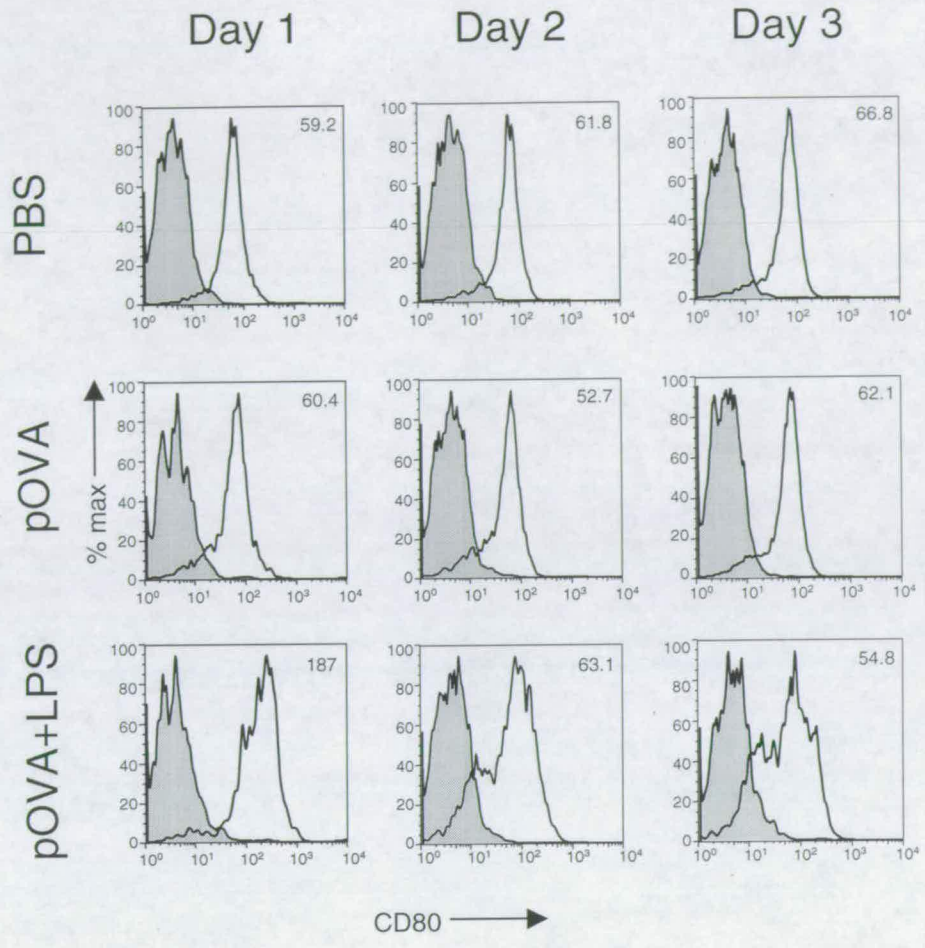

Fig. 4.17:
CD80 expression is upregulated on DC during the induction of immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD80 expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of CD80 stained DC are shown on each graph. These data are from one of six experiments giving consistent results.

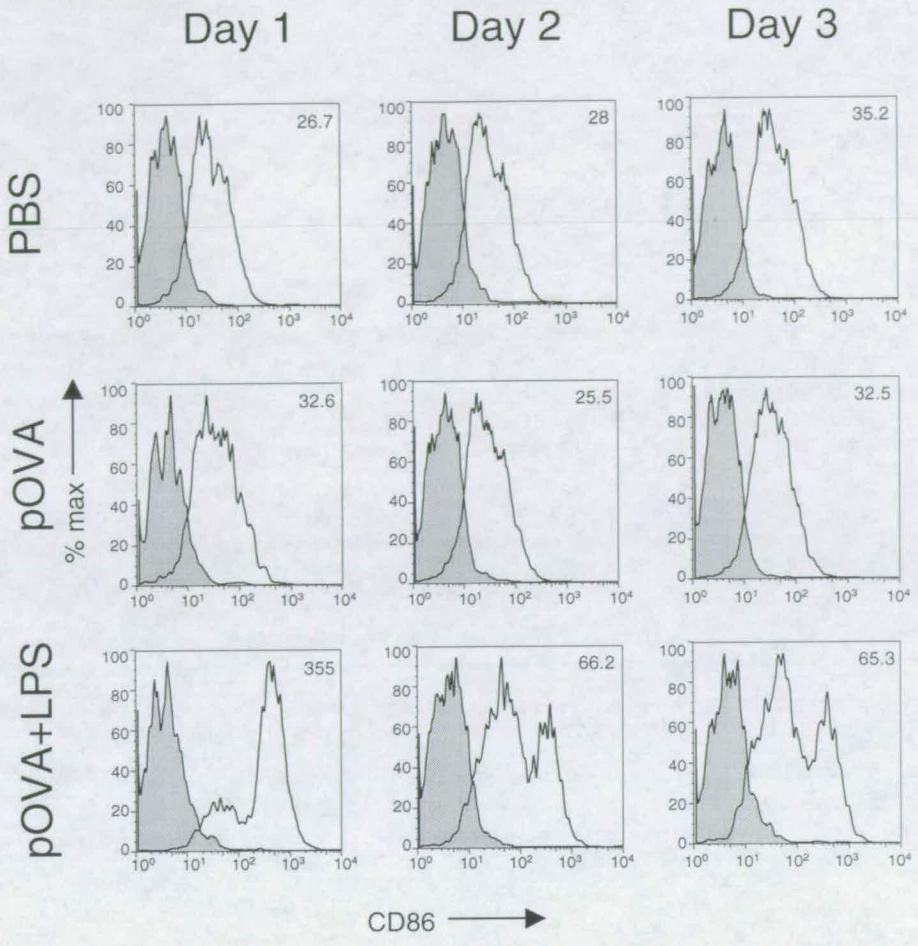

Fig. 4.18:
CD86 expression is upregulated on DC during the induction of immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD86 expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of CD86 stained DC are shown on each graph. These data are from one of five experiments giving consistent results.

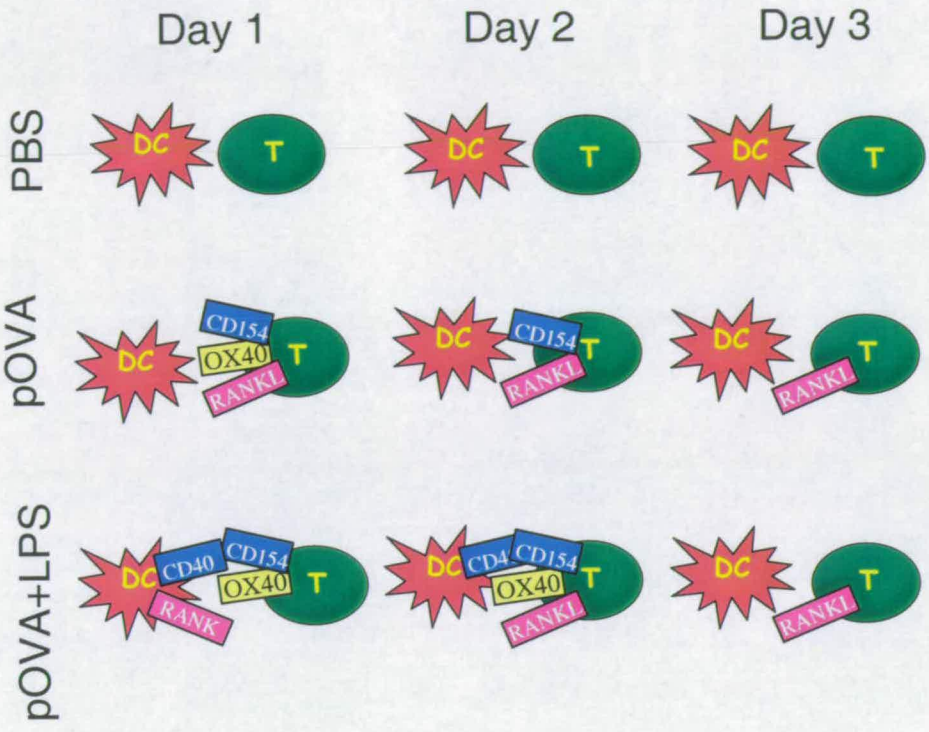

Fig. 4.19:
Summary of costimulatory molecule expression on DC and T cells during induction of tolerance or immunity
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen, and CD11c$^+$ cells were analysed by FACS for CD40, OX40L and RANK expression. Spleen cells were also analysed by FACS for CD154, OX40 and RANKL expression on Ly5.1$^+$ CD4$^+$ OT-II cells. These data are a summary from four experiments giving consistent trends of the timing of expression of these costimulatory molecules.

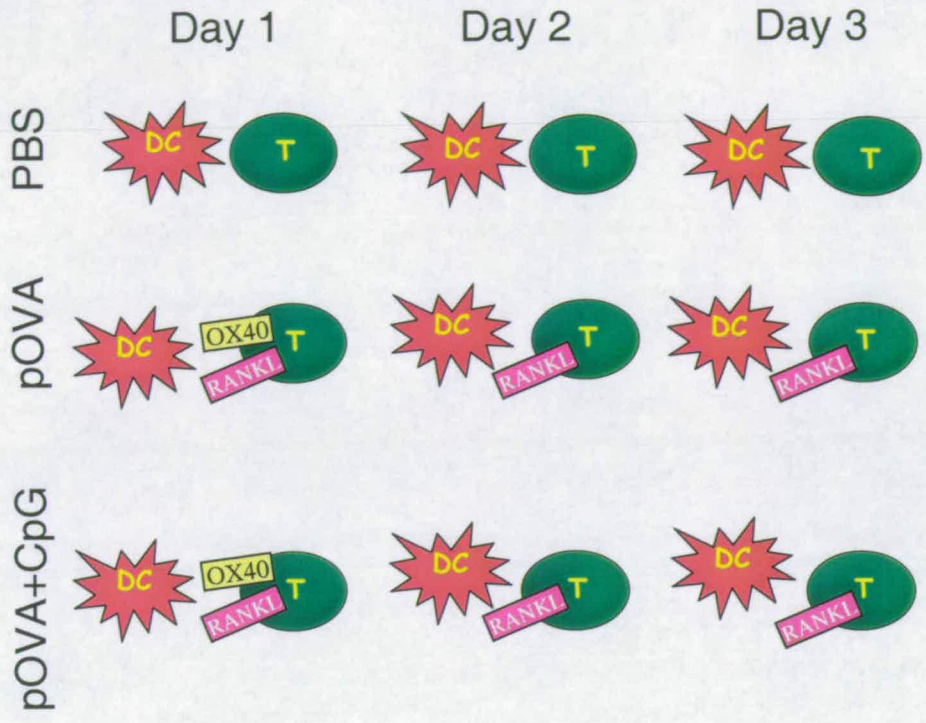

Fig. 4.20:
Summary of costimulatory molecule expression on DC and T cells after i.v. administration of pOVA alone, or pOVA+CpG
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+CpG or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen, and CD11c$^+$ cells were analysed by FACS for CD40, OX40L and RANK expression on days 1, 2 and 3. Spleen cells were also analysed by FACS for OX40 and RANKL expression by Ly5.1$^+$ CD4$^+$ OT-II cells. These data are a summary from three experiments giving consistent trends of the timing of expression of these costimulatory molecules. Expression of CD154 was not investigated in these experiments.

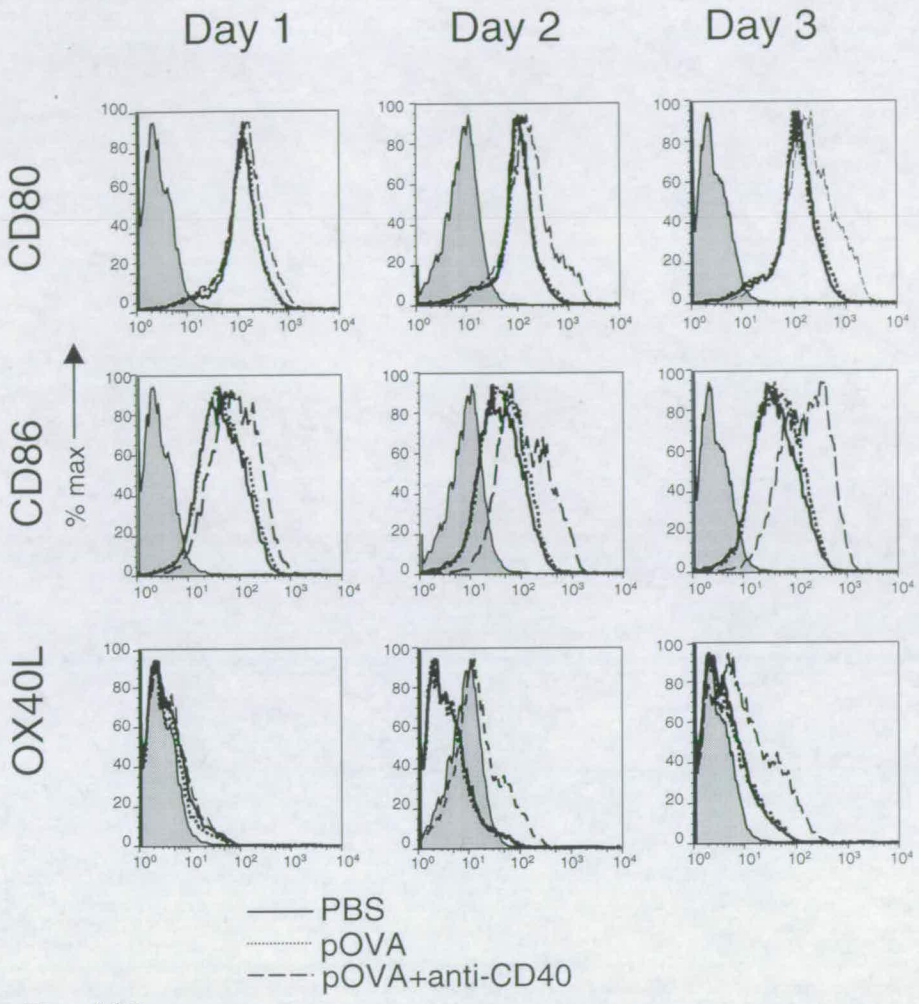

Fig. 4.21:
Expression of CD80, CD86 and OX40L after administration of agonistic anti-CD40 Ab
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA or pOVA+200µg anti-CD40.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen, and cells were analysed by FACS for OX40L, CD80 or CD86 expression on days 1, 2 and 3. Filled histograms are DC from mice that received pOVA+antiCD40, stained with streptavidin-APC alone. These data are from one of two experiments where administration of anti-CD40 successfully prevented the induction of T cell tolerance.

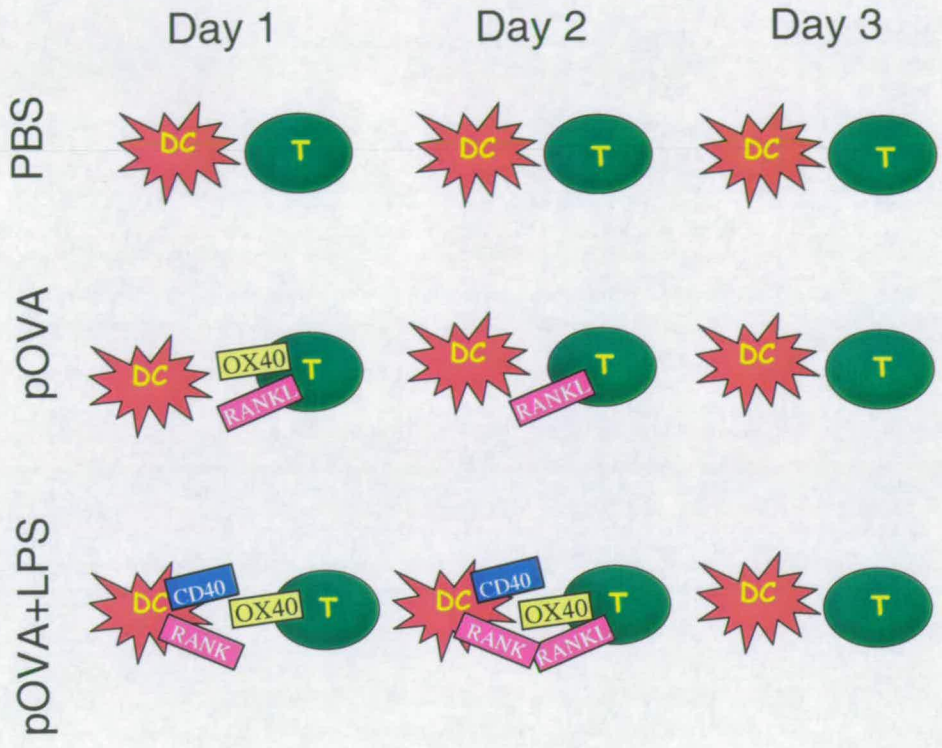

Fig. 4.22:
Summary of costimulatory molecule expression on DC and CD154-deficient T cells during induction of tolerance or immunity
$CD154^{-/-}$ mice received $CD154^{-/-}$xOT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, $CD11c^{+}$ DC were purified from the spleen, and $CD11c^{+}$ cells were analysed by FACS for CD40, OX40L, RANK. Spleen cells were also analysed by FACS for OX40 and RANKL expression by $V\alpha2^{+}$, $V\beta5^{+}$ OT-II cells. These data are a summary from three experiments giving consistent trends of the timing of expression of these costimulatory molecules.

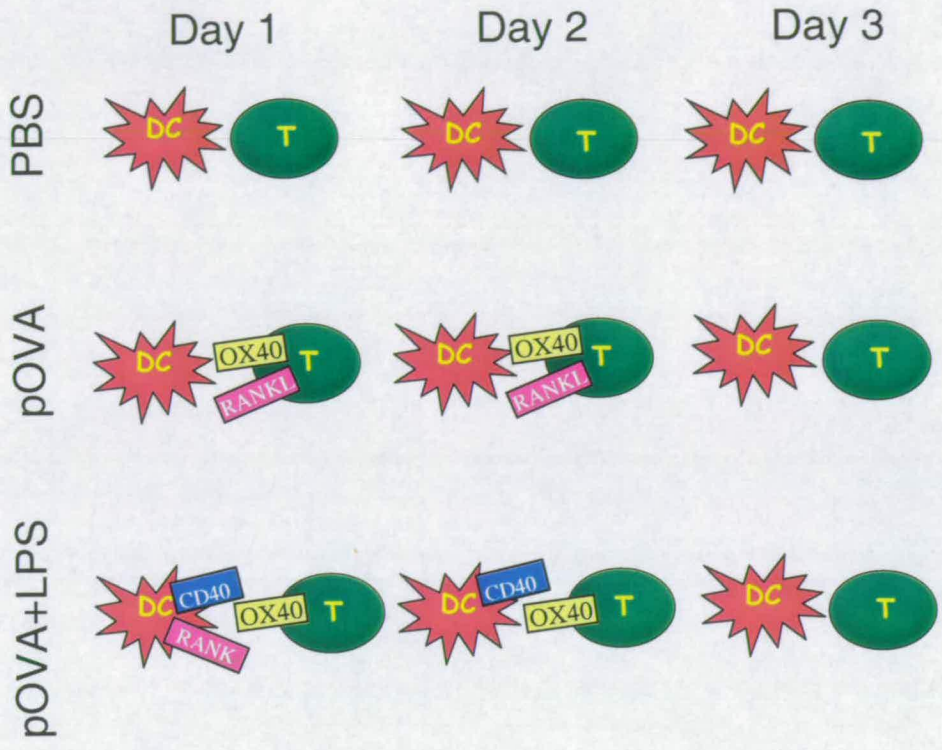

Fig. 4.23:
Summary of costimulatory molecule expression on DC and T cells during induction of tolerance or immunity in B cell deficient mice
μMT mice received OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, $CD11c^+$ DC were purified from the spleen, and $CD11c^+$ cells were analysed by FACS for CD40, OX40L and RANK expression. Spleen cells were also analysed by FACS for OX40 and RANKL expression by $Ly5.1^+$ $CD4^+$ OT-II cells. These data are a summary from two experiments giving consistent trends of the timing of expression of these costimulatory molecules. Expression of CD154 was not investigated in these experiments.

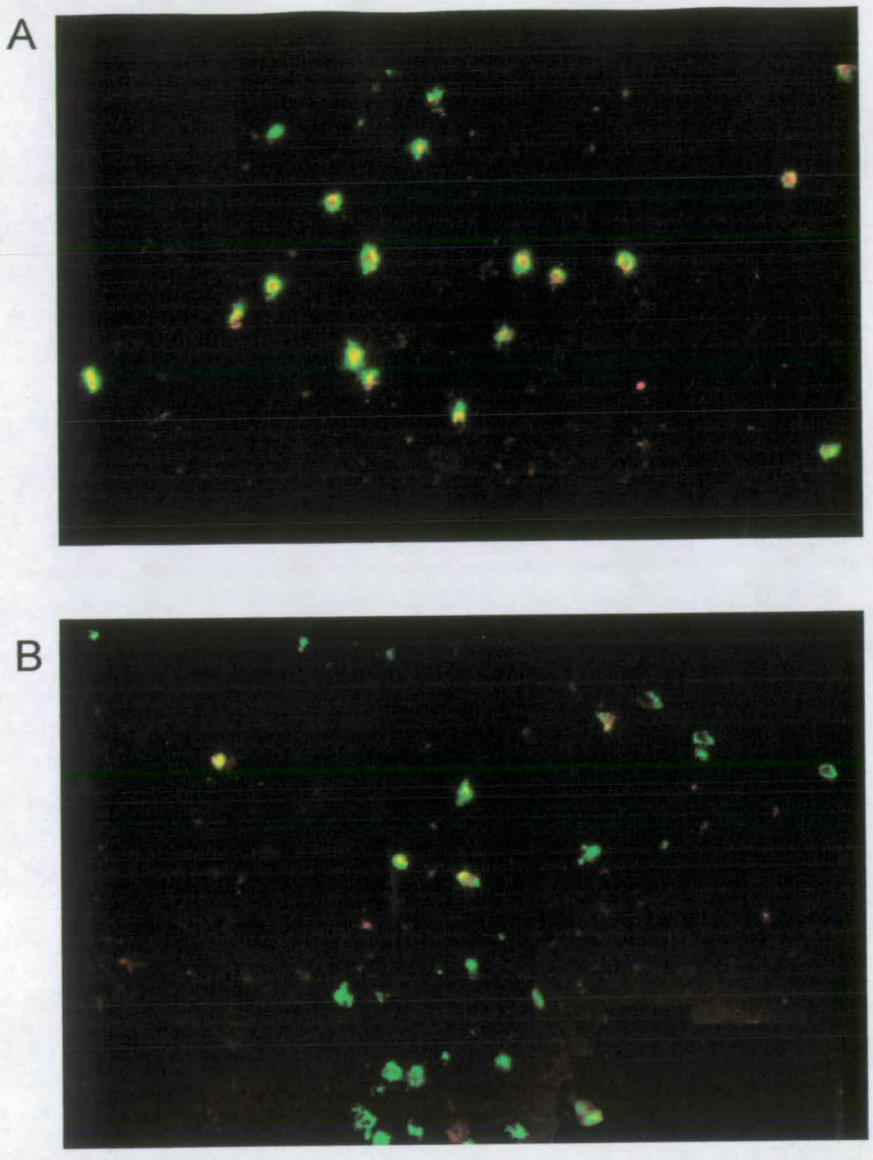

Fig. 4.24:
Identification of CT orange-labelled cells during induction of tolerance of immunity
B6 mice received CT orange-labelled, Ly5.1^{+} OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
12 and 36 hours post Ag administration, mice were sacrificed, samples of the spleen were snap-frozen in liquid nitrogen. CT orange-labelled cells are shown in red, and sections were stained for Ly5.1 (shown in green). Shown are samples from mice 12 hours (A) and 36 hours (B) after administration of pOVA alone. These results are from one preliminary experiment.

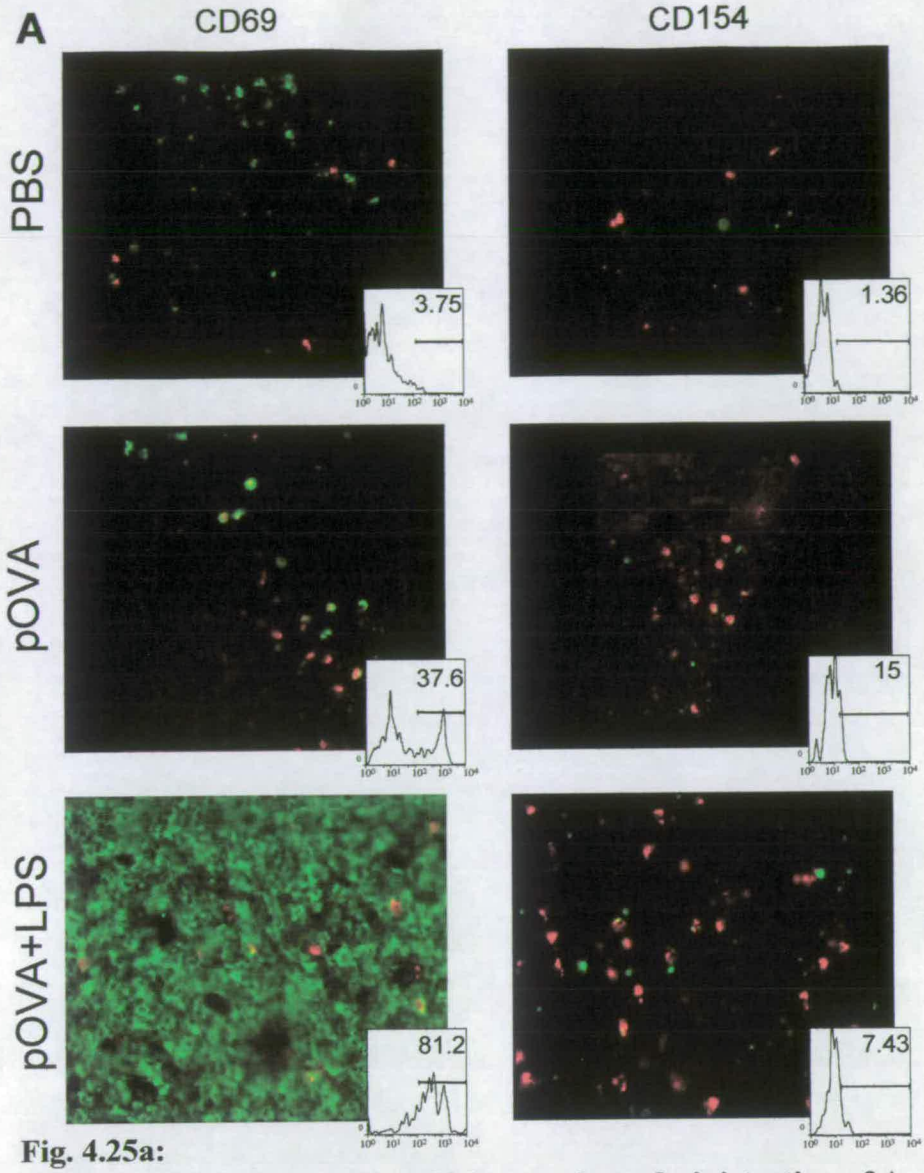

Fig. 4.25a:
CD69 and CD154 expression 12 hours after administration of Ag
B6 mice received CT orange-labelled, Ly5.1^{+} OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
12 post Ag administration, mice were sacrificed,and samples of the spleen were snap-frozen in liquid nitrogen. CT orange-labelled cells are shown in red, and sections were stained for CD69 and CD154 as indicated (in green). Corresponding FACS analysis is shown in lower right corners of pictures. These results are from one preliminary experiment.

