An Investigation into the Immunostimulatory Effects of Lipopolysaccharide on Lymphocytes and Dendritic Cells *in Vivo*.

By

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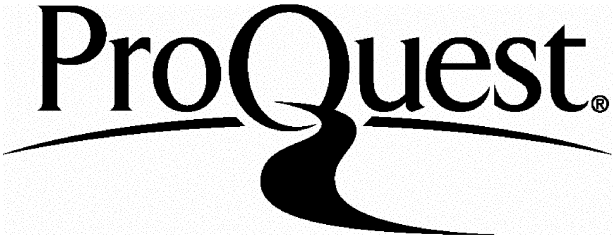

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ABSTRACT

Protein antigens alone are poorly immunogenic and require adjuvants to generate a vigorous immune response. Poor adjuvanticity may contribute to some vaccines conferring a relatively short duration of protection compared with natural infection.

At present, there are few adjuvants available for use in human vaccines. The best experimental adjuvants include components of microorganisms, the activity of which is likely to be related to their ability to mimic natural infection. In this regard, Lipopolysaccharide (LPS) has long been known to act as an adjuvant. However, the mechanism by which LPS enhances the generation of effector and memory T cells during the immune response remains undetermined. We have examined whether the immunostimulatory activity of LPS is associated with effects on both naïve lymphocytes and antigen presenting cells.

We have primarily focused on the possibility that LPS may act in part through the alteration of cell adhesion and co-stimulatory molecules on naïve T cells or antigen presenting cells (APC), altering their ability to respond to, or present specific antigen respectively. In this thesis we report that T cells and antigen presenting cells (DC) isolated from LPS-injected mice had increased cell surface expression of a number of activation markers, including Ly6A/E, CD54, CD95 and CD86. The activation was transient, and for DC was followed by extreme cell loss in the spleen mirrored by a 4-fold increase in the lymph nodes. The phenotypic changes exhibited by T cells were induced indirectly by LPS and could be mimicked by LPS-induced cytokines or supernatant harvested from LPS-stimulated dendritic cells. The data raise the possibility that non-specific "partial" activation of T cells and DC by LPS may affect the generation of an antigen-specific response.

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ABBREVIATIONS

AICD	Activation induced cell death
APC	Antigen presenting cells
APL	Altered peptide ligand
BCR	B cell receptor
BLP	Bacterial lipopeptide
BM	Bone marrow
bp	Base-pair
BrdU	2-bromo-2-deoxyuridine
cDNA	Complementary deoxyribose nucleic acid
CFA	Complete Freund's adjuvant
CGG	Chicken gammaglobulin
Con A	Concanavalin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAG	Diacyl glycerol
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbant assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FADD	Fas associated death domain
FDC	Follicular dendritic cell
GAP	GTPase activating protein
GC	Germinal centre
GCDC	Germinal centre dendritic cell
GEF	Guanyl nucleotide exchange factor
GP	Glycoprotein
GPI	Glycosylphosphatidylinositol
Grm	Granzyme
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein

i.p.	Intraperitoneal
i.v.	Intravenous
ICAM	Intercellular adhesion molecule
ICOS	Inducible co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Inositol phosphate
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
kB	Kilobase
LAT	Linker for activation of T cells
LBP	LPS-binding protein
LC	Langerhans Cell
LCMV	Lymphocyte choriomeningitis virus
LFA	Lymphocyte function-associated antigen
LN	Lymph node
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MMTV	Mouse mammary tumour virus
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteases
MPLA	Monophosphoryl Lipid A
NF-AT	Nuclear factor of actviated T cells
OVA	Ovalbumin
PALS	Periarterial lymphoid sheath
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononucleocytes
PBS	Phosphate-buffered saline

PGN	Peptidoglycan
PH	Plextrin homology
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLC-γ1	Phospholipase C-γ1
PMA	Phorbol myristate acetate
Poly I:C	Polyinosinic-polycytidylic acid
PRR	Pathogen recognition receptor
PTK	Protein tyrosine kinase
RANK	receptor activator of NF-kB
RNA	Ribose nucleic acid
SH2	Src-homology 2
SHP-2	SH2-containing tyrosine phosphatase-2
SLP-76	SH2 domain-containing Leukocyte Protein of 76 kDa
SMAC	Supramolecular activation clusters
SRBC	Sheep red blood cell
TCR	T cell receptor
TG	Transgenic
TGF-β	Transforming growth factor beta
T_H	T helper cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRANCE	TNF-related activation-induced cytokine
ZAP-70	ζ-associated phosphoprotein

Amino acid symbols

Amino Acid	**Three-letter symbol**	**One letter symbol**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1: Introduction

1.1 Overview of the immune system

The immune system has evolved due to the dynamic selective pressures imposed upon the host to protect itself from invading infectious micro-organisms. The networks of specialised cell types that have resulted from co-evolution between host and pathogen may be described as either "innate" or "adaptive". The innate immune system (e.g. macrophages and dendritic cells) use constitutively expressed or rapidly induced germline-encoded proteins to recognise microbes and provide immediate defence mechanisms. For example, macrophages possess multiple receptors that are able to bind directly to molecules within yeast and bacterial cell walls (e.g. lipopolysaccharide), engulf and digest the microbes and produce biologically active proteins called cytokines. Innate immune receptors have been selected by evolutionary forces determined by the rate of change of the pathogen. Thus, the relative genetic inflexibility of these receptors meant that mutating microbes could evade detection by the innate immune system.

The adaptive immune system provides a clonal recognition system that generates its receptors by somatic mechanisms. T and B lymphocytes express a diverse repertoire of receptors that recognise a wide range of substances collectively called antigens. As opposed to the innate pathogen recognition receptors, these adaptive antigen receptors are not selected due to evolution but are generated somatically without regard to the target. Therefore, a pitfall of the adaptive immune system is that whilst it can create huge numbers of potentially pathogen-

specific receptors, there is also an accompanying problem of possible "self-reactivity" with host tissue antigens.

Therefore, undesired B and T cell clones are removed at the developmental stage of lymphocyte production. Furthermore, only T cells bearing receptors (TCRs) that recognise antigen that has been processed into fragments called peptides, in the context of self major histocompatibility (MHC) molecules are selected in the thymus for survival. There are two classes of MHC molecules, which differ in their cell type distribution and antigen presentation to T cells. MHC class I is found on the surface of virtually all nucleated cells, whilst MHC class II expression is normally restricted to specialised immune cell types such as dendritic cells, B cells and activated macrophages. MHC class I molecules present peptides derived from endogenous antigen generated in the cytosol to T cells expressing the CD8 subset marker. Conversely, MHC class II molecules present peptides processed via the endocytic pathway from internalised exogenous antigens to T cells of the CD4 lineage. Under appropriate conditions, presentation of microbial peptides by MHC expressing cells stimulates T cells to differentiate from their "resting" naïve status to cells with specialised effector functions.

Effector $CD8^+$ T cells are able to directly kill virally infected cells, whilst activated $CD4^+$ T cells produce cytokines that assist B cell antibody synthesis or stimulate the cytotoxic activity of other cells (e.g. macrophages). Secreted antibodies can bind directly to neutralise virus or activate innate immune mechanisms to destroy bacteria. However, unlike innate defensive mechanisms,

adaptive responses require 1-2 weeks to develop. This is because the frequency of antigen-specific clones is initially very low and therefore generating a protective response involves massive clonal replication.

When the infection is cleared the vast majority of these expanded clones are destined to die by a process called apoptosis. However, a small number of T and B lymphocytes remain as "memory" cells. These cells are maintained for long periods so that upon subsequent infection with the same micro-organism, a more rapid protective response may be generated by re-activating antigen-experienced memory cells.

Therefore, the adaptive immune response may be viewed as "compressing evolution into two weeks" (Fearon, 1997). The antigen receptor repertoire is selected by microbes and is therefore not predetermined, thus compensating for the limitations of innate immunity (reviewed in Medzhitov and Janeway, 1997).

In the following sections, activation of the adaptive immune response and its initiation by dendritic cells will be discussed with particular emphasis on the molecules involved in T cell activation. The emerging paradigm surrounding pathogen recognition will be reviewed with the focus on recent developments that have significantly advanced our understanding of the enigmatic molecular requirements for recognition of the bacterial component, lipopolysaccharide.

1.2 T cell activation

1.2A. Antigen recognition

Naïve T cells require activation via their T cell receptor (TCR) to develop into effector cells. This involves the physical interaction between the antigen-specific α/β chains of the TCR and proteolytically processed short (8-15 residues) peptide antigens presented in the context of self-MHC molecules expressed on antigen-presenting cells (APC). However, the α/β subunits have only short cytoplasmic domains that are inadequate for the direct coupling of the TCR to the intracellular signalling machinery. Association with signal transducing subunits, ε, γ, δ, and ζ (CD3-ζ complex), which possess larger cytoplasmic domains, allows initiation of the protein-tyrosine phosphorylation cascade necessary for activating T cells (reviewed in Weiss and Littman, 1994). The CD3-ζ subunits possess no intrinsic enzyme activity but most likely function as initial sites of protein tyrosine kinase (PTK) activity. Indispensable immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic tails of the CD3 and ζ chains are phosphorylated by lck, the src-family PTK.

Lck is associated with the co-receptors CD4 and CD8, inferring a possible mechanism for co-receptor function (Luo and Sefton, 1990; Shaw *et al.*, 1989). Direct binding of MHC class I or MHC class II by CD8 and CD4, respectively, may not only stabilise the TCR-MHC interaction but also assist accumulation of lck at the recognition site. In support of this theory, interruption of CD4-MHC

class II interactions by anti-CD4 monoclonal antibodies (mAbs) resulted in a lack of association of CD4/lck with the TCR complex and T cell unresponsiveness to peptide stimulation *in vitro* (Acuto and Cantrell, 2000).

Therefore, close association of lck with ITAMs within the TCR complex would most likely result in their phosphorylation. This initial phosphorylation event makes the ITAMs competent to associate with ZAP70 via tandem src-homology 2 (SH2) domains, a cardinal feature of Syk family PTKs (Duplay *et al.*, 1994) (reviewed in Chu *et al.*, 1998). Thus, phosphorylated ITAMs act as high affinity docking sites for SH2 domains within ZAP-70, recruiting the PTK to the TCR. Lck also binds to ZAP70 via its SH2 domain and the consequent phosphorylation of ZAP70 enhances Syk catalytic activity (Chan *et al.*, 1995). Lck/ZAP70 association has been suggested as an alternative mechanism for co-receptor function. In this model, the high affinity of CD4 or CD8 for MHC will stabilise lck:ZAP70 interactions thus amplifying phosphorylation reactions and the ZAP70 activation loop (Acuto and Cantrell, 2000) (see figure 1.1.(a)).

The substrates for ZAP-70 are the adaptor (or scaffold) proteins LAT and SLP-76 (Zhang *et al.*, 1998; Wardenburg *et al.*, 1996). Phosphorylated LAT is thought to directly activate phospholipase C-γ1 (PLC-γ1) whilst SLP-76 may activate the same enzyme but via the phosphorylation of Itk (a Tec family kinase) (Kane *et al.*, 2000 and reviewed in Tomlinson *et al.*, 2000). Exactly how PLC-γ1 is activated by the numerous cytoplasmic adaptor proteins implicated in TCR signalling is not fully understood. However, it is clear that TCR activation of PLC-γ1 requires both PTKs (Lck, ZAP70 and Tec kinases i.e. Itk) and adaptor

proteins (LAT and SLP-76). LAT is able to interact with PLC-γ1 directly through its SH2 domain, whilst SLP-76 may act as a "scaffold" for the assembly of the PLC-γ1 signalling complex (reviewed in Kane *et al.*, 2000).

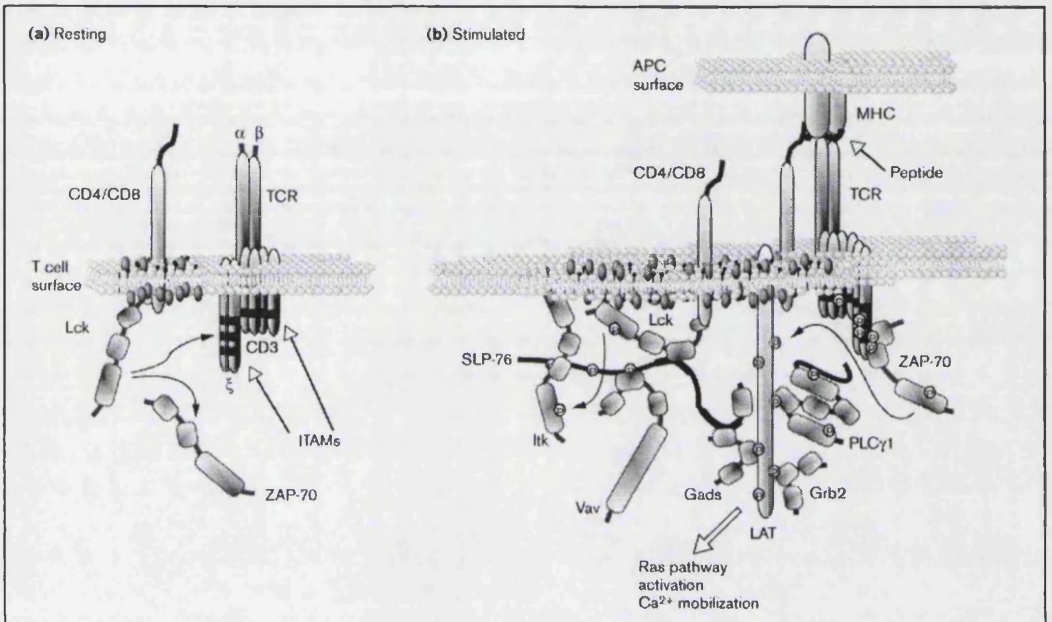


Figure 1.1. Intracellular signalling events in TCR activation. **(a)** The basic signalling units in resting T cells: interaction between the TCR and MHC-peptide complexes initiates tyrosine phosphorylation (represented by black thin arrows) of ITAMs and ZAP-70 by Lck. **(b)** Activated ZAP-70 phosphorylates the adaptor proteins e.g. LAT and SLP-76 that trigger a number of different downstream signalling events.
(Adapted from Kane *et.al.*, 2000)

The importance of PLC-γ1 activity appears to be more fully understood than the mechanism of its activation. PI 4,5-bisphosphate (IP_2) is hydrolysed by PLC-γ1 resulting in inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These second messengers recruit divergent signalling systems that are critical for the production of nuclear transcription factor subunits.

IP_3 is responsible for the rapid increase in intracellular calcium, $[Ca^{2+}]_i$, associated with T cell activation. This subsequently stimulates calcineurin, a

calcium-dependent serine phosphatase. Inhibition of calcineurin activity by the drug cyclosporin A has been used to great effect in organ transplants to suppress T cell-mediated graft rejection. Finally, calcineurin activates the nuclear factor of activated T cells (NF-AT) transcription system (Weiss and Littman, 1994; Kane *et al.*, 2000).

The other product of PLC-γ1, DAG, is thought to be the primary activator of protein kinase C (PKC), which in turn regulates the activation of the small GTPase Ras. GTP-bound Ras interacts directly with the serine/thronine kinase Raf-1, which regulates the kinase cascade of Mek and the MAP kinase, Erk. The Raf/Mek/Erk protein kinase cascade controls the level of the Jun/Fos dimeric transcription factor known as AP1. The co-ordinated activation of AP1 and NF-AT transcription factors results in the efficient transcription of the interleukin (IL) -2 gene, the protein product of which drives T cell proliferation.

Ras activation appears to be regulated by GTP hydrolysis, controlled by GTPase activating proteins (GAPs), and guanyl nucleotide exchange, which is limited by the activity guanyl nucleotide exchange factors (GEFs) (Kane *et al.*, 2000). DAG-activated PKC appears to assist Ras activation by inhibiting GAPs. Furthermore, the ability of the DAG analogue phorbol myristate acetate (PMA) to activate Ras has been ascribed to PKC-dependent inhibition of Ras GAPs (Ebinu *et al.*, 2000). Ras activity may also be enhanced by the recruitment of the GEF Sos to the signalling complex by tyrosine phosphorylated adaptor proteins such as LAT, Grb2 and SLP76 (Acuto and Cantrell, 2000).

Whilst the model for the proximal and distal signalling events after TCR stimulation is being continually modified and enhanced, a conceptual model for interpreting this complexity in terms of the T cell response remains elusive. How is it that subtle differences in the affinity of the TCR ligand may induce a variety of cellular responses? Neumeister Kersh *et al.* suggested that the presence of multiple potential tyrosine phosphorylation sites on CD3-ζ chains variations in TCR-ligand affinities may be translated into discrete phosphoforms, and by inference, into distinct immunological processes (Kersh *et al.*, 1998; reviewed in Malissen, 1998).

An alternative model, kinetic proofreading, has been proposed by McKeithan to explain how a low affinity receptor like the TCR can distinguish between small differences in antigen (McKeithan, 1995). Originally used to describe the precision of DNA synthesis, this modified T cell model assumes that a minimum amount of time is required for the establishment of multi-protein signalling complexes. Therefore, self-antigens may be of too low affinity to engage the TCR for the threshold duration required for generating the complete signalling complex. In contrast, higher affinity foreign antigen, with a lower "off"-rate (or dissociation rate) may stimulate the TCR with sufficient strength that the terminal reaction is accomplished and the T cell becomes fully activated. Therefore, the elaborate construction of the T cell signalling cascade may enable the T cell to discriminate precisely between foreign and self-antigen (reviewed in Shaw and Dustin, 1997).

Lanzavecchia *et al.* also addressed the "high sensitivity-low affinity" paradox of T cell antigen recognition. Their serial triggering model takes into consideration the accumulative effect of signalling via multiple TCR engagements. They have also proposed that this model may explain how T cells can be activated by only a very small number of specific ligands distributed randomly on the surface of the APC. On the basis of the number of TCRs downregulated after T cell-APC interactions they estimated that a single peptide-MHC complex could serially trigger around 200 TCRs (Valitutti *et al.*, 1995). Furthermore, under their experimental conditions they consistently observed an activation threshold of between 6-8,000 TCRs for both resting T cells and T cell clones. However, this threshold could be lowered by antigen-independent ligation of the surface receptor CD28 (Viola and Lanzavecchia, 1996). This implies that other molecules within the T cell membrane may facilitate signalling events controlled by the TCR.

1.2B. Co-stimulation

Indeed, activation via the TCR is currently viewed as only one of a number of signals required for the development of a fully functional activated T cell. The concept of a "two signal" model was first developed by Bretscher and Cohn (Bretscher and Cohn, 1970) to describe paralysis of B cell function when the antigen receptor was stimulated in the absence of a "second signal" from another cell type. This model was later modified by Lafferty and Cunningham (Lafferty and Cunningham, 1975) to explain T cell activation. Thus, the consequences of antigen-specific T cell stimuli discussed above have been defined as "signal one"

whilst non-TCR derived co-stimuli have been collectively labelled "signal two" (reviewed in Van Gool *et al.*, 1996). The two signal model's central tenet is that T cells stimulated with antigen (signal one) in the absence of co-stimulatory factors (signal two) become "anergic" (Schwartz, 1990) or non-responsive to subsequent antigenic challenge. The precise mechanisms by which signal two is delivered are not fully defined. However, a distinction has been drawn between adhesion molecules (e.g. integrins) that function by assisting the cell to cell interactions between T cells and the APC and "classical" co-stimulatory ligand-receptor interactions that initiate their own second messenger cascade which synergise with the downstream events of TCR signalling.