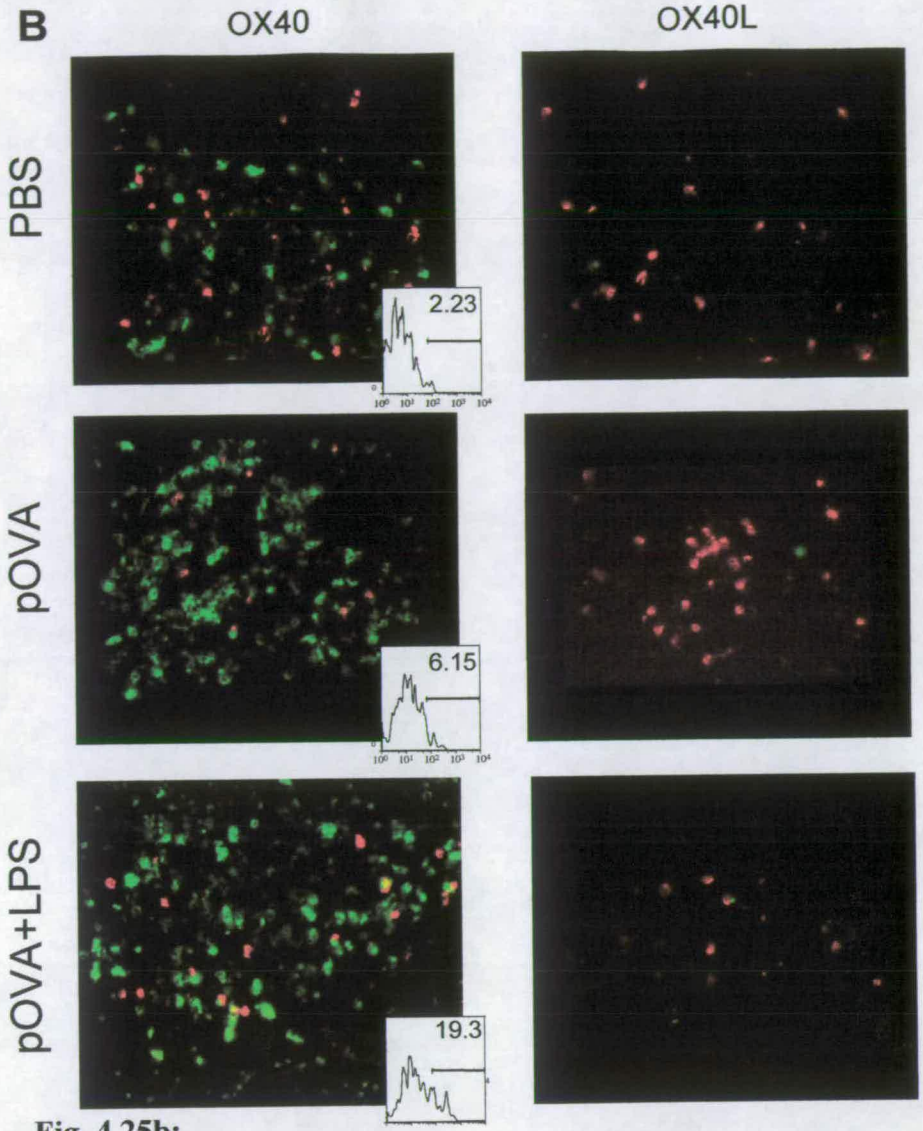

Fig. 4.25b:
OX40 and OX40L expression 12 hours after administration of Ag
B6 mice received CT orange-labelled, Ly5.1^{+} OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
12 post Ag administration, mice were sacrificed,and samples of the spleen were snap-frozen in liquid nitrogen. CT orange-labelled cells are shown in red, and sections were stained for OX40 and OX40L as indicated (in green). Corresponding FACS analysis for OX40 is shown in lower right corners of pictures. These results are from one preliminary experiment.

	CD69		CD154		OX40		OX40L	
	CD69$^+$/ total cells	% CD69$^+$	CD154$^+$/ total cells	% CD154$^+$	OX40$^+$/ total cells	% OX40$^+$	OX40L$^+$/ total cells	% OX40L$^+$
PBS	3/19	**15.8**	3/36	**8.3**	4/61	**6.6**	0/51	**0**
pOVA	16/34	**47.1**	18/70	**25.7**	20/72	**27.8**	0/93	**0**
pOVA+LPS	14/21	**66.6**	33/119	**27.7**	34/116	**29.3**	1/62	**0**

Fig. 4.26:
Summary of expression of costimulatory molecules as seen through histology
B6 mice received CT orange-labelled, Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
12 hours post Ag administration, mice were sacrificed, samples of the spleen were snap-frozen in liquid nitrogen. Tissue sections were stained for CD69, CD154, OX40 and OX40L. Pictures were taken, and CT$^+$ cells were analysed for expression of these markers. The numbers shown are marker$^+$ cells out of CT$^+$ cells (or in the case of OX40L, very little OX40L staining was observed), and percentage of marker$^+$ cells calculated from these counts are shown in bold. These data are from one preliminary experiment.

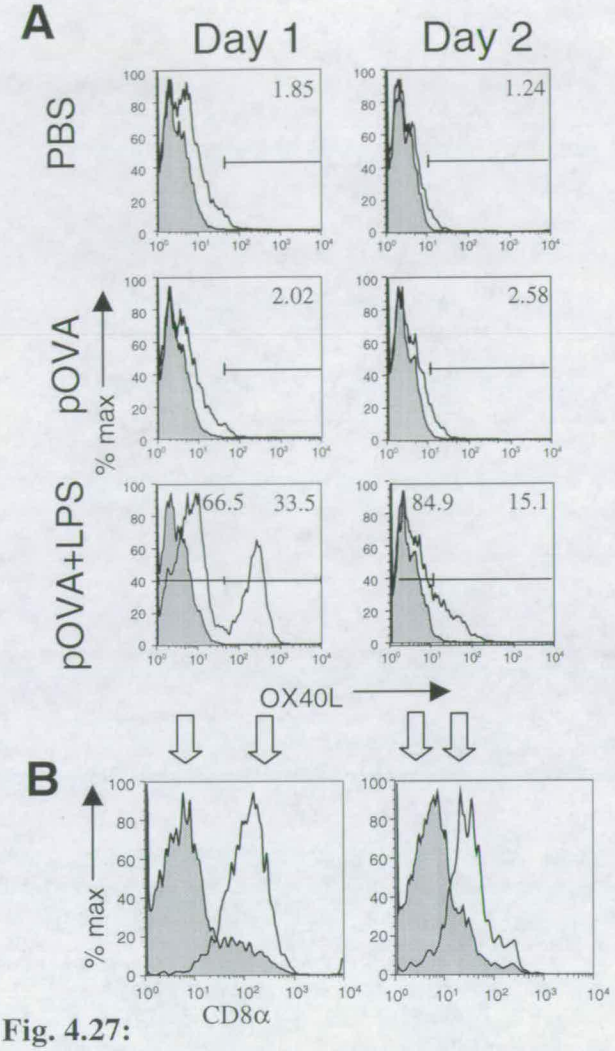

Fig. 4.27:
Induction of OX40L on CD8α⁺ DC by an unidentified compound
B6 mice received Ly5.1⁺ OT-II cells one day prior to administration of pOVA, pOVA+contaminated LPS or PBS. Shown are days 1 and 2 only.
A: On days 1, 2 and 3 post Ag administration, CD11c⁺ DC were purified from the spleen. CD11c⁺ cells were analysed by FACS for OX40L expression on days 1, 2 and 3 (filled histograms are CD11c⁺ cells stained with streptavidin-APC alone). Median fluorescence values of OX40L stained DC are shown on each graph. Shown are days 1 and 2.
B: CD8α expression is examined in the pOVA+contaminated LPS group on OX40L⁺ cells or OX40L⁻ (filled histograms are from OX40L⁻, empty lines OX40L⁺). These data are from one of three experiments giving consistent results.

5. CHAPTER 5 – Systemic administration of antigen-loaded CD40-deficient DC mimics peptide-induced tolerance

5.1. Introduction

The previous chapters provided evidence for the importance of the CD40-CD154 interaction in the induction of T cell immunity. Administration of pOVA and LPS, leading to effective immunity, resulted in increased expression of CD40 on DC. Exogenous ligation of CD40 induced expression of CD80, CD86 and OX40L on DC, and prevented the induction of T cell tolerance. This indicates that low levels of CD40 during tolerance induction, and the possible subsequent lack of CD40-CD154 interaction, may have been responsible for the induction of tolerance. In order to determine whether the predominant effect of CD40 ligation was through upregulation of CD80/86, a system was needed in which CD80 and CD86 were expressed at high levels on DC, while CD40 was either present or absent. If signals downstream of the CD40-CD154 interaction other than CD80/CD86 (for example OX40L) were crucial for induction of immunity, the lack of CD40 should override the presence of CD80/CD86, and result in the induction of tolerance.

Previous studies, which addressed the importance of CD40 using s.c. administration of Ag-loaded CD40-deficient or –sufficient DC, concluded that, whilst $CD40^{-/-}$ DC failed to lead to productive T cell immunity, T cell tolerance was also not evident (Miga et al., 2001). However a key factor for the induction of effective T cell tolerance with soluble Ag is the use of a systemic route (Anderton, 2001), implicating the spleen to be important in this process. One would therefore have predicted that the previous study using the subcutaneous route would not lead to a global tolerogenic effect. This chapter describes a series of experiments, in which CD40-deficient splenic DC (spDC) were administered i.v., testing the hypothesis that a lack of CD40 on DC in the spleen would induce Ag-specific T cell tolerance.

5.2. Approach

Throughout these experiments, B6 and CD40-deficient mice were used as sources of DC. These DC were loaded with Ag *ex-vivo*, before being used to activate T cells either *in vitro* or *in vivo*. Initially, pMOG was used to load the DC, but with the arrival of the OT-II adoptive transfer system in the lab, pOVA was used in most of the subsequent experiments, allowing the analysis of Ag-specific T cells. Early experiments, which were repeated with both pMOG and pOVA (without prior adoptive transfer of T cells), showed corresponding results, irrespective of the Ag used. The relevance of the findings from the adoptive transfer system were tested using the EAE model.

The experimental outline of this chapter is shown in Figure 5.1.

5.3. Comparison of T cell activation by bone marrow-derived DC and splenic DC *in vitro*

Using spleens from B6 and CD40-deficient mice as sources of DC, the consequences of the lack of CD40 expression by DC on the kinetics of T cell activation and expansion were examined. In order to show the viability and Ag-presenting capacity of spDC after purification, *in vitro* T cell activation by spDC and bone marrow-derived DC (bm-DC) from B6 mice were compared. SpDC were purified by positive selection using anti-CD11c-conjugated MACS beads, and bm-derived DC were grown from bm of B6 or $CD40^{-/-}$ mice for 7 days in the presence of GM-CSF. DC were loaded with pOVA in the presence of LPS for 2hrs, and cultured with Ag-specific T cells for 2 days. At lower DC:T cell ratios (i.e. fewer DC per T cell), bm-derived and spDC induced similar levels of T cell proliferation. At higher DC:T cell ratios, spDC induced more T cell proliferation compared to bm-derived DC (Fig. 5.2). The chosen method of purification for DC from the spleen thus left DC able to effectively present pOVA to Ag-specific T cells.

Although bm-derived DC can easily be obtained in larger numbers, spDC were used in subsequent experiments, since the spleen is arguably the most important organ for induction of systemic T cell tolerance, and DC from the spleen are very likely to be involved in this process. DC are very potent initiators of immune responses. Only low numbers are needed to carry out *in vitro* or *in vivo* experiments, therefore, the rarity of DC in the spleen (approximately 1% of total spleen cells) did not present a problem in this series of experiments.

5.4. B6 and $CD40^{-/-}$ splenic DC have similar phenotypic characteristics

So as to exclude the possibility that subsequent results were due to variation in expression of other costimulatory molecules or in the proportions of DC subsets, the

surface phenotype of splenic DC isolated from B6 or CD40$^{-/-}$ mice was analysed. The DC were treated as for subsequent use in T cell activation studies, i.e. positive selection using anti-CD11c-conjugated beads and cultured in the presence of LPS for 2 hrs. Similar percentages of CD8α^+ and CD4$^+$ DC subsets were found in each DC population (Fig. 5.3, bottom panel). Similar levels of CD80, CD86 and MHC class II were also found on the two DC populations (Fig. 5.3 top and middle panel). A large proportion of the DC displayed a mature phenotype, as illustrated by CD80, CD86 and MHC class II expression, suggesting that the procedure used resulted in significant DC activation (Fig. 5.3). The only difference between CD40-sufficient and CD40-deficient DC is the level of CD40, and subsequent differences in ability to activate Ag-reactive T cells are therefore likely to be due to the presence or absence of CD40.

5.5. Comparing the ability of B6 and CD40$^{-/-}$ splenic DC to induce T cell activation and expansion *in vitro*

The previous chapters have highlighted the importance of CD40-CD154 during T cell activation and tolerance. In an attempt to develop an *in vitro* model of T cell unresponsiveness, experiments were designed to test whether CD40$^{-/-}$ DC could activate a primary T response *in vitro*.

5.5.1. CD40$^{-/-}$ spDC are able to induce T cell activation and expansion *in vitro*

OT-II mice were crossed with CD40$^{-/-}$ mice and CD4$^+$ T cells were purified from these OT II x CD40$^{-/-}$ mice, by positive selection using anti-CD4-conjugated MACS beads. This use of CD4$^+$ cells from OT II x CD40$^{-/-}$ mice ensured that any contaminating CD4$^+$ DC would be CD40$^{-/-}$. The purified CD4$^+$ cells were CFSE-labelled, and cultured with pOVA-pulsed DC (purified from B6 or CD40$^{-/-}$ mice and loaded with 50μM pOVA in the presence of LPS) in a 10:1 T cell:DC ratio. The CFSE profile was assessed after 2, 3, 4, and 5 days of culture (Fig. 5.4), together with expression of CD69, CD25, OX40 and RANKL on pOVA-reactive T cells (Fig.

5.5). CD69 and CD25 were used as markers of T cell activation, and together with the costimulatory molecules OX40 and RANKL, expression of this set of molecules was investigated to reveal possible differences in the ability of B6 or $CD40^{-/-}$ DC to direct the outcome of T cell activation.

Stimulation with $CD40^{-/-}$ spDC consistently led to a lower percentage of T cells entering division at early time-points (day 3 of culture) compared with cultures using B6 DC (Fig. 5.4). By day 3 of the experiment shown, about twice as many T cells had undergone cell division when stimulated with B6 DC, compared with those stimulated with $CD40^{-/-}$ DC (Fig. 5.4, middle panel). This was reflected in the percentages of T cells showing upregulation of CD69 and CD25 at days 2 and 3, and in the percentages of T cells showing upregulation of OX40 and RANKL on day 3 (Fig. 5.5). The results shown are gated on live cells, but it must be mentioned that cultures with $CD40^{-/-}$ DC had greater numbers of dead cells at later time-points, i.e. day 5, presumably from T cells that had not divided in response to Ag stimulation. Although down-regulation of levels of CD69, CD25, OX40 and RANKL expression occurred more rapidly after stimulation with $CD40^{-/-}$ DC compared to B6 DC (Fig. 5.5), those T cells that had entered the proliferative cycle had undergone similar numbers of cell divisions in both groups, irrespective of the DC used for stimulation (Fig. 5.4, overlays). In summary, these experiments suggest that $CD40^{-/-}$ DC were less efficient at triggering initial $CD4^+$ T cell expansion *in vitro*, but that this defect was overcome with time, possibly due to other costimulatory molecules, compensating for the lack of CD40 on $CD40^{-/-}$ DC.

5.5.2. Varying the number of B6 and $CD40^{-/-}$ spDC does not differentially affect their ability to induce T cell expansion *in vitro*

The number of DC presenting pOVA to Ag-specific T cells had a great effect on T cell proliferation (Fig. 5.2). To ensure that the lack of CD40 on DC is not overcome by the number of DC present during T cell stimulation, the number of DC was decreased from the previous 10:1 ratio, while the number of T cells was kept

constant, at $4x10^4$ per well. The T cell:DC ratios were decreased 5 and 10 fold, giving 50:1 and 100:1 T cell:DC ratios, and DC were co-cultured with T cells as before. This reduction in DC number did not show any additional effects of CD40 deficiency on DC, neither with respect to T cell expansion (Fig. 5.6), nor T cell costimulatory molecule expression (Fig. 5.7). The difference in the ability of B6 and $CD40^{-/-}$ DC to stimulate T cells, which was observed at higher DC numbers, was still seen at lower numbers of DC. The overall percentages of T cells undergoing cell division and showing upregulation of costimulatory molecules (CD69 and OX40 are shown in Fig. 5.7, but the same effect was seen for CD25 and RANKL) was decreased after culture with $CD40^{-/-}$ DC, but T cells stimulated by CD40-deficient DC underwent the same number of cell divisions as those stimulated by B6 DC. The presence of CD40 on DC is therefore not crucial for efficient T cell activation *in vitro*.

5.6. CD40-deficient splenic DC induce abortive T cell activation and subsequent unresponsiveness *in vivo*

The previous chapter showed that T cells go through a phase of activation before reaching a tolerant state *in vivo*. The above data shows the ability of CD40-deficient DC to activate T cells *in vitro*, but did not provide evidence for unresponsiveness to rechallenge.

5.6.1. $CD40^{-/-}$ spDC do not support sustained Ag-specific T cell activation *in vivo*

In order to test whether activation by $CD40^{-/-}$ DC renders T cells unresponsive to rechallenge with the same Ag, DC from $CD40^{-/-}$ or B6 mice were purified as before, and pulsed *in vitro* with pOVA, prior to i.v. administration to naïve B6 mice (initially without adoptive transfer of pOVA-reactive T cells). After six days, peripheral LN and spleen cells were examined for *ex vivo* recall responses to pOVA. Ag-specific proliferation was observed in lymphoid populations taken from mice that had received pOVA-pulsed B6 DC, but not those that had received $CD40^{-/-}$ DC (Fig. 5.8).

Exogenous ligation of OX40 prevented the induction of tolerance (Chapter 3) and OX40 is upregulated on the T cell during both induction of tolerance and immunity (Chapter 4). Since one effect of CD40 ligation is the upregulation of OX40L on DC (Chapter 4), which signals to T cells via OX40, resulting in upregulation of the survival factors Bcl-2 and Bcl-xL (Rogers et al., 2001), one could reason that provision of OX40-signalling using an agonistic anti-OX40 monoclonal antibody might overcome the deficit in T cell activation seen after $CD40^{-/-}$ DC administration. However, no pOVA-reactive proliferative response in lymphoid populations from mice treated using this approach could be detected (Fig. 5.8), which correlated with data shown in Chapter 3, where provision of anti-OX40 at the time of primary Ag administration did not result in effective T cell responses (Fig. 3.7).

5.6.2. $CD40^{-/-}$ spDC induce unresponsiveness to subsequent rechallenge with Ag in adjuvant *in vivo*

Primary *in vivo* stimulation with Ag-loaded $CD40^{-/-}$ DC resulted in a lack of T cell responsiveness when T cells were restimulated *in vitro*. However, a more rigorous assessment of potential tolerance induction required analysis of T cell reactivity upon subsequent *in vivo* rechallenge with Ag in adjuvant. Mice therefore received pOVA-loaded DC as before, and were then immunised with pOVA in CFA 6 days after administration of DC. Recall proliferative responses to a dose range of pOVA were tested 10 days after challenge with pOVA in CFA. Lymphoid populations from mice that initially received $CD40^{-/-}$ DC were clearly impaired in their ability to proliferate to pOVA *in vitro* compared with those from mice that had received B6 DC, or from control mice that received no DC (Fig. 5.9). In this setting, the provision of anti-OX40 at the time of $CD40^{-/-}$ DC administration allowed *in vitro* recall responsiveness to be evident after immunisation with pOVA in CFA. Again, this is reminiscent of data obtained in Chapter 3, where T cells responded to *in vivo* rechallenge with Ag in adjuvant after previous tolerisation (Fig. 3.10). The absence of CD40 on DC rendered T cells unresponsive, but this unresponsiveness could be rescued by exogenous ligation of OX40.

5.6.3. CD40$^{-/-}$ spDC induce abortive T cell activation *in vivo*

A lack of T cell responsiveness may reflect an absence of Ag-reactive cells or their functional inactivation. To address this issue, the expansion and phenotypic changes of pOVA-specific T cells were followed *in vivo* using the OT-II adoptive transfer system. Naïve B6 mice received CFSE-labelled OT-II T cells on day –1, followed on day 0 by an i.v. injection of pOVA-pulsed B6 or CD40$^{-/-}$ DC as described above. Flow cytometry revealed similar changes in the levels of CD69 expression by transferred OT-II T cells in the first three days after administration of either B6 or CD40$^{-/-}$ DC. However, although CD40$^{-/-}$ DC induced T cell proliferation *in vivo*, this was not sustained past three days after DC administration (Fig. 5.10). OT-II T cell numbers were greatly reduced at the day 4 time-point in the spleens of mice that received CD40$^{-/-}$ DC, compared with a continued T cell expansion from day 3 to 4 in mice that received B6 DC (Fig. 5.10).

5.6.4. Reduced IL-2 and IFNγ production and reduced proliferative capacity after exposure to Ag-loaded CD40$^{-/-}$ spDC

The data presented above showed that the number of Ag-reactive T cells rapidly decreased after exposure to Ag on CD40$^{-/-}$ DC (Fig. 5.10). To determine whether disruption of CD40 signalling not only influenced survival of Ag-specific T cells, but also affected T cell effector function, i.e. T cell cytokine-producing capacity, intracellular cytokine production by OT-II T cells 6 days after DC administration was measured. Lymphoid cells received mitogenic stimulation with PMA and ionomycin for 4 hours before intracellular cytokine production was determined by FACS staining. Production of both IL-2 and IFN-γ was clearly evident after administration of B6 DC. Fewer OT-II cells were producing IL-2 and IFN-γ, however, after CD40$^{-/-}$ DC administration, although the percentages of Ly5.1^{+}, CD4^{+} T cells producing IL-2 and IFN-γ were still much higher than in PBS controls (Fig. 5.11b and 5.11c). Production of IL-4 and IL-10 was minimal after administration of either DC group (Fig. 5.11d and 5.11e). Consistent with earlier findings using B6 mice without OT-II transfer (Fig. 5.8), it was found that lymphoid populations from

mice harbouring OT-II T cells showed a greatly reduced proliferative capacity upon *in vitro* culture with pOVA after $CD40^{-/-}$ DC administration compared with those from mice receiving B6 DC (Fig. 5.12). The response in the $CD40^{-/-}$ DC group showed a low level of proliferation mirroring that seen in control mice that received PBS rather than DC (Fig. 5.12).

5.6.5. $CD40^{-/-}$ spDC induce unresponsiveness in the adoptive transfer model

The results obtained using the adoptive transfer system correlated with those from naïve B6 mice. After transfer of OT-II cells, mice received DC and were immunised with pOVA/CFA on day 6. Ten days after immunisation, diminished *in vitro* recall proliferative responses to pOVA and a decreased frequency of pOVA-reactive T cells in mice that received $CD40^{-/-}$ DC were found in DLN (Fig. 5.13a and b). Collectively, the data looking at T cell expansion in response to Ag encounter on DC lacking CD40 suggest that administration of $CD40^{-/-}$ DC results in an abortive activation of Ag-reactive T cells, with the remaining cells being less able to produce IL-2, IFN-γ, or to proliferate in response to Ag.

5.7. Administration of CD40-deficient spDC protects from CNS autoimmune pathology

The data thus far had indicated that exposure to Ag in the absence of the CD40:CD154 interaction induced a transient T cell activation followed by a substantial loss in Ag-reactive T cell numbers. Those T cells that did persist had a reduced ability to proliferate and produce cytokines. The net effect of these processes was to render the mouse relatively unresponsive to subsequent immunogenic challenge *in vivo*. To determine the significance of this unresponsiveness in a pathogenic setting, the capacity of autoantigen-loaded $CD40^{-/-}$ DC to modulate the development of pMOG-induced EAE was tested. Mice received either B6 or $CD40^{-/-}$ DC that had been loaded *in vitro* with the 50μM pMOG and 6 days later EAE was induced by immunization with pMOG/CFA. Both B6 and $CD40^{-/-}$ DC consistently

conferred a degree of protection against EAE compared to mice that did not receive DC. However, $CD40^{-/-}$ DC clearly gave a more pronounced protection than B6 DC (Fig. 5.14).

5.8. Inducing tolerance through administration of $CD40^{-/-}$ bone marrow-derived DC gives inconsistent results

Although the spleen is arguably the most important organ for induction of T cell tolerance, as previously mentioned, it was decided to investigate the tolerogenic potential of bm-derived $CD40^{-/-}$ DC in a similar fashion to spDC as shown above. Human blood-derived monocytes, which are currently used in many clinical trials for enhancing immune responses to tumours (Bocchia et al., 2000), show similar characteristics to bm-DC (Shortman and Liu, 2002). DC in which RelB function is inhibited lack CD40 expression (Martin et al., 2003). If bm-DC lacking CD40 have the same tolerogenic potential as $CD40^{-/-}$ spDC, the possible therapeutic benefits associated with blood-derived monocytes, which could be differentiated into DC lacking CD40, and could be grown for each patient individually, are considerable.

5.8.1. B6 and $CD40^{-/-}$ bone marrow-derived DC have similar phenotypic characteristics

After one week of *in vitro* culture with GM-CSF, bm-DC from B6 or $CD40^{-/-}$ mice were cultured in the presence of LPS for 2 hrs. DC were then stained for DC subset markers CD8α and CD4, and their relative maturity assessed by staining for the costimulatory molecules CD80, CD86 and CD40, as well as by staining for MHC class II expression. Bone marrow-derived cells were $CD8\alpha^-$ and $CD4^-$, and both B6 and $CD40^{-/-}$ DC displayed similar surface levels of CD80, CD86 and MHC class II (Fig. 5.15).

5.8.2. Bone marrow-derived CD40$^{-/-}$ DC do not support Ag-specific T cell proliferation *in vivo*

DC grown from CD40$^{-/-}$ or B6 mice were pulsed *in vitro* with pMOG in the presence of LPS, prior to i.v. administration to naïve B6 mice. After six days, peripheral LN and spleen cells were examined for *in vitro* recall responses to pMOG. Ag-specific proliferation was observed in lymphoid populations taken from mice that had received pMOG-pulsed B6 DC, but not those that had received CD40$^{-/-}$ DC (Fig. 5.16), consistent with findings using spDC (Fig. 5.8).