The CD28/B7 system is perhaps the most widely studied and characterised T cell co-stimulatory pathway. Ligation of the CD28 receptor enhances cytokine gene expression and mRNA stabilisation, whilst interruption of CD28 signalling on T cells may result in suppression of the response or even antigen-specific tolerance. Evidence suggests that CD28 co-stimulation functions by integrating and synergising with distal TCR-derived signals, particularly the activation of nuclear factors such as AP-1, which is known to be important for IL-2 production. Hence, IL-2 gene expression and AP-1 trans-activation are sub-optimal in the absence of co-stimulation (Michel *et al.*, 2000; Li *et al.*, 2001 and reviewed in Lenschow *et al.*, 1996).

However, CD28 signalling may itself be regulated by a homologous receptor, CD152 (or CTLA-4, cytotoxic lymphocyte antigen 4). CTLA-4 deficient mice have a profound lymphoproliferative disease and die within 3-4 weeks of birth,

implying an essential role for this molecule in negatively downregulating T cell responses. The mechanism for CTLA-4 activity may be threefold: (a) competitive inhibition with CD28; (b) interruption of the CD28 signalling cascade; (c) inhibition of T cell responses independently of CD28 by dephosphorylation of TCR-associated kinases.

For example, both CD28 and CTLA-4 are able to bind CD80 (B7-1) and CD86 (B7-2), although CTLA-4 has a 10-20 fold higher affinity for the B7 ligands than CD28. Furthermore, CD28 is constitutively expressed on T cells whereas CD152 expression is induced on T cells approximately 2-6 days after activation. Therefore, the difference in their affinities for the same ligand and the kinetics of their expression may result in a decrease in CD28 activity after T cell activation has been initiated.

CTLA-4 also possesses SH2 domains that are thought to be bound by either SH2-containing tyrosine phosphatase-2 (SHP-2) or phosphatidylinositol 3-kinase (PI3K). CTLA-4-associated SHP-2 phosphatase activity may disrupt TCR signalling by inactivating the PTK cascade, whilst binding of PI3K perhaps prevents the involvement of this kinase in CD28 signal transduction.

Certainly, there is a large body of *in vitro* and *in vivo* evidence for the role of CTLA-4 as an immune attenuator (reviewed in Thompson and Allison, 1997; Oosterwegel *et al.*, 1999). However, recently the relative importance of CTLA-4 has been re-evaluated using TCR transgenic mixed bone marrow chimeras where 50% of T cells express CTLA-4 and 50% are CTLA-4-deficient. These mice

were able to mount normal responses to either LCMV, *Leishmania major* or mouse mammary tumour virus (MMTV). Thus, CTLA-4 was not required for the downregulation of the antigen-specific T cell response to these pathogens. However, these experiments do not rule out the possibility that CTLA-4 may have functioned indirectly (i.e., on bystander cells) by inducing the release of soluble immunosuppressive factors (e.g. tumour growth factor-β or TGF-β) (Bachmann *et al.*, 2001).

Studies by Li *et al.* (2001) using CD28 antibodies to block the activation of $CD4^+$ T cells, indicated that CD28 signals are not absolutely required for TCR γ and ζ chain phosphorylation, IP_3 and DAG production, $[Ca^{2+}]_i$ flux nor the dephosphorylation and activation of NF-AT. Therefore, it may be possible that intense TCR stimulation (e.g. soluble peptide/MHC tetramers or immoblised peptide/MHC complexes) bypasses the requirement for a second signal.

Evidence for the stringency of CD28 involvement in T cell activation is contradictory. The phenotype of the CD28 knockout mice suggests that $CD4^+$ T cells are more dependent on CD28 co-stimulation than $CD8^+$ T cells. CD28-deficient mice show reduced $CD4^+$ T cell activity whilst $CD8^+$ T cell cytotoxicity remains normal (Sharpe, 1995). For example, repeated injection of lymphocytic choriomeningitis viral (LCMV) antigens into $CD28^{-/-}$ mice rescued $CD8^+$ T cells from anergy. However, withdrawal of signal one, rapidly re-set the requirement for signal two, thus implying that signal one duration determines the relative need for signal two (Kundig *et al.*, 1996). In an alternative model, the $CD8^+$ T cell response to peptides of varying affinity, from strong agonist to antagonist,

were compared in the presence or absence of CD28. T cell unresponsiveness was induced when CD28 was absent even when stimulating with the strongest agonist. Conversely, when CD28 was present, the weak agonists (or altered peptide ligands) failed to induce T cell unresponsiveness. Therefore in this system at least, signal two and not signal one was the critical factor in determining the T cell response (Bachmann *et al.*, 1999).

CD40 ligand (CD154) is not constitutively expressed on T cells but is induced upon TCR activation. Binding its receptor, CD40, on APCs stimulates the upregulation of B7, hence, CD40L-CD40 interactions are perceived as amplifiers of T cell responses compared to the supposed initiating role for the CD28-B7 system (Howland *et al.*, 2000). The requirement for CD40L accessory signals also appears to differ depending on the type of antigenic challenge and subset of T cells responding. Lack of functional expression of CD40L on human and murine T cells results in severe defects in B cell responses. Murine experiments using adoptively transferred antigen-specific CD40L $^{-/-}$ $CD4^+$ T cells showed that this molecule was required for T cell expansion in response to antigenic challenge *in vivo* (Grewell and Flavell, 1995).

However, the experiments mentioned have all been investigating $CD4^+$ T cell responses. According to Whitmire *et al.* (1999) there appears to be a striking difference between the requirements of $CD8^+$ and $CD4^+$ T cells for CD40-CD40L co-stimulation. CD40L $^{-/-}$ mice generated a vigorous anti-viral $CD8^+$ T cell response but 10-fold fewer virus-specific $CD4^+$ T cells than in $CD40L^{+/+}$ mice.

Taken together, the studies mentioned above show a differential requirement of purified model proteins and infectious virus for co-stimulation. One of the characteristics of the $CD8^+$ T cell response to LCMV is that it is relatively $CD4^+$ independent. Therefore, perhaps some viruses have inherent adjuvant properties that condition APC to be able to stimulate $CD8^+$ T cells directly, bypassing CD4 "help". Alternatively, viral antigenic stimulus is sufficient to activate the T cell via signal one alone (Bachmann *et al.*, 1998).

Another possible factor may be that "co-stimulation-independent responses" recruit additional unknown or partially characterised receptor:ligand pathways. In support of this, the recently identified murine homologue of human B7-H1 (itself a quite recently discovered homologue of B7) was shown to be important for co-stimulating CD28 independent T cell responses. Both murine and human B7-H1 enhance T cell proliferation and secretion of IL-10 (Dong *et al.*, 1999) (Tamura *et al.*, 2001). Like B7-1H, the new member of the CD28 receptor family, inducible co-stimulator or ICOS, has similar activity upon ligation but has been reported not to bind B7-1H (Hutloff *et al.*, 1999; Aicher *et al.*, 2000). ICOS binds to another murine homologue of B7, B7h, which is expressed on resting B cells and can be induced on nonlymphoid cells by LPS or tumour necrosis factor-α (TNF-α) (Swallow *et al.*, 1999). Initially identified on human T cells, murine ICOS expression was subsequently characterised as limited to activated T cells and germinal centres *in vivo*. It has been reported that ICOS lacks the binding motif necessary to interact with B7 and that B7h is not bound by either CD28 or CTLA-4. Therefore, it would appear that the ICOS:B7h and

CD28:CTLA-4:B7 systems are entirely independent co-stimulatory pathways (reviewed in Abbas and Sharpe, 1999).

Another consideration is the possible involvement of adhesion molecules in facilitating TCR-MHC interactions. It has been difficult to discriminate the adhesion role of receptor-ligand interactions, such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), from their putative co-stimulatory activity. For example, fibroblast cell lines transfected with I-E^k and ICAM-1 (CD54) have been shown to stimulate naïve TCR transgenic T cells to respond to specific peptide *in vitro*. It was argued that this may not be solely attributed to adhesion since APC bearing only CD54 enhanced (i.e. co-stimulated) the T cell proliferative response to immobilised anti-CD3 mAbs (Dubey *et al.*, 1995). A more recent study has demonstrated that co-immobilised CD3 mAbs and soluble CD54 enhances IL-2 production by T cells. Furthermore, Chen *et al.* (1999) reported a much greater effect of CD54 co-stimulation on $CD8^+$ T cell IL-2 production than on $CD4^+$ T cell IL-2 secretion. This differential effect on the activation of $CD8^+$ and $CD4^+$ T cells is perhaps reflected by the expression of CD54 *in vivo*. In contrast to B7, CD54 is expressed on MHC class I-bearing hematopoietic and non-hematopoietic cells rather than just being limited to professional APCs. Thus CD54-LFA-1 interactions may also be involved in $CD8^+$ CTL recognition of their targets.

This idea is supported by a study using LFA-1-deficient $CD8^+$ T cells that express peptide specific transgenic TCRs. In the 2C model, in which T cells can recognise either allogeneic L^d molecules or syngeneic K^b molecules and peptide,

the lack of functional LFA-1 profoundly diminished proliferative responses to allogeneic BALB/c splenocytes or high affinity peptides. Addition of exogenous IL-2 partially restored proliferation to peptide, implying that LFA-1 may be required for optimal IL-2 production. Cytolytic activity of $CD8^+$ $LFA\text{-}1^{-/-}$ T cells for a variety of targets was also impaired, suggesting that LFA-1 is important for both T cell activation and effector function (Shier *et al.*, 1999). However, the results derived from the 2C system differ to conclusions drawn from work by Bachmann *et al.* using LFA-1 deficient T cells expressing transgenic TCRs specific for viral antigens. In these experiments, LFA-1 deficiency also reduced proliferation and cytolytic activity in response to peptides derived from LCMV GP. However, in contrast to the 2C data, LFA-1 deficiency in the LCMV system could be compensated by increasing the amount of antigen 100-fold (Bachmann *et al.*, 1997).

One of the hallmarks of a co-stimulatory molecule has been defined as the recruitment of signalling molecules which result in increased IL-2 production (Watts and DeBenedette, 1999). In this regard, LFA-1 binding to ICAM-1 has recently been reported to increase IL-2 mRNA levels and protein production by $CD8^+$ T cells (Ni *et al.*, 2001). Furthermore, Ni *et al.* went on to reveal that ligation of LFA-1 upregulates PI3K activity and that LFA-1 co-stimulation is dependent on this enzyme. CD28 interaction with B7 also results in the recruitment and activation of PI3K, perhaps suggesting a shared function with LFA-1.

The products of PI3K are phosphorylated inositides (PI) at the 3 position on the inositol ring, i.e. PI-3-monophosphate (PIP), PI-(3,4)-biphosphate (PI2P) and PI(3,4,5,)-triphosphate (PI3P). These D3-phosphoinositides are able to regulate GEF function by binding to plextrin homology domains (PH) domains contained within the proteins. For example, PI3P binds to the PH domain of the Rho family GEF Vav-1, recruiting it to the plasma membrane where it is tyrosine phosphorylated. Active Vav-1 indirectly regulates actin re-organisation by modulating Rho family GTPase (Rac, Rho and Cdc42) activity. Hence, lack of Vav-1 in T cells results in defective actin polymerisation, whilst Vav-1 overexpression induced abundant lamellipodia formation. This signalling pathway may also be initiated by TCR signalling (reviewed in Acuto and Cantrell, 2000). Thus, it would appear that PI3K links the TCR, CD28 and LFA-1 to the actin cytoskeleton of lymphocytes.

This is in agreement with current experimental data on T lymphocyte cyto-architecture. For example, LFA-1 cross-linking by CD54 has been shown to polarise intracellular talin, an actin binding protein, to the area of T cell-APC cell to cell contact. Furthermore, TCR signalling induced microtubule organising centre (MTOC) reorientation which was facilitated by CD28 engagement (Sedwick *et al.*, 1999). Polarisation of the cytoskeleton appears to drive receptor accumulation at the T cell-APC interface. This was demonstrated by using beads coated with antibodies to CD54. Bead loaded T cells were mixed with peptide-pulsed APCs and within minutes the beads were observed to move from the posterior of the T cell to the newly formed area of contact between the T cell and APC. Both antibodies to CD54 or CD80 and CD86, and the PI3K inhibitor,

wortmannin, blocked bead movement. Furthermore, CHO cells transfected with I-E^k and either CD54 or CD86 gene contructs were competent stimulators of bead movement on T cells. Taken together these experiments suggest that LFA-1-CD54 and CD28-B7 are both important pathways for the movement of the T cell cortical actin cytoskeleton towards the T cell-APC interface.

Therefore, the mechanism for co-stimulation may be viewed through two, not necessarily mutually exclusive conceptual models: (1) co-stimulatory signals integrate with the TCR signalling cascade to amplify gene expression in the nucleus. (2) Co-stimulation induces the accumulation of cytoskeleton-linked immunoreceptors to the T-cell-APC interface, thus increasing receptor density and signal amplification.

1.2C. The immunological synapse

Over the past 5 years there has been an increasingly intense focus on the accumulation of T cell activation-associated molecules at the T cell-APC interface. Monks *et al.* (1998) first demonstrated that clustering of talin, LFA-1 and PKC at the site of contact between T cells and APCs was not random but segregated into three-dimensional domains. Using digital imaging of antigen-specific T cell-APC conjugates, they were able to observe that talin and LFA-1 were localised to the contact area but excluded from the central region, which was selectively enriched for PKC. Thus, Monks *et al.* re-labelled the contact domain, "supramolecular activation clusters" (SMACs), and further described SMACs as spatially segregated into peripheral SMAC (p-SMAC) and central

SMAC (c-SMAC). The distinct spatial organisation described by Monks *et al.* was not restricted to LFA-1, talin and PKC. Subsequent work has characterised the differential localisation of a number of molecules (reviewed in van der Merwe *et al.*, 2000 and illustrated in figure 1.2.). Briefly, the TCR and proximal signalling molecules localised to the central cluster, and was surrounded by two concentric rings containing adhesion molecules (e.g. CD2 and LFA-1). Furthermore, molecules possessing large extracellular glycocalyx e.g. CD43 and CD45 were excluded from the SMAC, thus minimising steric hindrance between the T cell and APC.

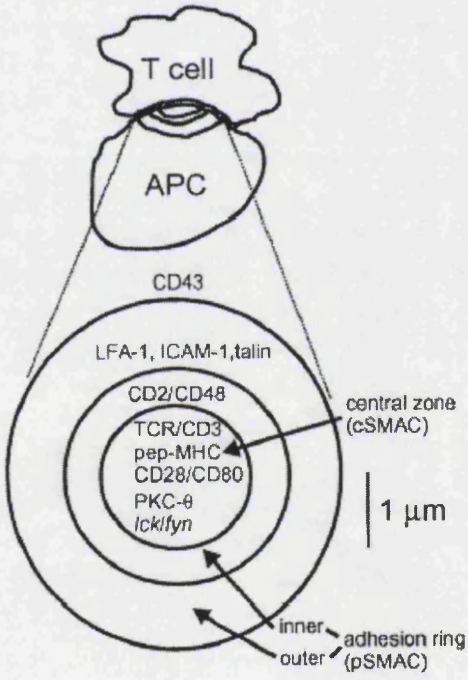


Figure 1.2. The spatial organisation of SMACs. Upon T cell-APC interactions both cell types reorganise surface proteins into discrete concentric rings labelled the central SMAC (cSMAC) and the peripheral SMAC (pSMAC). The cSMAC primarly contains molecules involved in antigen-recognition and signal transduction. Typically, adhesion molecules localise to the outer pSMAC.

(Adapted from van der Merwe *et al.*, 2000)

The formation of SMACs was subsequently reported to occur in discrete temporal stages. Grakoui *et al.* observed the kinetics of fluorescently labelled MHC-peptide and CD54 accumulation in T cell junctions (Grakoui *et al.*, 1999). For optimal imaging conditions, the APC was replaced with lipid bilayers containing glycosylphosphatidylinositol or GPI-anchored MHC-peptide and

CD54 to allow free diffusion. Therefore, any accumulation of these molecules could be attributed to the movement of their respective receptors on the T cell. Initially, newly formed T cell-APC contact zones were described as a broad central zone of CD54 accumulation surrounded by a concentric ring of MHC-peptide complexes. Eventually, distribution of these two molecules resembled that reported by Monks *et al.*, i.e. the engaged MHC-peptide complexes formed a central cluster whilst CD54 became peripherally localised. Thus, Grakoui *et al.* labelled the early distribution pattern the "immature immunological synapse" and the later arrangement, the "mature immunological synapse". They also proposed a multi-step model for synapse formation: (1) junction formation. LFA-1 forms the central anchor of the synapse and allows TCR sampling of the MHC-peptide complex. (2) MHC-peptide transport to the central zone of the synapse. (3) Stabilisation. Clustered MHC-peptide molecules are "locked in" by flanking adhesion molecules (see figure 1.3.).

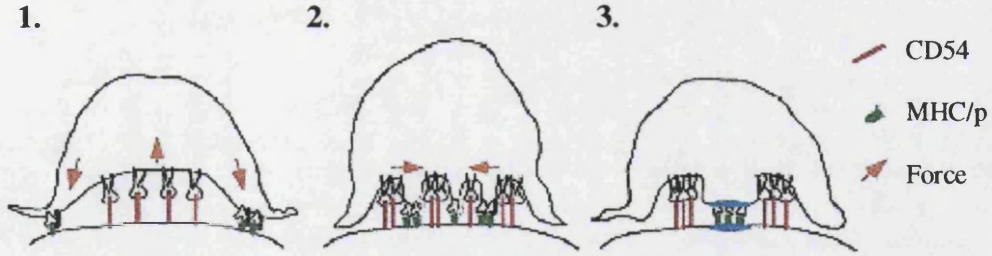


Figure 1.3. Formation of the immunological synapse. **Stage 1** - TCR sampling of surface MHC-peptide complexes is facilitated by integrin-mediated cell-cell adhesion. **Stage 2** - Upon successful TCR binding of antigen, MHC-peptide complexes are concentrated within a central zone. **Stage 3** – The immunological synapse is stabilised by further organisation of adhesion molecules. (Adapted from van der Merwe *et al.*, 2000)

These events occur simultaneously with reorganisation of the actin cytoskeleton with specialised microdomains or "rafts" within the plasma membrane. Rafts are composed of tightly packed cholesterol and sphingolipids; the sphingolipids

exhibit stronger lateral cohesion than glycerolphospholipids and preferentially associate with cholesterol molecules, which function as spacers. Simons & Ikonen's "raft hypothesis" propounds that these lipid microdomains exist within the plasma membrane and serve as rafts for the transport of selected molecules or as "relay stations" for signalling complexes (Simons and Ikonen, 1997). Therefore, according to this model, the accumulation of rafts at the immunological synapse (IS) may be an important mechanism for the amplification of the TCR signalling.