5.8.3. Bone marrow-derived CD40$^{-/-}$ DC inconsistently induce unresponsiveness *in vivo*

As with spDC, the above findings indicated a lack of T cell responsiveness as a result of primary *in vivo* stimulation with Ag-loaded bm-derived CD40$^{-/-}$ DC. However, rechallenge with Ag in adjuvant, which consistently resulted in decreased pOVA-specific proliferation in after administration with CD40$^{-/-}$ spDC, gave inconsistent results when bm-derived CD40$^{-/-}$ DC were used (Fig. 5.17). In one experiment, administration of CD40$^{-/-}$ DC gave an Ag-non-specific reduction in T cell proliferation, while in a separate experiment, administration of both B6 and CD40$^{-/-}$ DC resulted in a modest decrease in proliferative responses to pMOG (Fig. 5.17). Even several repeats of the experiment did not lead to clarification whether or not administration of CD40$^{-/-}$ bm-derived DC could lead to Ag-specific T cell unresponsiveness.

5.9. Summary

The experiments described in this chapter tested the prediction that Ag presentation by DC lacking CD40 would drive Ag-reactive T cell towards unresponsiveness. It was shown that CD40 expression on spDC was neither necessary for T cell proliferation *in vitro*, nor was it crucial for initial T cell expansion *in vivo*. However, it was essential for sustaining *in vivo* T cell expansion past day 3 after DC administration, after which a decrease in Ag-specific T cells was observed. This correlated with a decreased proportion of T cells producing IL-2 and IFN-γ when stimulated by $CD40^{-/-}$ spDC. Importantly these deficits were evident even though the $CD40^{-/-}$ DC had elevated expression of other costimulatory molecules (CD80, CD86), and this effect is most likely due to the lack of CD40 on DC, disrupting further down-stream events (other than CD80/CD86). Reduced proliferative recall responses were found after administration of $CD40^{-/-}$ spDC, even after rigorous assessment of tolerance by administration of Ag in a strong adjuvant. Similar to data obtained in chapter 3, exogenous ligation of OX40 did not rescue responsiveness during initial T cell priming, but allowed effective recall responses after rechallenge in the presence of a strong adjuvant. Using EAE, it was shown that administration of CD40-deficient DC conferred effective protection against the development of autoimmune pathology. From these data, it is concluded that the systemic administration of CD40-deficient spDC leads to inhibition of T cell activation and a deletional form of tolerance that is reminiscent of that seen following administration of soluble Ag.

The chapter concludes by exploring the potentially similar tolerogenic effects of $CD40^{-/-}$ bm-derived DC. Although the initial results appeared promising, and $CD40^{-/-}$ bm-derived DC did not support T cell proliferation *in vivo*, and more rigorous assessment of tolerogenic potential gave inconsistent results. Nevertheless, this avenue of research possesses great therapeutic possibilities, and may be worth further investigation.

5.10. Discussion

Presentation of peptide-MHC complexes by resting DC is generally accepted to be the key to T cell tolerance induction (Steinman and Nussenzweig, 2002). The finding that administration of Ag-loaded, CD40-deficient splenic DC resulted in T cell tolerance further highlighted the importance of CD40-CD154 during the decision between tolerance and immunity. This fits with data obtained in Chapters 3 and 4, which showed that activation of DC by CD40 prevented tolerance induction, and that expression levels of CD40 were increased during the induction of immunity compared to tolerance induction (Chapter 4). The system used in this chapter provided a tool to specifically look at the impact of CD40-deficiency during T cell activation. The finding that $CD40^{-/-}$ DC induced initial T cell expansion, but were unable to sustain this expansion compared to their CD40-sufficient counterparts, is consistent with an earlier study that gave DC by a s.c. route (Miga et al., 2001). This previous study, however, did not report any unresponsiveness in the Ag-reactive T cells that persisted after $CD40^{-/-}$ DC administration. The obvious difference between these two studies is that the route of DC administration. In our series of experiments, it was deliberately chosen to give DC i.v., allowing access to the spleen, based on previous experience that systemic delivery is most effective at inducing T cell tolerance with peptide (Anderton et al., 1998). Inefficient T cell priming after s.c. injection of $CD40^{-/-}$ DC has been reported to be associated with accelerated loss of DC from the draining lymph node. Studies of tolerance induction with peptides indicate that, whilst establishment of a fully unresponsive state takes 4 to 5 days (Liu and Wraith, 1995), the key events that initiate the tolerogenic program probably occur within hours of TCR recognition (Hoyne et al., 1996; Zell et al., 2001). This being the case, it could be argued that the issue of how many days B6 versus $CD40^{-/-}$ DC persist in the spleen after i.v. administration is unlikely to be the key to the T cell unresponsiveness observed.

The results clearly show that $CD40^{-/-}$ DC provoked a proliferative burst in Ag-reactive T cells that lasts for three days. This is reminiscent of the data obtained in Chapter 4, showing that pOVA-reactive T cells underwent several rounds of cell

division after administration of soluble peptide before becoming tolerant. The soluble peptide was most likely presented by resting DC, expressing lower levels of CD40, compared to DC after administration of Ag and LPS. Therefore the initial proliferation seen using DC which completely lacked expression of CD40, fits with other experimental systems that lead to T cell tolerance. Even though the percentage of cells producing IL-2 and IFN-γ is reduced after administration of $CD40^{-/-}$ DC, it is still greatly increased compared to PBS controls. However, the proliferative responses were greatly reduced after administration of $CD40^{-/-}$ DC compared to PBS controls, suggesting proliferative unresponsiveness, but not a complete lack of effector function. This has previously been observed after administration of soluble peptide (Malvey et al., 1998), and is again reminiscent of tolerance induction by soluble peptide explored in previous chapters.

The results suggest that the decreased IL-2 production in T cells stimulated with $CD40^{-/-}$ DC leads to an abortive immune response, and T cell death. This is supported by the sudden decrease in Ag-reactive T cells 4 days after priming with $CD40^{-/-}$ DC. Consistent with inducing a form of deletional tolerance, administration of $CD40^{-/-}$ DC protected from EAE. This matches a recent report in which administration of Ag-loaded $RelB^{-/-}$ DC, which also lack $CD40^{-/-}$ expression, induced Ag-specific T cell tolerance (Martin et al., 2003). However, this study reported the induction of an IL-10 producing regulatory T cell population. It is unlikely that this explains the protection against EAE we observe, since $CD40^{-/-}$ DC did not induce an IL-10 producing T cell population in our OT-II adoptive transfer system. Interestingly, although pOVA-pulsed B6 DC did not appear to result in decreased proliferative capacity upon rechallenge, giving pMOG-pulsed B6 DC prior to EAE induction consistently gave some protection from disease. Similar data have been obtained using LPS-treated, pMOG-pulsed, bone marrow-derived DC (S. Anderton, unpublished observations). T cells can become exhausted upon repeated Ag-stimulation (Lenardo et al., 1999), resulting in reduced capacity to respond to rechallenge. Administration of B6 DC and subsequent challenge with a strong adjuvant such as CFA may therefore drive some high avidity self-reactive T cells

into activation-induced cell death, reducing the ability to induce disease. Alternatively, LPS-treated DC could have expanded an already existing population of T reg, which has been observed in other systems (Yamazaki et al., 2003), which then conferred some protection against autoimmune pathology.

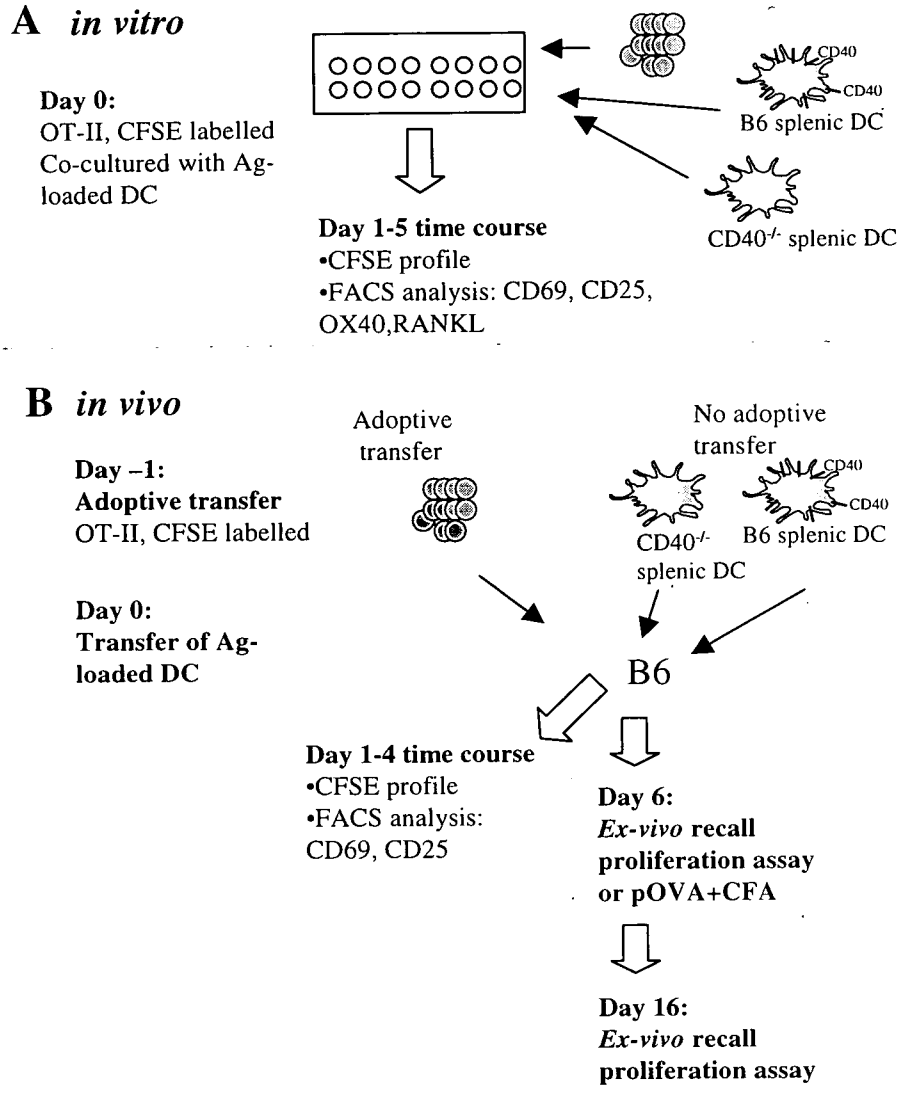

Figure 5.1:
Experimental outline for experiments of chapter 5
A: $CD4^+$ pOVA-reactive OT-II cells were CFSE labelled and co-cultured with LPS-treated, pOVA-loaded B6 or $CD40^{-/-}$ DC, and analysed by FACS on days 1,2,3,4,5 and 6.
B: Some mice received CFSE-labelled, $CD4^+$ OT-II cells on day-1, before LPS-treated, pOVA-loaded B6 or $CD40^{-/-}$ DC on day 0. In the case of an adoptive transfer, mice were sacrificed on 1,2,3 and 4 for time course analysis of expression of costimulatory markers. In either case, mice were tested for pOVA-specific recall responses on day 6, or received pOVA/CFA on day 6 and were tested for pOVA-specific recall responses on day 16.

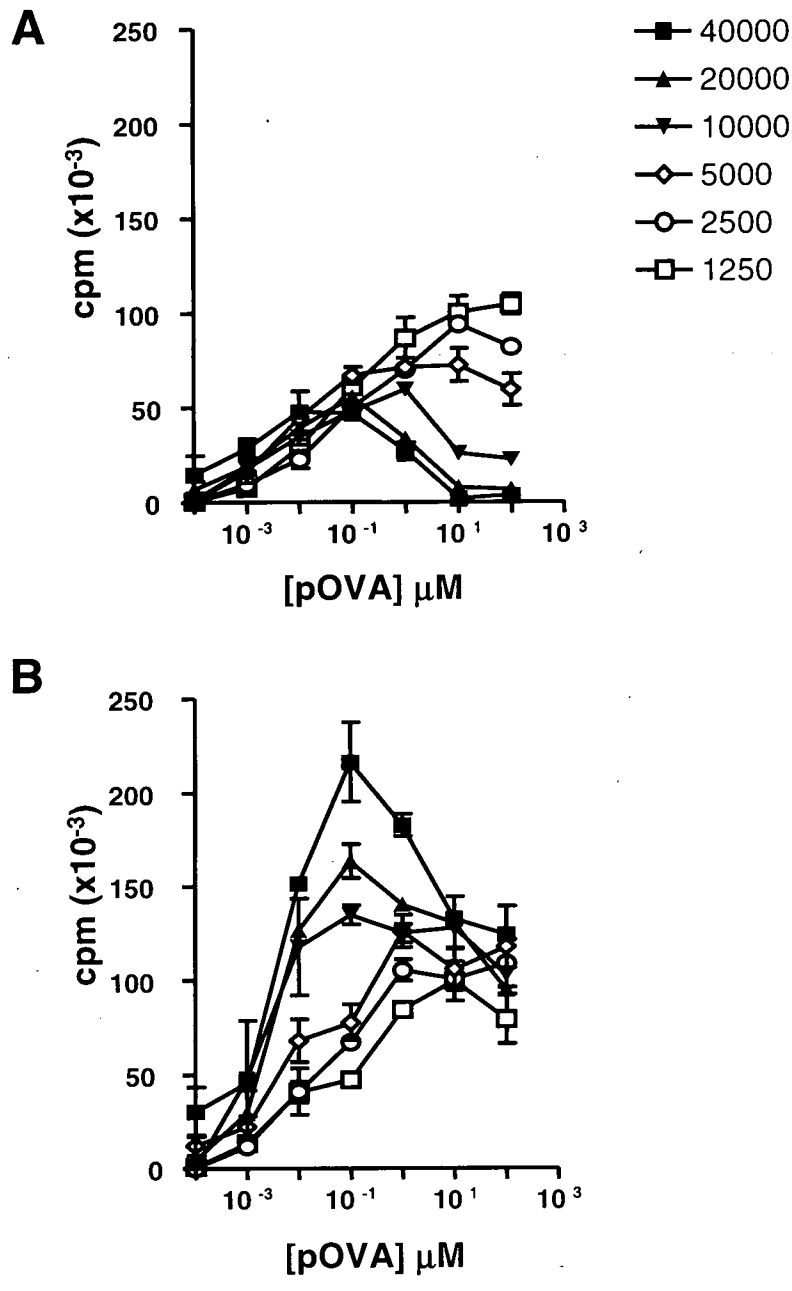

Figure 5.2:
Comparison of T cell activation by bone marrow-derived and splenic DC *in vitro*
CD4-purified OT-II cells were co-cultured with bm-derived DC (A) or splenic DC (B), and assessed for pOVA-specific proliferative responses. Figures in the legend refer to numbers of DC/well. $4x10^4$ T cells/well were used. Background levels ranged from 105-5646. This experiment was carried out once.

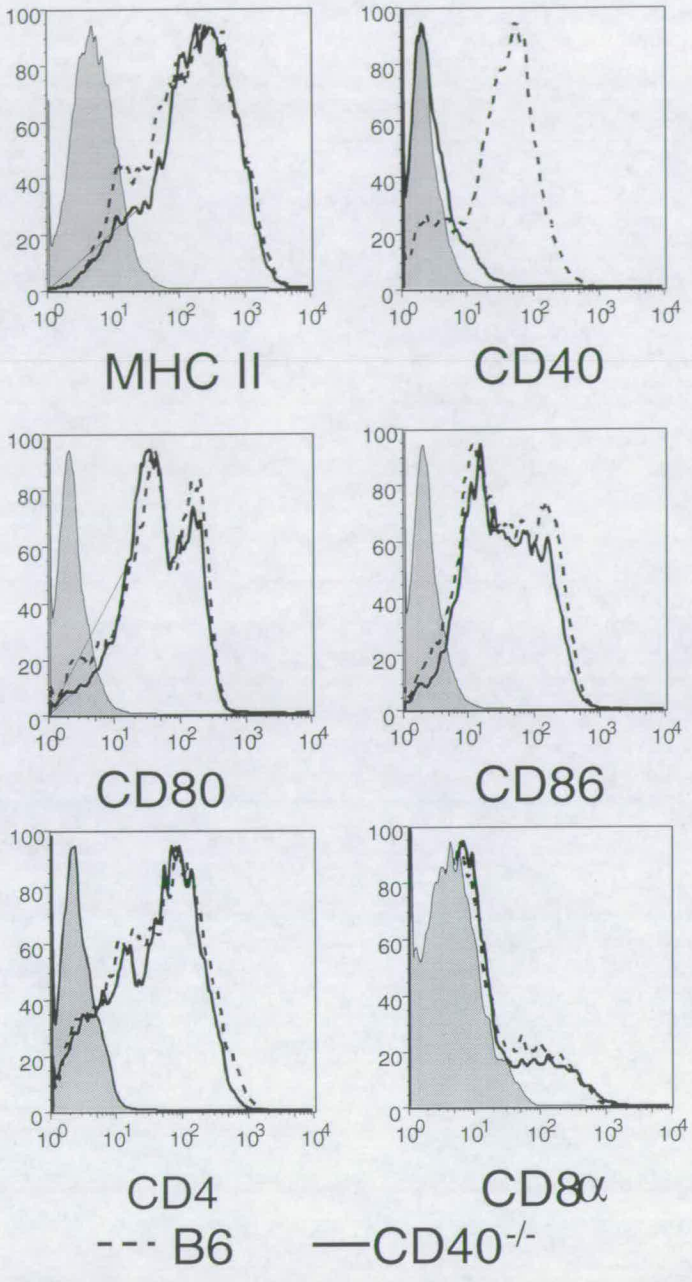

Figure 5.3:
Splenic DC from B6 and CD40$^{-/-}$ mice display similar phenotypic characteristics

Spleens were treated with collagenase and DNAse, DC were purified as described in materials and methods, cultured with LPS for 2 hours at 37°C, and stained for MHC class II, CD40, CD80, CD86, CD8α and CD4. CD11c^{+} DC were analysed for expression of these molecules. These data are from one of two experiments giving consistent results.

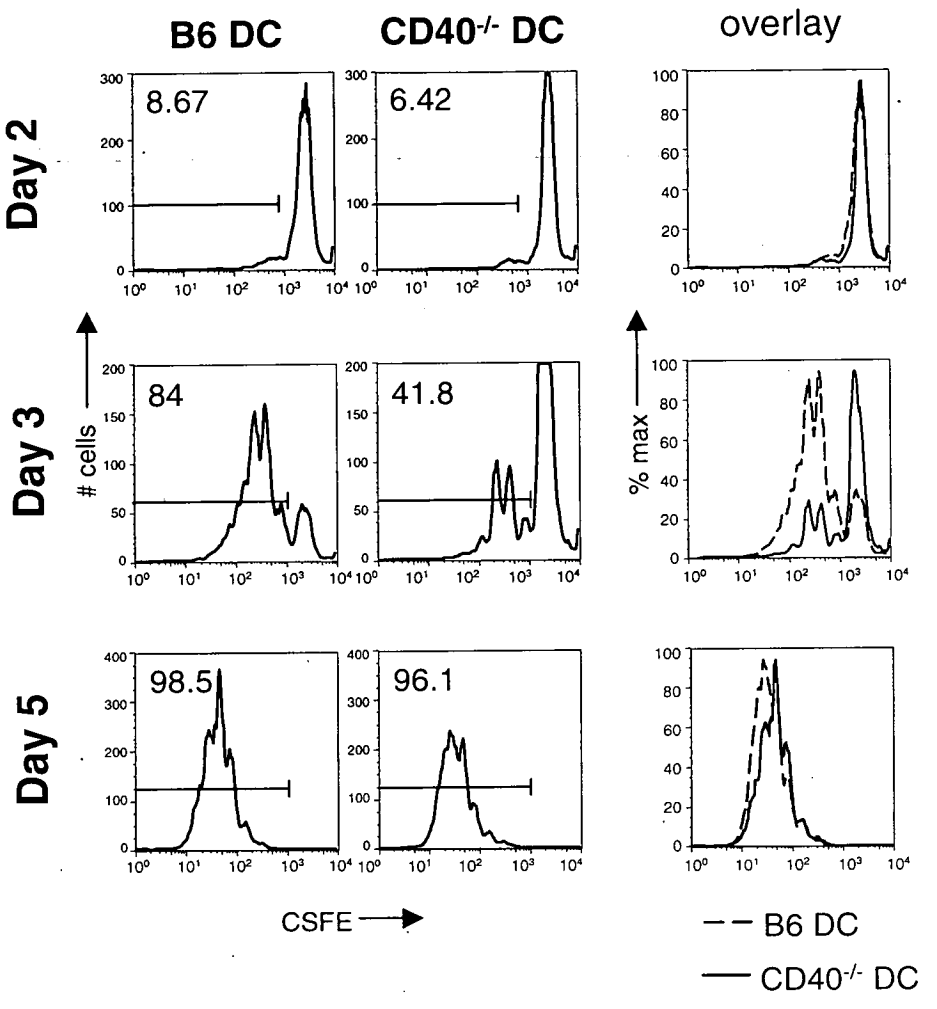

Figure 5.4:
B6 and CD40$^{-/-}$ spDC show similar T cell activation capacities *in vitro*
CFSE-labelled CD4^{+} OT-II T cells were cultured with Ag-loaded splenic DC (10:1 T/DC ratio) and analysed at the times indicated. These data are from one of three experiments giving consistent results.

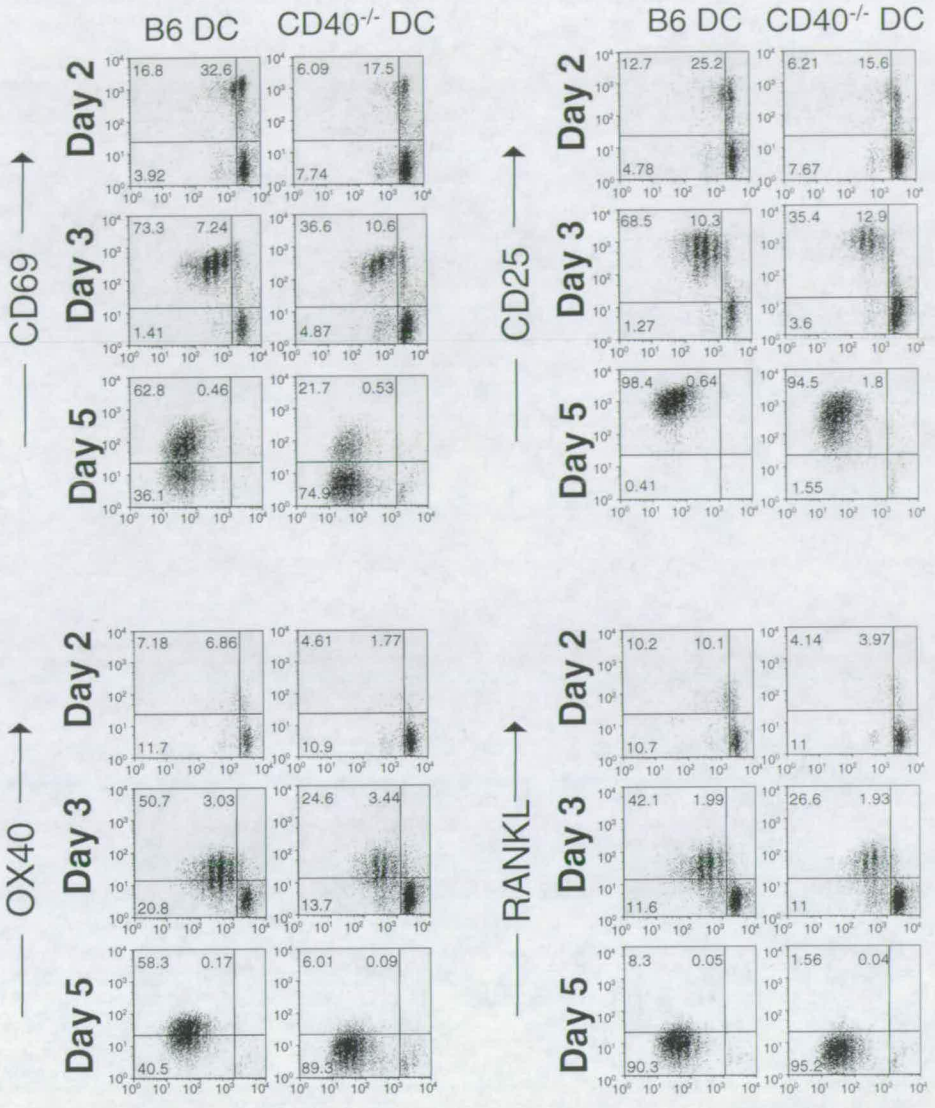

Figure 5.5:
Comparing costimulatory molecule expression induced by B6 and CD40$^{-/-}$ spDC

CFSE-labelled CD4^{+} OT-II T cells ($4x10^4$ cells per well) were cultured with Ag-loaded splenic DC ($4x10^3$ DC per well). At the indicated time-points T cells were sampled and expression of CD69, CD25, OX40 and RANKL analysed. Percentages shown on graphs refer to percentages of cells in top left and right, and bottom left quadrants. These data are from one of three experiments giving consistent results.

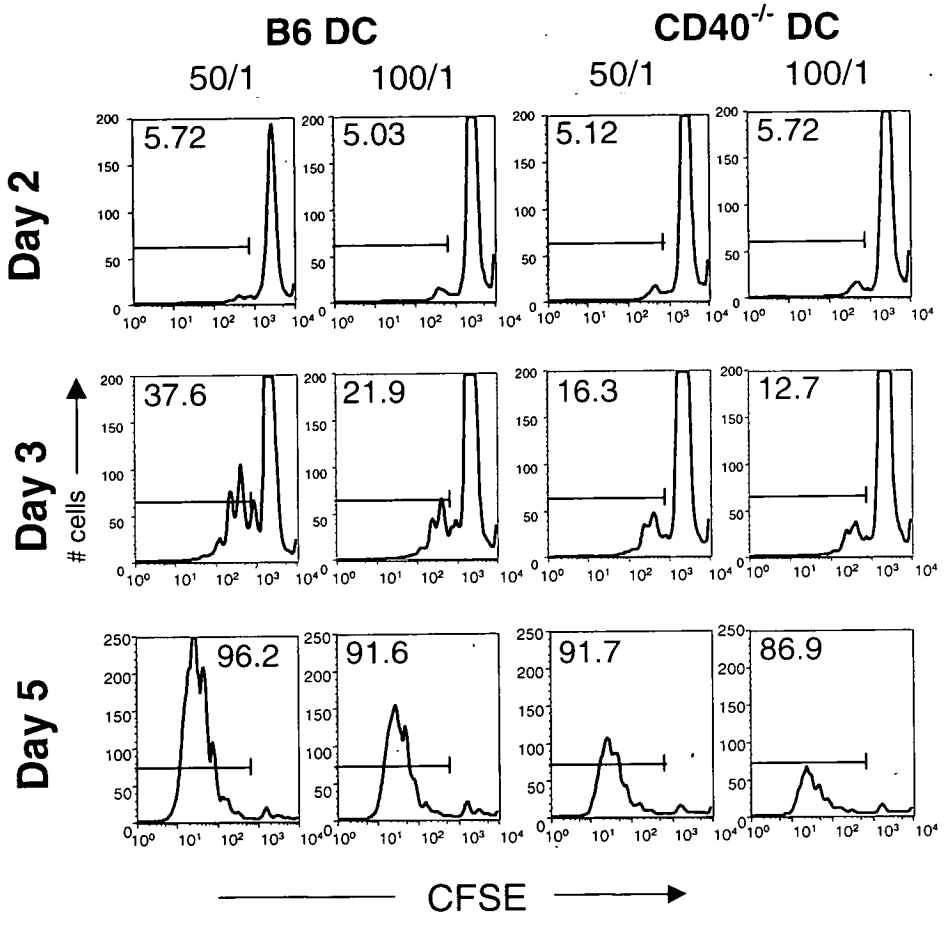

Figure 5.6:
Varying the number of B6 and CD40$^{-/-}$ spDC does not differentially affect their ability to induce T cell expansion *in vitro*
CFSE-labelled CD4^{+} OT-II T cells ($4x10^4$ cells per well) were cultured with varying numbers of Ag-loaded splenic DC (50/1 or 100/1 T/DC ratios) and analysed at the times indicated. Numbers shown on graphs refer to percentages of divided cells. These data are from one of two experiments giving consistent results.