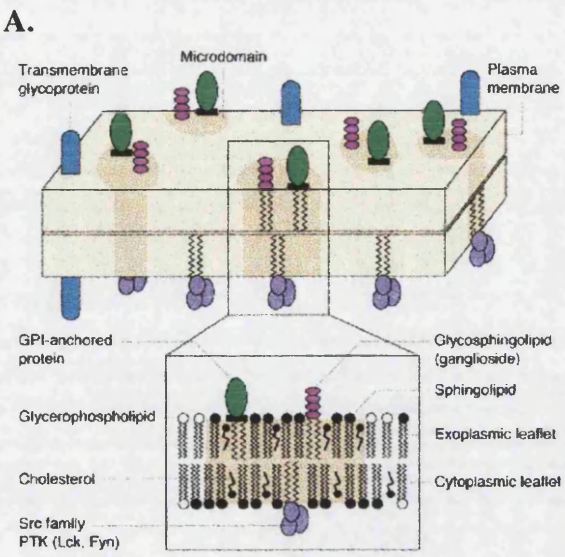
It has recently been suggested that CD28 costimulation depends on the recruitment of rafts to the IS. T cell stimulation via anti-CD28 antibody has been reported to induce the clustering of rafts rich in T cell signalling molecules (e.g. Lck, LAT, Ras and the PLC-γ1 substrate, IP_2) at the site of TCR engagement (Viola *et al.*, 1999). Under resting conditions the TCR-CD3 complex is excluded from the rafts but upon engagement of the TCR, the rafts become permissive to TCR translocation into the lipid microdomains. Disruption of membrane compartmentalism has been shown to inhibit the early signalling events of T cell activation. Preincubation of T cells with the raft inhibitor nystatin decreased tyrosine phosphorylation of CD3ζ and PLC-γ1. Thus, membrane compartmentalism appears to be a pre-requisite for efficient TCR signal transduction (Xavier *et al.*, 1998; reviewed in Viola, 2001).

Therefore, it would appear that the raft hypothesis has been corroborated by functional *in vitro* data. However, the physiological relevance of these membrane structures has been much questioned since the isolation of rafts from

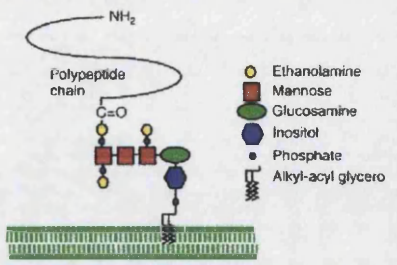
the cell membrane requires the use of mild detergents. Therefore, it's possible that rafts may form because of detergent treatment and do not represent the actual organisation on intact membranes.

Evidence supporting the existence of rafts in living cells has been independently reported by Varma *et al.* and Friedrichson *et al.* Both studies relied on the fact that GPI-anchored proteins are enriched within rafts. Indeed, the presence of a GPI-anchor has been reported to specifically target a protein to lipid rafts. Both papers, via different techniques, showed that TM-proteins were randomly distributed and that GPI-linked proteins were focused into microdomains. Collectively they estimated that a single raft would be approximately 70nm in diameter and contains at least 15 GPI-linked molecules (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998; reviewed in Viola and Lanzavecchia, 1999 and illustrated in figure 1.4.).

GPI-anchored proteins are not passive passengers on these rafts and may even be important in recruiting lipid rafts to the TCR activation cap. Moran and Morcelli demonstrated that co-engagement of CD48 (a GPI-linked molecule) and the TCR resulted in a number of events that facilitated T cell activation. ζ-chains preferentially associated with the lipid rafts as a consequence of actin polymerisation induced by CD48/CD3 ligation. Collectively these events resulted in enhanced tyrosine phoshporylation of the ζ chains probably due to the increased association with PTKs (e.g. Lck) enriched within the rafts (Moran and Miceli, 1998).



(Adapted from Ilangumaran *et al.*, 2000)



(Adapted from Horejsi *et al.*, 1999)

Figure 1.4. A. GPI-anchored molecules are organised into lipid rafts or microdomains characterised by the presence of gangliosides, sphingolipids and cholesterol. **B.** Schematic diagram of a GPI-anchored molecule.

Enhanced tyrosine phosphorylation may have been due to the direct interaction between GPI-anchored proteins and Src family kinases. GPI-anchored proteins have been shown to co-precipitate with lck and fyn in T cell detergent lysates. Since GPI-proteins are not linked to the plasma membrane via a transmembranous domain, the exact nature of the interaction with PTKs is unclear. However, it has been suggested that the fatty acid residues present in GPI-anchors are long enough to penetrate into the opposing cytoplasmic leaflet and thus interact with the aliphatic chains of the cytoplasmic PTK signalling molecules. How this translates into efficient tyrosine phosphorylation upon GPI-protein cross-linking is also not understood. The present model is that cross-linking GPI-anchored proteins leads to the aggregation of PTK in sufficient concentration to induce auto-phosphorylation (reviewed in Horejsi *et al.*, 1999).

An interesting feature of this diverse group of proteins (see table 1) is that cross-linking by antibodies results in signal transduction characterised by elevated

$[Ca^{2+}]_i$, tyrosine phosphorylation and T cell proliferation and effector function. The physiological relevance of ligating with antibodies remains debatable. However, experiments studying the effect of altered expression of GPI-anchored proteins *in vivo* certainly point towards a role for GPI-linked molecules in T cell activation. For example, activation of human T cells derived from GPI-deficient, paroxysomal nocturnal haemoglobinuria (PNH) patients is reduced compared to normal T cells stimulated *in vitro* (Romagnoli and Bron, 1999). PNH is a haemolytic disorder caused by aberrant complement activity in the absence of functional CD59. The disorder is also accompanied by suppressed T cell responses e.g. reduced proliferation to allogeneic APC *in vitro*. Furthermore, T cell lines derived from patients with PNH display significantly decreased activation of Lck when stimulated with anti-CD3ε *in vitro* (Romagnoli and Bron, 1999).

Table 1. GPI-anchored surface proteins are functionally diverse.

Name	**Description**	**Function**
CD14	LPS receptor	Receptor for the bacterial cell wall component LPS
CD16b	Fc_γ receptor type 3	IgG receptor
CD24	Heat stable antigen	Unclear
CD48		Adhesion
CD52		Unclear
CD55	Decay accelerating factor	Complement-protecting factor
CD58	LFA-3	Adhesion
CD59		Complement-protecting factor
CD73	5' nucleotidase	Ectoenzyme

Name	Description	Function
CD87	Urokinase-type plasminogen activator receptor (uPAR)	Protease receptor
CD90	Thy-1	Unclear role in adhesion
CD157	ADP-ribosyl cyclase	Ectoenzyme
Ly-6	Multi-gene family	Unclear
RT-6	Arginine ADP-ribosyl transferase	Ectoenzyme

One family of GPI-linked molecules that may be of particular interest with regard to T cell activation are the Ly-6 proteins. In this respect, Henderson *et al.* showed that Ly-6A (a.k.a. Ly-6A/E, TAP or Sca-1) may even be able to induce signals that can substitute for those generated from the TCR. Normally, Ly-6A is expressed on stem cells and mature peripheral T cells, but expression is switched off in the thymus. Using the T cell specific human CD2 promoter, transgenic mice were created that expressed Ly-6A in the thymus. The result was a substantial but incomplete block in thymocyte development at the $CD3^-CD4^-CD8^-CD44^+CD25^+$ stage. The Ly6A.2 transgenic mice were also crossed with MHC class I and II-deficient mice. Lack of MHC expression prevented the development of mature $CD4^+$ and $CD8^+$ T cells. However, dysregulated Ly-6A.2 expression was able to partially compensate for the absence of MHC-dependent positive selection since $CD4^+CD8^-$ (but not $CD4^-CD8^+$) T cells developed in Tg^+MHC^- mice. Therefore in these studies, overexpression of Ly-6A.2 could initiate signalling in T cells that normally use their antigen-specific α/β TCR (Bamezai *et al.*, 1995; Henderson *et al.*, 1998). Furthermore, thymocytes derived

from Ly-A.2 transgenic mice spontaneously aggregate in a Ly-6A-dependent manner, suggesting a possible role in cell-cell adhesion.

Conversely, inhibiting Ly-6A expression produced a profound loss of T cell response to stimuli *in vitro*. Antisense oligonucleotides complementary to the 5' end of the mRNA encoding the Ly-6A protein could block surface expression by 60-80%. This level of suppression was sufficient to prevent T cells proliferating to anti-CD3ε or Concanavalin A (Con A) or re-stimulation of antigen-primed T cells *in vitro* (Flood *et al.*, 1990). Downregulation of Ly-6A expression was subsequently shown to decrease TCR β chain transcription and PTK activity (Lee *et al.*, 1994). Correlatively, loss of TCR expression results in an inability to activate T cells by cross-linking Ly-6A (Bamezai *et al.*, 1988).

In contrast to the majority of the published *in vitro* data, mature peripheral T cells derived from Ly-6A-deficient mice actually proliferated more vigorously to antigens and mitogens than wild-type littermates. Ly-6A null splenocytes proliferated at significantly higher levels to stimulation with Con A, allo-antigen and anti-CD3ε mAbs. Moreover, T cells primed with a model protein antigen *in vivo* proliferated at higher levels on subsequent re-challenge with the same antigen *in vitro* compared to T cells from primed wild type littermates. Thus, the results from the antisense experiments and Ly-6A knockout experiments appear to contradict each other. However, the disparity between data may reflect two very different experimental conditions. In the Ly6-A knockout mice, Ly-6A had never been expressed allowing the development of redundancy, or perhaps in this case, overcompensation by another molecule. In the case of antisense-mediated

inhibition of Ly-6A expression, the rapid transition *in vitro* may not allow for the emergence of redundancy. Either way, it is clear that Ly-6A (or Ly-6A/E, as it will be subsequently referred to) may play an important role in regulating T cell activation.

1.2D. T cell differentiation and effector function.

Both CD4 and CD8 T cell responses may be broken down into three phases: (1) activation and clonal expansion, (2) effector function and (3) stability or memory. Each phase is tightly regulated with the default pathway being programmed cell death. T cells isolated from their *in vivo* context are programmed to die unless they receive survival signals either in the form of CD28 signalling or cytokines such as IL-6 and IL-7. These cytokines are constitutively produced *in vivo* and so may in part account for the relatively long lifespan of T cells *in vivo* (reviewed in Marrack *et al.*, 1998).

Providing that T cells receive the appropriate stimuli to overcome the default pathway, substantial antigen-driven expansion occurs followed by differentiation into effector cells. $CD4^+$ T cell effectors have been primarily characterised as non-lytic cytokine producers, whilst $CD8^+$ T cell effectors are normally referred to as cytotoxic T lymphocytes or CTL due to their potent cytotoxicity. However, it is important to mention that these effector properties may not be totally restricted to one particular T cell subset. For example, $CD4^+$ T cells have been reported to possess the cytolytic machinery necessary for lysis of target cells and

$CD8^+$ T cells have been shown to be efficient producers of cytokines (Harty *et al.*, 2000)

As antigen levels decline, effector activity subsides. The fate for the vast majority of activated T cells (>95%) is apoptosis, which occurs through both activation induced cell death (AICD) and passive cell death (Refaeli *et al.*, 1998; Van Parijs *et al.*, 1998). Apoptosis of effector T cells is important for regulating cell numbers and restoring homoeostasis (Ahmed and Gray, 1996). Death signals for AICD are conveyed by Fas ligand binding to the Fas receptor (CD95) and by TNF-α binding to the TNF receptor type I. By contrast, "passive cell death" occurs as a result of a lack of signalling; specifically the loss of contact with growth factors as these become limiting late in an immune response. These growth factors are thought to be required for the up-regulation of anti-apoptotic molecules that are down-regulated on T cell activation. Both AICD and passive cell death (or "death by neglect" (Van Parijs *et al.*, 1998)) are ultmiately mediated by the activation of a network of intracellular proteases called the caspase cascade. For example, Fas and TNF-α activate caspase-8 and caspase-9, respectively. These "gatekeeper" caspases activate even more caspases resulting in destruction of subcellular structures, organelles and the genome, all ultimately contributing to the death of the T cell (Hanahan and Weinberg, 2000).

However, a small fraction of antigen-experienced cells escapes apoptosis and these enter a stable pool of memory cells that may persist for many years. The ability to generate an accelerated T memory cell response contributes to the protective immunity to a subsequent encounter with the same pathogen. The

augmented memory response upon re-exposure to antigen has been suggested to be due to the increased frequency of antigen-specific T cells and also to qualitative changes in memory T cells (Ahmed and Gray, 1996).

The above paragraphs have described general properties of T cell responses. The following sections will detail I. Polarisation of $CD4^+$ T cell responses and helper function and II. $CD8^+$ T cell effector mechanisms.

I. $CD4^+$ T cell helper function.

$CD4^+$ T cell heterogeneity was first demonstrated in a seminal study by Mosmann *et al.* (1986). They identified two distinct murine $CD4^+$ T cell clones on the basis of their cytokine profile. "T_H1" clones produced IL-2 and interferon-γ (IFN-γ), whilst T_H2 clones produced IL-4 and IL-5. Subsequent work has defined TNF-α as a "T_H1 cytokine" and IL-6 and IL13 as "T_H2 cytokines" (reviewed in Abbas *et al.*, 1996). Furthermore, cytokines produced by T_H1 cells could block activation of T_H2 cells and *vice versa*. The result of cross-regulation tends to be that a $CD4^+$ T cell response becomes progressively polarised as the response proceeds. Further developments in the sensitivity of cytokine assays have shown that individual cells may display multifarious and heterogeneous cytokine production. Thus, a third $CD4^+$ T cell subset that produces a mixture of cytokines was labelled T_H0 (Abbas *et al.*, 1996). It is important to note that activated $CD4^+$ T cells may become permanently differentiated to a particular cytokine profile. The polarised pattern of cytokine production may persist indefinitely *in vivo* as memory cells (Swain, 1994).

What determines the dominant polarity of a $CD4^+$ T cell response has been a considerable area of interest in recent years. The cytokines present during T cell activation are known to influence the type of response generated e.g. IL-2 drives T_H1 proliferation whilst IL-4 production enhances the development of a T_H2-type response. However, it has been recently proposed that APC deliver a polarising signal to naïve $CD4^+$ T cells. This may be due to a combination of signal two (i.e. the expression levels of co-stimulatory molecules such as B7 and CD54) and a cytokine-derived "third" signal. A major focus of attention has been the non-redundant role of IL-12 in the production of a functional T_H1 response. A T_H1-deficient phenotype has been demonstrated in IL-12-deficient mice (Kobayashi *et al.*, 1996) and IL-12 receptor-deficient humans (Dorman and Holland, 2000 Dec). Furthermore, suppression of IL-12 production has been shown to preferentially induce a T_H2 type response. Therefore, APCs may be important for delivering signal one: TCR triggering, signal two: co-stimulation and signal 3: polarisation (Kalinski *et al.*, 1999).

The T cell derived cytokines IL-10 and TGF-β have been reported to be major suppressors of APC IL-12 production. Originally this T cell response was described as T_H2-like, however subsequent work has characterised a distinct regulatory $CD4^+$ T cell termed T reg cells or Tr1 (formerly the unpopular T suppressor cells). Tr1 cells have been shown to reside within the $CD45RB^{lo}$ $CD4^+$ T cell population and depend upon IL-10 and TGF-β for their regulation of IL-12 production. IL-10 has also been shown to down-regulate co-stimulatory molecules such as CD54 and B7. Thus, Tr1 cells may be able to dampen a

potentially destructive T_H1 response by acting at the level of the APC (reviewed in Mason and Powrie, 1998).

APC may also be important for the induction of chemokine receptors that are necessary for the interaction of T cells with B lymphocytes *in vivo*. $CD4^+$ T cells first interact with professional APCs in the T cell rich areas of the lymph nodes and spleen (i.e. the paracortex and periarteriolar lymphoid sheath or PALS). Activated T cells are induced to lose expression of CCR7 (which is required for entry into the T cell areas) and gain CXCR5, which permits migration into the B cell-rich areas. This allows $CD4^+$ T cells to recognise peptide-MHC complexes expressed on the surface of antigen-specific B cells and provide "help" for antibody production (reviewed in Jenkins *et al.*, 2001).

$CD4^+$ T cell help is a critical step in the production of different antibody isotypes by activated B cells. In this regard, T_H1 and T_H2 $CD4^+$ T cell selectively induce the switching of different isotypes. For example, T_H1-dependent isotypes are IgG2a and IgG3 in mice (homologous to IgG1 and IgG3 in humans), whilst T_H2 cells characteristically induce IgE and/or IgG1 in mice (or IgG4 in humans). Consequently, T_H1-type responses are typically characterised by the activation of microbicidal macrophages (e.g. by IFN-γ) and the recruitment of the complement system. In contrast, T_H2-type responses activate anti-parasitic cell types (e.g. IL-5 activated eosinophils) that are important for the elimination of helminths (Abbas *et al.*, 1996).

A vital molecule for mediating $CD4^+$ T cell help appears to be CD40L, since B cell responses in CD40L-deficient mice are devoid of non-IgM isotypes (Xu *et al.*, 1994). Furthermore, CD40L also appears to be important for $CD4^+$ T cell help of $CD8^+$ CTL responses, but in contrast to T cell-B cell interactions, CD40L seems to act via a third cell type. $CD4^+$ T cell help for $CD8^+$ T cell responses has been shown to require APCs that present antigen in the context of both MHC class I and MHC class II. CD4 T cell help was also demonstrated to be blocked by the absence of CD40 signalling or replaced by stimulating APC with anti-CD40 mAb. Therefore, Ridge *et al.* proposed that via CD40-CD40L interactions, $CD4^+$ T cells could "condition" APC to acquire the capacity to stimulate $CD8^+$ T cells (Ridge *et al.*, 1998; reviewed in Kalamis and Walker, 1998).

Recently, it has been suggested by Gerloni *et al.* (2000) that $CD4^+$ T cells may also be able to help $CD4^+$ T cell responses. They showed that a strong $CD4^+$ T cell response to a particular *Plasmodium falciparum* peptide could facilitate the induction of a $CD4^+$ T cell response to a "weaker" tumour-derived peptide. Similar to $CD4^+$ T cell help of $CD8^+$ T cell response, $CD4^+$ T cell help of $CD4^+$ T cell response could be abrogated by inhibiting CD40 signalling. Therefore, it is conceivable that this novel role for CD4 T cells is also mediated by CD40-activation of APCs (den Haan and Bevan, 2000).

II. $CD8^+$ cytotoxic T lymphocytes

$CD8^+$ T lymphocytes are primarily activated by cytosolic infections i.e. viruses, intracytoplasmic bacteria and protozoa. This is because priming of $CD8^+$ T cells occurs via the presentation of short pathogen-derived peptides processed by the endogenous MHC class I presentation pathway. However, professional APC appear to have the capacity to cross-present exogenous antigens (i.e. non-cytosolic pathogens such as phagosomal-bacteria) via the MHC class I restricted pathway.

Activated $CD8^+$ T lymphocytes undergo massive clonal expansion. Our comprehension of the magnitude of the $CD8^+$ T cell clonal burst has been greatly assisted by recent developments in techniques for the numeration of antigen-specific $CD8^+$ T cells. MHC class I-peptide tetramers in particular have been instrumental in measuring the kinetics of antigen-specific $CD8^+$ T cell responses. Using pathogen-specific tetramers, the proportion of pathogen-specific $CD8^+$ T cells within secondary lymphoid organs has been observed to increase from undetectable levels in the naïve host to around 2% of total $CD8^+$ T cells after bacterial infections (Busch *et al.*, 1998). Furthermore, in certain viral infections, antigen-specific $CD8^+$ T cells have been shown to account for greater than 50% of the $CD8^+$ T cells within an infected spleen (Butz and Bevan, 1998). Upon clearance of the pathogen, $CD8^+$ T cell numbers decline to approximately 5-10% of the initial clonal burst size and are maintained as antigen-hyperreactive memory cells for the lifetime of the host (Murali-Krishna *et al.*, 1998).