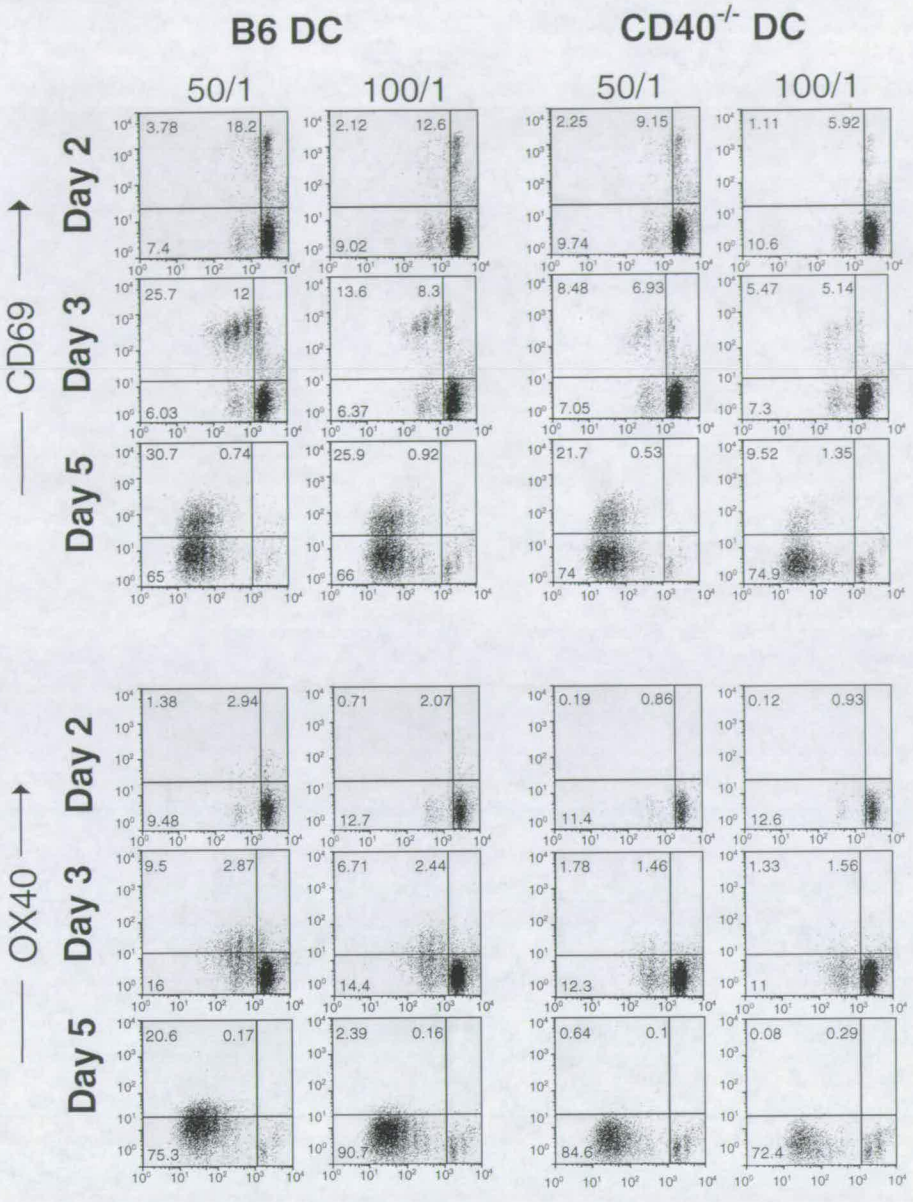

Figure 5.7:
Comparing costimulatory molecule expression induced by varying numbers of B6 and CD40$^{-/-}$ spDC

CFSE-labelled CD4^{+} OT-II T cells ($4x10^{4}$ cells per well) were cultured with varying numbers of Ag-loaded splenic DC. At the indicated time-points T cells were sampled and expression of CD69, CD25, OX40 and RANKL analysed. Shown are the expression of CD69 and OX40. Percentages shown on graphs refer to percentages of cells in top left and right, and bottom left quadrants.These data are from one of two experiments giving consistent results.

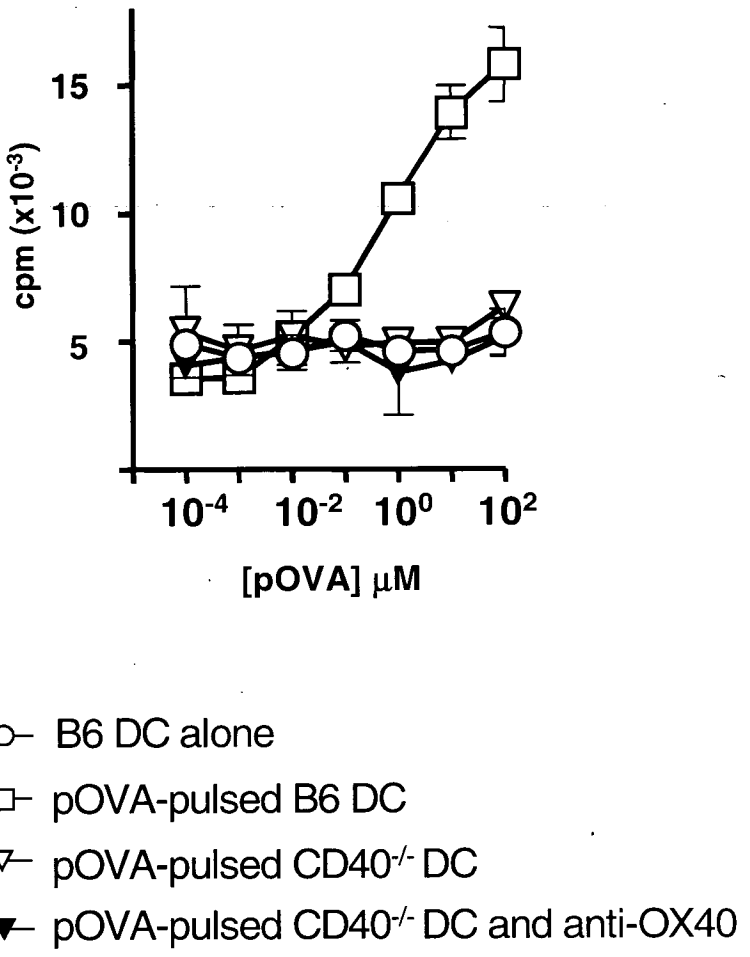

Figure 5.8:
CD40$^{-/-}$ spDC do not support Ag-specific T cell proliferation *in vivo*
B6 or CD40$^{-/-}$ DC were purified and loaded with pOVA as described in materials and methods, before i.v. transfer into naïve B6 recipients. Six days after DC transfer, splenic lymphoid populations were tested for proliferative responses to a dose range of pOVA. These data are from one of three experiments giving consistent results.

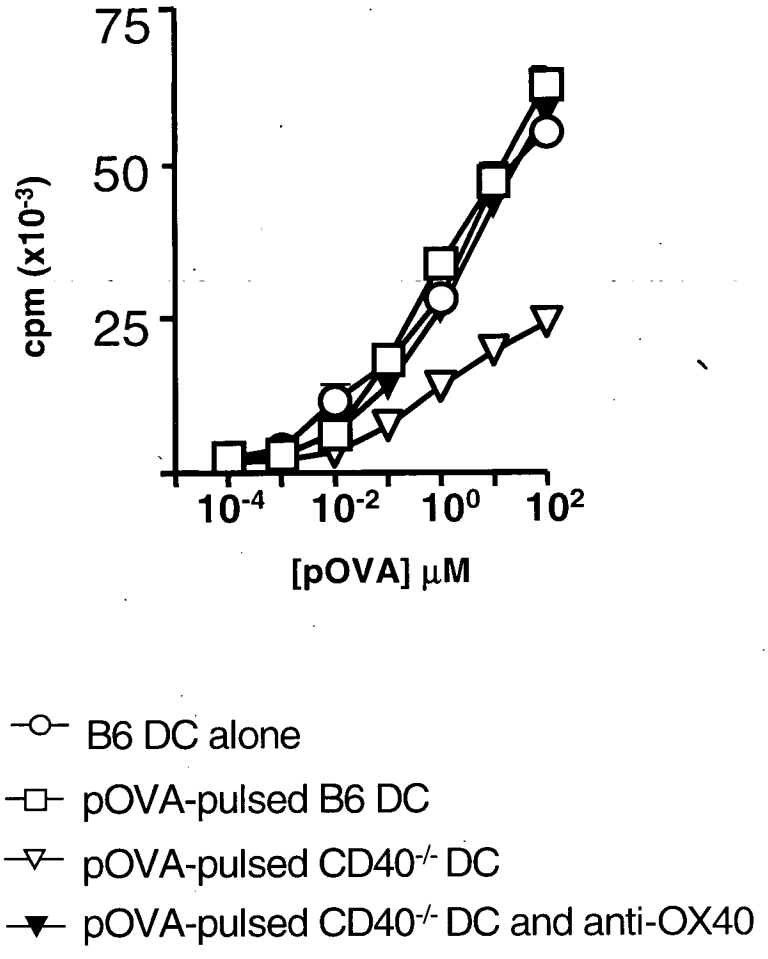

Figure 5.9:
CD40$^{-/-}$ spDC administration leads to T cell unresponsiveness *in vivo*
B6 or CD40$^{-/-}$ DC were purified and loaded with pOVA as described in materials and methods, before transfer into naïve B6 recipients on day 0. Mice were immunised on day 6 with pOVA/CFA and draining lymph nodes assessed 10 days later for proliferative responses to a dose range of pOVA. As indicated some groups also received anti-OX40 at the time of DC administration and 2 days later. The effects seen with anti-OX40 were not found when using an isotype control antibody. These data are from one of three experiments giving consistent results.

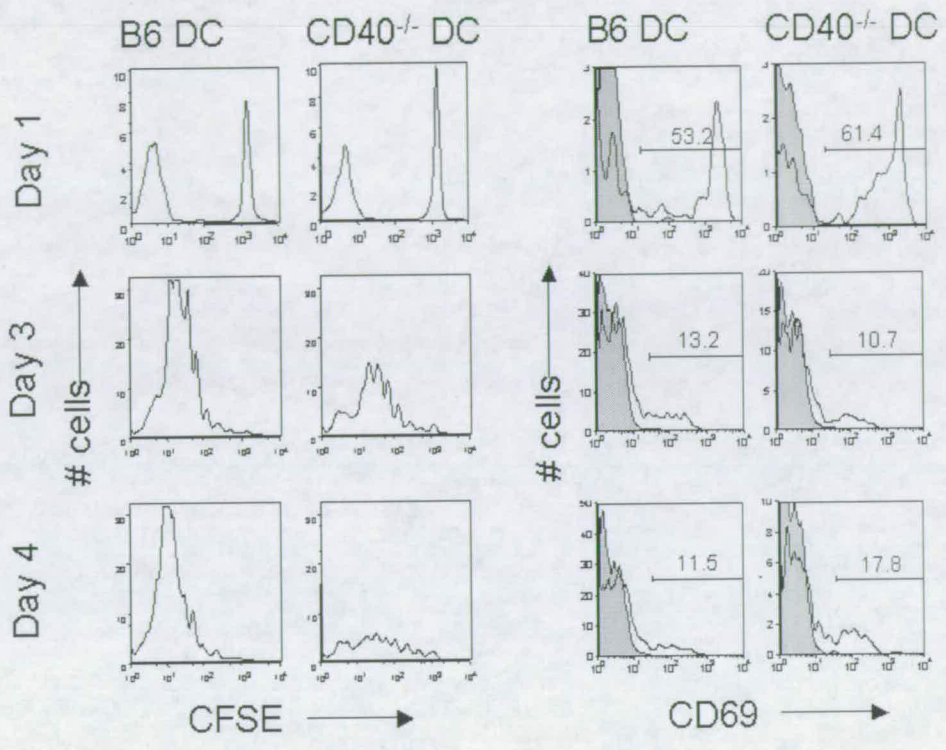

Figure 5.10:
CD40$^{-/-}$ spDC do not support sustained T cell expansion *in vivo*
B6 mice were seeded with CFSE-labelled OT-II cells one day prior to administration of DC. On days 1,3 and 4 post DC administration, lymphoid cells (spleen shown here) were analysed by FACS for CFSE profile and CD69 expression by Vβ5+Vα2+ OT-II cells (filled lines are Vβ5+Vα2+ cells that were stained with streptavidin-APC alone). These data are from one of three experiments giving consistent results.

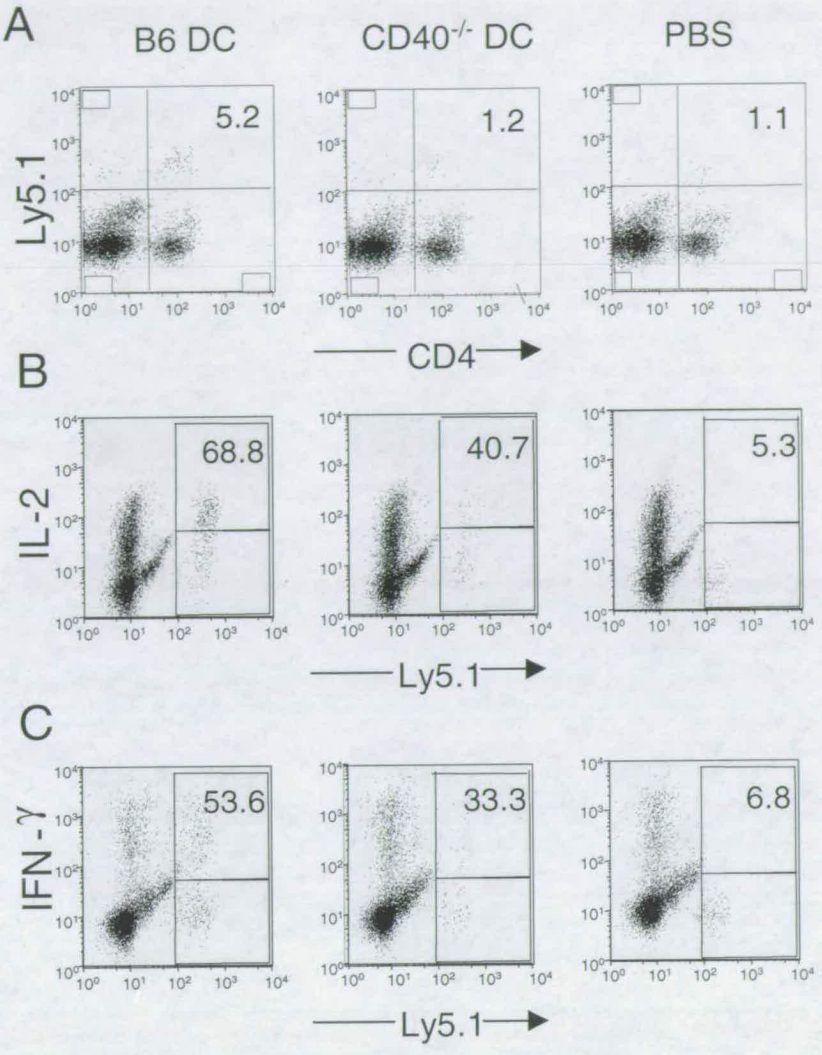

Figure 5.11a-c:
Reduced cytokine production after administration of Ag-loaded CD40$^{-/-}$ spDC

B6 mice were given Ly5.1$^+$ OT-II cells one day prior to administration of DC. Lymphoid populations (spleen shown here) were analysed on day 6. OT-II cells were identified by gating on CD4$^+$, Ly5.1$^+$ cells (A, percentages of CD4$^+$ cells that were Ly5.1$^+$ shown) and counterstained for IL-2 (B), or IFN-γ (C, percentages of CD4$^+$Ly5.1$^+$ cells that were cytokine-positive shown). These data are from one of three experiments giving consistent results.

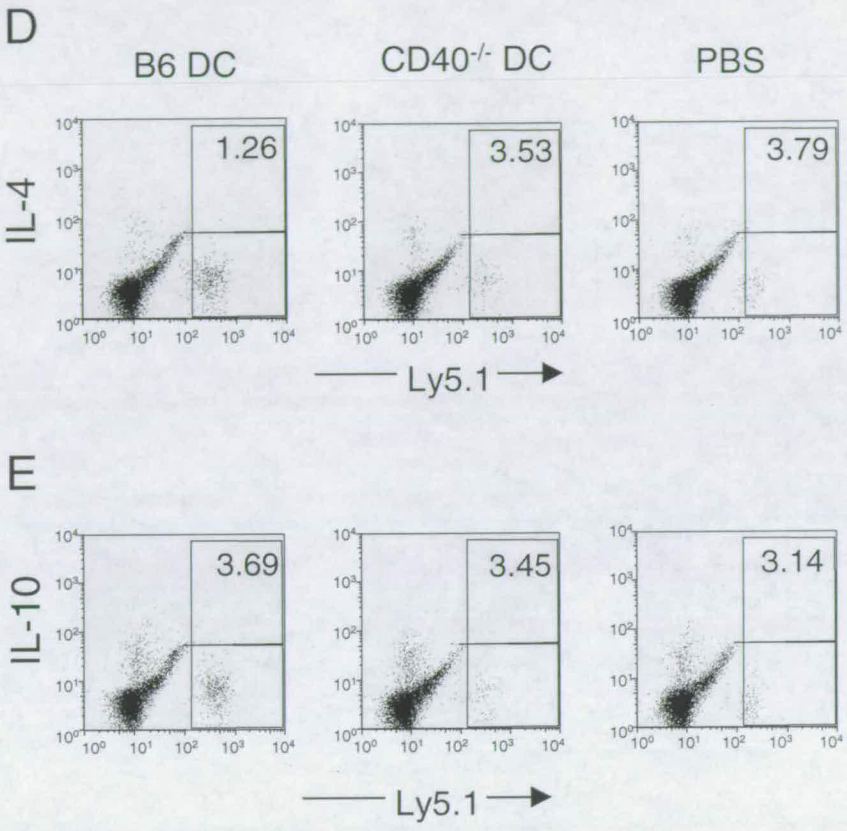

Figure 5.11d,e:
Reduced cytokine production after administration of Ag-loaded CD40$^{-/-}$ spDC

B6 mice were given Ly5.1^{+} OT-II cells one day prior to administration of DC. Lymphoid populations (spleen shown here) were analysed on day 6. OT-II cells were identified by gating on CD4^{+}, Ly5.1^{+} cells as in Fig. 511a-c, and counterstained for IL-4 (D), or IL-10 (E, percentages of CD4^{+}Ly5.1^{+} cells that were cytokine-positive shown). These data are from one of three experiments giving consistent results.

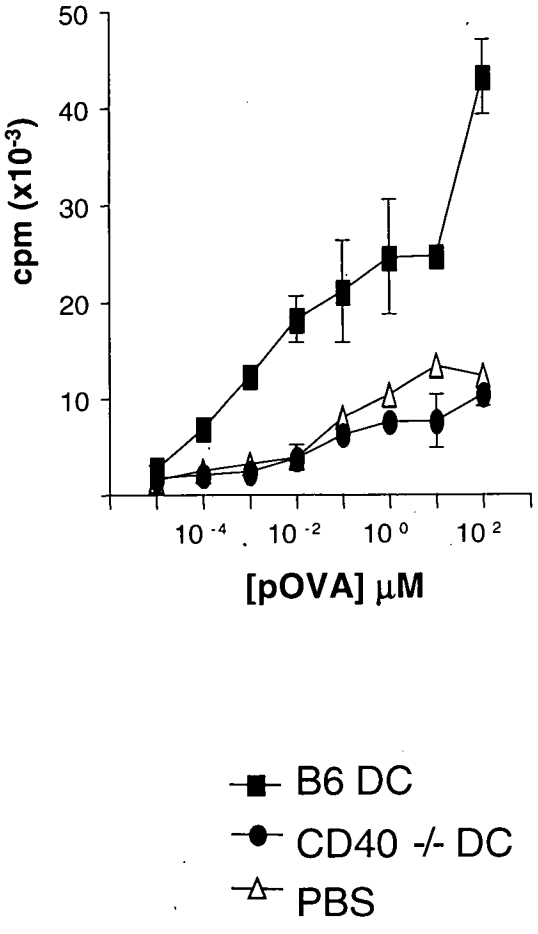

Figure 5.12:
Reduced T cell proliferation after administration of Ag-loaded CD40$^{-/-}$ spDC
B6 mice were given Ly5.1^{+} OT-II cells one day prior to administration of DC on day 0. B6 or CD40$^{-/-}$ DC were purified and loaded with pOVA as described in materials and methods, before transfer into naïve B6 recipients. Six days after DC transfer, splenic lymphoid populations were tested for proliferative responses to a dose range of pOVA. These data are from one of two experiments giving consistent results.

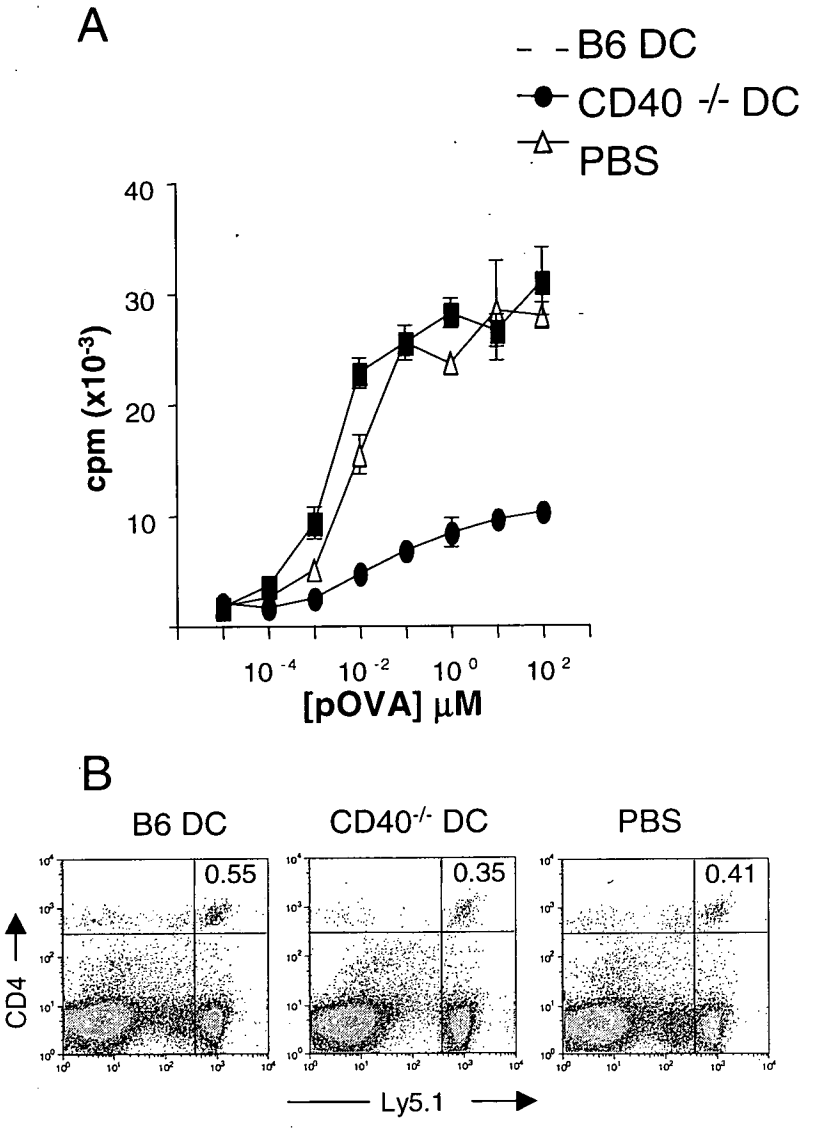

Figure 5.13:
CD40$^{-/-}$ spDC induce unresponsiveness to subsequent rechallenge *in vivo* after adoptive transfer of Ag-specific T cells

A: B6 mice were given Ly5.1^{+} OT-II cells one day prior to administration of DC on day 0 as before (Fig. 5.12). Mice were immunised with pOVA in CFA on day 6 and *in vitro* recall responses to pOVA in the DLN were assessed on day 16. These data are from one of several experiments giving consistent results.

B: OT-II cells were identified by gating on CD4^{+}, Ly5.1^{+} cells on day 16. Percentages of OT-II cells in DLN are shown.

These data are from one of two experiments giving consistent results.

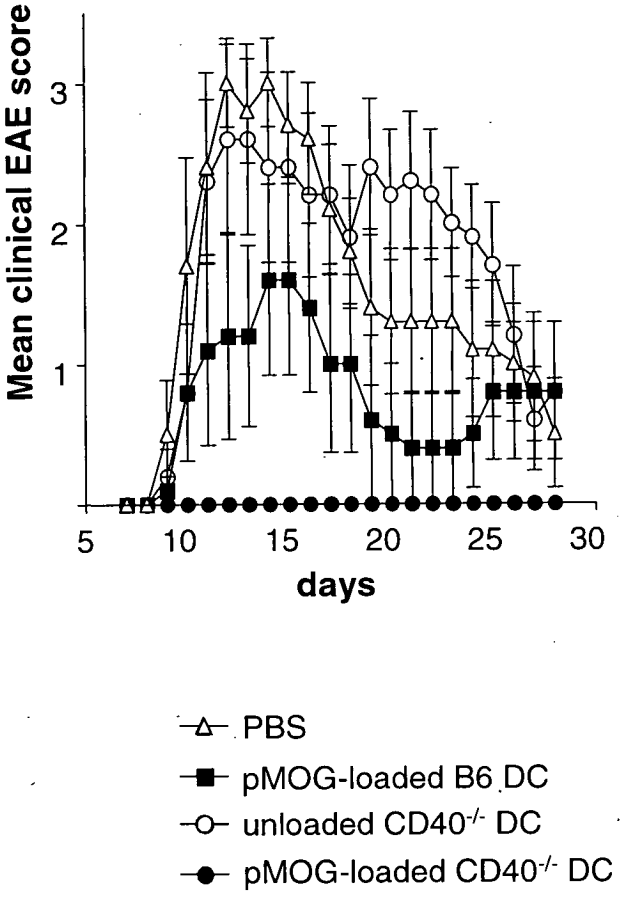

Figure 5.14 :
CD40$^{-/-}$ spDC protect from autoimmune CNS pathology
Mice received pMOG-loaded B6 or CD40$^{-/-}$ DC 6 days prior to induction of EAE with pMOG in CFA. (p values determined by Mann-Whitney U test were: PBS vs. B6: 0.0025, PBS vs. CD40$^{-/-}$ +pMOG: <0.0001, B6 vs. CD40$^{-/-}$+pMOG: <0.0001.) These data are from one of two experiments giving consistent results.

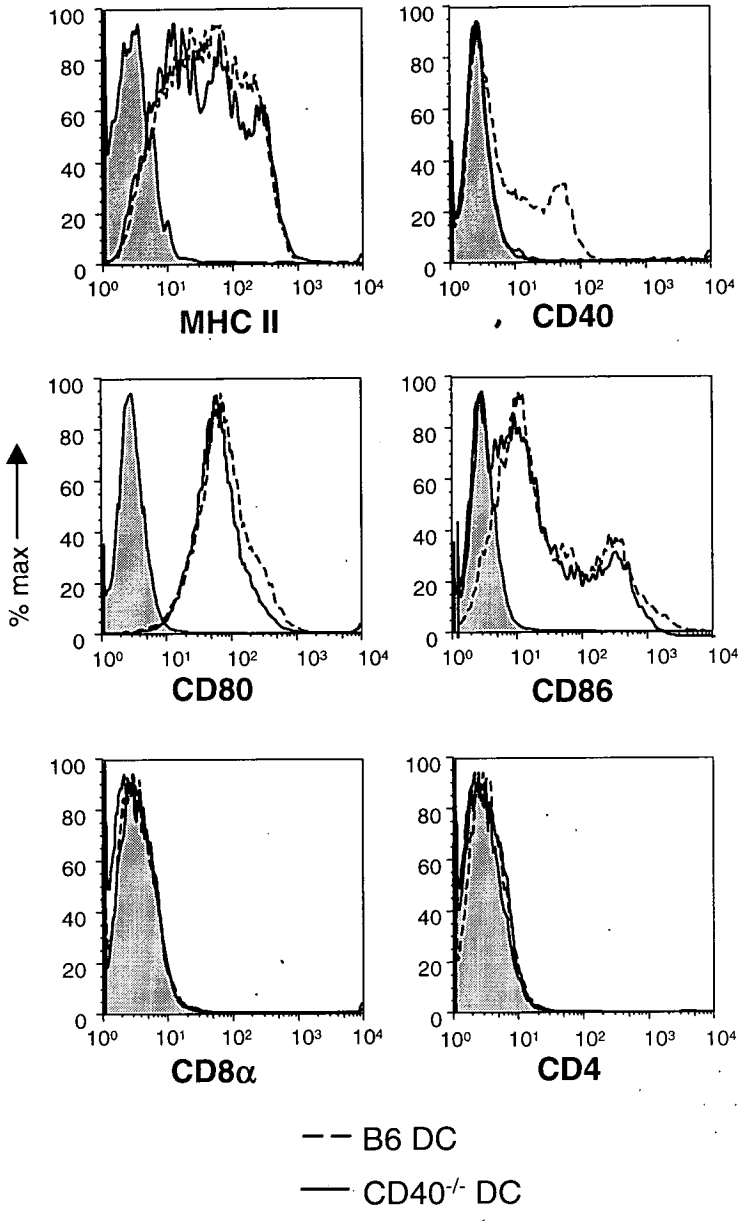

Figure 5.15:
Bm-DC from B6 and CD40$^{-/-}$ mice display similar phenotypic characteristics
B6 and CD40$^{-/-}$ bone marrow cells were grown for 7 days in the presence of GM-CSF, cultured with LPS for 2 hours at 37°C, and stained for MHC class II, CD40, CD80, CD86, CD8α and CD4. CD11c^{+} DC were analysed for expression of these molecules. These data are from one of two experiments giving consistent results.

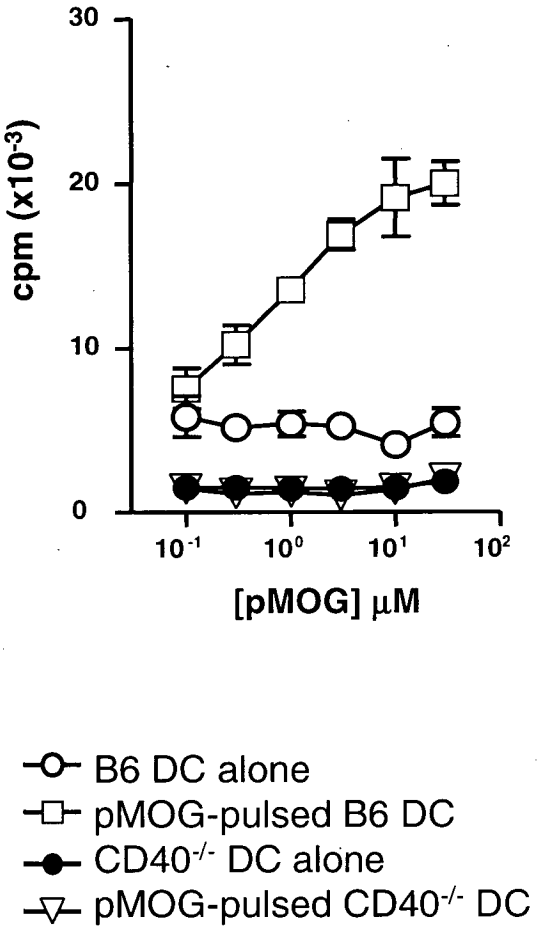

Figure 5.16:
CD40$^{-/-}$ bmDC do not support Ag-specific T cell proliferation *in vivo*
B6 or CD40$^{-/-}$ DC were grown and loaded with pMOG as described in materials and methods, before transfer into naïve B6 recipients. Six days after DC transfer, splenic lymphoid populations were tested for proliferative responses to a dose range of pMOG. These data are from one of three experiments giving consistent results.

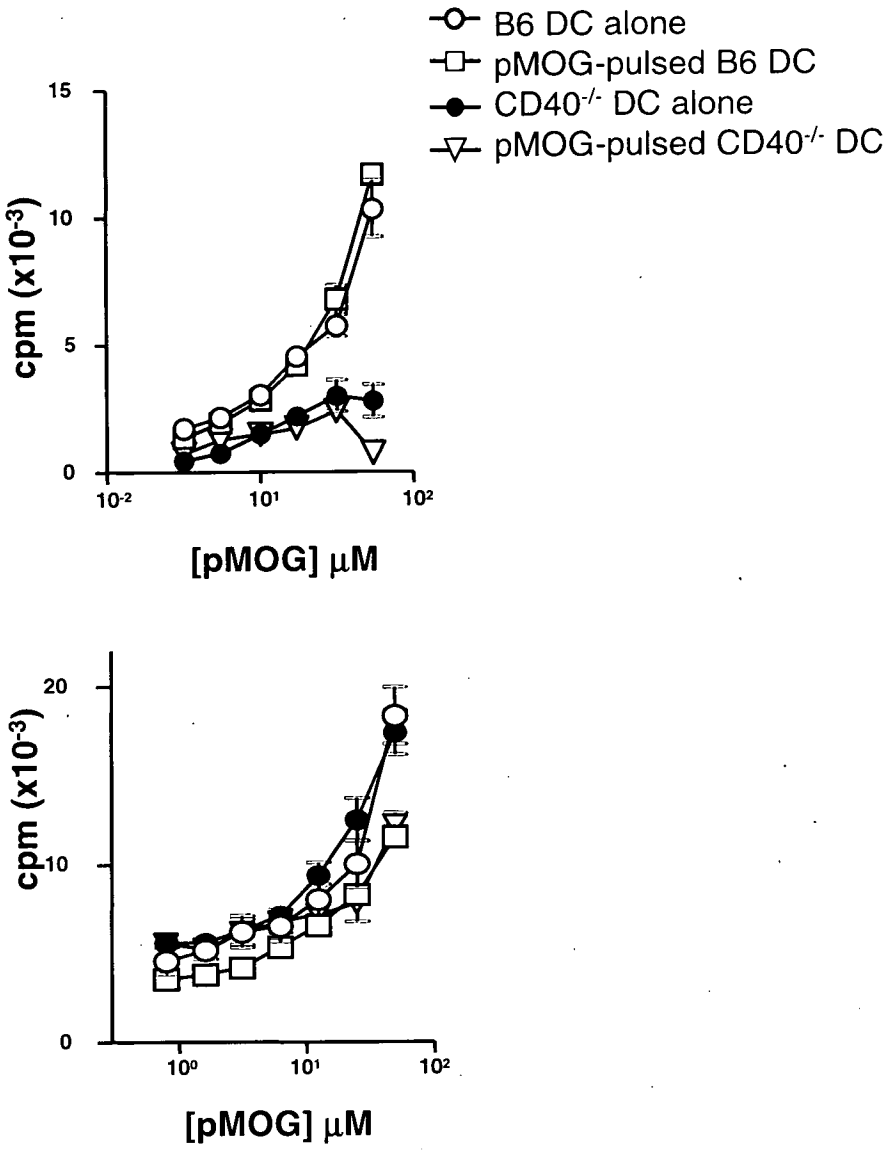

Figure 5.17:
Bone marrow-derived CD40$^{-/-}$ DC inconsistently induce unresponsiveness *in vivo*
B6 or CD40$^{-/-}$ DC were grown and loaded with pMOG as described in materials and methods, before transfer into naïve B6 recipients on day 0. Mice were immunised on day 6 with pMOG/CFA and draining lymph nodes assessed 10 days later for proliferative responses to a dose range of pMOG. Shown are two examples of the inconsistent results obtained.

6. CHAPTER 6 – Conclusions and Discussion

This thesis tested the hypothesis that the CD40-CD154 and the OX40-OX40L interactions are pivotal in the decision between T cell tolerance and T cell immunity.

The results can be summarised as follows:

- Exogenous ligation of both CD40 and OX40 prevented the induction of T cell tolerance after administration of a tolerogenic stimulus.

- The activation state of the DC, illustrated by the expression levels of CD40 and RANK was the primary factor in the decision between tolerance and immunity. Increased levels of CD40 and RANK resulted in effective immunity, while during the induction of tolerance, CD40 and RANK levels on DC remained at steady-state levels.

- Exposure to spDC lacking expression of CD40 resulted in T cell tolerance reminiscent of that induced by administration of soluble peptide, and effectively protected mice from autoimmune pathology.

From these results, it can be concluded that the CD40-CD154 interaction plays a pivotal role in the decision between tolerance and immunity. A lack of this interaction leads to tolerance, probably also due to subsequent lack of the OX40-OX40L interaction, while signalling through CD40 leading to DC activation results in T cell immunity.

6.1. Which molecules constitute the checkpoint in the decision between tolerance and immunity?

Both peptide-induced tolerance and tolerance to peripherally expressed auto-Ag involve an initial phase of T cell activation and proliferation (Huang et al., 2003; Kearney et al., 1994). This implicates the costimulatory molecules, that are important for sustained T cell activation, as key players in the immunity versus tolerance checkpoint. The idea that regulation of CD40 expression might represent a general mechanism of maintaining peripheral tolerance is an attractive one, and is supported by an increasing amount of data. Interference with the CD40:CD40L interaction using blocking anti-CD40L prevents effective T cell priming, sometimes leading to a tolerant state (Buhlmann et al., 1995; Honey et al., 1999; Tang et al., 1997). Ligation of CD40 on the other hand can induce effective immune responses in a number of systems (Diehl et al., 1999; Grohmann et al., 2001; Hawiger et al., 2001; Sotomayor et al., 1999).

In accord with the data obtained in other systems (Diehl et al., 1999; Grohmann et al., 2001; Hawiger et al., 2001; Sotomayor et al., 1999), the results presented here show that exogenous stimulation of CD40 can prevent the induction of tolerance (Chapter 3), and ligation of CD40 leads to upregulation of OX40L, CD80 and CD86 by DC (Chapter 4). Conversely, administration of splenic DC lacking CD40 induces T cell deletion and subsequent tolerance, and protects from development of autoimmune pathology (Chapter 5). These results support a model of tolerance induction in which failure to provide sufficient ligation of CD40 on DC uncouples downstream T cell:DC dialogue involving other members of the TNF and TNF-receptor families. Efficient CD40 ligation drives the upregulation of OX40L by the DC (Fillatreau and Gray, 2003), allowing ligation of OX40 that is upregulated by the T cell following activation through the TCR. OX40 signalling results in accumulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL promoting T cell survival (Rogers et al., 2001). Moreover, exogenous ligation of OX40 on T cells also prevented the induction of tolerance, in this study (Chapter 3) and another (Bansal-

Pakala et al., 2001), further supporting the hypothesis that the CD40-CD154 and OX40-OX40L interactions are pivotal in the decision between tolerance and immunity.

A role for the CD28/CTLA-4-CD80/CD86 interaction in this decision has previously been suggested. $CD28^{-/-}$ mice prime poorly (Linsley and Ledbetter, 1993), and T cells lacking CD28 fail to expand upon immunisation (Howland et al., 2000). The CD28-CD80/CD86 interaction is thus crucial for initial T cell activation. Since a T cell has to become activated and go through several rounds of division in order to become tolerant, those costimulatory molecules which are important for initial T cell activation are unlikely to determine the outcome of the immune response, suggesting that the initial interaction between CD28 and CD80/86 is not disrupted en route to tolerance.

However, a role for CTLA-4 for the induction of tolerance cannot be excluded. Expression of CD80 and CD86 increased during the induction of immunity, similar to levels of CD40 (Chapter 4). CD80/CD86 bind to CTLA-4 on activated T cells, and it is accepted that CTLA-4 plays a role in the maintenance of peripheral tolerance. CTLA-4-deficient mice develop lymphoproliferative diseases, suggesting CTLA-4 plays a role in limiting autoimmune responses. In some systems, T cells lacking CTLA-4 can be tolerised (Frauwirth et al., 2000; Frauwirth et al., 2001), while in others, CTLA-4 was required for tolerance induction (Greenwald et al., 2001; Perez et al., 1997). Most data investigating $CD4^+$ T cell tolerance suggests that CTLA-4 is required for inhibition of proliferation of the activated T cell and subsequent tolerance, suggesting that CD80/CD86 signalling to the T cell via CTLA-4 is required for tolerance induction. CD80 and CD86 levels increased during immunity, but were found at basal levels during tolerance induction. All activated T cells upregulate CTLA-4 after activation (Oosterwegel et al., 1999), and signalling through CD80 and CD86 acts as a negative regulator for T cell activation. It is possible that basal CD80 and CD86 signalling via CTLA-4 in the absence of positive signals to the T cell results in tolerance induction. During the induction of immunity,

the positive signals outweight the negative signals and en effective immune response is initiated. Data on the expression of CTLA-4 during tolerance induction *in vivo* exists, showing that CTLA-4 expression peaked 2 days after i.n. administration of soluble peptide, and had decreased to basal levels by day 6 (Metzler et al., 1999). CD80 and CD86 expression was highest at day 1 after i.v. Ag administration in the system used here, and decreased to basal levels by day 4. It will be important to investigate the timing of expression of CTLA-4 in our system in order to further comment on the precise role of CTLA-4 in the induction of tolerance.

Nevertheless, it was shown here that the absence of CD40 on DC, even in the presence of high levels of CD80 and CD86 resulted in T cell tolerance (Chapter 5). This indicates that tolerance can be induced in the presence of strong CD28 signalling (i.e. tolerance probably arises from disruption of other CD40-dependant costimulatory events such as OX40-OX40L signalling). Although CD80 and CD86 signalling via CTLA-4 may have a role in tolerance induction, the absence of CD40 has a far greater effect on the outcome of the immune response. If the main effect of lack of CD40 signalling is a lack of OX40L upregulation, experiments using DC from OX40L-deficient mice should also induce tolerance. These mice have recently been acquired, allowing us to further test our hypothesis in the near future. Others have shown that the presence of CD80 and CD86 on immunising APC is required for the induction of T cell immunity (Lohr et al., 2003), and the presence of basal CD80 and CD86 expression is required for maintenance of peripheral tolerance and the T reg population (Lohr et al., 2003; Paust et al., 2004). A picture emerges where CD80 and CD86 are important in the maintenance of peripheral tolerance, possibly through inducing T reg populations and/or through interactions with CTLA-4. CD40 ligation on the other hand, leads to DC activation and upregulation of costimulatory molecules on the DC, and acts as a "master regulator" for the decision between immunity and tolerance.

6.2. Ligation of CD40 versus provision of LPS in the prevention of tolerance

It has previously been suggested that LPS provides a qualitatively different signal from CD40 and/or OX40 costimulation (Maxwell et al., 2002). The data presented here provides further evidence that although ligation of CD40 or provision of LPS both lead to prevention of tolerance, they do so in different manners. Provision of agonistic anti-CD40 has previously been shown to not only enhance immune responses (Maxwell et al., 1999), but also to convert tolerogenic Ag presentation by DC into immunogenic presentation in some systems (Hawiger et al., 2001), and to induce effective tumour immunity in others (Diehl et al., 1999; Grohmann et al., 2001). It has also been shown that an effective $CD8^+$ T cell response develops after administration of soluble Ag and anti-CD40 (Lefrancois et al., 2000). However, it was shown here that co-administration of soluble Ag and anti-CD40 did not by itself result in effective $CD4^+$ T cell immunity (Chapter 3). Nonetheless, exogenous ligation of CD40 appeared to prevent the tolerance program, and subsequent immunisation with Ag/CFA thus allowed efficient recall responses.

Exogenous ligation of CD40 induced OX40L expression. OX40 was expressed at high levels on day 1 after administration of soluble Ag and anti-CD40, and at lower levels on day 2, while OX40L was expressed on day 2 and 3 after administration of soluble Ag and anti-CD40. This gives a time window in which the T cell could have received a survival signal from the DC, preventing induction of tolerance. In contrast, co-administration of LPS and soluble Ag led to effective T cell immunity, as has been shown in other systems (Maxwell et al., 2002). Administration of LPS increased expression of CD80, CD86, CD40 and RANK, but expression of OX40L could not be detected. Whether this was simply due to practical limitation in our FACS and histological approaches remains to be investigated. Nevertheless, upregulation of all the other costimulatory molecules at a time when their ligands were also present on the T cells allowed effective T cell activation.

Although early data suggested that CD40 ligation of DC alone was optimal to induce DC maturation and licensing to induce an effective T cell response (Mackey et al., 1998), more recent studies show that co-ligation of TLR and CD40 is necessary to induce IL-12 p70 production (i.e. functional IL-12) *in vitro* or *in vivo* (Schulz et al., 2000). Co-ligation of CD40 and TLR also enhances the frequency of peptide-reactive $CD8^+$ T cells by more than tenfold (Quezada et al., 2004). The importance of combined TLR and CD40 signalling becomes evident when considering the histological impact of TLR ligation on DC migration. LPS administration induces rapid DC migration from the splenic marginal zone into PALS (De Smedt et al., 1996), and once in the T cell area, DC have a short half life unless they receive CD154 signalling (Miga et al., 2001). This may explain why, although CD40 ligation activated DC *in vivo* and induced increased or *de novo* expression of CD80, CD86 and OX40L, subsequent TLR ligation through components found in CFA seemed to be necessary to induce an effective immune response. This again raises the question of when the administered antibodies are having their effects, which remains to be investigated.

6.3. RANKL: An indication for retrograde signalling inducing T cell tolerance?

While Chapter 5 shows that the lack of CD40 led to tolerance, Chapter 3 shows that provision of CD40 and OX40 signals (i.e. providing a signal that was lacking) could prevent tolerance. Chapter 4, on the other hand, suggests that tolerance may be more than just the absence of signalling from the DC. The early and increased expression of RANKL by T cells heading for tolerance was a surprising finding, and it is intriguing to speculate what the significance of this tolerance-specific effect may be. It could be that RANKL might have been expressed early on T cells during the induction of tolerance because a signal from the DC is missing, which is needed to control the correct timing of RANKL expression. It is clear from the data presented here as well as other studies of tolerance induction with soluble Ag, that transient T cell proliferation is intrinsic to the tolerance program (Hoyne et al., 1996; Switzer et

al., 1998). Tolerance may therefore be an active choice of the immune system, rather than just a lack of costimulatory signalling. Another possibility is thus the existence of a second receptor for RANKL on the DC. Retrograde signalling from the DC to the T cell could (possibly in the absence of other signals, such as CD154 or OX40) deliver negative signals to the T cell resulting in tolerance - an exciting option.

The existence of a second receptor for RANKL was therefore investigated. Incubation of DC with a recombinant RANKL with a histidine tag, followed by incubation with a PE-conjugated anti-histidine antibody, was used in an attempt to identify a possibly second receptor for RANKL. Unfortunately, this approach did not result in staining on DC known to express RANK, and was therefore not used any further. A RANKL fusion protein has already been shown to enhance mucosal tolerance induction *in vivo*, and incubation with DC isolated from the spleen or mesenteric lymph nodes (MLN) showed differential DC cytokine production in response to RANKL, even though both sets of DC expressed similar levels of RANK (Williamson et al., 2002). Splenic DC showed IL-12 production, while MLN DC showed IL-10 production (Williamson et al., 2002), which was interpreted to show differential responsiveness to RANKL. However, it may be that MLN DC, although very likely predisposed to inducing tolerance rather than immunity due to their surroundings, might express a second receptor to RANKL, which further promotes tolerance rather than immunity. A RANK fusion protein, which has been used previously to look at its effects on osteoclastic bone resorption (Oyajobi et al., 2001), may further elucidate whether or not signalling through RANKL could indeed give tolerogenic signals to T cells.

6.4. Lessons learned from comparing *in vitro* and *in vivo* models

Although the *in vitro* requirements for T cell activation have been studied extensively, the initiation of an immune response *in vivo* takes place within a complex microenvironment, in which the interplay between DC and T cells is

restricted by anatomical constraints. Data obtained from *in vitro* experiments may therefore not always directly correlate with those from *in vivo* models, which is further illustrated by results presented here. Chapter 4 showed the timing of expression of CD154, OX40 and RANKL on T cells after administration of soluble Ag with or without LPS. CD154 expression had previously been investigated *in vitro* after CD3 stimulation, and was found to peak at 6-8 hours, returning to resting levels between 24 and 48 hours (Roy et al., 1993). *In vivo* however, expression was found at day 1 (approximately 18 hours after Ag administration), but levels stayed increased until at least 48 hours.

A greater contrast between *in vitro* and *in vivo* findings were observed looking at expression of OX40. OX40 was reported to be upregulated later than CD154, peaking at 3-4 days after initial T cell activation *in vitro* (Gramaglia et al., 1998). As clearly shown in Chapter 4, OX40 was upregulated *in vivo* within 24 hours of Ag administration, but had disappeared again by day 3. Similarly, RANKL was suggested to act sequentially after CD154, having been found to peak in expression 48 hours after *in vitro* T cell stimulation, and remaining high in expression until 96 hours (Josien et al., 1999). In the system used here, RANKL was found to be expressed at day 1 after administration of Ag alone, and at day 2 after administration of Ag and LPS, and had greatly decreased in expression by day 3 (i.e. well before 96 hours). These differences show that the actual *in vivo* expression of costimulatory molecules greatly differs from *in vitro* experiments, and probably also varies when different immunisation routes are used. Intravenous administration of soluble Ag allows almost immediate access of Ag for uptake and/or binding to DC, while s.c. administration of Ag/CFA is likely to require DC activation at the site of CFA administration, and subsequent migration to DLN, before efficient T cell activation.

Chapter 5 describes attempts to design an *in vitro* model to investigate the importance of CD40 on DC for T cell priming. Stimulating T cells *in vitro* with B6 or $CD40^{-/-}$ DC showed CD69 expression over period of several days, and OX40 and RANKL expression peaked by day 3 after stimulation, and was still found by day 4

(not shown). *In vivo* however, CD69 expression peaked at day 1, and by day 4, a large proportion of T cells activated by $CD40^{-/-}$ DC had disappeared. The findings of *in vitro* experiments may give an indication of the requirements for T cell activation *in vivo*, but testing of *in vitro* findings in an *in vivo* setting is essential. Nevertheless, there are of course potential benefits to the development of appropriate *in vitro* models of T cell activation versus tolerance, particularly considering animal welfare, and further research should be conducted in an effort to increase the validity of such models.

Identification of the molecules and pathways that drive the T cell response towards tolerance rather than immunity is crucial for future design of therapy. A number of human trials have shown the effectiveness of administration of allergen peptides to reduce allergy symptoms (Norman et al., 1996; Oldfield et al., 2002). Mouse studies have already indicated the therapeutic potential of targeting costimulatory molecules in either preventing disease or inducing effective immunity. The results presented in this thesis provide important information on the kinetics of the basic molecular interactions leading to immunity versus tolerance *in vivo*. These data will hopefully contribute to the rational design of therapies that either boost or block immune responses, and which may allow treatment of autoimmunity, allergy or tumours in the future.