Since MHC class I molecules are expressed on virtually all nucleated cells, activated $CD8^+$ T cells are important in mediating pathogen clearance by lysing cells infected with intracellular pathogens. $CD8^+$ effector cells i.e. CTL, possess a variety of molecular mechanisms for the cytolysis of their targets including the granzyme/perforin pathway and CD95-CD95L mediated cell death; $CD8^+$ T cells can also mediate pathogen clearance through the secretion of cytokines.

The proposed function of perforin is to form pores within the membrane of the target cell, thus allowing the delivery of granule enzymes, granzyme A and B (GrmA and GrmB), into the target cell. Both the granzyme and CD95 pathways induce the caspase cascade in the target cell, resulting in DNA fragmentation and apoptosis. Cytokines on the other hand appear to function by disrupting infection through interference of pathogen attachment or limiting intracellular replication (Harty *et al.*, 2000).

Classically, IFN-γ and TNF-α have been the cytokines associated with $CD8^+$ T cell effector function. $CD8^+$ T cell derived cytokines are particularly effective for the elimination of non-lytic virus. For example, IFN-γ and TNF-α were shown to be very effective in shutting down hepatitis B viral gene expression in hepatocytes, thus lessening the potential risk of immunopathology to essential organs such as the liver (Guidotti *et al.*, 1996). However, it is now understood that like $CD4^+$ T cells, $CD8^+$ T cells may produce a variety of cytokines. Furthermore, in the presence of "polarising" cytokines, $CD8^+$ T lymphocytes differentiate into type 1 (IFN-γ, IL-2, TNF-α) and type 2 (IL-4, IL-5, IL-6 and IL-10) cytokine-secreting cells, or T_C1 and T_C2 as they have subsequently been

labelled. Recently, it has been suggested that the heterogeneity of human $CD8^+$ T cells may be extended to cytolytic function. Co-staining of both intracellular perforin and cytokines revealed that activated human $CD8^+$ T lymphocytes either expressed cytokines (e.g. IFN-γ and TNF-α) or perforin, implying possible functional segregation (Sandberg *et al.*, 2001).

Analogous to $CD4^+$ T lymphocyte differentiation, IL-4 has been shown to drive T_C2 $CD8^+$ T cell differentiation. Cross-regulation of cytokine production has also been observed for the suppression of T_C1-type responses by IL-4. TGF-β also inhibits the development of T_C1 $CD8^+$ T cells but curiously, when TGF-β and IL-4 are added together no inhibitory effect is observed (Erard *et al.*, 1999). However, the primary differentiation factor for the T_C1 subset has been characterised as IL-12. Moreover, IL-12 has been demonstrated to be important for efficient clonal expansion, possibly providing an essential "third" signal necessary for full activation of naïve $CD8^+$ T cells (Curtsinger *et al.*, 1999).

In addition to acting as polarising signals during antigen-specific $CD8^+$ (and $CD4^+$) T cell activation, some of the same cytokines have been shown to exert "bystander" effects on non-antigen-stimulated $CD8^+$ T cells. For example, IL-12 has been reported to selectively promote the bystander proliferation of memory phenotype ($CD44^{hi}$) $CD8^+$ T cells *in vivo*. The capacity of IL-12 to induce bystander proliferation is also shared with type I (α/β) interferons (IFN-I), IFN-γ, IL-18 and IL-15. However, amongst these cytokines IL-15 is the only one able to stimulate the proliferation of purified $CD44^{hi}$ $CD8^+$ T cells *in vitro*, implying that IL-15 signals T cells directly and may act as the final "effector" cytokine in

pathways initiated by the other cytokines *in vivo* (Zhang *et al.*, 1998). Consistent with this idea is the fact that IL-15 expression is induced by each of the other cytokines (directly or indirectly) and the observation that the responding cell population expresses high levels of the IL-15 receptor. Furthermore, the paucity of memory phenotype T cells in IL-15Rα- and IL-15-deficient mice indicates that IL-15 is required for the maintenance of memory $CD8^+$ T cells *in vivo*.

In the case of IL-12 or IL-18, the cytokine cascade necessary for bystander T cell activation appears to require IFN-γ. Using IFN-I receptor- and IFN-γ-deficient mice, Tough *et al.* (2001) demonstrated that IL-12 and IL-18-induced $CD8^+$ T cell proliferation was dependent on IFN-γ and not IFN-I. A recent study by Lertmemongkolchai *et al.* supports a physiological role for this type of T cell activation pathway *in vivo*. Host survival of an infection with the gram-negative bacterium *Burkholderia pseudomallei* has been reported to be heavily dependent on the rapid production of IFN-γ (Santanirand *et al.*, 1999). The predominant source of IFN-γ was subsequently shown to be natural killer (NK) cells and $CD8^+$ T cells. Strikingly, IFN-γ production by $CD44^{hi}$ $CD8^+$ T cells was detected within 15 hours after infection and was inhibited by neutralising the activity of IL-12 and IL-18 (Lertmemongkolchai *et al.*, 2001). Therefore, bystander T cell activation may also play an important role in rapidly mobilising a non-specific anti-bacterial response *in vivo* whilst the antigen-specific adaptive response is being initiated.

1.3 Dendritic cell activation

1.3A. Dendritic cell life cycle

The previous section has discussed the potent effector mechanisms of the adaptive immune system. Common to the primary response of both T- and B-lymphocytes is the requirement for instruction and regulation by a third cell type: the dendritic cell (DC). Bone marrow DC progenitors yield precursors that circulate in the blood and home to peripheral tissues as "immature" DC. Upon exposure to infectious agents, DC capture antigen and migrate to lymphoid organs where they initiate adaptive immune responses (see figure 1.5.).

Broadly speaking, DC development may be separated into four sequential phases: (1) bone marrow progenitors. (2) precursor DC that circulate through the blood and lymphatics and may respond directly to pathogens by releasing large quantities of cytokines e.g. IFN-I that may limit dissemination of the infection, possibly in a manner comparable to the plasmacytoid DC characterised in humans (Cella *et al.*, 1999). (3) immature DC, tissue-residing sentinels specialised for antigen capture. (4) mature DC, terminally differentiated "professional" antigen-presenting cells, located within lymphocyte enriched secondary lymphoid organs (reviewed in Banchereau *et al.*, 2000).

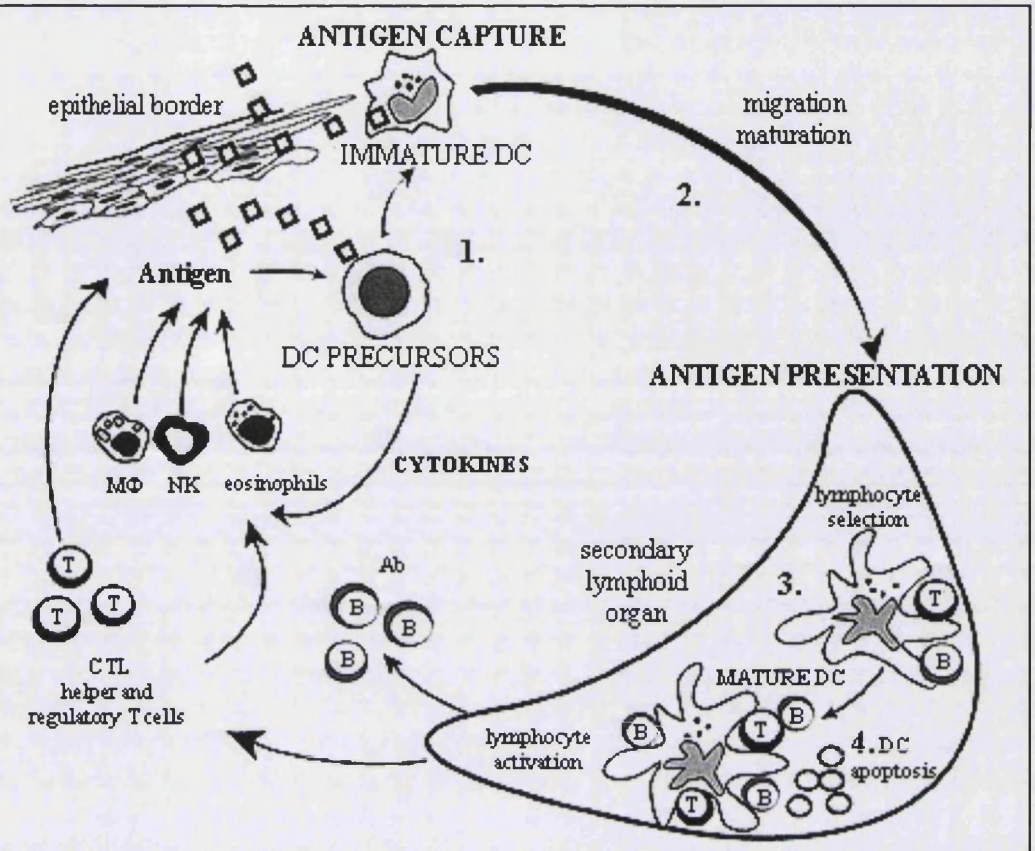


Figure 1.5. A schematic diagram summarising the life cycle of dendritic cells. **1.** Circulating precursor DC seed peripheral tissues but may also respond directly to pathogens, producing cytokines such as IFN-α. **2.** Immature DC capture antigen and migrate to secondary lymphoid organs. **3.** Mature DC interact with antigen-specific T and B lymphocytes to initiate adaptive immune responses. **4.** Finally, following lymphocyte activation, DC are thought to undergo apoptosis. (Adapted from Banchereau *et al.*, 2000)

1.3B.Murine DC subsets

Murine DC are frequently described as being of either myeloid or lymphoid in origin. Accordingly, DC and macrophages may be generated by stimulating precursors with granulocyte/macrophage colony-stimulating factor (GM-CSF), implying a common myeloid developmental pathway (Inaba *et al,* 1992). By contrast, adoptive transfer of lymphoid precursors into irradiated hosts allowed the development of T and B lymphocytes, NK cells and DC expressing CD8α

homodimers, supporting a common lymphoid pathway (Wu *et al.*, 1996). In fact, CD8α has been commonly used as a marker to distinguish these two lineages, as it was thought to be expressed on lymphoid- but not myeloid-lineage DC. However, the recent demonstration by Manz *et al.* (2001) that both CD8α positive and negative DC can be produced from either myeloid or lymphoid progenitors calls this distinction into question. Nevertheless, experiments in knockout mice support the notion that different developmental pathways lead to $CD8\alpha^+$ versus $CD8\alpha^-$ DC. Thus, mice deficient for the transcription factors Rel B (Burkly *et al.*, 1995) and PU.1 (Guerriero *et al.*, 2000) possess $CD8\alpha^-$ but not $CD8\alpha^+$ DC, whilst the reverse is true for Ikaros-deficient mice (Wu *et al.*, 1997).

While the exact significance of CD8α expression with respect to DC lineage remains unclear, this marker has been used to demonstrate that phenotypically distinct DC are distributed differently in lymphoid tissues. In particular, $CD8\alpha^+$ "lymphoid" DC appear to selectively localise to the T cell rich areas of the PALS in the spleen, whilst $CD8\alpha^-$ "myeloid" DC can be found in the marginal zone (Vremec and Shortman, 1997). However, $CD8\alpha^-$ DC may be induced to accumulate within the PALS by proinflammatory signals (De Smedt *et al.*, 1996; Sousa *et al.*, 1997). The presence of $CD8\alpha^+$ DC in the thymus was taken to suggest that lymphoid DC may have a tolerising role *in vivo*. However, both myeloid and lymphoid have been reported to efficiently stimulate antigen specific T cells *in vitro* and *in vivo*.

CD8α, along with several other markers, has been used to show the existence of multiple phenotypically distinct DC subpopulations. Amongst mouse DC,

CD11b generally shows reciprocal expression to CD8α, and thus has been used as a marker of "myeloid" DC (Vremec and Shortman, 1997). More recently, the discovery of a major DC population in the LN (Salomon *et al.*, 1998) and spleen (Vremec *et al.*, 2000) that expresses CD4 has lead to the delineation of three DC subtypes: $CD4^+CD8\alpha^-$, $CD4^+CD8\alpha^+$ and $CD4^-CD8\alpha^-$. Recent work suggests that the subtypes represent the product of distinct DC lineages, with "none being the precursor of the other" (Kamath *et al.*, 2000). Further functional analysis of these DC subtypes suggests that they may possess distinctive patterns of cytokine secretion. Compared to $CD4^+CD8\alpha^+$ and $CD4^-CD8^-$ splenic DC, $CD4^-CD8\alpha^+$ DC were the main producers of IL-12 and IFN-α in response to activation by microbial products such as lipopolysaccharide (LPS) and bacterial DNA. Similarly, activated DC isolated from the LN showed a similar pattern of cytokine secretion, with the intensity of $CD8\alpha^+$ expression correlating with IL-12 production. By contrast, the major subtype producing IFN-γ was identified as the $CD4^-CD8\alpha^-$ DC (Hochrein *et al.*, 2001).

The interpretation of DC subset characteristics has been further complicated by the recent report that DC are able to "pick up" CD4 and CD8 from T cells (Vremec *et al.*, 2000). "Authentic" CD8α and CD4 expression on LN DC was visualised by constructing bone marrow chimeras containing a mixture of wild type Ly5.1 bone marrow cells and Ly5.2 bone marrow cells that were either CD8α null or CD4 null. Comparing the difference in CD4 and CD8α expression on wild type and null bone marrow derived DC revealed that only a small proportion of DC expressing low levels of CD8α was due to "pick up" from associated T cells. In contrast, CD4 expression on wild type DC appeared to be

predominantly due to "pick up", with only the high level expression representing genuine surface expression of CD4 on LN DC.

Excluding this artefact, Henri *et al.* recently described three subsets ($CD4^+8^-$ $DEC\text{-}205^-$, $CD4^-8^-DEC\text{-}205^-$, $CD4^-CD8^+DEC\text{-}205^+$) present both in the spleen and LN. Two additional DC subsets exist in the LN that lack CD4 but express CD8α at moderate intensities. The first subtype expresses intermediate levels of DEC-205, is found ubiquitously in LN and may represent dermal or "interstitial" DC. By contrast, the distribution of a second DC subtype (which expressed high levels of DEC-205) was found to be mostly limited to the lymph nodes draining the skin. Therefore, it was proposed that this subset may correspond to the mature form of human epithelial DC or Langerhans cells (LC). Indeed, both subsets were demonstrated to be emigrants from the skin, migrating to the lymph node upon activation (Henri *et al.*, 2001).

1.3C. Dendritic cell maturation

It is now understood that DC migration from peripheral to lymphoid tissues is just one part of a continuous maturation process that may be initiated by antigen deposition and inflammation.

I. Recruitment of DC precursors

Progenitor DC have been shown to rapidly accumulate in the respiratory mucosa just hours after an airway challenge with soluble antigen (Xia *et al.*, 1995), LPS

(Schon-Hengrad *et al.*, 1991) or bacteria (McWilliam *et al.*, 1994). Furthermore, as DC precursors present in the blood are constitutively located at the interface of blood and skin, upon local inflammation, blood DC are rapidly recruited to cutaneous tissues. The trafficking of circulating precursor DC to the site of inflammation or source of antigen is largely controlled by soluble chemotactic factors called chemokines. Immature DC express a number of chemokine receptors (e.g. CCR1-6) and therefore are responsive to a broad spectrum of chemokines which facilitate migration. For example, LC expressing CCR6 infiltrate inflamed epithelia in response to increasing concentrations of macrophage inflammatory protein-3α (MIP-3α) (reviewed in Caux *et al.*, 2000 and illustrated in figure 1.6.).

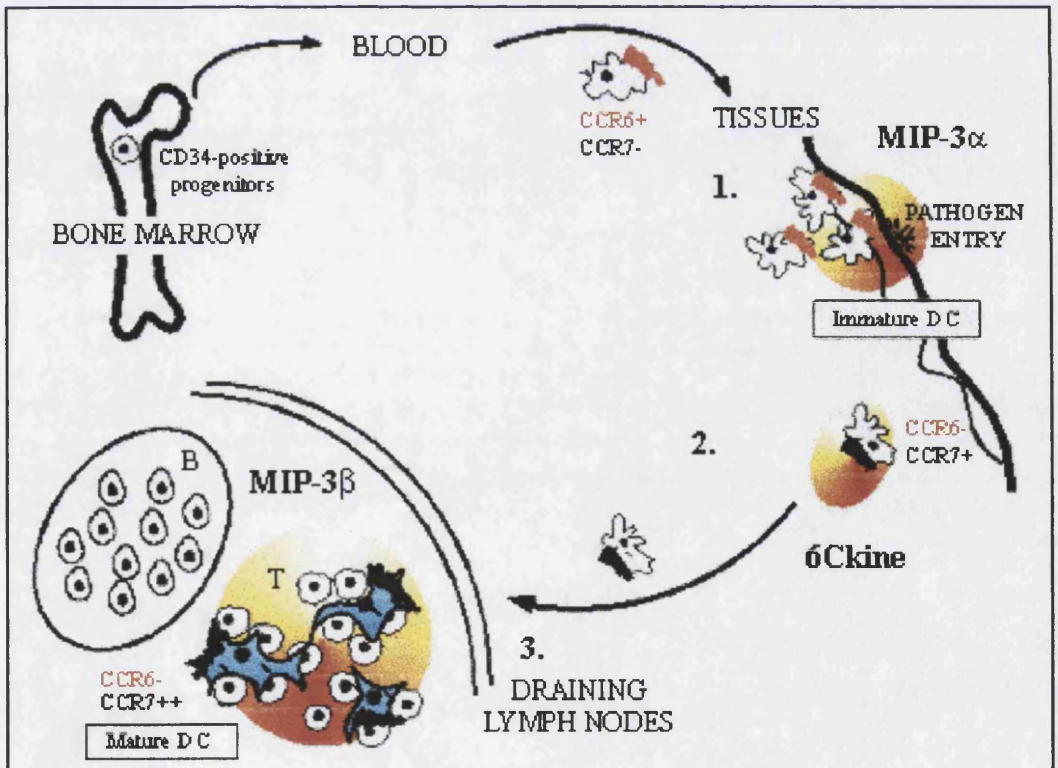


Figure 1.6. The role of chemokines in dendritic cell migration. **1.** Immature LC are attracted to inflamed epithelia by CCR6 binding of MIP-3α. **2.** Upon antigen capture, activated LC downregulate CCR6 and enter the draining lymphatic vessels. This process is dependent on expression of CCR7 and binding of its ligand, 6Ckine, which is present on lymphatic cell walls. **3.** Mature LC migrate to the draining lymph nodes and enter the T cell areas via CCR7 interaction with MIP-3β. (Adapted from Banchereau *et al.*, 2000)

DC recruitment across endothelium also requires several adhesion events, initially involving selectin-supported "rolling" across the endothelia (e.g. via E- and P-selectin) and followed by stronger integrin-mediated adhesion (e.g. CD18). Immature LC uniquely express E-cadherin which allows entry to the epidermis but upon activation this must be downregulated to permit emigration from the skin. DC also produce enzymes such as matrix metalloproteases (MMP) and collagenase type IV that are probably required for breaching basal membranes and the extracellular matrix (Banchereau *et al.*, 2000).

II. Antigen capture and processing

In situ in the skin, airways and LN, DC appear to be star-shaped or stellate. Electron microscopy has revealed that DC possess long processes (>10μm) that when visualised under live conditions, via phase contrast, appear to bend, extend and retract in a manner that befits the phagocytic function of DC (Banchereau and Steinman, 1998).