7. CHAPTER 7 - References

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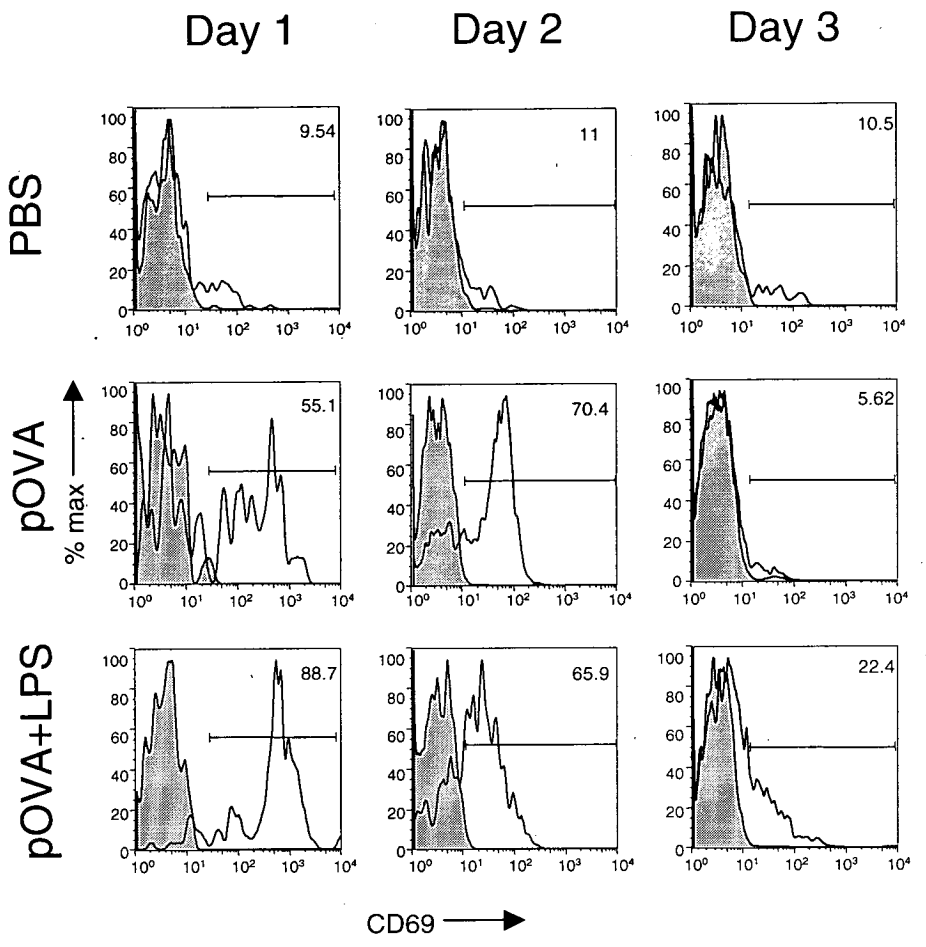
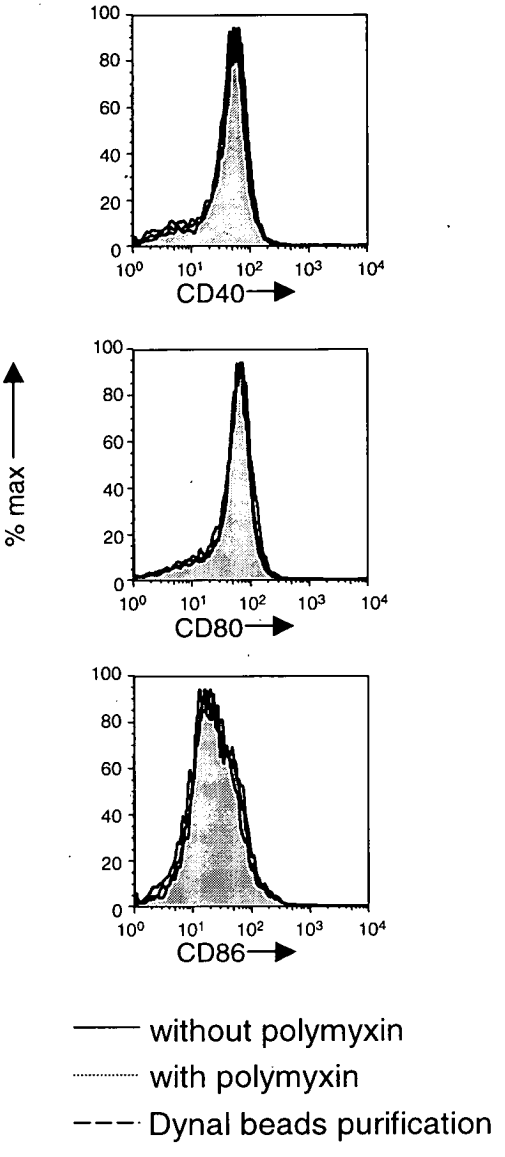
8. CHAPTER 8 – Appendix

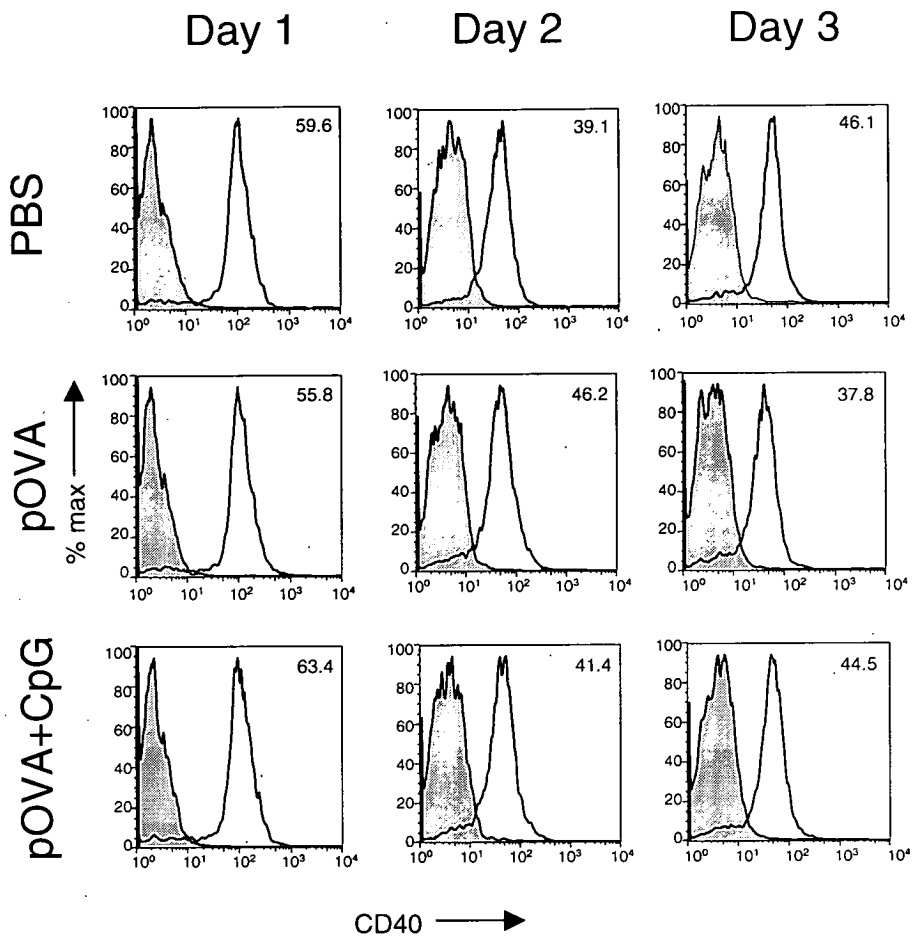
8.1. Appendix Figures



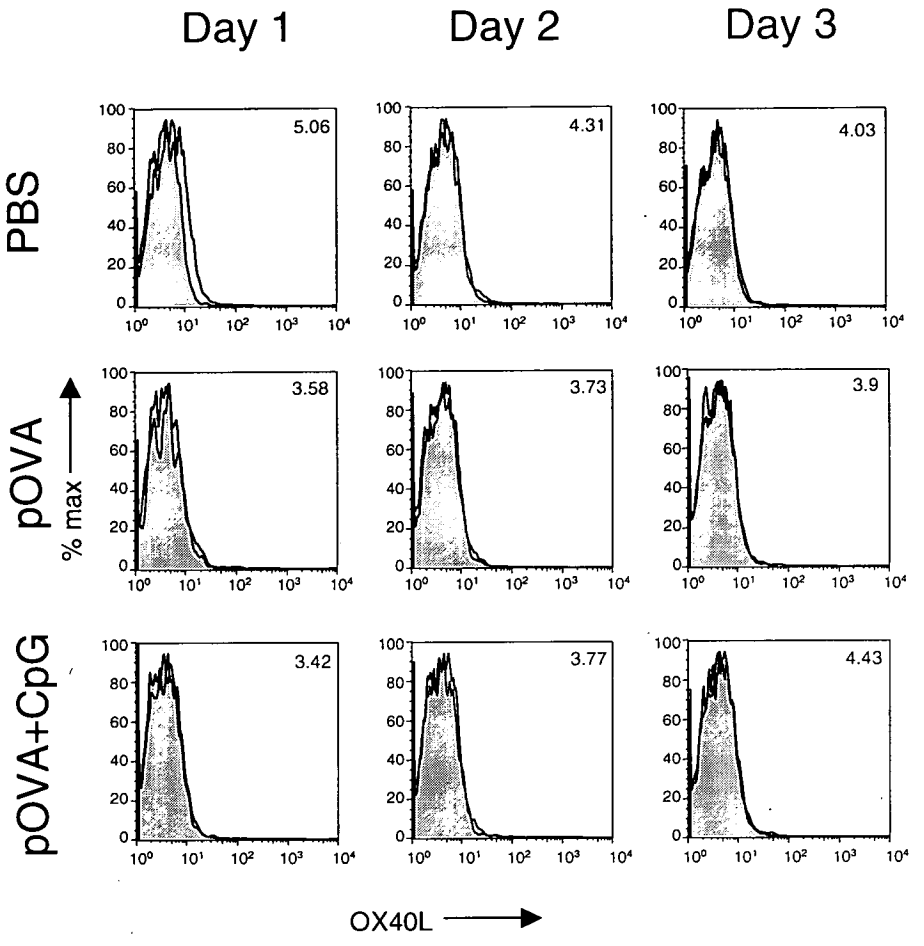
Appendix Fig. 1:
CD69 expression on T cells after administration of pOVA, pOVA+LPS or PBS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for CD69 expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for CD69 are shown on each graph. These data are from one of several experiments giving consistent results.



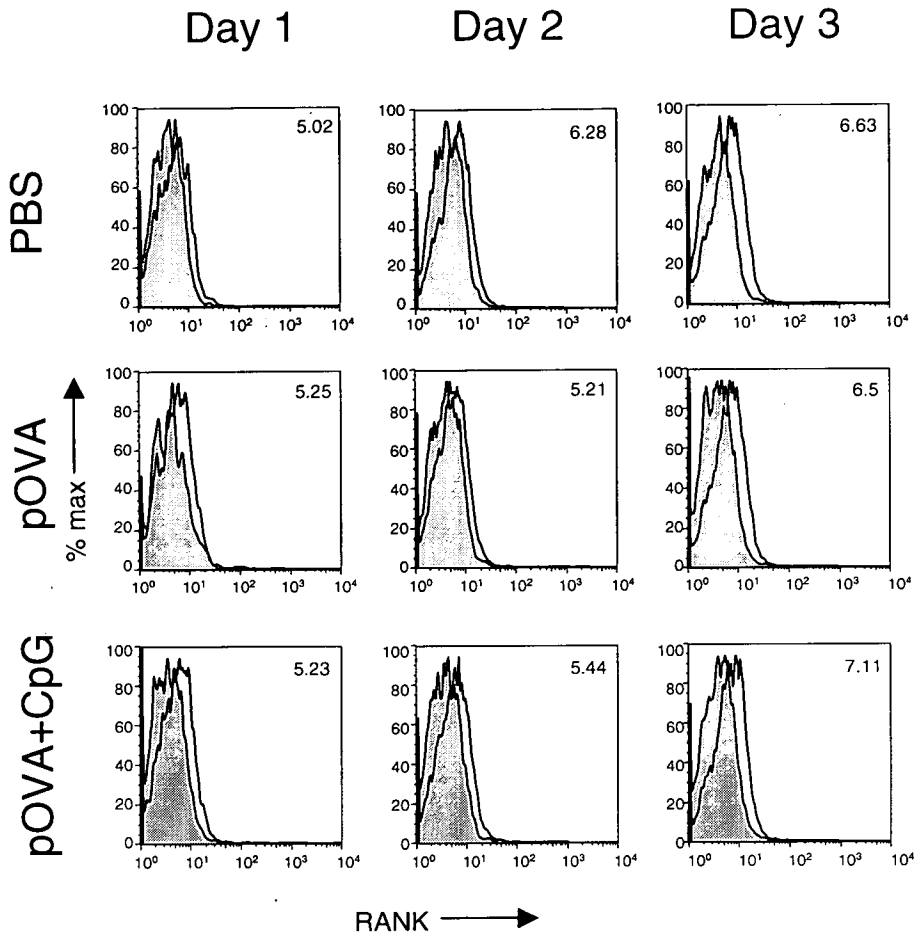
Appendix Fig. 2:
DC phenotype after purification in the presence of polymyxin, or or after negative selection using Dynal beads
DC were purified from naïve B6 mice, either by positive selection in the absence (full lines) or the presence of polymyxin (dotted lines) or by negative selection using Dynal beads (dashed lines). $CD11c^+$ DC were analysed for expression of CD40, CD80 and CD86. Filled histograms are $CD11c^+$ cells stained with an isotype control antibody.



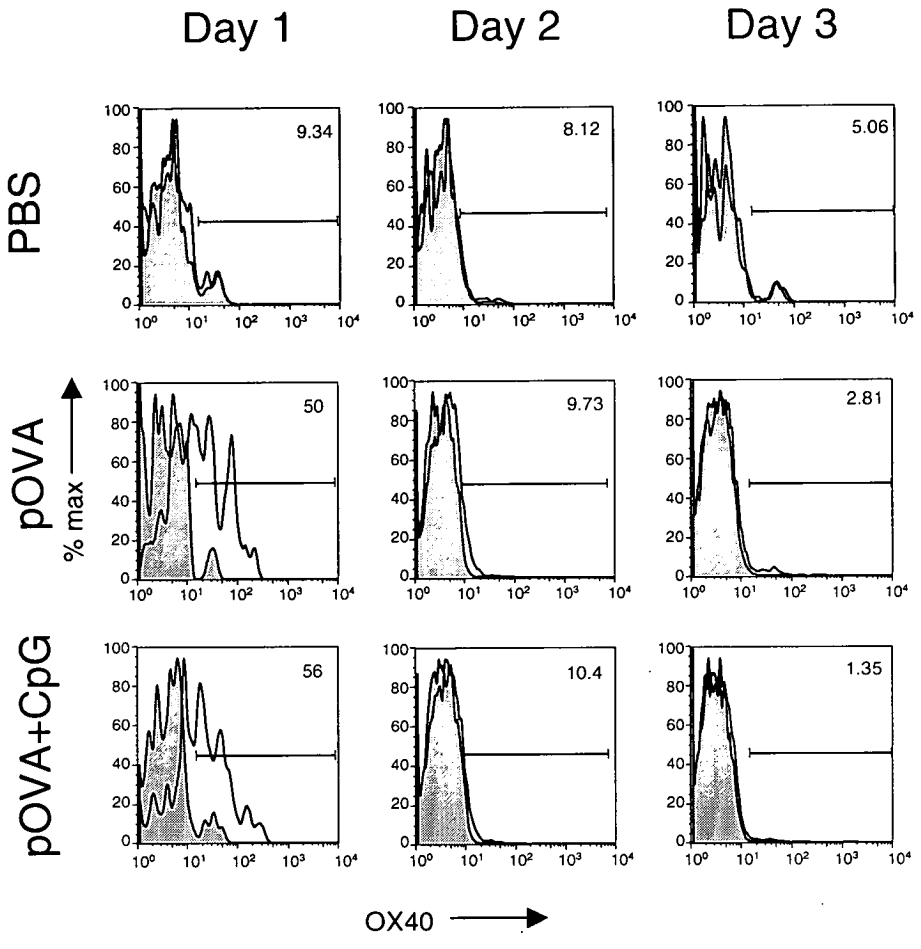
Appendix Fig. 3:
CD40 expression on DC after administration of pOVA, pOVA+CpG or PBS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+CpG or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD40 expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of CD40 stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



Appendix Fig.4:
OX40L expression on DC after administration of pOVA, pOVA+CpG or PBS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+CpG or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for OX40L expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of OX40L stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



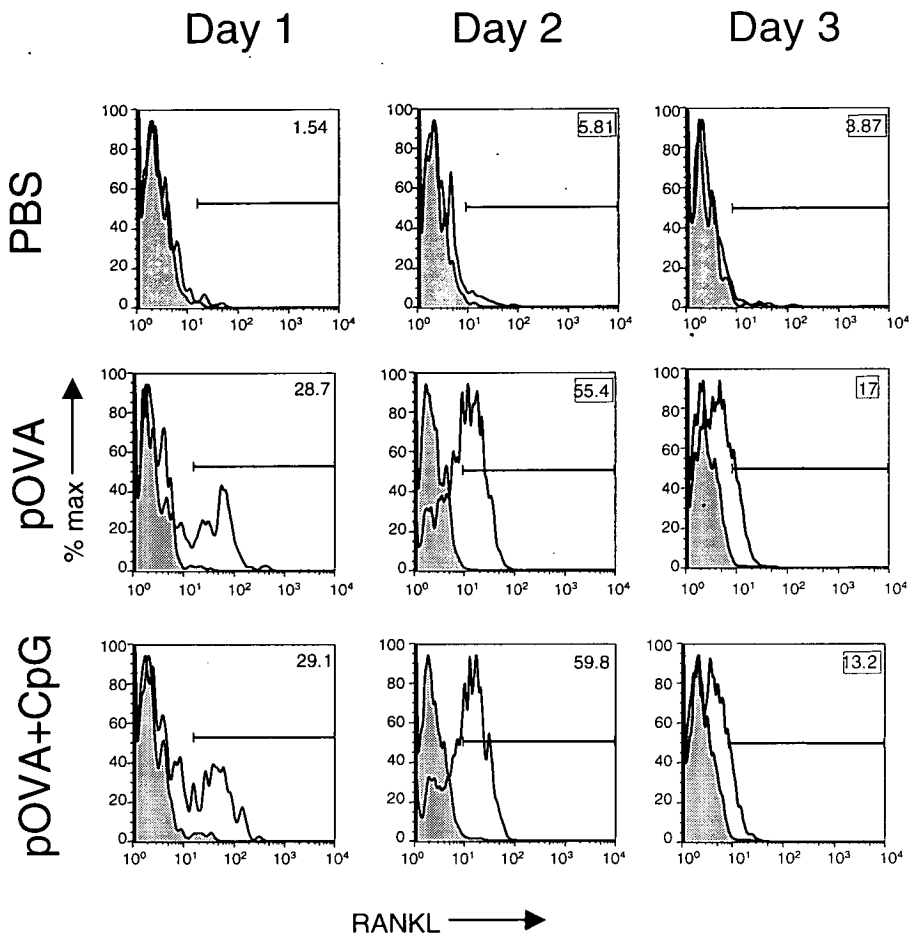
Appendix Fig. 5:
RANK expression on DC after administration of pOVA, pOVA+CpG or PBS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+CpG or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for RANK expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of RANK stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



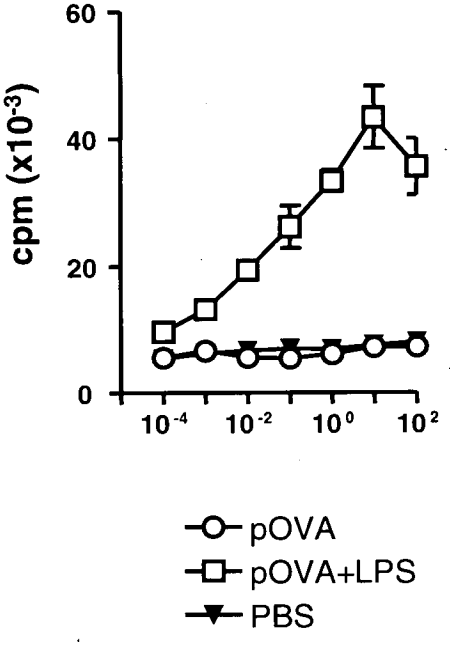
Appendix Fig. 6:
OX40 expression on T cells after administration of pOVA, pOVA+CpG or PBS

B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+CpG or PBS.

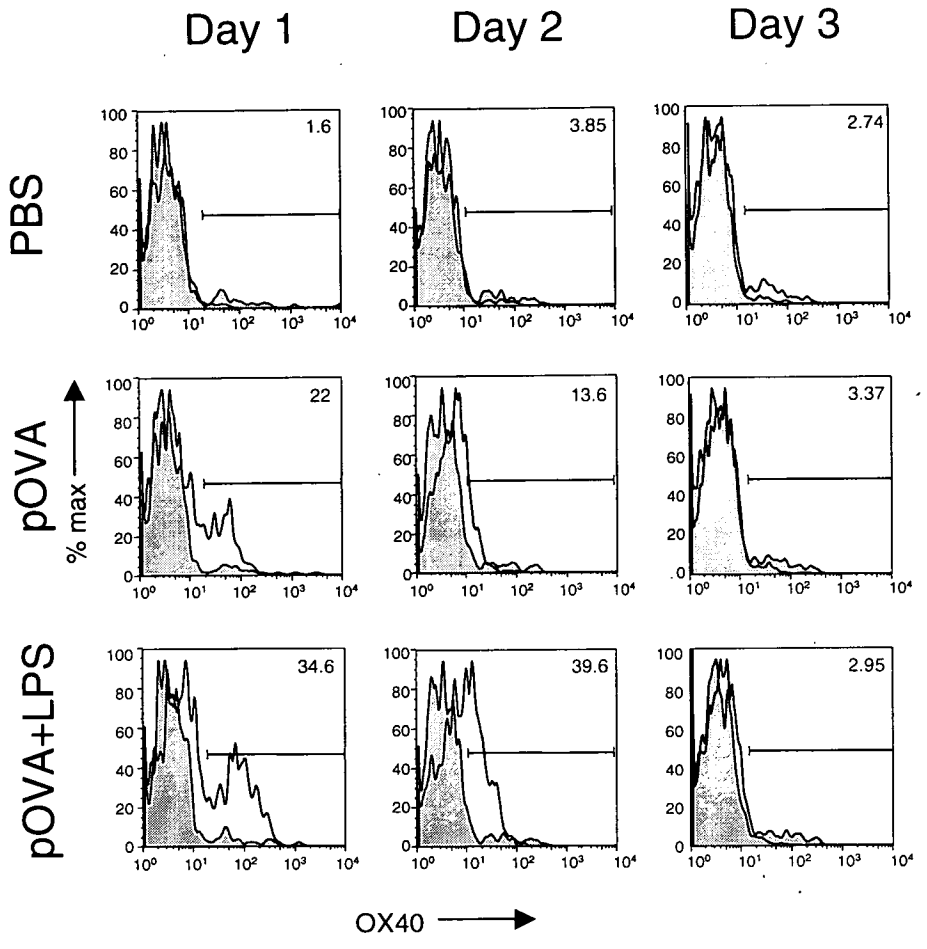
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for OX40 expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for RANKL are shown on each graph. These data are from one of several experiments giving consistent results.



Appendix Fig. 7:
RANKL expression on T cells after administration of pOVA, pOVA+CpG or PBS
B6 mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+CpG or PBS.
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for RANKL expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for RANKL are shown on each graph. These data are from one of several experiments giving consistent results.

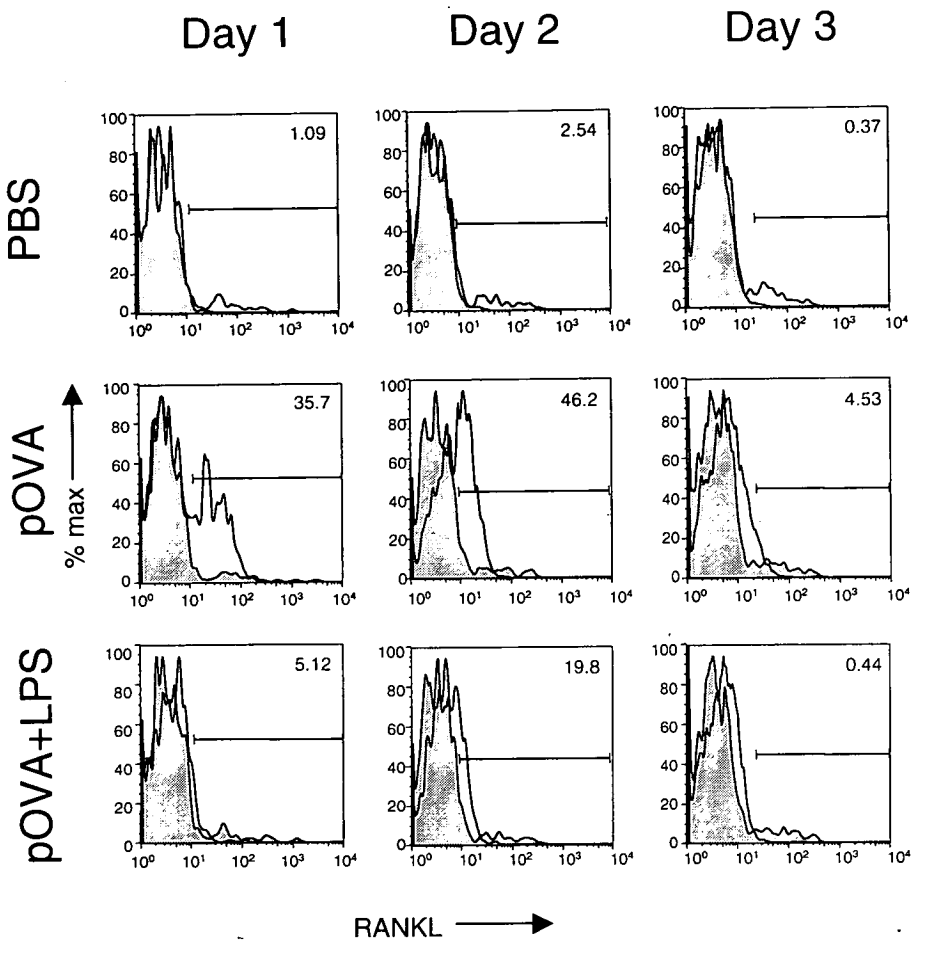


Appendix Fig. 8:
Administration of pOVA+LPS leads to successful priming in CD154$^{-/-}$ mice
Naïve CD154$^{-/-}$ mice received a single dose of 500µg pOVA, pOVA+50µg LPS, or PBS i.v. on day 0. Spleen were assessed 5 days later for proliferative responses to a dose range of pOVA. Background levels were as follows: pOVA: 4862, pOVA+LPS: 6504, PBS: 4837.



Appendix Fig. 9:
Tolerance of CD154$^{-/-}$ T cells is not due to the absence of OX40 on T cells

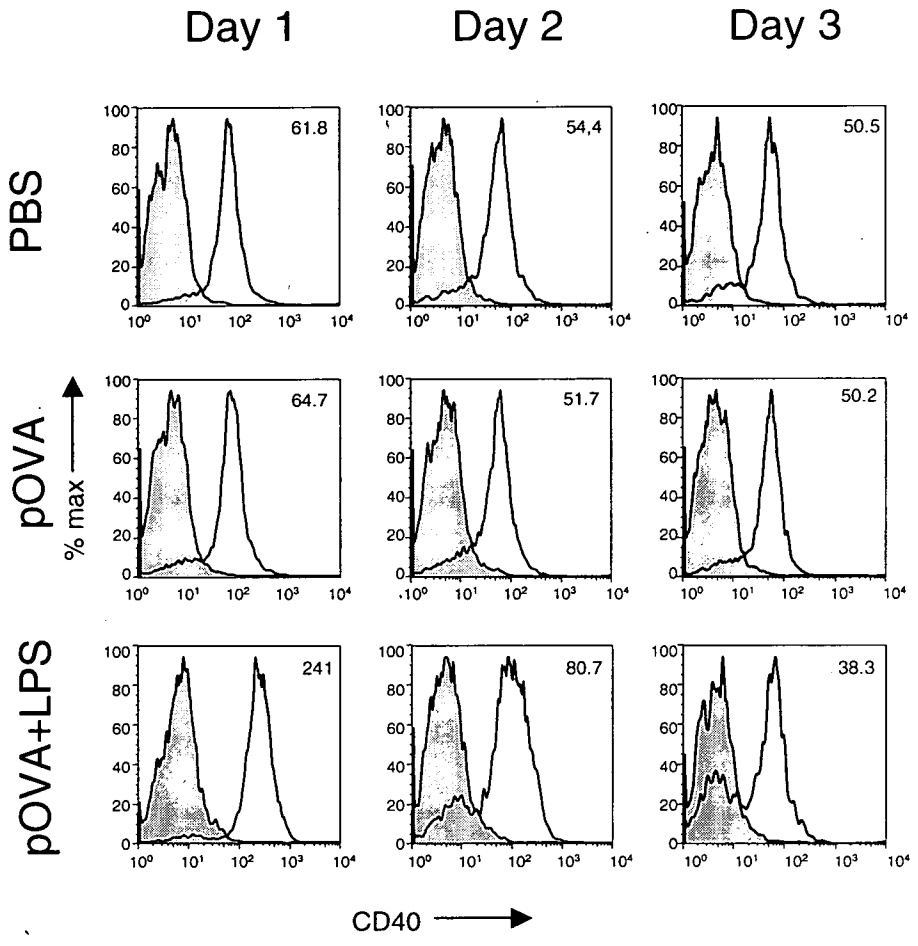
CD154$^{-/-}$ mice received CD154$^{-/-}$xOT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for OX40 expression by Vα2$^+$, Vβ5$^+$ CD4$^+$ OT-II cells (filled histograms are Vα2$^+$, Vβ5$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Vα2$^+$, Vβ5$^+$ CD4$^+$ OT-II cells positive for OX40 are shown on each graph. These data are from one of several experiments giving consistent results.