Immature DC have several mechanisms for the capture of antigen at their disposal: (1) macropinocytosis, the sampling of relatively large volumes of extracellular fluid by the formation of large pinocytic vesicles; (2) receptor-mediated adsorptive endocytosis e.g. mannose receptor, DEC-205 or Fcγ receptor uptake of immune complexes; (3) phagocytosis of e.g. particulate antigens, zymosan and *Staphylococus aureus*. Consequently, DC are so efficient at antigen uptake that picomolar quantities are sufficient for antigen presentation

compared to the micromolar amounts required by other APCs e.g. macrophages. However, in contrast to the phagocytic activity of macrophages, LC have been reported to be unable to phagocytose opsonised red blood cells (Reis e Sousa *et al.*, 1993). Probably these respective differences reflect the specialisation of these cell types, with the primary role of DC being antigen presentation and the principal function of macrophages being "scavenging" (reviewed in Austyn, 1996).

Consistent with this idea, the lysosomal system in DCs appears to be different to that in macrophages in its capacity to attenuate protein degradation. The default endocytic pathway for protein antigen ingested by macrophages is rapid lysosomal degradation to amino acids. In contrast, DC have the ability to sequester antigen for long periods of time in MHC class II enriched lysosomes. However, antigen capture and internalisation is not necessarily sufficient for the formation and transport of MHC class II-peptide complexes. Inaba *et al.* showed that internalised antigen could reach the processing endocytic compartments but required an additional maturation signal for conversion to immunogenic complexes (Inaba *et al.*, 2000). Manickasingham *et al.* proposed that adjuvants such as LPS may provide this signal, since presentation of a model antigen was enhanced by the co-administration of LPS and antigen compared to antigen alone (Manickasingham and Sousa, 2000; reviewed in Manickasingham and Reis e Sousa, 2001). In tandem, endocytosis is downregulated, perhaps in order to assist the accumulation of MHC complex at the cell surface. Furthermore, peptide-MHC complexes are transported to the cell surface together with CD86

in clusters, where they remain associated within membrane micro-domains (Turley *et al.*, 2000).

Like MHC class II, the expression of MHC class I is also upregulated by DC maturation. Typically, the MHC class I pathway is considered to present endogenous cellular antigens. For example, influenza virus may infect DC and produce viral proteins in the cytosol, which may be subjected to proteosomal proteolysis. The peptides produced are then translocated via TAP transporters into the lumen of the endoplasmic reticulum (ER) for loading onto MHC class I molecules. However, not all pathogens infect and replicate within DC and so non-conventional mechanisms exist for MHC class I presentation. For example, antigen captured and internalised via Fcγ receptors are allowed to egress from endocytic organelles and into the cytosol where they may be processed by the classical MHC I pathway. Alternatively, immature DC may phagocytose infected dying cells that contain viral antigen and present to $CD8^+$ T cells via MHC class I (reviewed in Mellman and Steinman, 2001).

III. Migration

As intimated, immature DC are very efficient at antigen capture and processing. However, upon receiving pathogen related maturation signals (e.g. LPS, bacterial DNA, double stranded RNA) the expression of MHC-peptide complexes and co-stimulatory molecules at the cell surface is increased (e.g. B7, CD40, CD54) whilst endocytic activity declines. Furthermore, the responsiveness of DC to chemokines is dramatically altered due to changes in chemokine receptor

expression. DC are desensitised to local chemokine production (e.g. MIP-3α) by down-regulation of their receptors. Concomittantly, CCR7 is rapidly expressed on the cell surface of "maturing" DC. In contrast to the ligands of the previously expressed chemokine receptors, the ligands for CCR7, MIP-3β and 6Ckine, have no chemotactic activity. Instead 6Ckine is expressed on lymphatic vessels draining non-lymphoid tissue and so may direct DC that have escaped from the local chemokine gradient to the lymphatics system. Therefore, the changes in chemokine receptor expression predispose DC to emigrate from the site of inflammation.

It has been known for some time that upon activation DC migrate from peripheral tissue via afferent lymphatics to the T cell areas of the draining lymph nodes (Kupiec-Weglinski *et al.*, 1988). However, the crucial role of CCR7 in mediating entry of activated DC into the LN and localisation to the paracortex has only recently been understood. Mice lacking CCR7 or its ligand 6Ckine have a deficiency in DC homing to lymph nodes. Production of 6Ckine at the site of entry to the lymph node, called the high endothelial venules or HEV, is likely to be important for DC migration. Furthermore, the observation that the expression of MIP-3β is restricted to the T cell areas suggests that CCR7 is required for the specific localisation of migrated DC (see figure 1.6.) (Caux *et al.*, 2000; Banchereau *et al.*, 2000).

1.3D. Lymphocyte Priming

Mature DC that have migrated to the LN typically have a life span of around 2-3 days (Lanzavecchia and Sallusto, 2001). During this period, DC may elicit vigorous antigen-specific $CD4^+$ T cell responses. The potent T cell stimulatory activity of DC is probably not due to the expression of a single molecule but to the high density of surface MHC-peptide complexes and co-stimulatory molecules. For example, DC possess 10 to 100 fold more MHC-peptide complexes on their surface than other APCs like B cells and monocytes. Furthermore, evidence suggests an "instructive" model for DC-$CD4^+$ T cell interactions since different microenvironments created by $CD8\alpha^+$ and $CD8\alpha^-$ DC subtypes appear to determine the type of $CD4^+$ T cell cytokine response. For example, administration of antigen-pulsed $CD8\alpha^-$ DC was shown to induce a T_H2-type of response whereas injection of $CD8\alpha^+$ DC resulted in the development of a T_H1-type response. IL-12 was demonstrated to be crucial for promotion of T_H1 differentiation by "lymphoid" DC (Maldonado-Lopez *et al.*, 1999). At present the mechanism for the induction of T_H2 cytokines by $CD8\alpha^-$ DC remains unclear.

T cell-DC interactions have often been referred to as a "dialogue" since T cell signals have also been shown to activate DC. Members of the TNF superfamily (i.e. CD40L, TRANCE, LIGHT, OX40L and 4-1BB) in particular appear to be important participants of this dialogue. For example, CD40L (CD154) expressed on activated $CD4^+$ T cells has been shown to stimulate DC to upregulate expression of CD80/CD86 and cytokines (e.g. IL-12). Similarly, TNF-related

activation-induced cytokine (TRANCE) is expressed on activated T cells and can trigger DC (by engagement of RANK) to secrete IL-12 and inhibit apoptosis. In this respect, the activity of TRANCE may explain the redundancy for T cell function observed in CD40L-deficient mice (Lanzavecchia and Sallusto, 2001).

Triggering of CD40 on DC also results in the expression of other members of the TNF superfamily such as OX40L and 4-1BB. Together these molecules preferentially induce $CD8^+$ T cell cytotoxic responses *in vivo*. LIGHT, a recently identified member of the TNF superfamily, has also been reported to co-stimulate CD40L-induced DC maturation, resulting in enhancement of the primary antigen-specific CTL response. Together, the data largely vindicates the sequential model for DC-T cell interactions proposed by Ridge *et al.* to explain the dependency of some $CD8^+$ T cell responses on $CD4^+$ T cell help (Ridge *et al.*, 1998). In their model, DC are initially stimulated by $CD4^+$ T cells, which in turn drive further DC maturation. This process of reciprocal stimulation enables the DC to directly activate $CD8^+$ T cells.

DC are also important in regulating B cell responses by a number of mechanisms. Indirectly, the priming of $CD4^+$ T cells is important for providing the T cell help necessary for B cell responses to T-dependent antigens. Finally, DC also activate $CD4^+$ T cells via OX40L to express the chemokine receptor CXCR5, which facilitates the entry of T helper cells into the B cell areas of the LN. DC may also be able to directly activate naïve and memory B cells (see section 1.4) (Banchereau *et al.*, 2000).

In summary, activation of DC by stimuli from pathogens and inflammatory cytokines are integrated into distinct signals that are required by the adaptive immune system for context discrimination.

1.4 B cell activation

1.4A. B cell antigen receptor structure and function

The B cell antigen receptor (BCR) is a multimeric complex consisting of an immunoglobulin (Ig) receptor that recognises unprocessed "native" antigen and trans-membranous signalling subunits, CD79a (Ig-α) and CD79b (Ig-β) (reviewed in De Franco, 2000). The membrane bound IgM receptor possesses a highly variable antigen-binding portion and an invariant region within the constant μ heavy chain. The prevailing view is that binding of oligovalent antigen to the BCR initiates receptor clustering which results in activation of intracellular signalling cascades. Multivalent antigens aggregate mIgM molecules which induces the phosphorylation of ITAMs in the cytoplasmic domains of Ig-α and Ig-β by Src-family tyrosine kinases (e.g lyn, fyn, lck). Dual tyrosine phosophorylation then creates a specific binding site for the SH2 domains of the PTK, Syk/ZAP-70 (Campbell, 1999). In addition, Reth *et al.* (2000) have recently proposed a more complex model for activation of the BCR. In their hypothesis, multiple BCR complexes are pre-organised in an oligomeric configuration that segregates the ITAMs from the Src kinases. However, upon encountering specific antigen, the BCRs reorganise to allow the PTKs access to the ITAM phosphorylation sites.

Activation of B cells through the BCR is highly analogous to TCR-mediated T cell activation, in that neither antigen receptor has intrinsic PTK activity and must recruit cytoplasmic PTKs for initiation of intracellular signalling pathways. The major signalling events triggered by BCR clustering on mature B cells are also initiated during T cell activation i.e. activation of PLCγ and Ras (Campbell, 1999) (see section 1.2A for more detail). Furthermore, recent work has described the translocation of clustered BCRs to the kinase-rich lipid rafts (Cheng *et al.*, 1999). Similar to T cell activation via the TCR, movement to lipid rafts may be an important event in the initiation of proximal signalling cascades (see section 1.2C.).

BCR signalling may also be modulated by constitutive association with co-receptors. For example, CD22 has been shown to regulate $[Ca^{2+}]_i$ mobilisation after ligation of the BCR via immunoreceptor tyrosine-based inhibition motifs (ITIMs). Similar to ITAMs, ITIMs are located within the cytoplasmic region of CD22, but instead of PTK recruitment; ITIMs activate SH2-containing phosphatases (SHP) that downregulate B cell activity (Sato *et al.*, 1998).

In contrast to the effects of CD22, the signalling threshold for B cell activation is significantly decreased when the BCR is co-ligated with CD19 (Tedder *et al.*, 1997). CD81, Leu13 and CD21 (complement receptor 2) form a noncovalent complex with CD19. Thus, B cell responses to complement-coupled antigens may be enhanced by co-ligation of the BCR and the CD19 complex. In addition, CD19 may also function as an adaptor molecule for BCR signalling since

ligation of the BCR phosphorylates CD19, which results in recruitment and activation of Vav and PI-3K (reviewed in Tsubata, 1999).

1.4B. B cell clonal expansion and germinal centre formation

Antigen-binding by the B cell initiates a complex series of cellular interactions that ultimately determine the fate of the B cell. Activation of the naïve B cell heightens its sensitivity to apoptosis and in doing so imparts a dependency for survival signals. Initially these signals are provided by activated $CD4^+$ T helper cells. Therefore, a two-signal model may be envisaged where inappropriate activation of immature B cells by antigen alone (signal one) is likely to lead to cell suicide but provision of T-cell derived signal two promotes survival (Liu and Banchereau, 1996).

Antigen captured by the BCR is internalised by receptor-mediated endocytosis, targeted to the endosomal-processing pathway and presented to T cells in the context of MHC class II (reviewed in Lui *et al.*, 1997). Antigen engagement may also be necessary for upregulation of T cell ligands (e.g. CD86 and CD80) and localisation to the outer PALS region (Jacob *et al.*, 1991). Thus, activation of the naïve B cell induces a number of changes that facilitate the collaboration between T and B cells at the interface between the T cell and B cell zones of secondary lymphoid organs (Liu *et al.*, 1991). A proportion of activated B cells remain at this location and form extrafollicular foci. Within these foci are clonally expanding B cells that preferentially differentiate to antibody-secreting plasma cells. This initial phase of antibody production (of predominantly IgM

isotype) by short-lived plasma cells represents the immediate B cell effector function. IgM is particularly efficient at activating innate effector mechanisms such as the complement system and phagocytes. Thus, the early B cell response is primarily required for antigen fixation (in the form of immune complexes), uptake and clearance (McHeyzer-Williams and Ahmed, 1999).

A subset of the activated B cells migrates to the B cell rich areas and initiates the formation of specialised follicles called germinal centres (GC) (Kelsoe, 1996). B cells recruited to the GC undergo massive and rapid clonal expansion that ultimately results in regional polarisation into the "dark zone" and the "light zone" (see figure 1.7.). The dark zone consists mainly of centroblasts: rapidly dividing B cells undergoing targeted mutation of the B cell genome at a rate of $10^{-3} - 10^{-4}$ per base pair per generation. Somatic hypermutation diversifies the genes responsible for determining the affinity of the antigen-binding region of an antibody (Reynaud *et al.*, 1996; Hogdkin and Basten, 1995). In tandem, genes encoding different constant heavy chains are deleted from the genome, as demonstrated by the appearance of non-replicating excision DNA called "switch circles" (Iwasato *et al.*, 1990; von Schwedler *et al.*, 1990). The process is called isotype switching and is required for the expression of non-IgM antibody isotypes e.g. IgG, IgA, IgE (Zhang *et al.*, 1994).

The progeny of centroblasts are non-replicating centrocytes that re-express the mutated BCR for positive selection. Antigen-binding ability is tested by competition for antigen presentation by follicular dendritic cells (FDC) present in the light zone (reviewed in Cyster *et al.*, 2000). FDC are distinct from

"interdigitating" DC in that (a) they are generated by local differentiation of mesenchymal cells and so are non-hematopoietic in origin and (b) FDC bind immune complexes (via FcγRs) and antigens coated in complement fragments, but unlike DC, there is little evidence to suggest internalisation and processing of captured antigen. In fact the contrary appears to be the case as FDC have been reported to retain unprocessed antigen on their cell surface for prolonged periods, acting as antigen reservoirs for the GC reaction. Thus FDC do not present antigen in the context of MHC class II and consequently do not stimulate T cell activation. The role of FDC appears to be the capture and presentation of intact antigen to B cells, and the selection of centrocytes expressing high affinity antibody (Van Rooijen, 1993).

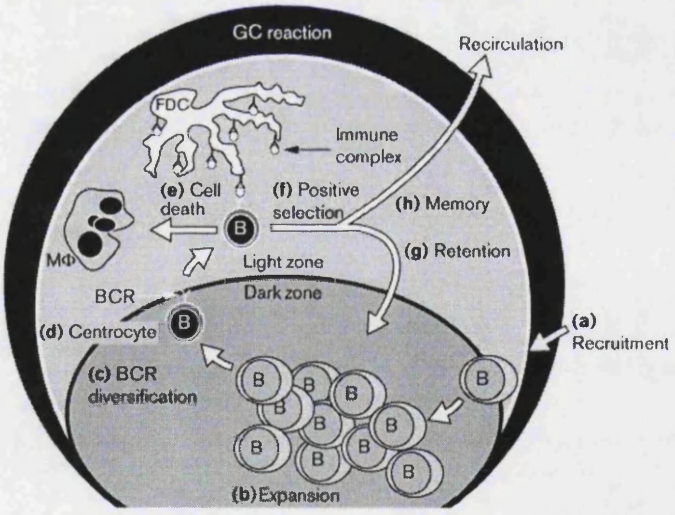


Figure 1.7. A schematic illustration of the germinal centre reaction. **(a)** Recruitment of activated B cells to the GC reaction is initiated in the T cell-rich areas of the secondary lymphoid organs. **(b)** Rapid B cell clonal expansion then ensues in the B cell-rich areas forming a secondary follicle, which polarises into "dark" and "light" zones. **(c)** Centroblasts expand and mutate their BCR, thus increasing BCR diversity. **(d)** Centrocytes enter the light zone and re-express their mutated BCR. **(e)** Centrocytes expressing BCRs with poor affinity for antigen expressed on the surface of FDC undergo apoptosis and are eliminated by phagocytosis. **(f)** High affinity binding of antigen by the centrocyte promotes survival (i.e. positive selection). **(g)** A fraction of positively selected centrocytes re-enter the GC reaction, whilst **(h)** others differentiate into memory B cells or plasma cells. (Adapted from McHeyzer-Williams and Ahmed, 1999 #534])

However, diminished binding of antigen by centrocytes results in apoptosis and engulfment by resident macrophages, whilst successful centrocytes expressing a BCR with an "improved fit" survive to exit the GC; a process called affinity maturation. Positive selection of centrocytes has been reported to require T cells, since in their absence germinal centres are aborted (de Vinuesa *et al.*, 2000). This is probably due to failed centrocyte selection that prevents re-entry of centrocytes into the dark zone. Thus, the pool of proliferating centroblasts cannot be replenished and the GC reaction becomes exhausted. Normally, however, in the absence of further antigenic challenge the GC reaction eventually wanes of its own accord around 21-24 day post-immunisation with GCs typically absent after 32 days (Kelsoe, 1996) (see figure 1.7.).

1.4C. B cell differentiation

CD40L has been demonstrated to be crucial for the development of B cell responses. Absence of functional CD40 ligand prevents the formation of germinal centres and consequently isotype switching and somatic hypermutation cannot be initiated. Furthermore, blocking CD40 ligation triggers the termination of established GC. Thus, the interaction between CD40L (expressed on activated T cells) and CD40 (expressed on B cells) appears to be an absolute requirement for the initiation and maintenance of B cell activation. Evidence also supports the role of CD40-CD40L interactions in determining the differentiation status of B cells exiting the GC (van Kooten and Banchereau, 1997).

Classically, two post-GC B cell differentiation states have been described: antibody secreting cells (called plasma cells) and non-antibody secreting precursor memory B cells. Plasma cells are believed to home to the bone marrow where they can last for long periods, secreting high affinity neutralising antibody in the absence of detectable antigen. In fact, long-lived plasma cells appear relatively unresponsive to subsequent exposure to antigen since they do not proliferate upon secondary challenge. In contrast, human memory B cells, identified by CD27 and CD148 expression (on human B cells) (Tangye *et al.*, 1998), are found circulating the blood and secondary lymphoid organs in a quiescent state and only secrete antibody upon re-exposure to antigen. However, the secondary antibody response is massive compared to the primary response (around 10 times greater) and is characterised by a variety of downstream isotypes (McHeyzer-Williams and Ahmed, 1999).

In vitro evidence suggests that the essential factor that determines whether activated B cells differentiate into plasma cells or memory cells appears to be engagement of CD40 on B cells. Prolonged CD40 triggering preferentially induces B cell to differentiate into memory cells, whilst interruption of CD40 signalling selectively stimulates the production of plasma cells (Arpin *et al.*, 1995).

By contrast, dendritic cells of hematopoietic origin have recently been demonstrated to be involved in the differentiation of naïve and memory B cells to plasma cells. This may in part be due to the secretion of cytokines such as IL-12 (Dubois *et al.*, 1998). The presence of IgA switch circles in B cells co-cultured

with anti-CD40 and DC *in vitro* suggest that DC also have the ability to induce isotype switching. Furthermore, a distinct DC subset has been characterised in human B cell follicles. These GCDC, as they have been called, stimulate GC B cell proliferation, isotype switching to IgG1 and differentiation to plasma cells (Dubois *et al.*, 1999). MacPherson *et al.* have also reported that in both rats and mice, LN and splenic DC may retain native antigen on its surface for at least 36 hours *in vitro*. They also showed that the adoptive transfer of antigen-pulsed DC was sufficient for the induction of isotype switching and antibody production (MacPherson *et al.*, 1999). In conclusion, antigen-bearing DC have been suggested to act as a platform for early interactions between antigen-specific T and B cells; although a model analogous to the conditioning of DC for $CD8^+$ T cell activation may also be a possibility (Banchereau *et al.*, 2000).

1.5 Pathogen recognition

1.5A. LPS structure and activity

Lipopolysaccharide (LPS), a potent natural adjuvant for adaptive immune responses (Khoruts *et al.*, 1998), is a vital constituent of the outer membrane of gram-negative bacteria. LPS-deficient bacteria have been generated *in vitro* but they may only survive in the artificial conditions of the laboratory (Svein Andersen, personal communication). Thus, LPS is a highly conserved molecule amongst gram-negative bacteria and consequently the immune response has evolved to mount vigorous inflammatory responses to it, subsequently labelled "endotoxic" activity. However, the structure of LPS is by no means

homogeneous between bacterial strains and as a result its biological activity may vary (Darveau, 1998; Schromm *et al.*, 2000).

Briefly, the structure of LPS may be broadly separated into three distinct units: (1) the hydrophilic core, (2) the O-antigen domain and (3) the hydrophobic lipid A (see figure 1.8.). The core region is a conserved non-repeating oligosaccharide that is itself segregated into two domains: the outer core (which consists of primarily glucose and galactose) and the inner core. The inner core contains carbohydrates that are unique to LPS such as $_{L}$-*glycero*-$_{D}$-*manno*-heptose and 3-deoxy-$_{D}$-*manno*-octulosonic acid (KDO) which is linked directly to lipid A. The O-antigen domain is a repeating oligosaccharide of around 40 residues long attached to the core region and is a characteristic of "smooth" bacteria. It is highly antigenic and normally the focus of the LPS-specific immune responses. Perhaps to facilitate evasion of the immune system, the O-antigen is a highly variable structure and may not even be expressed by some bacteria (termed rough bacteria).

However, it has long been recognised that the endotoxic properties of LPS are essentially due to lipid A: a broad term for a family of phosphorylated, acylated glucosamine disaccharides (see fig 1.8) that anchor LPS to the outer membrane. The distinctive structure of lipid A most probably confers resistance of the outer membrane to phospholipases. The general chemical configuration of lipid A is highly conserved between gram-negative bacteria, although the amount and position of fatty acid acylation and phosphorylation may vary. Relatively minor differences in lipid A fatty acid composition has been shown to profoundly affect

the biological activity of LPS. For example, bacteria with few fatty acids and phosphates attached to their lipid A possess poor endotoxicity, whilst deacylated LPS completely loses its biological activity. Therefore, it seems likely that the lipid A acyl groups are involved in interactions with the initial mediator of the LPS-induced host inflammatory response (reviewed in (Darveau, 1998; Henderson *et al.*, 1996; Raetz, 1990; Ultevich and Tobias, 1995)).

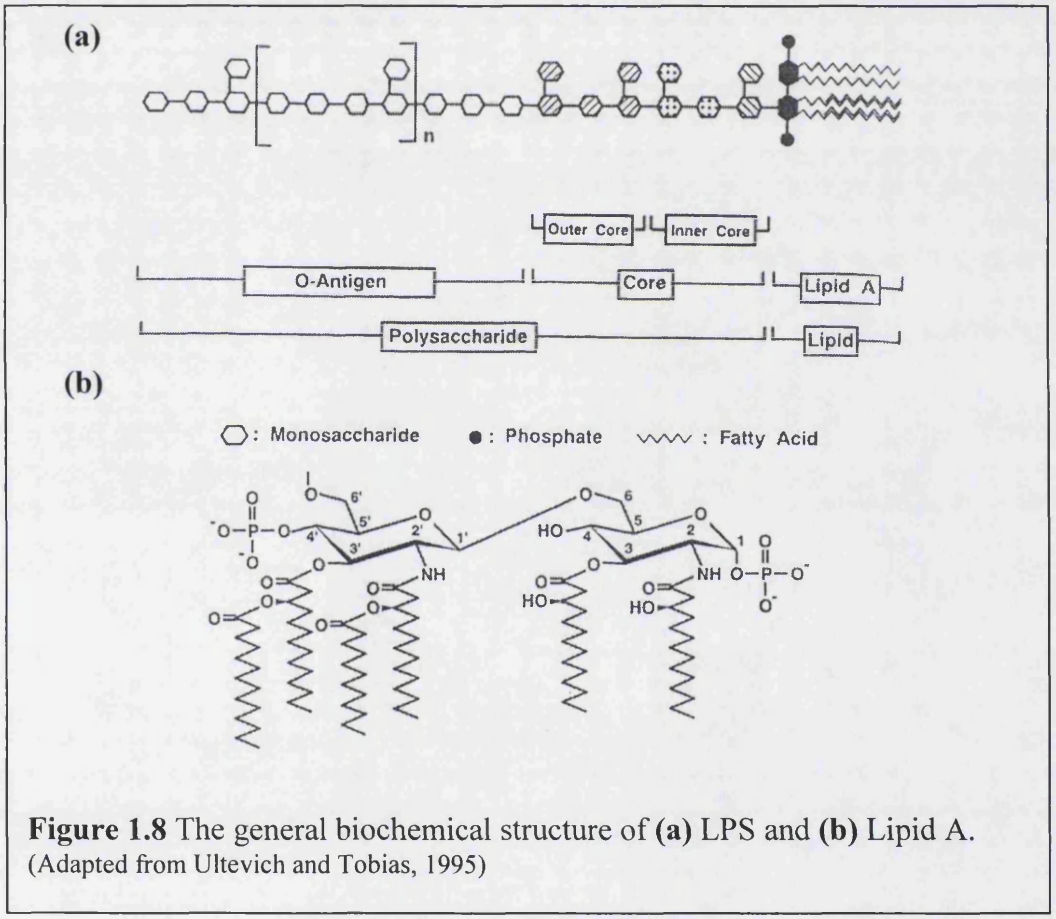


Figure 1.8 The general biochemical structure of **(a)** LPS and **(b)** Lipid A. (Adapted from Ultevich and Tobias, 1995)

1.5B. LBP and CD14 paradigm

Recent data suggests that lipid A interacts with LPS-binding protein (LBP) via its acyl chains. Within minutes of intravenous injection, approximately half of the LPS molecules are cleared from the circulation by leukocytes and lipoproteins.

Whilst LPS-stimulated leukocytes initiate an inflammatory response, lipoprotein-bound LPS essentially becomes inactivated. LBP is crucial in facilitating both of these mechanisms of LPS uptake, as suggested by the analysis of LBP-deficient mice (Heinrich *et al.*, 2001). Thus, LBP has the paradoxical dual function of facilitating the inflammatory response to LPS and conversely ameliorating it by neutralisation of endotoxin. LBP is required as an "opsonin" for both of these processes because LPS exists either within the bacterial outer membrane or in aggregates (or micelles) due to the amphiphilic nature of free LPS. Thus, LBP is thought to function by releasing LPS from aggregates or bacterial membranes and supplying it to various "acceptor molecules" in its monomeric form (Vest *et al.*, 2000; reviewed in Ultevich and Tobias, 1999).

The primary acceptor molecule for initiating LPS inflammatory molecules is thought to be CD14. Myeloid cells such as monocytes, macrophages and granulocytes express CD14 on the cell membrane as a GPI-linked protein. A soluble form of CD14 (sCD14) may also be found in the blood, possibly due to proteolytic cleavage of membrane bound CD14 from mononuclear cells. sCD14 is thought to enable responses to LPS by a variety of cells that do not normally express the GPI-anchored CD14 (e.g. endothelial and epithelial cells) (Frey *et al.*, 1992). Targeted gene-deletion studies have shown that the LBP/CD14 pathway is the essential LPS recognition mechanism for the innate immune system. However, the activity of sCD14 implies that mCD14 is not directly involved in signal transduction but instead presents LPS to an additional co-receptor. Thus, the purpose of LBP and CD14 may be to present disaggregated

LPS to receptors that are intrinsically coupled to the transmembrane signalling complex.

1.5C. TLR2 and TLR4 as LPS receptors

The discovery of the first LPS signalling receptor was initially carried out using transfection studies on LPS-unresponsive cell types. Transfection of human Toll-like receptor (TLR) 2 (but not TLR4) into a kidney cell line was shown to be sufficient for LPS-responsiveness. Furthermore, consistent with the statement above, the maximal LPS response required LBP and CD14 (Yang *et al.*, 1998).

In contrast, genetic studies have demonstrated that the *Tlr4* gene, and not the *Tlr2* gene, is required for sensing LPS. Two groups independently discovered that the LPS-hyporesponsiveness of mouse strains C3H/HeJ and C57BL/10ScCr was due to mutations within the *Tlr4* gene. C3H/HeJ mice were shown to possess a single nucleotide substitution which resulted in non-functional TLR4 (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). It was subsequently determined that this mutant results in the substitution of a proline residue with a histidine residue within the crucial signalling region of the Toll/IL-1 receptor homology (TIR) domain (O' Neill, 2001). TIR domains are a defining characteristic of Toll receptors and are present within all the cytoplasmic regions of 10 human TLRs and 9 *Drosophila* Tolls (see 1.6D.).

The importance of the *Tlr4* gene for LPS signalling was also demonstrated by the generation of TLR4-deficient mice. These mice appeared to be almost identical

in phenotype to the LPS-hyporesponsive C3H/HeJ mouse strain (Hoshino *et al.*, 1999). The lack of redundancy in $TLR4^{-/-}$ mice suggests that under *in vivo* conditions, TLR2 may not be involved in LPS signalling. This is in agreement with data from TLR-2 deficient mice where the LPS response was unaffected by the absence of TLR2, but activation via peptidoglycan (PGN) was obliterated (Underhill *et al.*, 1999).

The possible role of TLR2 in mediating LPS responses remains controversial. Hirschfeld *et al.* (2000) suggested that activation of TLR2 by LPS maybe due to low concentrations of highly active protein contaminants present in commercial preparations of LPS. More recently, it has been reported that cellular activation by LPS extracted from the eubacterium *Leptospira interrogans*, using the same protocol recommended by Hirschfeld *et al.*, requires TLR2 and not TLR4. However, at present the precise structure of Leptospiral LPS is not known and so it is possible that marked differences in lipid A structure of Gram-negative and Spirochetal LPS may contribute to the different TLR usage. In support of this idea, LPS extracted from *Prophyromonas gingivalis* is structurally distinct from *E. coli* LPS and acts through TLR2 and not TLR4 (Hirschfeld *et al.*, 2001).

Thus, it would appear that these two TLRs exhibit specificity for different structures of LPS, perhaps suggesting a direct physical interaction between LPS and the TLR. Alternatively, it has been suggested that the poor affinity for LPS implies that LPS is not the physiological ligand for TLR2 or TLR4. A system analogous to Drosophila Toll ligation has been proposed, where CD14/LBP-bound LPS stimulates the production of a protein ligand. At present this

hypothesis seems unlikely because CD14 does not have any proteolytic activity and currently there is no mammalian homologue of Spätzle, the *drosophila* protein ligand for Toll. Furthermore, human TLR4 has been shown to discriminate between lipid A and partially-deacylated lipid A, whereas murine TLR4 does not. The ability to discriminate between these two lipid A molecules implies intimate contact between receptor and ligand (reviewed in Beutler, 2000).

Therefore, a more plausible explanation is that accessory proteins are required to stabilise the CD14/TLR/LPS complex. Compatible with this idea is the reported activity of a secreted TLR4 "helper" molecule, MD-2. The association of MD-2 with TLR4 has been shown to enable TLR4-transfected cells lines to respond to LPS, thus possibly explaining the discrepancies between the data obtained from transfection and genetic studies. MD-2 has also been reported to broaden the number of ligands recognised by TLR2 and TLR4. Thus in the presence of MD-2, TLR-2 may respond to LPS, lipid A and gram-negative bacteria and TLR4 may recognise lipoteichoic acid or LTA (but not PGN or lipoprotein) (Dziarski *et al.*, 2001).

The current consensus appears to be that TLR4 is the predominant mammalian sensor for LPS. TLR2, on the other hand, has a broad range of ligands (e.g. PGN, lipopeptide, LTA) that does not normally include Gram-negative LPS unless assisted by the presence of accessory proteins.

1.5D. Pattern recognition receptors: The IL-1R/TLR superfamily

The IL-1R/TLR superfamily defines an expanding number of mammalian IL-1R homologues, the majority of which possess a consensus sequence for the TIR domain (the exceptions being BP105, IL-1R II and its vaccinia ortholog B15R). Within this superfamily there is considerable diversity of the extracellular domains of family members. However the structures of these receptors may be broadly separated into three groups: (1) those with Ig domains (e.g. IL-1RI, IL-1RII, IL-18), (2) those with leucine-rich repeat (LRR) domains (TLRs 1-10) and (3) those with no extracellular domain (e.g. MyD88) (reviewed in O' Neill and Dinarello, 2000).

Myeloid differentiation factor 88 (MyD88) in particular is an essential member of this superfamily in that deficiency of this cytoplasmic adaptor molecule results in a lack of responsiveness to IL-1, IL-18 and LPS. The TIR domain within MyD88 is thought to associate homotypically to initiate proximal signalling events which ultimately result in the activation of the NF-κB pathway. MyD88 also possesses an N-terminal death domain that may induce apoptosis by recruitment of Fas-associated death domain (FADD) and subsequent activation of caspase 8 (reviewed in Imler and Hoffmann, 2001).

Although there is a high degree of homology between TIR domains of various TLRs, conformational differences exist which may affect subsequent signalling events. Furthermore, dimerisation of the cytoplasmic domains of TLR4 is required for signal transduction and TNF-α production by macrophages, whereas

similar TLR2 homodimers were non-functional (Ozinsky *et al.*, 2000). Moreover, in contrast to TLR4, TLR2 is able to form combinatorial associations with other TLR proteins (e.g. TLR6 or TLR1), perhaps explaining the broad ligand specificity for TLR2 compared with TLR4. For example, TLR6 cooperation with TLR2 has been reported to be required for responses to bacterial lipoproteins (e.g. mycoplasmal macrophage activating lipopeptide-2 (Takeuchi *et al.*, 2001) and outer surface protein A (Bulut *et al.*, 2001)), phenol-soluble modulin (secreted by *Staphylococcus epidermis* (Hajjar *et al.*, 2001)), soluble tuberculosis factor (Bulut *et al.*, 2001), Gram-positive bacteria and the yeast cell-wall particle, zymosan (Ozinsky *et al.*, 2000) (see figure 1.9.). In addition, TLR5 and TLR9 has been shown to recognise flagellin and bacterial DNA CpG motifs respectively, although the interaction with other TLRs has not yet been determined (Hayashi *et al.*, 2001; Bauer *et al.*, 2001).

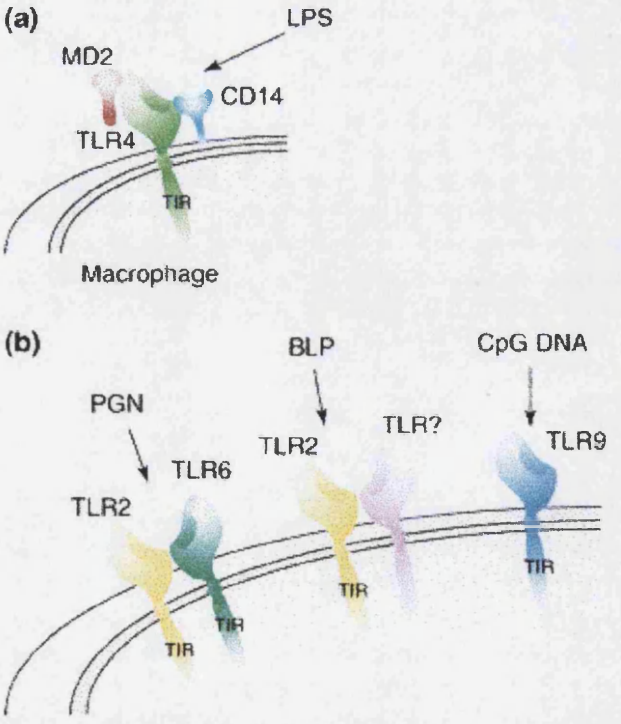


Figure 1.9. (a) Schematic representation of the LPS signalling complex: CD14, TLR4 and MD2. **(b)** TLRs recognise a diverse set of microbial ligands, due perhaps in part due to the formation of heterodimers. (PGN = peptidoglycan, BLP = bacterial lipoprotein)

(Adapted from Imler and Hoffmann, 2001)

Collectively, the rapid progress in our understanding of TLR structure and function over the last 5 years has largely justified the concept of pattern recognition receptors (PRRs) proposed some 12 years ago (Janeway, 1989). Medzhitov and Janeway hypothesised that the primary targets of innate immune recognition by PRRs were pathogen-associated molecular patterns (PAMPs). PAMPs were defined as (1) microbial in origin, (2) a conserved molecular pattern necessary for survival of the microbe, (3) shared by multiple microorganisms and (4) possibly representing a "molecular signature" of a type of pathogen e.g. LPS and Gram-negative bacterium (Medzhitov and Janeway, 2000).

The 10 mammalian TLRs isolated so far could potentially recognise a huge number of PAMPs, facilitated by heterodimerisation and accessory molecules. Furthermore, the targets of TLR currently determined are mostly ideal candidates for PAMPs. For example, flagellin is crucial for bacterial motility and has conserved domains shared by Gram negative and Gram-positive bacteria. Thus, recognition of flagellin by TLR5 may represent the danger signal that allows the innate immune system to respond to a diverse set of motile microbes (Hayashi *et al.*, 2001).

TLRs may indeed be involved in sensing "danger signals" as defined by P. Matzinger. The central belief of the "danger hypothesis" is that the immune system has evolved to respond to "danger signals" rather than distinguishing self-tissue antigens from foreign antigens. Danger signals were defined as "anything that causes cell stress or necrotic cell death" (Matzinger, 1994). Recently two

groups have proposed that TLRs are activated by both of these "danger signals". The danger theory predicts that the immune system is activated by damaged necrotic cells but not by apoptosis. In support of this central tenet, Li *et al.* have reported that necrotic cells (a cellular "signature" of disease), but not apoptotic cells, activate the NF-κB pathway in macrophages via TLR2 (Li *et al.*, 2001). Further support for the concept that TLRs can sense "danger signals" has been provided by the demonstration that heat shock protein 60 (hsp 60), which is thought to be produced during cell stress, induces inflammation through TLR2 and TLR4 (Vabulas *et al.*, 2001). Therefore, current evidence suggests that the family of TLRs, a set of germ-line encoded pattern recognition receptors, may have evolved to detect the presence of infectious organisms and the cellular damage that they may induce.

1.6. Aims and Objectives

The previous sections have discussed recent advances in our understanding of the cellular and molecular requirements for the activation of T cells, B cells and dendritic cells. This information will be key to the development of modern vaccines that elicit vigorous and long-lasting immune responses to purified antigens. However, purification of soluble antigens for use in vaccines may result in poor immunogenicity, raising a requirement for "help" in the form of any number of reagents collectively called adjuvants (a term derived from the latin for help, *adjuvare*) (Gupta and Siber, 1995). In this respect, it is well recognised that injection of a foreign antigen alone generally does not induce effective immunity, whereas co-administration with (for example) Freund's

complete adjuvant (CFA), a crude concoction of mineral oil and mycobacterial products, may stimulate robust humoral and cell mediated responses. Furthermore, the activity of some "traditional" vaccines (e.g. typhoid, diptheria, pertussis and cholera) has been partly attributed to contamination with the powerful natural adjuvant, lipopolysaccharide (LPS).