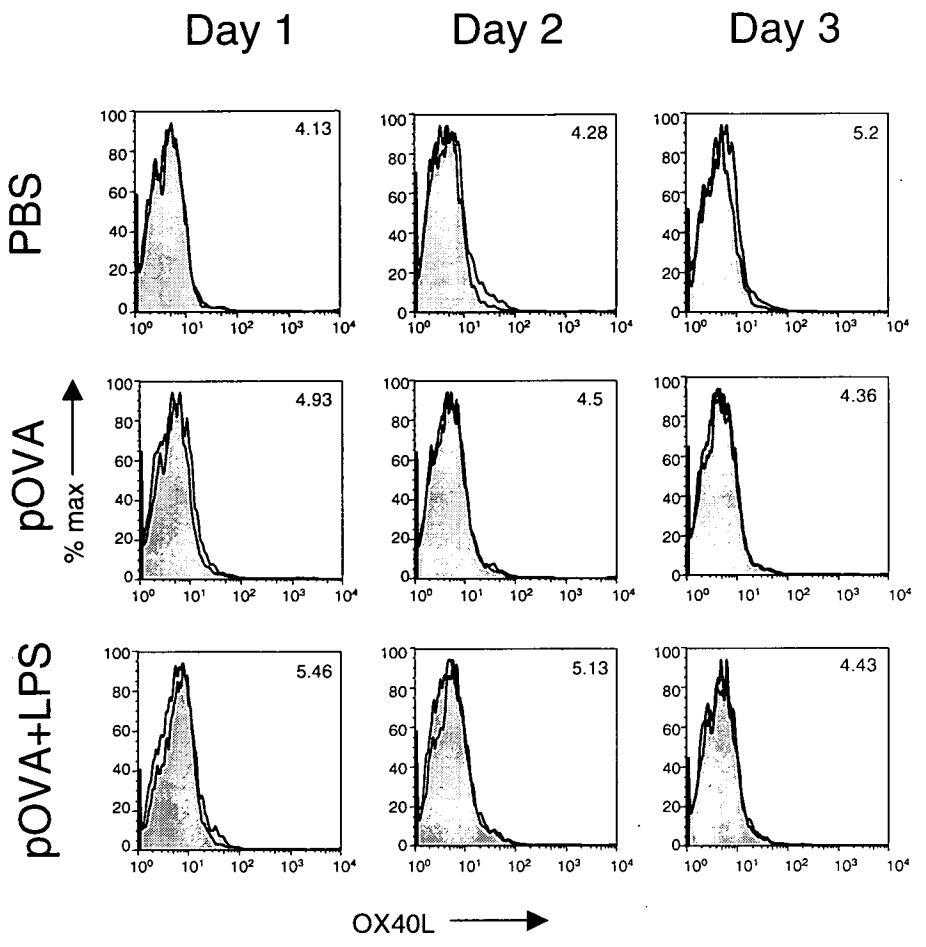
Appendix Fig. 10:
Tolerance of CD154$^{-/-}$ T cells is not due to the absence of RANKL on T cells

CD154$^{-/-}$ mice received CD154$^{-/-}$xOT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.

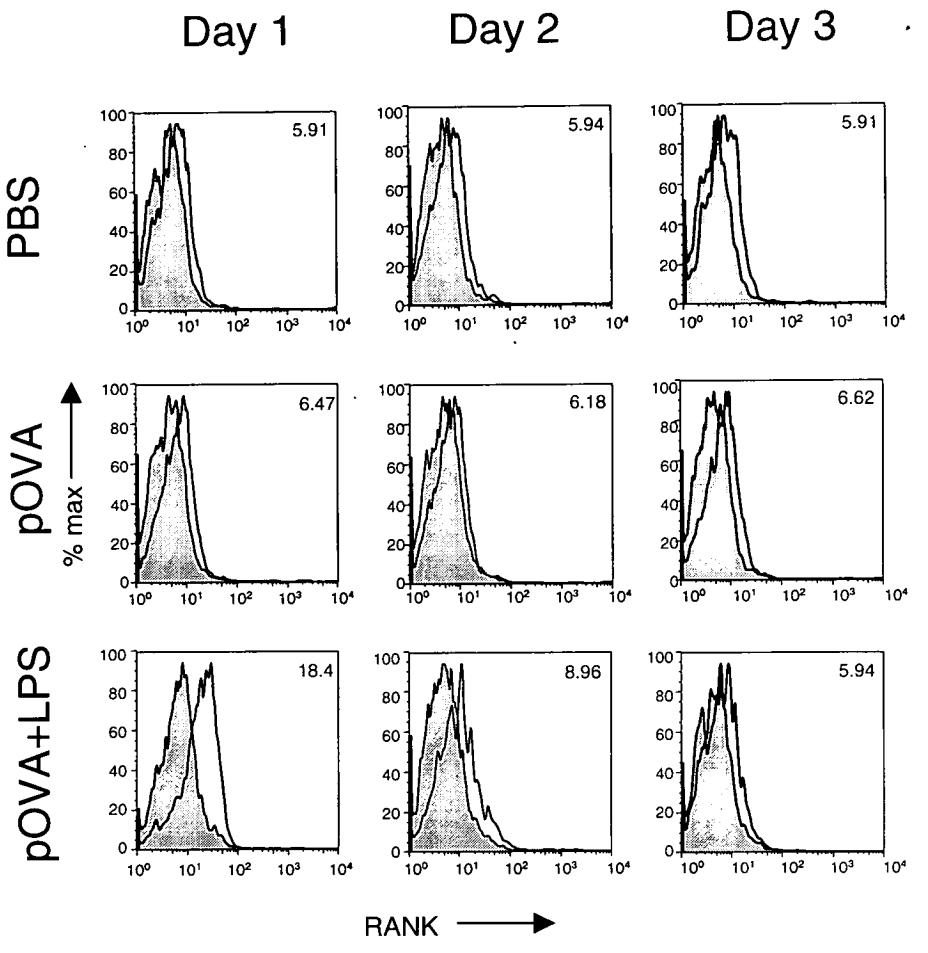
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for RANKL expression by Vα2^{+}, Vβ5^{+} CD4^{+} OT-II cells (filled histograms are Vα2^{+}, Vβ5^{+} CD4^{+} OT-II cells stained with streptavidin-APC alone). Percentages of Vα2^{+}, Vβ5^{+} CD4^{+} OT-II cells positive for RANKL are shown on each graph. These data are from one of several experiments giving consistent results.



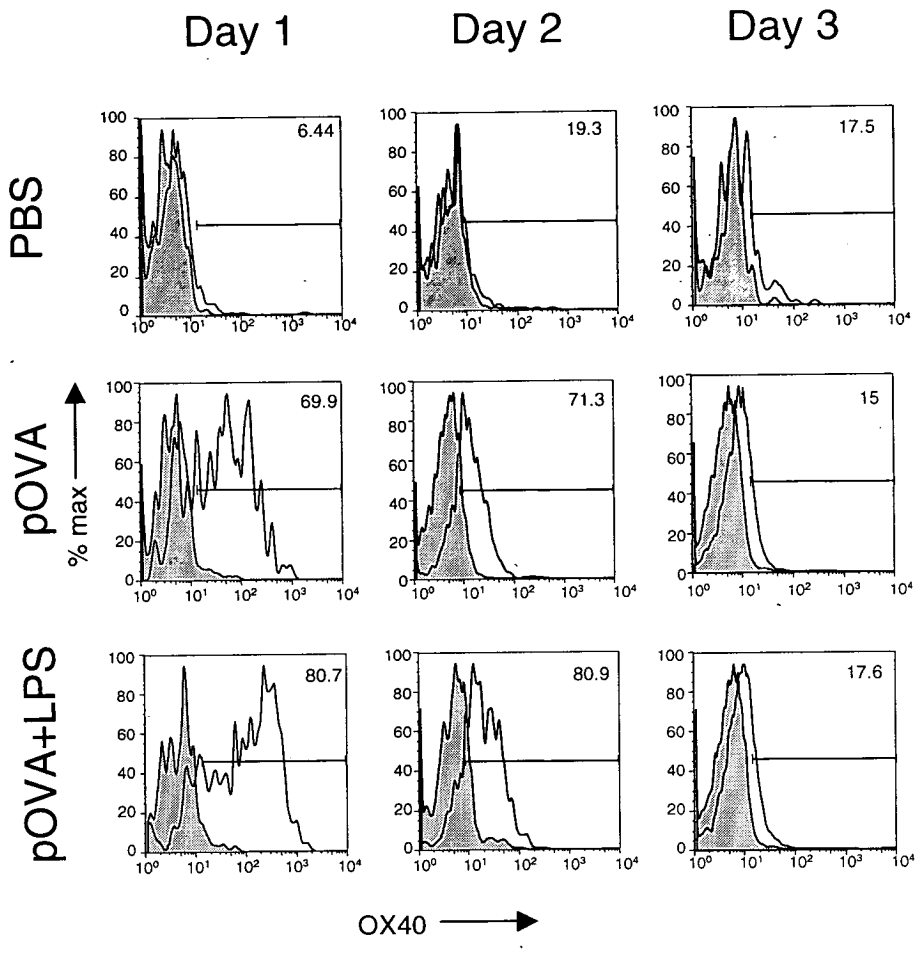
Appendix Fig 11:
CD40 expression is upregulated on DC during the induction of immunity in CD154$^{-/-}$ mice
CD154$^{-/-}$ mice received CD154$^{-/-}$xOT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c^{+} DC were purified from the spleen. CD11c^{+} cells were analysed by FACS for CD40 expression on days 1, 2 and 3 (filled histograms are CD11c^{+} cells stained with an isotype control antibody). Median fluorescence values of CD40 stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



Appendix Fig.12:
OX40L expression on DC during the induction of tolerance or immunity in CD154$^{-/-}$ mice
CD154$^{-/-}$ mice received CD154$^{-/-}$xOT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c^{+} DC were purified from the spleen. CD11c^{+} cells were analysed by FACS for OX40L expression on days 1, 2 and 3 (filled histograms are CD11c^{+} cells stained with an isotype control antibody). Median fluorescence values of OX40L stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



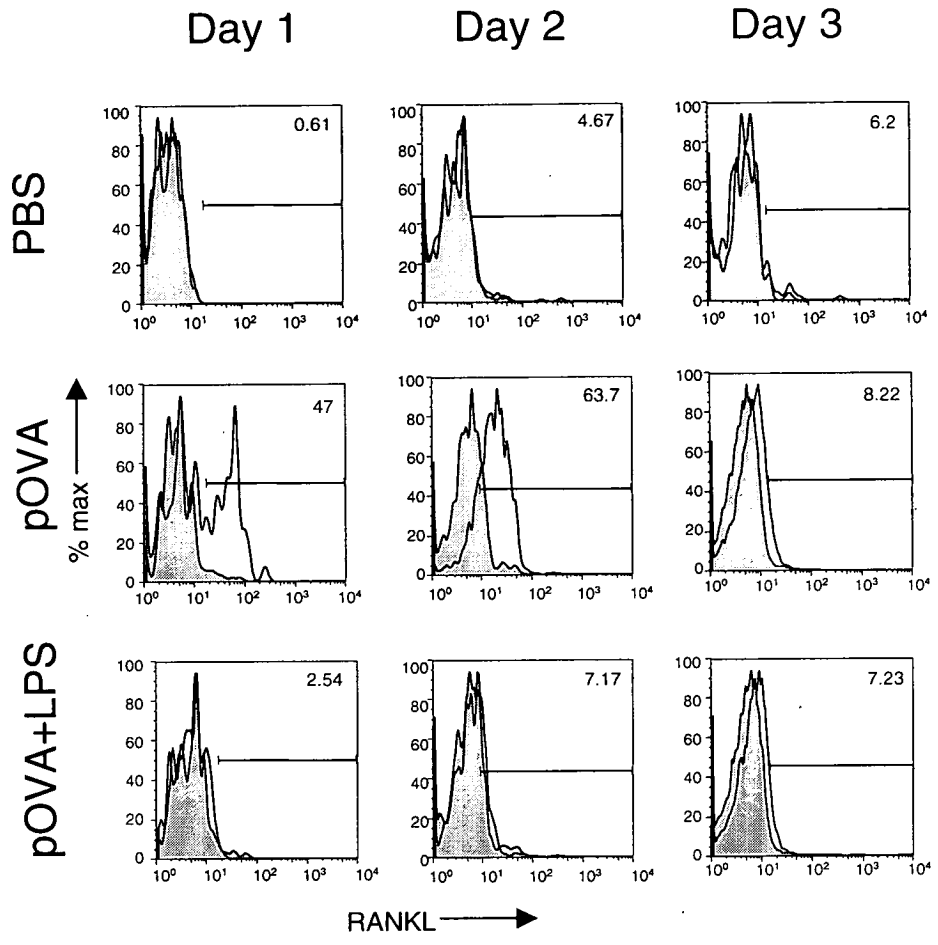
Appendix Fig. 13:
RANK expression is upregulated on DC during the induction of immunity in CD154$^{-/-}$ mice
CD154$^{-/-}$ mice received CD154$^{-/-}$xOT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c^{+} DC were purified from the spleen. CD11c^{+} cells were analysed by FACS for OX40L expression on days 1, 2 and 3 (filled histograms are CD11c^{+} cells stained with an isotype control antibody). Median fluorescence values of RANK stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



Appendix Fig. 14:
Tolerance in B cell-deficient mice in not due to the absence of OX40 on T cells

μMT mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.

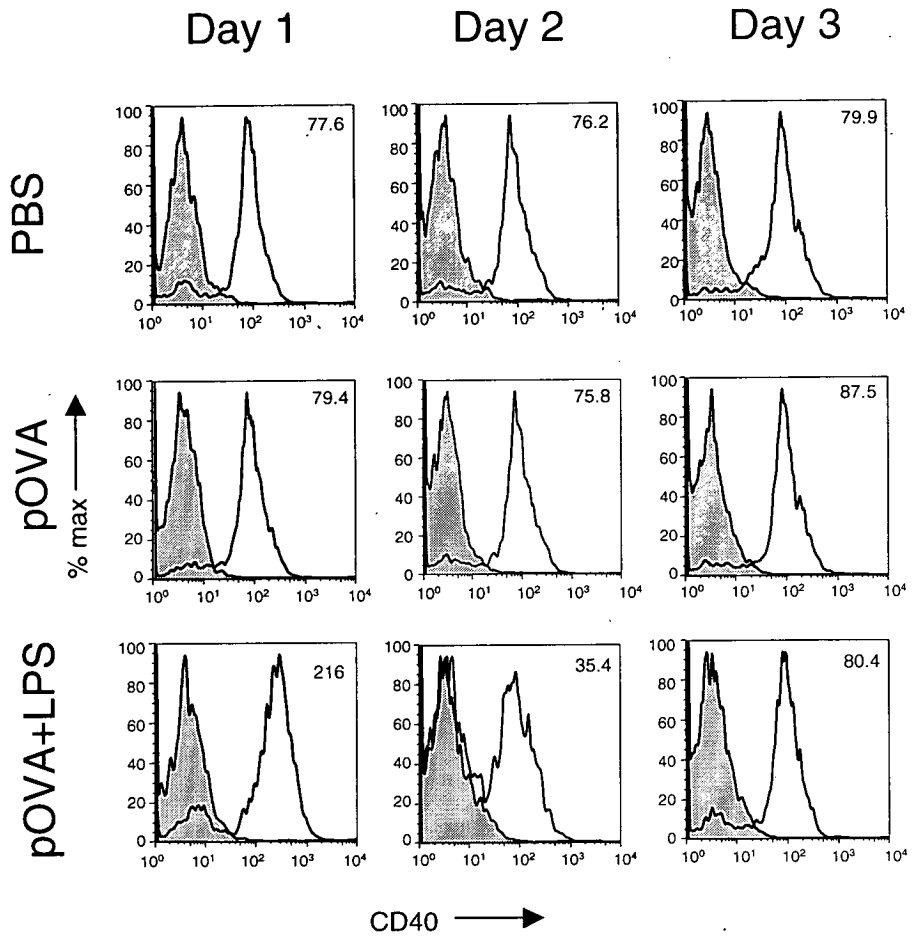
On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for OX40 expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for OX40 are shown on each graph. These data are from one of several experiments giving consistent results.



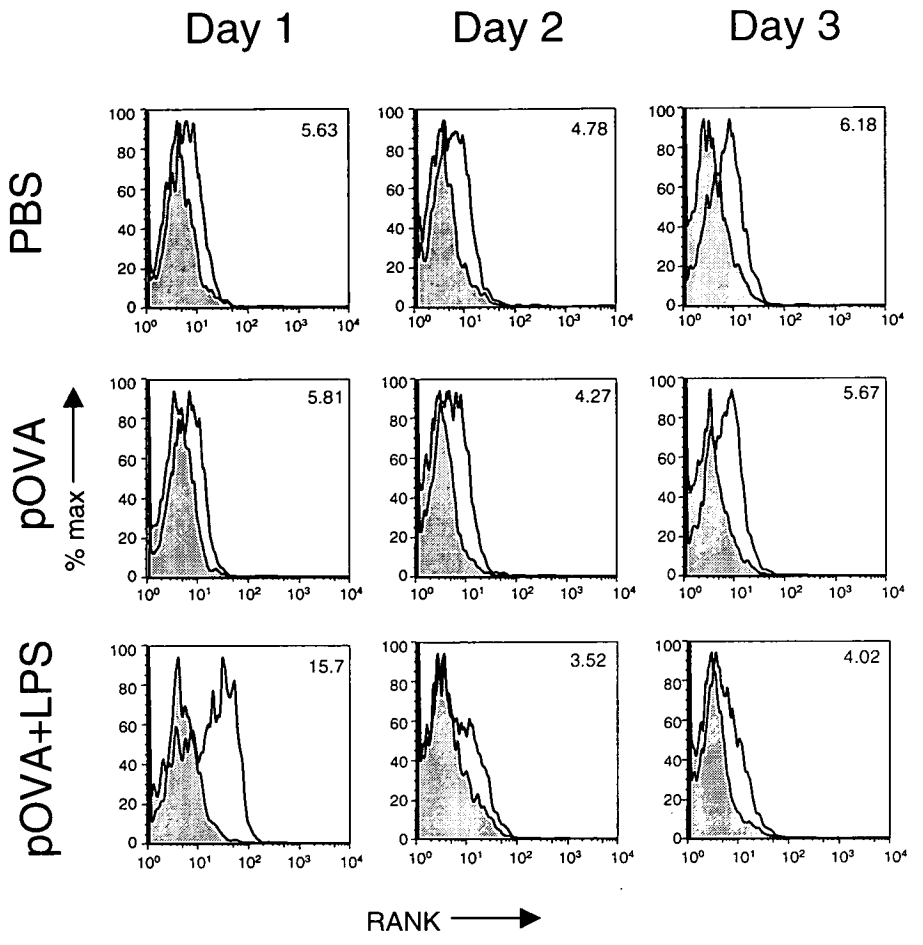
Appendix Fig. 15:
Tolerance in B cell-deficient mice is not due to the absence of RANKL on T cells

μMT mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.

On days 1, 2 and 3 post Ag administration, spleen cells were analysed by FACS for RANKL expression by Ly5.1$^+$ CD4$^+$ OT-II cells (filled histograms are Ly5.1$^+$ CD4$^+$ OT-II cells stained with streptavidin-APC alone). Percentages of Ly5.1$^+$ CD4$^+$ OT-II cells positive for RANKL are shown on each graph. These data are from one of several experiments giving consistent results.



Appendix Fig. 16:
CD40 expression is upregulated on DC during the induction of immunity in B cell-deficient mice
μMT mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for CD40 expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of CD40 stained DC are shown on each graph. These data are from one of several experiments giving consistent results.



Appendix Fig. 17:
RANK expression is upregulated on DC during the induction of immunity in B cell-deficient mice
μMT mice received Ly5.1$^+$ OT-II cells one day prior to administration of pOVA, pOVA+LPS or PBS.
On days 1, 2 and 3 post Ag administration, CD11c$^+$ DC were purified from the spleen. CD11c$^+$ cells were analysed by FACS for RANK expression on days 1, 2 and 3 (filled histograms are CD11c$^+$ cells stained with an isotype control antibody). Median fluorescence values of RANK stained DC are shown on each graph. These data are from one of several experiments giving consistent results.

8.2. Publications

Systemic administration of antigen-loaded CD40-deficient dendritic cells mimics soluble antigen administration

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The decision to mount a T cell response to antigen (Ag) is dependent on the cellular context in which the Ag is presented. Activated dendritic cells (DC) are potent stimulators of immune responses, an ability which is linked to their high expression of several costimulatory molecules. In contrast, resting DC have been implicated in the generation of self tolerance, presumably due to their reduced costimulatory capacity. However, the precise molecular basis for the choice between Ag-induced immunity and unresponsiveness remains unclear. We show here that CD40 plays an important role in this decision. Systemic administration of Ag-loaded, CD40-deficient DC failed to induce a productive primary T cell expansion and rendered mice relatively unresponsive to subsequent immunization with Ag in adjuvant. Using a TCR-transgenic T cell transfer system, we found that $CD40^{-/-}$ DC triggered an initial T cell activation that could not be sustained, resulting in loss of Ag-reactive T cells and reduced cytokine production by those T cells that did persist. Furthermore, administration of $CD40^{-/-}$ DC that had been loaded with a central nervous system autoantigen was found to protect mice from autoimmune pathology. These data implicate the CD40:CD40L interaction as a key checkpoint in the development of T cell immunity rather than tolerance.

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Key words: Dendritic cell / CD40 / T cells / Tolerance / EAE

1 Introduction

The adaptive immune response is dependent on complex interactions between APC and $CD4^+$ T cells. *In vitro* and *in vivo* studies have shown that dendritic cells (DC) are the most efficient APC at activating naive T cells [1]. This capacity is linked to the ability of DC to sense pathogen-derived molecular triggers or inflammatory mediators, resulting in DC "activation" as characterized by increased expression of MHC and costimulatory molecules. Experimental provision of protein or peptide antigens in the absence of DC activation (by *in vivo* administration of soluble Ag) fails to induce sustained T cell activation, but instead leads to T cell tolerance [2, 3]. The consensus view is that the activation state of the DC is crucial in determining the outcome of antigenic challenge, *i.e.* the development of either productive T cell immunity or tolerance. Resting and activated DC show marked differences in their expression of various costimulatory molecules [4]. Notably, CD40 is expressed at very low levels by resting DC, but is rapidly up-regulated upon activation [5, 6]. Ligation of CD40 leads to further DC maturation and up-regulation of CD80, CD86 and OX40L which all serve to boost T cell activation and expansion [7–10].

Experiments using CD40- or CD40L-deficient mice have shown clear defects in T cell priming *in vivo* when this interaction is absent [11–13]. This has led to the view that ineffective DC stimulation through CD40 may, at least in part, account for T cell tolerance that results from administration of Ag in the absence of adjuvant. Consistent with this, triggering CD40 using agonistic anti-CD40 *in vivo* has been shown in several studies to convert a tolerogenic antigenic stimulus into an immunogenic one [14–16]. If the CD40:CD40L interaction is truly a critical checkpoint in the immunity versus tolerance decision, then administration of Ag-loaded CD40-deficient DC should lead to Ag-specific T cell tolerance reminiscent of that induced by soluble Ag. Previous studies that have addressed this by administration of DC s.c. concluded that, whilst $CD40^{-/-}$ DC failed to lead to productive T cell immunity, T cell tolerance was also not evident [17]. However, a key factor for the induction of effective T cell tolerance with soluble Ag is the use of a systemic route [3]. We would therefore have predicted that the previous

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Abbreviations: pOVA: 323–339 peptide of OVA **B6:** C57BL/6

study using the sub-cutaneous route would not lead to a global tolerogenic effect.

To re-address this question we have administered Ag-loaded CD40-sufficient or -deficient DC i.v. and assessed their influence on the fate of Ag-reactive T cells by using TCR-transgenic transfer systems and by monitoring the development of $CD4^+$ T cell-mediated CNS autoimmunity. We show that CD40 expression was not necessary for initial T cell expansion *in vivo*, but was essential for sustaining this expansion past day 3 after DC administration. This correlated with a decreased proportion of T cells producing IL-2 and IFN-γ when using $CD40^{-/-}$ DC. Importantly these deficits were evident even though the $CD40^{-/-}$ DC had elevated expression of other costimulatory molecules (CD80, CD86). Furthermore, using EAE, we show that this approach conferred effective protection against the development of autoimmune pathology. From these data, we conclude that the systemic administration of CD40-deficient DC leads to inhibition of T cell activation and deletional tolerance that is reminiscent of that seen following administration of soluble Ag.

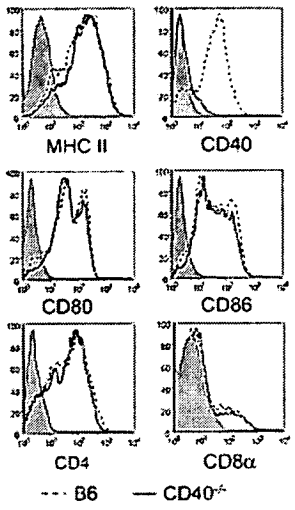

Fig. 1. Splenic DC from B6 and $CD40^{-/-}$ display similar phenotypic characteristics. Spleens were treated with collagenase and DNase, DC were purified as described in Sect. 4, cultured with LPS for 2 h at 37°C, and stained for CD80, CD86, CD40, OX40L, MHC class II, CD8α and CD4.

2 Results

2.1 Wild-type and $CD40^{-/-}$ splenic DC have similar phenotypic characteristics

Using wild-type and $CD40^{-/-}$ mice as sources of DC, we have examined the consequences of the lack of CD40 expression by DC on the kinetics of T cell activation and expansion. In order to exclude the possibility that subsequent results were due to variation in expression of other costimulatory molecules or in the proportions of DC subsets, we analyzed the surface phenotype of splenic DC isolated from C57BL/6 (B6) or $CD40^{-/-}$ mice. These two DC populations expressed similar levels of CD80 and CD86 immediately after purification (Fig. 1). Similar percentages of $CD8\alpha^+$ and $CD4^+$ DC subsets were also found in each population. A large proportion of DC displayed a mature phenotype, as illustrated by MHC class II expression and high levels of CD40 on B6 cells, suggesting that the purification procedure we used resulted in significant DC activation.