LPS, perhaps the most characterised microbial adjuvant, has potent immunomodulatory properties for T cells, B cells and DC (see 3.1, 4.1 and 5.1 for more detail). However, the mechanisms for LPS adjuvanticity are still far from clear. A common feature of adjuvants is the ability to modify the cytokine network. For example, LPS and lipid A are potent stimulators of T_H1 cytokine production whilst aluminium hydroxide (or alum) predominantly stimulates the secretion of T_H2-type cytokines (Cox and Coulter, 1997). Therefore, the immunomodulatory activity of LPS may in part be due to the induction of pro-inflammatory cytokines.

Indeed, recent advances suggest that activation through TLRs may be the initiating event for inflammatory responses. Furthermore, Jenkins *et al.* (2001) have proposed that the induction of inflammation may be a pre-requisite for successful T cell responses to purified "foreign" antigen *in vivo*. They also comment that *in vitro* experimental conditions may not be adequate for studying the effect of inflammation on adaptive immune responses.

Therefore, we decided to use LPS as a model microbial adjuvant to investigate its effects on lymphoid cells *in vivo*. Our primary objectives were to investigate

whether the *in vivo* administration of an adjuvant may affect (1) naïve lymphocytes, altering their ability to respond to specific antigen and (2) APC function *in vivo*.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Mice

SPF = Specific Pathogen Free (Animal Unit, Institute for Animal Health, UK.)

Strain	Description	Source
C57BL6	H-2^b	Charles River
BALB/c	H-2^d	Charles River
C3H/HeJ	LPS non-responder, H-2^k TLR4 mutants	Harlan
C3H/HeN	LPS normal responders, H-2^k	Harlan
IFN α/βR -/-	Type I interferon receptor knockout mice on 129 SvEv background	SPF
IFNγR -/-	Interferon gamma receptor knockout mice on a 129 SvEv background (H-2^b)	SPF
129 SvEv	Wild type control (H-2^b background)	SPF
DES	CD8 TCR transgenic specific for MHC K^b CBA1 H-2^k background	SPF
2C	CD8 TCR transgenic recognising H-$2L^d$ + LSPFPFDL peptide or H-$2K^b$ + SIYRYYGL peptide (C57BL6 H-2^b background)	SPF
DO11.10	CD4 TCR transgenic specific for $OVA_{323-339}$ peptide + I-A^d.	SPF

2.1.2. Reagents

Reagent	Supplier	Catalogue Number
Acetone	BDH	10003
Ammonium chloride	Sigma	A 5666
Ammonium sulphate	Sigma	A 2939
Bromodeoxyuridine (BrdU)	Sigma	B5002
Betaplate Scintillant	Wallac	SC/9200/21
Bovine Serum Albumin	Sigma	A4503
Diethylpyrocarbonate (DEPC)	Sigma	D5758
Ethanol Absolute	Sigma	E 7023
Ethylendiaminetetra-acetic acid disodium salt (EDTA)	BDH	100935V
Paraformaldehyde	Sigma	P6148
Polyoxyethylenesorbitan Monolaurate (Tween 20)	Sigma	P-1379
Sodium azide	Sigma	S 8032
Sodium bicarbonate	Sigma	S 6014
Sodium chloride	Sigma	S 5886
Sodium phosphate, dibasic anhydrous	Sigma	S 7907
Sodium phosphate, monobasic anhydrous	Sigma	S 8282
Sterile water	Baxter/3S Healthcare	TRF7114

2.1.3 Tissue culture reagents

Name	Supplier	Catalogue number
Antibiotic/Antimycotic solution (Pen/Strep)	Gibco BRL Life Tech.	15240-062
Chicken Egg Albumin (OVA)	Sigma	A7641
Chicken gammaglobulin (CGG)	Stratatech Scientific	003 000 002
Collagenase type 3	Lorne Laboratories	LS 004183
Concanavalin A (From *Canavalia ensiformis* or Jack Bean)	Sigma	C5275
Dimethyl Sulfoxide (DMSO)	Sigma	D-8879
Deoxyribosenuclease I (Dnase)	Sigma	D-25
Dynabeads M-459 sheep anti-rat IgG	Dynal UK	110.08
Dynabeads M-459 sheep anti-mouse IgG	Dynal UK	110.02
Fetal Bovine Serum Lot. 40Q1282F	Gibco BRL Life Tech.	10106-169
Gentamycin	Gibco BRL Life Tech.	15750-045
Guinea Pig Complement	VH Bio	CL4051
HBSS (Hank's Balanced Salt Solution)	Gibco BRL Life Tech.	14085-049
Hepes Buffer 1M	Gibco BRL Life Tech.	1530-056
Histopaque 1.077	Sigma	1.077-1
Histopaque 1.083	Sigma	1.083-1
Horse Serum	Gibco BRL Life Tech.	16050-098

Name	Supplier	Catalogue number
Lipopolysaccharide (*E.coli* serotype 055:B5)	Sigma	L-2880 L-4005
Lipopolysaccharide (*Neisseria meningitidis*)	A kind gift from Dr. Svein Andersson	N/A
Lipopolysaccharide (*Pseudomonas aeruginosa* serotype 10)	Sigma	L-7018
Lipopolysaccharide (*Salmonella enteritidis*)	Sigma	L-6761
Lipoteichoic Acid (*Staphylococcus aureus*)	Sigma	L-2515
2 – Mercapto-ethanol (2-Hydroxyethylmercaptan;β-mercaptoethanol = C_2H_6OS)	Sigma	M-7522
Anti-FITC Microbeads	Miltenyi Biotech	487-01
Monophosphoryl-Lipid A (MPL)	Sigma	L-6638
NCTC – 135 Medium with L-Glutamine	Gibco BRL Life Tech.	41350-018
Nycodenz powder	Gibco BRL Life Tech.	1002423N
Nycoprep 1.077A	Gibco BRL Life Tech	1002380N
Phorbol 12-myristate 13-acetate (PMA)	Sigma	P 8139
Polyinosinic-polycytidylic acid (PolyI:C)	Sigma	P1530

Name	Supplier	Catalogue number
Polymyxin 'B' sulfate, liquid	Gibco BRL Life Tech.	15350-28
RPMI 1640 Medium	Gibco RBL Life Tech	72400-021

2.1.4. Radioactive Isotopes

Tritiated [^{3}H] thymidine was purchased from Amersham Pharmacia Cat. No. TRA120

2.1.5 Recombinant Cytokines

Name	Supplier	Catalogue Number
Human IL-15	R & D Systems	247-IL-025
Murine IFN-α	Appigene	PMC4014
Murine IFN α/β	A kind gift from Giovanna Schiavonni	N/A
Murine IFNγ	R & D Systems	485 MI 100
Murine IL-12	R & D Systems	419-ML-010
Murine IL-18	Biosource	PMC0184

2.1.6. Cytokine ELISA Kits

Cytokine	Supplier	Catalogue number
TNF-α	R&D systems	MTA00
IFNγ	R&D systems	M1F00
IL-6	R&D systems	M6000
IL-4	R&D systems	404-ML-005
IL-12 p40	R&D systems	M1240
Il-12 p70	R&D systems	M1270
IL-18	R&D systems	M1800

2.1.7. Peptides

Name	Sequence	Supplier
$OVA_{323-339}$	ISQAVHAAHAEINEAGR	IAH
SYRGL (2C)	SIYRYYGL	IAH

2.1.8. Antibodies to murine surface antigens and second step reagents

Name	Clone	Isotype	Supplier of antibody or hybridoma	Conjugates	Cat. No.
B220	RA36B2	Rat IgG2a, κ	Pharmingen	None	01120D
BrdU	B44	IgG1	Becton Dickinson	FITC	347583
CD3ε	145.2C11	Hamster IgG2	Pharmingen	FITC PE	01084A 01085B
CD4	GK1.5	Rat/IgG2b	ATCC	None, Cy5, FITC	TIB-207
CD8α	YTS.169.4.2.1	Rat IgG2b	ECACC	None, Cy5, FITC	89040604
CD11a	2D7	Mouse IgG1, κ	Pharmingen	PE	01205A
CD11b	M1/70	Rat IgG2b	ATCC	None	TIB-128
CD11c	HL3	Armenian Hamster IgG1, λ	Pharmingen	FITC PE Biotin	553801 09705B 09702D

Name	Clone	Isotype	Supplier of antibody or hybridoma	Conjugates	Cat. No.
CD14	rmC5-3	Rat IgG1, κ	Pharmingen	FITC PE	09474D 553740
CD16/CD32	2.4G2	Rat IgG2b, κ	Pharmingen	None	553141
CD19	ID3	Rat IgG2a, κ	Pharmingen	FITC PE	09654D 09655A
CD28	37.51	SyrH IgG, λ	Pharmingen	None PE Biotin	01670D 01675B 01672D
CD40	3/23	Rat IgG2a, κ	Pharmingen	PE	09665B
CD44	IM7	Rat IgG2b, κ	Pharmingen	FITC PE	01224D 01225A
CD54	'3E2	ArmH IgG, κ	Pharmingen	PE	01545B
CD62L	MEL-14	Rat IgG2a, λ	ATCC	Biotin	HB132
CD69	H1.2F3	ArmH IgG, λ	Pharmingen	PE	01505B
CD80	16-10A1	Ham. IgG	Pharmingen	Biotin	09602D
CD86	GL1	Rat IgG2a	Pharmingen	PE	09275B

Name	Clone	Isotype	Supplier of antibody or hybridoma	Conjugates	Cat. No.
CD95	Jo2	ArmH IgG, λ	Pharmingen	None PE Biotin	15400D 15405B 15402D
CD95L	33	Mouse IgG1	Pharmingen	Biotin	09932D
Clonotypic TCR (2C)	1B2	Mouse IgG1	Dr. H.N. Eisen,	Biotin	N/A
Clonotypic TCR (DO11.10)	KJ1-26	Mouse IgG2a	Caltag	PE	MM7504-3
Erythrocyte	TER119	Rat IgG2b	Dr. T. Kina IFMS, Kyoto University	None	N/A
Gr-1 or Ly6G	RB68C5	Rat IgG	Prof. Ken Shortman, WEHI	None	N/A
I-Ab , I-Ad	28-16-8S	Mouse IgM, κ	ATCC Pharmingen	None PE	HB-35 06045A
I-Ab , I-Ad	M5/114.1 5.2	Rat IgG2b, κ	ATCC	None	TIB 120

Name	Clone	Isotype	Supplier of antibody or hybridoma	Conjugates	Cat. No.
LPAM-1	DATK32	Rat IgG2a, κ	Pharmingen	PE	09745B
Ly6A/E	D7	Rat IgG2a, κ	Pharmingen	None	01161A
				PE	01165B
	E13-161-7	Rat/IgG2a	ATCC	None	HB-215
Ly6C	AL-21	Rat IgM, κ	Pharmingen	FITC	01154D
				Biotin	01152D
Thy 1.2	30-H12	Rat, IgG2b κ	ATCC	None,FITC	TIB107
Streptavidin	N/A	N/A	Gibco	RED670	19543-016
			Pharmingen	APC	13409A
			Caltag	Cy5	SA1011
			Pharmingen	HRP	13047E

2.2. Methods

2.2.1. Mice

All the mice were either bred under specific pathogen free conditions at the Institute for Animal Health, Compton UK or supplied by Charles River UK, or Harlan UK (2.1.1.). For the majority of experiments C57/BL6 mice were used aged between 5 and 7 weeks of age. The culling of mice was invariably carried out using the Schedule 1 method of asphyxiation by a rising concentration of CO_2.

2.2.2. Injection protocol

Unless otherwise stated groups of three mice were injected intravenously with either 10μg LPS in 0.2ml PBS or 0.2ml PBS alone as the negative standard. Mice were then sacrificed between 1 hour and 1 week post injection, although the time point most frequently studied was 24 hours p.i. In a minor subset of experiments mice were injected intraperitoneally or subcutaneously.

Mice were also injected with either 50μg polyinosinic-polycytidylic acid in 0.2ml PBS, 100μg monophosphoryl Lipid A in 0.2ml PBS or 0.2ml PBS alone as the negative control. These mice were sacrificed 24 hours later.

Spleen and lymph nodes (LN) were harvested from dissected mice. In the vast majority of experiments removal of "total" LN refers to pooling of cervical,

facial, brachial, axillary, inguinal, and mesenteric lymph nodes. Where LPS was administered subcutaneously in the hindquarters, only the draining inguinal lymph nodes were removed.

2.2.3. The preparation of single cell suspensions from lymphoid tissue.

Two principle techniques were used for the preparation of single cell suspensions, which involved either abrasion or digestion of lymphoid tissue. In the abrasive method, tissues were gently disrupted to release cells using either a homogeniser or frosted glass slides. In the digestion method lymphoid tissue was cut up into small pieces with Castroviejo's Corneal Scissors. This was followed by a 20-minute digestion at room temperature with a mixture of Collagenase type III (2mg/sample) and DNase I (2mg/sample) in a final volume of 7 ml of RPMI 1640 medium + 5% FCS.

After the digestion or abrasion step, cell suspensions were sieved through a 70μm cell strainer to remove tissue fragments and spun down at 1200 rpm (300 x g) for 6 minutes. Erythrocytes in splenocyte preparations were lysed by means of a short two-minute incubation at room temperature with 2ml 0.83% ammonium chloride. After two more washes, first with 10ml RPMI + 5% FCS and then with 10 ml 1x PBS, the cell suspensions were resuspended in 10ml RPMI + 5% FCS medium, sieved and counted using a hemacytometer.

2.2.4. Immunofluorescence staining of cell surface markers.

A maximum of 1 x 10^6 cells were aliquoted into FACS (fluorescence activated cell sorting) tubes containing 2ml of FACS buffer (1x HBSS, 2% horse serum and 0.1% sodium azide). Following centrifugation (300g, 6 minutes, 4 °C) the supernatant was poured off and the pellet was resuspended in the residual FACS buffer (~100µl). F_c receptors were blocked by adding 10µl F_c block (anti-CD16/CD32 mAb diluted 1:250) followed by incubation of the cells at 4°C for 5-10 minutes. A mixture of diluted fluorescently conjugated monoclonal antibodies was added to each tube in a 10µl volume after which cells were incubated at 4°C for 20 minutes in the dark. After another wash step, the cells were resuspended in a small volume of buffer. For biotinylated antibodies a further step was required. A second layer was added, normally by incubating with 10µl/tube of streptavidin-RED670 (diluted 1:500) for 20 minutes at 4°C in the dark.

The stained cell surface markers were examined and quantified using a FACScalibur (Becton Dickinson) and analysed using either Cell Quest (Becton Dickinson) or Win MDI (Joseph Trotter) software. Cells with very low forward scatter (dead cells and debris) were excluded from analysis by drawing a gate around the population with the forward/side scatter profile of lymphoid cells.

2.2.5. Intracellular BrdU staining.

Mice were continuously administered the thymidine analogue, 2-bromo-2-deoxyuridine (BrdU) in their drinking water (0.8mg/ml) for between 1 and 7 days prior to sacrificing.

Following the usual procedure of tissue preparation and cell surface staining (2.2.3 and 2.2.4), the cells were washed in 2ml 1xPBS and resuspended in 500µl ice cold 0.15M aqueous NaCl. To fix the cells, 1.2ml ice-cold 95% ethanol was added dropwise and then cells were incubated on ice for 30 minutes. The cells were spun down and resuspended in 1ml of PBS containing 1% paraformaldehyde + 0.01% Tween-20 for 30 minutes at room temperature to permeablise the cell membrane. The penultimate step involved the addition of 1ml DNase solution (50 Kunitz units/ml DNase, 4.2mM $MgCl_2$, 0.15M NaCl and 0.01mM HCl) and incubating at room temperature for 10 minutes. Finally, the cells were stained intracellularly for BrdU incorporation (10µl anti-BrdU-FITC per tube, 30-minute incubation at room temperature), washed and resuspended in a small volume of 1x PBS ready for acquisition on the FACScalibur.

2.2.6. Depletion and purification techniques.

A. Fluorescence activated cell sorting

Between 1 x 10^6-1 x 10^8 cells were stained with 10µl of diluted antibody/1x10^6 cells and incubated on ice for 20 minutes. Unlike the previous staining protocol,

all steps took place using sodium azide free medium, namely RPMI + 5% FCS. The cells were subsequently washed repeatedly and resuspended at $2x10^7$ cells/ml. Prior to sorting, the cells were transferred to sterile FACS tubes and simultaneously sieved through a 40μm cell strainer. Highly purified or depleted fractions were obtained by fluorescence-activated cell sorting on the MoFlo (Cytomation, Fort Collins, CO). Sorting was performed by Andrew Worth.

B. Positive selection using MACS beads.

Between 1 x 10^6 and 1 x 10^9 cells were washed with preservative-free MACS buffer (0.5% BSA, 2mM EDTA in PBS, pH = 7.2) before being stained with a primary antibody conjugated to FITC (20 minutes on ice). After washing cells with MACS buffer, 10μl anti-FITC microbeads/$1x10^7$cells were added to each sample, which were then incubated on ice for 15 minutes. The cells were spun down, resuspended in 500μl MACS buffer and transferred to a pre-washed MACS column attached to a strong magnetic field. The column was washed by addition of 6ml of MACS buffer thus displacing the non-labelled cells from the column. The column was removed from the magnet and 5ml of buffer passed through to collect the magnetically labelled fraction.

C. Dynal bead depletion

Between 1 x 10^6 and 1 x 10^9 cells were washed with preservative-free RPMI + 5% FCS. Each sample was incubated on ice for 20 minutes with 500μl diluted antibody supernatant per 1 x 10^8 cells. Cells were subsequently washed with

RPMI + 5% FCS and resuspended in anti-rat Ig and/or anti-mouse Ig dynal beads (100µl per 1 x 10^7 positive cells). Samples were incubated for 30-minute at 4°C, with constant rotation. Following another wash step the pellets were resuspended in 1-3ml RPMI + 5% FCS and placed in a test tube rack which contained a strong magnet. The non-labelled population was collected carefully, making sure to avoid disrupting the aggregated positive fraction next to the magnet. The aggregated fraction was discarded whilst the harvested non-labelled cells were spun down and resuspended for counting.

D. Complement Killing

Splenocytes were spun down and resuspended in complement killing medium so that the final cellular concentration was 2 x 10^7 cells/ml. The medium consisted of antibody supernatants and guinea pig complement diluted 1:10 in RPMI with 5% FCS and 1% HEPES. Samples were subsequently incubated for 45 minutes in a 37°C water bath. After extensive washing the cells were resuspended in RPMI + 5% FCS, filtered and counted.

E. Cell depletion by plastic adherence.

The adherent properties of macrophages and dendritic cells were utilised for their removal from splenocyte preparations. 8 x 10^6 splenocytes were plated onto 100mm diameter plastic petri dishes at a concentration of 1 x10^6 cells/ml. Following a 2-hour incubation at 37°C, non-adherent cells were carefully

harvested. Plastic adherence was verified under the microscope before the adherent cells were discarded.

2.2.7. Purification and depletion of lymphoid subsets.

(For details on individual experiments see appendix 2.)