2.2 CD40-deficient splenic DC induce T cell activation and expansion *in vitro*

We next tested whether $CD40^{-/-}$ DC could activate a primary T cell response *in vitro* using OT-II T cells that recognize the 323–339 peptide of OVA (pOVA) [18]. $CD4^+$ OT-II T cells (from OT-II×$CD40^{-/-}$ mice to avoid contaminating $CD40^{+/+}$ cells) were CFSE-labeled, and cultured with pOVA-pulsed DC from B6 or $CD40^{-/-}$ mice. The CFSE profile was assessed after 2, 3, 4, and 5 days of culture, together with expression of CD69 and CD25 (Fig. 2). Stimulation with $CD40^{-/-}$ DC consistently led to a lower percentage of T cells undergoing division at early time points (days 2 and 3 of culture) compared with cultures using B6 DC. This was reflected in the percentages of T cells showing up-regulation of CD69 and CD25 at these time points (Fig. 2). However, those T cells that had entered the proliferative cycle had undergone similar numbers of cell divisions in both groups, irrespective of the DC used for stimulation. Moreover, although down-regulation of levels of CD69 expression occurred more rapidly after stimulation with $CD40^{-/-}$ DC compared to B6 DC, by day 5 of culture, a similar number of T cells in both sets of cultures had undergone substantial cell division (Fig. 2). In summary, these experiments suggest that $CD40^{-/-}$ DC were less efficient at triggering initial $CD4^+$ T cell expansion *in vitro*, but that this defect was overcome with time, possibly due to costimulation via CD80 and CD86, which were displayed at similar levels on both B6 and $CD40^{-/-}$ DC (Fig. 1). Experiments using a range of DC numbers indicated that these modest differences in T cell activation were no more pronounced when DC numbers were limiting (data not shown).

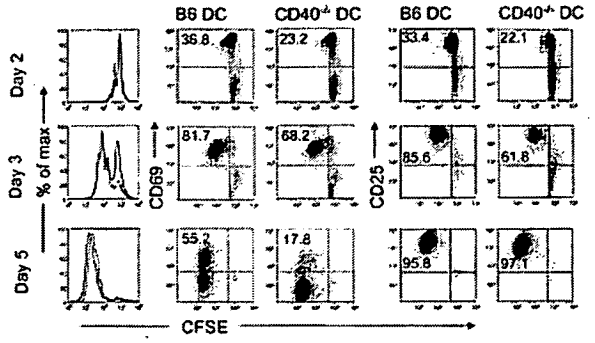

Fig. 2. B6 and CD40$^{-/-}$ DC show similar T cell activation capacities *in vitro*. CFSE-labeled CD4$^+$ OT-II T cells were cultured with Ag-loaded splenic DC. At the indicated time points T cells were sampled and expression of CD69 and CD25 analyzed. These data are from one of three experiments giving consistent results. Left hand panels: solid line: CD40$^{-/-}$ DC, dotted line: B6 DC. Percentages of T cells that had undergone one or more division(s) were: day 2, B6: 35.7%, CD40$^{-/-}$: 25.8%; day 3, B6: 78.7%, CD40$^{-/-}$: 57.2%. Percentages of T cells expressing activation markers are shown.

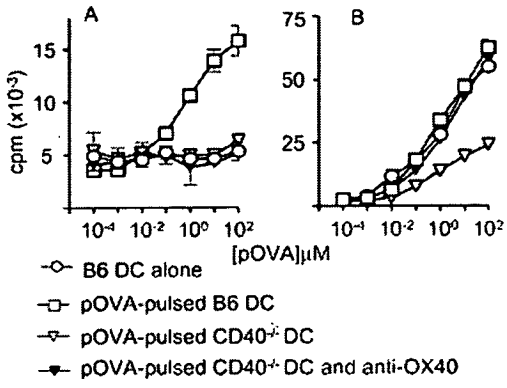

Fig. 3. CD40$^{-/-}$ DC administration leads to T cell unresponsiveness *in vivo*. B6 or CD40$^{-/-}$ DC were purified and loaded with pOVA as described in Sect. 4, before transfer into naive B6 recipients. Six days after DC transfer, splenic lymphoid populations were tested for proliferative responses to a dose range of pOVA (A). Further groups of mice were immunized on day 6 with pOVA/CFA and draining lymph nodes assessed 10 days later for proliferative responses to a dose range of pOVA (B). As indicated some groups also received anti-OX40 at the time of DC administration and 2 days later. The effects seen with anti-OX40 were not found when using an isotype control antibody. These data are from one of three experiments giving consistent results.

2.3 CD40-deficient splenic DC induce abortive T cell activation and subsequent unresponsiveness *in vivo*

Since CD40$^{-/-}$ DC appeared capable of supporting primary T cell expansion *in vitro*, we continued by testing their ability to initiate productive T cell immunity *in vivo*. DC from CD40$^{-/-}$ or B6 mice were pulsed *in vitro* with pOVA, prior to i.v. administration to naive B6 mice. After 6 days, peripheral LN and spleen cells were examined for *in vitro* recall responses to pOVA. Ag-specific proliferation was observed in lymphoid populations taken from mice that had received pOVA-pulsed B6 DC, but not those that had received CD40$^{-/-}$ DC (Fig. 3A). One effect of CD40 ligation is the up-regulation of OX40L on the surface of DC [10]. Ligation of OX40, (up-regulated on the T cell in response to TCR signaling) has been reported to lead to elevated T cell expression of survival factors such as Bcl-2 and Bcl-xL [19] and to prevent peripheral T cell tolerance induction [20]. We therefore reasoned that provision of OX40-signalling using an agonistic anti-OX40 monoclonal antibody might overcome the deficit in T cell activation seen after CD40$^{-/-}$ DC administration. However, we found no pOVA-reactive proliferative response in lymphoid populations from mice treated using this approach (Fig. 3A).

The above findings indicated a lack of T cell responsiveness as a result of primary *in vivo* stimulation with Ag-loaded CD40$^{-/-}$ DC. However, a more rigorous assessment of potential tolerance induction requires analysis of T cell reactivity upon subsequent *in vivo* rechallenge with Ag in adjuvant. We therefore immunized mice with pOVA in CFA 6 days after administration of DC. Lymphoid populations from mice that initially received CD40$^{-/-}$ DC were clearly impaired in their ability to proliferate to pOVA *in vitro* compared with those from mice that had received B6 DC (Fig. 3B), or from control mice that received no DC. In this setting, the provision of anti-OX40 at the time of CD40$^{-/-}$ DC administration allowed *in vitro* recall responsiveness to be evident after immunization with pOVA in CFA.

A lack of T cell responsiveness may reflect an absence of Ag-reactive cells or their functional inactivation. To address this issue we followed the expansion and phenotypic changes of pOVA-specific T cells *in vivo* using a transfer system in which naive B6 mice were seeded with CFSE-labeled OT-II T cells. One day after transfer, the recipient mice received an i.v. injection of pOVA-pulsed B6 or CD40$^{-/-}$ DC as described above. Flow cytometry revealed similar changes in the levels of CD69 expression by OT-II T cells in the first 3 days after administration of either B6 or CD40$^{-/-}$ DC. However, although CD40$^{-/-}$ DC induced T cell proliferation *in vivo*, this was not sustained past 3 days after DC administration (Fig. 4). OT-II T cell numbers were greatly reduced at the day 4 time

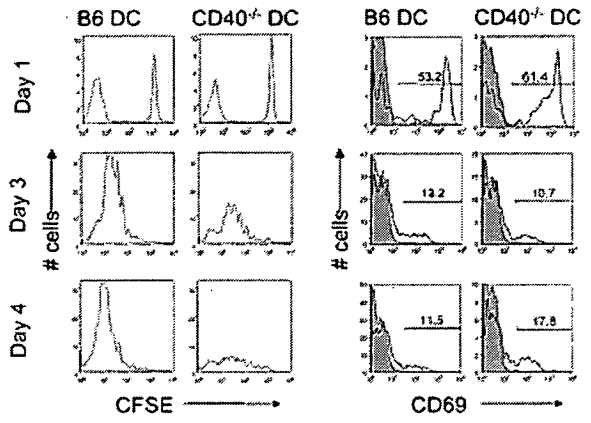

Fig. 4. CD40$^{-/-}$ DC do not support sustained T cell expansion *in vivo*. B6 mice were seeded with CFSE-labeled OT-II cells 1 day prior to administration of DC. On days 1, 3 and 4 post-DC administration, lymphoid cells (spleen shown here) were analyzed by FACS for CFSE profile and CD69 expression by Vβ5$^+$Vα2$^+$ OT-II cells (filled lines are Vβ5$^+$Vα2$^+$ cells that were stained with streptavidin-allophycocyanin alone). These data are from one of several experiments giving consistent results.

point in the spleens of mice that received CD40$^{-/-}$ DC, compared with a continued T cell expansion from day 3 to 4 in mice that received B6 DC (Fig. 4).

2.4 Reduced IL-2 and IFN-γ production and reduced proliferative capacity as a result of exposure to Ag-loaded CD40$^{-/-}$ DC

The above data indicated a more rapid loss of Ag-reactive cells after exposure to Ag on CD40$^{-/-}$ DC. To determine whether disruption of CD40 signaling also had an influence on T cell cytokine-producing capacity, we measured intracellular cytokine production by OT-II T cells 6 days after DC administration. We did not detect production of IL-10 and production of IL-4 was minimal after administration of either DC group (data not shown). Production of both IL-2 and IFN-γ was clearly evident after administration of B6 DC. Fewer OT-II cells were producing IL-2 and IFN-γ, however, after CD40$^{-/-}$ DC administration (Fig. 5B, C). Consistent with our earlier findings using B6 mice without OT-II transfer (Fig. 3), we found that lymphoid populations from mice harboring OT-II T cells showed a greatly reduced proliferative capacity upon *in vitro* culture with pOVA after CD40$^{-/-}$ DC administration compared with those from mice receiving B6 DC. The response in the CD40$^{-/-}$ DC group showed a low level of proliferation mirroring that seen in control mice that received PBS rather than DC (Fig. 5D).

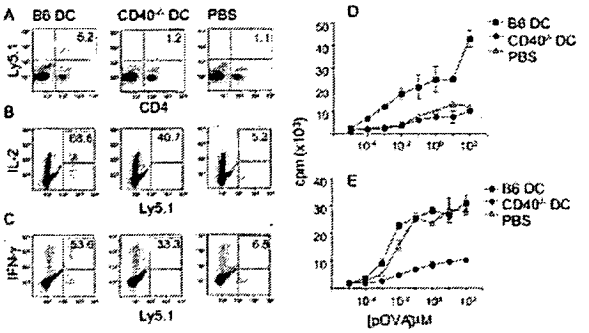

Fig. 5. Reduced T cell proliferation and cytokine production after administration of Ag-loaded CD40$^{-/-}$ DC. B6 mice were given Ly5.1$^+$ OT-II cells 1 day prior to administration of DC. Lymphoid populations (spleen shown here) were analyzed on day 6. OT-II cells were identified by gating on CD4$^+$Ly5.1$^+$ cells (A, percentages of CD4$^+$ cells that were Ly5.1$^+$ shown) and counterstained for IL-2 (B), or IFN-γ (C, percentages of CD4$^+$Ly5.1$^+$ cells that were cytokine-positive shown). (D) Proliferation in response to a dose range of pOVA. Further groups of mice were immunized with pOVA in CFA on day 6 and *in vitro* recall responses to pOVA were assessed 10 days later (E). These data are from one of several experiments giving consistent results.

As above, we then immunized mice with pOVA/CFA 6 days after they had received DC. Ten days after immunization, we found diminished *in vitro* recall proliferative responses to pOVA and a decreased frequency of pOVA-reactive T cells in mice that received CD40$^{-/-}$ DC, reminiscent of tolerance induced by soluble peptides (Fig. 5E). These data suggest that administration of CD40$^{-/-}$ DC results in an abortive activation of Ag-reactive T cells, with the remaining cells being less able to produce IL-2, IFN-γ, or to proliferate in response to Ag.

2.5 Administration of CD40-deficient DC protects from CNS autoimmune pathology

Our data thus far had indicated that exposure to Ag in the absence of the CD40:CD40L interaction induced a transient T cell activation followed by a substantial loss in Ag-reactive T cell numbers. Those T cells that did persist had a reduced ability to proliferate and produce cytokines. The net effect of these processes was to render the mouse relatively unresponsive to subsequent immunogenic challenge *in vivo*. To determine the significance of this unresponsiveness in a pathogenic setting, we tested the capacity of autoantigen-loaded CD40$^{-/-}$ DC to modulate the development of EAE induced with the p35-55 peptide of myelin oligodendrocyte glycoprotein (MOG) [21]. Mice received either B6 or CD40$^{-/-}$ DC

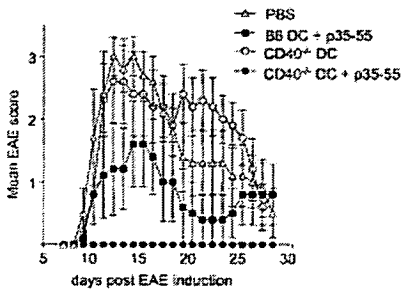

Fig. 6. $CD40^{-/-}$ DC protect from autoimmune CNS pathology. Mice received p35-55-loaded B6 or $CD40^{-/-}$ DC 7 days prior to induction of EAE with p35-55 in CFA. *p* values determined by Mann-Whitney U test were: PBS *vs.* B6: $p=0.0025$, PBS *vs.* $CD40^{-/-}$ + p35-55: $p<0.0001$, B6 *vs.* $CD40^{-/-}$ + p35-55: $p<0.0001$). These data are from one of two experiments giving consistent results.

that had been loaded *in vitro* with the p35-55 peptide and 6 days later EAE was induced by immunization with p35-55/CFA. Both B6 and $CD40^{-/-}$ DC consistently conferred a degree of protection against EAE compared to mice that did not receive DC. However, $CD40^{-/-}$ DC clearly gave a more pronounced protection than B6 DC (Fig. 6).

3 Discussion

Presentation of peptide-MHC complexes by resting DC is generally accepted to be the key to T cell tolerance induction [22]. The rapid activation and proliferation of Ag-reactive T cells that is consistently reported after soluble peptide administration [23–25] suggests that the initial interaction between CD28 and CD80/86 is not disrupted on route to tolerance. This is consistent with reports that the CD28-CD80/86 interaction is required for initial T cell activation, but that other receptor-ligand interactions are important in sustaining T cell expansion [26]. In contrast, exogenous stimulation of CD40 (using agonistic anti-CD40) can convert an Ag-specific intervention that would otherwise lead to tolerance into one that produces immunity [14, 15]. If CD40 truly is a crucial checkpoint between tolerance and immunity, administration of Ag-loaded APC that lack CD40 ought to lead to T cell tolerance. Alloantigen-specific tolerance has been reported after i.v. injection of either $CD40^{-/-}$ B cells into wild-type allogeneic hosts [27], or $CD40^{+/+}$ allogeneic B cells into $CD40L^{-/-}$ hosts [28]. However, the ability of $CD40^{-/-}$ DC to induce T cell tolerance after systemic administration had not been formally tested. Our data show that this approach leads to an abortive activation of Ag-reactive T cells that ultimately leads to an unresponsive state similar to that seen after systemic administration of peptide in solution. Moreover, administration of p35-55-loaded $CD40^{-/-}$ DC rendered mice resistant to subsequent attempts to induce EAE.

Previous studies have shown that ligation of CD40 leads to up-regulation of CD80 and CD86 by DC [7]. It is important to note that the splenic DC used for this study expressed high levels of both CD80 and CD86. We do not therefore believe that the failure of the $CD40^{-/-}$ DC to provoke a sustained T cell expansion *in vivo* stems from insufficient CD28 signaling. Instead our results support a model of tolerance induction in which failure to provide sufficient ligation of CD40 on DC uncouples downstream T cell:DC dialogue involving other members of the TNF and TNF receptor families. Efficient CD40 ligation drives the up-regulation of OX40L by the DC [10], allowing ligation of OX40 that is up-regulated by the T cell following activation through the TCR. OX40 signaling results in accumulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL promoting T cell survival [19]. Moreover, exogenous ligation of OX40 on T cells can override the tolerogenic program that follows soluble peptide administration, allowing T cells to remain Ag-responsive ([20], and our own unpublished observations). We found here that administration of Ag-loaded $CD40^{-/-}$ DC under the cover of agonistic anti-OX40 did not lead to an expansion of Ag-reactive T cells. However, provision of anti-OX40 did allow for the development of a productive immune response upon subsequent immunization with Ag/CFA. Therefore OX40 signaling appears to prevent the tolerance program, but does not in itself promote full T cell responsiveness.

Our finding that $CD40^{-/-}$ DC were unable to support a sustained T cell expansion compared with their CD40-sufficient counterparts is consistent with an earlier study in which DC were administered by a s.c. route [17]. This previous study, however, did not report any unresponsiveness in the Ag-reactive T cells that persisted after $CD40^{-/-}$ DC administration. The obvious difference between these two studies is that we deliberately chose to give our DC i.v., allowing access to the spleen, based on our previous experience that systemic delivery is most effective at inducing T cell tolerance with peptide [29]. Inefficient T cell priming after s.c. injection of $CD40^{-/-}$ DC has been reported to be associated with their accelerated loss from the draining lymph node. Studies of tolerance induction with peptides indicate that, whilst establishment of a fully unresponsive state takes 4 to 5 days [30], the key events that initiate the tolerogenic program probably occur within hours of TCR recognition [25, 31]. This being the case, we would argue that the issue of how many days B6 versus $CD40^{-/-}$ DC persist in the spleen after i.v. administration is unlikely to be the key to the T cell unresponsiveness we observe.

Our results suggest that the decreased IL-2 production in T cells stimulated with CD40$^{-/-}$ DC leads to an abortive immune response, and T cell death. This is supported by the sudden decrease in Ag-reactive T cells 4 days after priming with CD40$^{-/-}$ DC. Consistent with inducing a form of deletional tolerance, administration of CD40$^{-/-}$ DC protected from EAE. This matches a recent report in which administration of Ag-loaded RelB$^{-/-}$ DC, which also lack CD40$^{-/-}$ expression, induced Ag-specific T cell tolerance [32]. However, this study reported the induction of an IL-10-producing regulatory T cell population. It is unlikely that this explains the protection against EAE we observe, since CD40$^{-/-}$ DC did not induce an IL-10-producing T cell population in our OT-II adoptive transfer system. Interestingly, although pOVA-pulsed B6 DC did not appear to result in decreased proliferative capacity upon rechallenge, giving p35-55-pulsed B6 DC prior to EAE induction consistently gave some protection from disease. T cells can become exhausted upon repeated Ag-stimulation [33], resulting in reduced capacity to respond to rechallenge. Administration of B6 DC and subsequent challenge with a strong adjuvant such as CFA may therefore drive some high avidity self-reactive T cells into activation-induced cell death, reducing the ability to induce disease. Bone marrow-derived CD40$^{-/-}$ DC also lead to T cell unresponsiveness *in vivo* (data not shown), suggesting the use of cultured "tolerogenic" DC as a possible therapeutic approach in man.

Various mechanisms exist in the periphery in order to maintain T cell tolerance, and avoid autoimmune diseases. The idea that regulation of CD40 expression might represent a general mechanism of maintaining peripheral tolerance is an attractive one, and is supported by an increasing amount of data. Interference with the CD40:CD40L interaction using blocking anti-CD40L prevents effective T cell priming, sometimes leading to a tolerant state [28, 34, 35]. Recent data have shown that in the absence of inflammatory stimuli *in vivo*, DC play an important role in the maintenance of peripheral tolerance [22, 36–38]. The ability of DC to induce tolerance or immunity appears to depend on their maturation stage [39, 40]. Unmanipulated DC *in vivo* express little CD40 [6, 41], and DC constitutively take up Ag in the periphery and present them to T cells in lymphoid organs in a tolerogenic fashion, which can be converted into immunogenic presentation after DC activation through CD40 [14, 16]. Our results clearly show that CD40$^{-/-}$ DC provoke a proliferative burst in Ag-reactive T cells that lasts for 3 days. Several studies using the transfer of TCR-transgenic T cells into hosts that also express a transgenic Ag have shown an initial T cell expansion prior to the development of unresponsiveness [42–44]. This presumably is due to presentation of Ag by resting DC in the lymphoid organs draining the organ that expresses the transgenic Ag. Also it is clear from studies of tolerance induction with soluble Ag (another situation in which the Ag should be presented by resting DC) that transient T cell proliferation is intrinsic to the tolerance program [24, 25]. Therefore the initial proliferation we see using CD40$^{-/-}$ DC fits with other experimental systems that lead to T cell tolerance.

In summary we show that the systemic administration of Ag-loaded CD40$^{-/-}$ DC leads to abortive T cell activation and subsequent unresponsiveness reminiscent of that seen after systemic administration of soluble peptide Ag. This approach was robust enough to provide effective protection against CNS autoimmunity. These data provide insights into the effects of DC that may lead to the use of costimulation-deficient DC as a future therapeutic approach.

4 Materials and methods

4.1 Mice, Ag, immunizations and reagents

C57BL/6 (B6), CD40$^{-/-}$ (H-2^b) [45], B6 Ly5.1 congenic mice and OT-II mice expressing an A^b-restricted, transgenic pOVA-reactive TCR [18] were bred at the Institute of Cell, Animal and Population Biology, University of Edinburgh. OT-II×CD40$^{-/-}$ mice were generated by back-crossing OT-II with CD40$^{-/-}$ mice. Similarly, OT-II×Ly5.1 mice were generated by crossing OT-II mice with Ly5.1 mice. Sex-matched, 6–8-week-old mice were used for all experiments.

Peptides (pOVA and p35-55) were synthesized by the Advanced Biotechnology Centre, Imperial College London. For experiments analyzing T cell activation, mice were immunized s.c. with 20 μg pOVA emulsified in CFA (Sigma, Poole, GB). After 10 days, spleens and draining inguinal and popliteal LN were removed, disaggregated and used as a source of primed lymphoid populations. Tissue culture medium was RPMI 1640 medium containing 2 mM L-glutamine, 5×10^{-5} M 2-ME, 100 μg/ml penicillin, 100 U/ml streptomycin (all from Gibco, Life Technologies, Paisley, GB) (RPMI) supplemented with a source of serum. The OX-86 hybridoma, that produces anti-OX40, and the MAC-49 isotype control hybridoma were obtained from the European Collection of Cell Culture (ECACC, Wiltshire, GB).

4.2 Isolation of DC and naive T cell populations

CD4$^+$ T cells were isolated by positive selection from LN and spleen of naive OT-II×CD40$^{-/-}$ mice using anti-CD4-conjugated MACS beads (Miltenyi Biotec, Bisley, GB), according to the manufacturers instructions. Splenic DC were isolated from naive B6 or CD40$^{-/-}$ mice by initial digestion in 10 ml RPMI without serum, supplemented with 2.4 mg/ml collagenase D (Worthington Biochemical) and

1 mg/ml DNase (Sigma) for 30 min at 37°C. CD11c$^+$ cells were isolated by positive selection using anti-CD11c-conjugated MACS beads (Miltenyi Biotec), according to the manufacturers instructions, with a consistent purity of >85% CD11c$^+$ as confirmed by flow cytometry.

4.3 *In vitro* assays of primary T cell activation

Ag-loaded DC were prepared by incubation with 50 μM pOVA in RPMI plus 1% normal mouse serum at 37°C for 2 h in the presence of 0.1 μg/ml LPS (Sigma). Naive OT-II×CD40$^{-/-}$ T cells were labeled with 5 μM CFSE (Molecular Probes), prior to culture with pOVA-loaded DC (4×10^4 T cells + 4×10^3 DC per well) in 96-well flat-bottom plates (Becton Dickinson) using RPMI + 5% FCS. Cells were harvested for FACS analysis as described in the figure legends.

4.4 Cell transfers

DC were purified from naive B6 or CD40$^{-/-}$ mice as described above and pulsed with either pOVA or p35-55 (50 μM) in the presence of LPS as above for 2 h prior to i.v. injection into B6 recipients (5×10^5 DC/mouse). Some mice also received 200 μg anti-OX40 on the day of DC injection and 2 days later, as indicated. For T cell transfers, single cell suspensions were prepared from LN of naive OT-II or OT-II×Ly5.1 donor mice. In some cases, LN cells from naive OT-II mice were labeled with CFSE as above before 5×10^6 cells were injected i.v. into syngeneic B6 recipients.

4.5 Flow cytometric analysis

All samples were incubated with the 2.4.G2 anti-Fc receptor antibody to prevent nonspecific binding via Fc receptors, prior to incubations with other antibodies. All samples were collected on a Becton Dickinson FACScan (Mountain View, CA) flow cytometer and analyzed using FlowJo Software (TreeStar, USA). All antibodies were obtained from BD PharMingen (Oxford, GB) unless stated otherwise. DC purity was assessed with FITC-conjugated anti-CD11c. Costimulatory molecules on DC were identified using biotinylated primary antibodies specific for CD80, CD86, CD40, or OX40L, followed by streptavidin-allophycocyanin conjugate.

In experiments involving transfer of OT-II LNC the pOVA-reactive T cells were identified using biotinylated anti-Vβ5 followed by allophycocyanin- or FITC-conjugated streptavidin and PE-conjugated anti-Vα2. Transferred OT-II×Ly5.1 cells were identified using allophycocyanin-conjugated anti-CD4 together with FITC-conjugated anti-Ly5.1. T cell activation was assessed using biotinylated anti-CD69, anti-CD25, or anti-OX40, all followed by allophycocyanin-conjugated streptavidin.

Intracellular cytokine production was measured *ex vivo* in splenocytes by flow cytometric staining as described [46]. Briefly, splenocytes were stimulated with PMA/ionomycin in the presence of GolgiStop (BD PharMingen) for 4 h at 37°C, before staining with biotinylated anti-Vβ5 followed by allophycocyanin-conjugated streptavidin, and FITC-conjugated anti-Vα2, or allophycocyanin-conjugated anti-CD4 together with FITC-conjugated anti-Ly5.1, as indicated. Cells were then fixed for 20 min on ice, washed with cyto-perm wash buffer (BD PharMingen) and stained with PE-conjugated anti-IL-2, anti-IL-4, anti-IL-10 or anti-IFN-γ for 30 min on ice.

4.6 Recall proliferation assays

Lymphoid cell suspensions were cultured in 96-well flat-bottom microtiter plates (Becton Dickinson) at 6×10^5 LN cells/well or 8×10^5 spleen cells/well using X-vivo15 serum-free medium (BioWhittaker, Maidenhead, GB) supplemented with 2 mM L-glutamine and 5×10^{-5} M 2-ME. Cultures were stimulated with a dose range of pOVA or p35-55 for 48 h prior to addition of [^{3}H]dThd (0.5 μCi/well). After a further 18 h, cultures were harvested and dThd incorporation measured using a liquid scintillation β-counter (Wallac). Results are expressed as mean cpm of triplicate cultures. In some experiments, spleen cells were labeled with CFSE as above and stimulated for 72 h with 1 μM pOVA before flow cytometric assessment of proliferation.

4.7 Induction and assessment of EAE

EAE was induced with s.c. injection of 100 μg p35-55 in a total of 100 μl CFA containing 1 mg heat-killed *Mycobacterium tuberculosis* (50 μl into each hind leg). Mice also received 200 ng pertussis toxin (ECACC) i.p. in 0.5 ml PBS on the same day and 2 days later. Clinical signs of EAE were assessed using the following scoring index: 0, no signs; 1, flaccid tail: 2, impaired righting reflex and/or impaired gate; 3, partial hind leg paralysis; 4, total hind leg paralysis; 5, hind and fore leg paralysis; 6, moribund or dead. Differences in total disease burden between groups were determined using the Mann-Whitney U test.

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