A. Purification and depletion of T cells.

For a small number of experiments lymphocytes were labelled with anti-Thy1.2-FITC mAb and T cells were purified by sorting on the MoFlo. This technique generated high purity T cells but for the majority of experiments negative selection using dynal beads was a more desirable approach, especially for the purification of CD8 or CD4 T cell subsets.

T cells were negatively selected by Dynal bead depletion as described above (2.2.6C.). Starting cell populations were incubated with a mixture of primary antibodies, which were derived from supernatants of the following hybridomas: RB68C5 (anti-Gr-1), TER119 (anti-erythrocyte), M5/114.15.2 (anti-MHC class II), M1/70 (anti-CD11b) and RA36B2 (anti-B220). In addition anti-CD4 (GK1.5) or anti-CD8 (YTS. 169.4) was added to the antibody cocktail when purifying CD8 or CD4 T cells respectively. Dynal bead depletion consistently produced a level of purity > 95% (See appendix 1), together with high yields and more importantly, minimal loss of viability.

Depletion of T cells was also achieved using dynal beads. Antibody supernatant 30-H12 (Thy1.2) was used to deplete T cells. Antibody supernatant GK1.5 or YTS.169.4 depleted CD4 T cells or CD8 T cells, respectively. Depletion was verified by FACS staining (see appendix 1).

B. Purification and depletion of B cells.

Pure B cells were acquired by Dynal bead depletion of cells labelled with RB68C5 (ant-Gr-1) TER119 (anti-erythrocyte), M1/70 (anti-CD11b), GK1.5 (anti-CD4), YTS. 169.430 (anti-CD8) and 30-H12 (anti-Thy1.2). Depletion using dynal beads consistently yielded cell preparations that were > 95% $CD19^+$ (as determined by FACS analysis, see appendix 1).

C. Purification of $CD11c^+$ dendritic cells.

Splenocyte suspensions were prepared using the collagenase/DNase technique (2.2.3) followed by resuspension in 5ml liquid Nycodenz (ρ = 1.077). The colloid was then carefully overlaid onto 5ml Nycodenz. The third and final layer consisted of FCS + 9.1mM EDTA. The layered column was spun at 3,100rpm (1700 x g) for 30 minutes at 20°C. The top 3ml of the gradient; which was enriched for low-density cells, including dendritic cells, was collected. Enrichment by density centrifugation varied greatly between different batches of laboratory prepared Nycodenz solutions since osmolarity was not standardised. From the enriched population (see appendix 1), $CD11c^+$ dendritic cells were purified either by fluorescence activated cell sorting or by MACS separation.

The FACS method required increased enrichment by the depletion of T cells and B cells using dynal beads. The remaining cells were stained for CD11c-FITC and then positively selected on the MoFlo under semi-sterile conditions. Autoflourescent cells were observed by displaying a live dotplot of FL1 (CD11c-FITC) v FL2 (no fluorochrome present). Cells that were not truly CD11c+ but were simply autoflourescing in both FL1 and FL2 were not included in the positive fraction. By using this method we could confidently estimate that the final purity of $CD11c^+$ cells was > 95% (see appendix 1).

The MACS separation technique involved staining with CD11c-FITC mAb followed by anti-FITC MACS microbeads (2.2.6B). This method was also able to discriminate between true $CD11c^+$ cells and autofluorescent cells, although this procedure was only able to enrich $CD11c^+$ dendritic cells to between 90-95% purity (see appendix 1). Furthermore, yields were typically very poor in comparison to the FACS method.

D. Depletion of $CD11c^+$ dendritic cells.

LN suspensions were prepared by collagenase digestion as described previously. Erythrocytes were lysed by the usual method (2.2.3). The remaining cells were stained with CD11c-FITC for negative depletion on the MoFlo. To avoid the depletion of auto-fluorescent cells, only cells that were fluorescent in FL1 were depleted. Cells that fluoresced in both FL1 and FL2 were not excluded from the sample (see appendix 1).

2.2.8. *In vitro* proliferation assays.

All proliferation assays *in vitro* were cultured in "MLR" medium (10% FCS, 10mM HEPES, 250μg/ml Gentamicin, 100U/ml penicillin, 100μg/ml streptomycin, 5% NCTC and 50μM 2 – mercapto-ethanol). In particular experiments, the endotoxin inhibitor polymyxin B sulphate was added to the medium at a final concentration of 100U/ml. Polymyxin B sulphate appeared to have non-specific inhibitory effects on proliferation and therefore the inclusion of this compound was not always practical.

Assays were cultured at 37°C for up to 7 days. Proliferation was assessed by pulsing with tritiated thymidine (1μCi per well) for up to 16 hours. The Tomtec 96 well harvester was used to transfer the contents of each well onto 96 well filtermats. After air drying for 1 hour the filtermats were placed in sample bags, bathed in β-scintillant and sealed. Proliferation was then quantified as counts per minute (cpm) by the Wallac β-counter.

A. Mixed Leukocyte Reactions.

1.5 x 10^5 LN cells from either LPS or PBS injected C57BL6 mice were seeded into 96-well plates in 100μl aliquots. A further 100μl of irradiated BALB/c stimulators were added to each well to make a final volume of 200μl/well. For the greater number of experiments BALB/c splenocytes irradiated at 3,000 RADS were used as stimulators at a concentration of 5 x 10^5 cells/well. Thus, the stimulator to responder ratio was 3.3:1.

In other experiments splenocyte stimulators were derived from PBS or LPS injected mice. In this case, either total splenocytes were used at 5 x 10^6 cells/ml or purified $CD11c^+$ dendritic cells seeded between 4 x 10^5 and 1.56 x 10^3 cells per well.

The plates were cultured at 37°C for between 2 and 7 days and proliferation was determined as described above.

B. CD4 T cell peptide stimulation *in vitro*.

OVA peptide responders were prepared by the usual methods from lymph nodes removed from DO11.10 transgenic mice. CD4 T cells derived from these mice express a clonotypic TCR which binds to the $OVA_{323-339}$ peptide in the context of MHC class II ($I\text{-}A^d$). 1.5 x 10^5 responders were seeded into 96 well plates in a 100µl volume. Where multiple populations of responders were derived from PBS or LPS treated mice, the percentage of CD4 T cells that expressed the transgenic TCR was determined by FACS analysis prior to plating. Cellular concentrations were adjusted to standardise the number of transgenic T cells per well.

100µl of stimulators was added to each well. Depending on the aims of the experiment, the stimulators were irradiated (3,000 RAD) BALB/c splenocytes from untreated mice, irradiated (3,000 RAD) BALB/c splenocytes derived from PBS or LPS-injected mice or purified BALB/c splenic $CD11c^+$ dendritic cells

derived from PBS or LPS-injected mice. Total splenocytes were seeded at a responder to stimulator ratio of 1:3.3. Splenic DC were seeded at ratios between 1:1 and 320:1.

Lyophilised peptide $OVA_{323-339}$ was reconstituted in RPMI medium at a concentration of 1mg/ml. The peptide was serially diluted and 10µl aliquots pipetted into each well. The final concentrations of peptide used are indicated for individual experiments.

The plates were cultured at 37°C for between 2 to 7 days and pulsed with tritiated thymidine as previously described (2.2.8).

C. CD8 T cell peptide stimulation *in vitro*.

CD8 T cell peptide responders were prepared by the usual methods from lymph nodes removed from 2C transgenic mice. CD8 T cells derived from these mice express a clonotypic TCR which binds to the 2C peptide (SIYRYYGL) in the context of syngeneic MHC class I ($I\text{-}K^b$) (Udaka *et al.*, 1996). 1.5×10^5 responders were seeded into 96 well plates in a 100µl volume. Where multiple populations of responders were derived from PBS or LPS treated mice, the percentage of CD8 T cells expressing the transgenic TCR was determined by FACS analysis prior to plating. Cellular concentrations were adjusted to standardise the number of transgenic T cells per well.

100μl of stimulators was added to each well. Depending on the aims of the experiment the stimulators were irradiated (3,000 RAD) C57BL6 splenocytes from untreated mice, irradiated (3,000 RAD) C57BL6 splenocytes derived from PBS or LPS-injected mice or purified C57BL6 splenic $CD11c^+$ dendritic cells derived from PBS or LPS-injected mice. Total splenocytes were seeded at a responder to stimulator ratio of 1:3.3. Splenic DC were seeded at ratios between 1:1 and 320:1.

Lyophilised 2C peptide was reconstituted in RPMI medium at a concentration of 1mg/ml. The peptide was serially diluted and 10μl aliquots pipetted into each well. The final concentrations of peptide used are indicated for individual experiments.

The plates were cultured at 37°C for between 2 to 7 days and pulsed with tritiated thymidine as previously described (2.2.8).

D. T cell mitogenic stimulation.

Mice were injected with PBS or LPS (2.2.2.) and culled 24 hour later. The LN and spleen were removed and cell suspensions were prepared by the usual techniques (2.2.3.). Different numbers of cells were seeded into wells of a 96 well plate in a volume of 100μl. 100μl of Concanavalin A (Con A) was added to every well at a final concentration of between 10μg/ml and 0.1μg/ml.

The plates were cultured at 37°C for between 2 and 4 days and pulsed with [^{3}H]-thymidine as described previously (2.2.8.)

E. B cell mitogen stimulation.

Cell suspensions were prepared from LN removed from PBS or LPS-treated mice. B cells were purified by negative selection (2.2.7 B). A maximum of 1.5 x 10^5 LN cells or B cells was seeded into each well in a 100µl volume. 100µl of one of two B cell mitogens were added to each well: LPS (0.5µg/ml to 50µg/ml) or anti-IgM cross-linking antibody (0.1µg/ml to 100µg/ml). The assays were cultured at 37°C for between 2 and 4 days and pulsed with tritiated thymidine 2.2.8.).

F. Stimulation with immobilised antibodies.

50µl of antibody was aliquoted per well into 96 well plates and incubated at 37°C for a minimum of 8 hours. Non-immobilised antibody was then removed from the plates by repeatedly washing with PBS. 1 x 10^5 cells were added per well and cultured for between 2 and 3 days before being pulsed with tritiated thymidine (2.2.8.).

Antibodies used in this assay were anti-CD3 (0.1-50µg/ml), anti-CD28 (1-10µg/ml), anti-CD95 (10µg/ml) and anti-Ly6A/E (1-10µg/ml).

G. Ly6A/E-mediated proliferation.

CD4 or CD8 T cells were purified by negative selection and labelled with rat anti-mouse Ly6A/E antibody. Either 10µl of clone D7 was added per 1 x 10^6 T cells or 500µl of clone E13-161-7 per 1 x 10^8 T cells; both of these antibodies are IgG2a. The lymphocytes were incubated on ice for 30 minutes, washed and resuspended in MLR medium at a concentration of 1 x 10^6 cells/ml. 2 x 10^5 cells were seeded per well in a 200µl volume. 10µl of PMA (5ng/ml final concentration) was added to all of the wells. To a third of the wells there was no further addition of reagents. To the remaining two thirds of the wells, either 10µl of secondary cross-linking antibody (rabbit anti-rat IgG) or negative control antibody (rabbit anti-mouse IgG) was added per well.

The T cells were cultured for 2 days at 37°C and pulsed with tritiated thymidine (2.2.8.).

2.2.9. Cytokine detection *in vitro.*

A. Generation of splenocyte and DC supernatant.

Mice were injected with either PBS or LPS (2.2.2.) and sacrificed 1 hour post injection. Cell suspensions were prepared from their spleens by the usual technique (2.2.3.). Either total splenocytes or splenic dendritic cells (isolated as per 2.2.7C.) were cultured in MLR medium at 1 x 10^6 cells per well in a 1ml volume. The supernatant was harvested 16 hours later.

In some experiments the supernatant was used to stimulate lymphocytes *in vitro*. To prevent contamination of cells between cultures the supernatant was filter sterilised through a 0.2μm syringe filter. The supernatant was then added to cultures of LN-derived lymphocytes for 16 hours, after which the cells were surface stained to determine phenotype.

B. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed using kits supplied by R&D Systems. Provided in the kits were plates pre-coated with analyte-specific antibody (capture antibody). 50μl of supernatant was added to each well and diluted 1:2 with the buffered protein solution provided. In tandem, serially diluted standards and control samples were added to the plates. The plates were then covered and incubated for 2 hours at room temperature. To remove any unbound analyte, the wells were aspirated and washed three times with buffered surfactant.

100μl of horseradish peroxidase (HRP) conjugated antibody was added per well. This antibody was specific for the analyte and formed an enzyme- (HRP) linked analyte-antibody complex. After two hours the wells were washed and 100μl of substrate (hydrogen peroxide and chromagen) was added to every well. During the 30-minute incubation the H_2O_2 was reduced allowing the soluble chromagen to be spectrophotometrically detected. The resulting blue colour was turned yellow by the addition of 100μl of stop solution (HCl).

The Molecular Devices SpectraMax microplate reader quantified the substrates optical density (λ = 450nm). SoftMax Pro software was used to plot the standard curve and calculate the concentration of analyte in pg/ml.

C. Interferon Bioassay

This protocol was adapted from Tovey *et al.* (Tovey *et al.*, 1974). 100µl of each supernatant was added per well. 50µl was transferred across subsequent wells containing 50µl of medium thus performing a 1 in 2 serial dilution. 1 x10^5 L929 cells were seeded in each well in a 50µl volume and incubated at 37°C. After 18 hours, the medium was removed and the wells were rinsed in PBS to remove FCS. 100µl of diluted Encephalomyocarditis virus (10^5 pfu/ml) was added to each well. Plates were incubated for 48 hours at 37°C after which the medium was removed and the wells were washed twice with PBS. Cells were fixed with 100µl of formaldehyde at ambient temperature for 1 hour. Wells were then washed, dried and stained with 100µl/well of 0.1% crystal violet. After 5 minutes the wells were rinsed with water and dried.

N.B. The assay relies on IFN generating a virus-resistant state within the cells, rather than the direct inhibition of virus infection or replication. In the absence of neutralising antibodies, this assay did not distinguish between IFN-α, -β or -γ.

2.2.10. Detection of the antibody response to CGG *in vivo.*

A. Injection protocol

C57BL/6 mice were injected with PBS or LPS i.v. 24 hours later half of the mice were primed with 100μg CGG in PBS s.c. All mice were culled and bled 10 days later. Blood samples were left for approximately an hour to coaggulate and the serum was collected by pelleting the aggregates (14,000rpm, 15 minutes) and harvesting the supernatant. Serum samples were stored at -20°C.

B. Assay of serum antibody by ELISA.

Wells of 96-well plates were coated with CGG (5μg/ml in carbonate buffer [pH 9.6] by overnight incubation at ambient temperature. The wells were blocked with PBS containing 4% powdered milk for 1 hour at 37°C and then washed three times with PBS-tween 20 (0.05%). Twelve-fold serial dilutions of sera in PBS-1% milk were added to the wells for 1 hour at room temperature. The plates were then washed three times to remove any unbound non-specific antibody. Bound CGG-specific antibody was then detected by forming an enzyme-linked antibody complex. Briefly, biotinylated rat anti-mouse isotype specific antibodies (IgM [R6-60.2], IgG1 [A85-1], IgG2a [R19-15], IgG2b [12-3], IgG3 [R40-82] or IgE [R35-72] (Becton Dickinson) were added to the wells for 1 hour (room temperature), washed and then incubated for another hour, also at room temperature, with streptavidin-horseradish peroxidase. OPD tablets (Sigma, P 9187) were used as the peroxidase substrate. The reaction was

stopped by the addition of 50µl 3M HCl. Optical densities were read at 492nm on a SPECTRAmax microplate reader (Molecular Devices). Results are expressed as optical density values and reciprocal endpoint titres. The latter values were determined by using a threshold of positivity (i.e. a value greater than three times the standard deviation) automatically calculated by Excel. Thus, for each sample, the endpoint titres were estimated as the first dilution below the threshold of positivity.

Chapter 3: The phenotypic and functional alteration of T cells by LPS

3.1. Introduction

Lipopolysaccharide (LPS) was demonstrated to possess adjuvanticity almost 50 years ago. Early studies focussed on the augmentation of the antibody response to model antigens co-administered with LPS. Seminal experiments conducted by Johnson *et. al.* (1956) used LPS purified from various gram-negative bacteria to enhance the *in vivo* antibody response to ovalbumin. Other studies into murine antibody production to sheep red blood cells (SRBC) provided evidence that T cells were not involved in the potent polyclonal activation of B cells by LPS *in vitro* (Hoffman *et al.*, 1975). However, in the presence of antigen, LPS improved antigen-specific antibody response *in vitro* and *in vivo* in a T cell-dependent manner (Armerding and Katz, 1973; Goodman and Weigle, 1979; McGhee *et al.*, 1979).

This raised the possibility that LPS did not only affect the humoral response but could have major effects on various T cell effector functions. For example, LPS was described to increase the cytotoxicity of human PBL and murine CTL *in vitro* (Narayan and Sundharadas, 1978; Schacter *et al.*, 1981). Furthermore, T cell-mediated responses such as delayed type hypersensitivity to SRBC were enhanced by LPS (Lagrange and Mackaness, 1975).

Administration of LPS *in vivo* did not always elevate T cell responses. For example, allograft implantation followed immediately by LPS injection resulted

in delayed rejection (Winchurch *et al.*, 1982). Moreover, high doses of LPS (100μg) given to mice i.p. induced massive apoptosis and hence the disappearance of CD4 and CD8 T cells in the spleen (Castro *et al.*, 1998). Work by the same authors showed that a lower dose of LPS (10μg) led to the activation of T cells as indicated by the expression of CD69 and CD25 and a corresponding increase in size (Castro *et al.*, 1998).

Tough *et. al.* demonstrated that under similar *in vivo* conditions, LPS stimulated both naïve ($CD44^{lo}$) and memory phenotype ($CD44^{hi}$) T cells to up-regulate CD69 expression. Furthermore, low doses of injected LPS (10ng) selectively stimulated $CD44^{hi}$ $CD8^{+}$ T cells to divide *in vivo* (Tough *et al.*, 1997). This effect could be mimicked by the Type I Interferon inducing agent, poly I:C or by IFN-I itself (Tough *et al.*, 1996). Unlike IFN-I, IL-15 not only selectively stimulated "memory" phenotype $CD8^{+}$ T cells to proliferate *in vivo*, but also stimulated purified T cells to divide *in vitro*. Therefore, IL-15 was postulated to be the effector cytokine mediating T cell bystander activation *in vivo* (Zhang *et al.*, 1998).

Activation of T cells by LPS *in vivo* also conferred a larger proliferative response to antigen *in vivo*. T cells incorporated twice as much tritiated thymidine (administered i.v.) when LPS was co-injected with SRBCs compared to SRBC alone (Mita *et al.*, 1982). This observation suggested that LPS could act as an adjuvant by increasing the expansion phase of the T cell response to antigen. Subsequent work showed that the secondary T cell response to OVA *in vitro* was

both greater in magnitude and possessed more rapid kinetics when the *in vivo* priming regime included both LPS and OVA (Milner *et al.*, 1983).

Providing evidence for the effect of LPS on the *in vivo* behaviour of T cells was facilitated by the advent of clonotypic T cell receptor transgenic mice. Adoptive transfers of antigen-specific transgenic T cells revealed the pleiotropic effects of LPS on T cell stimulation by antigen *in vivo*. The number of adoptively transferred $CD4^+$ transgenic TCR^+ ($KJ1\text{-}26^+$) T cells retrieved from the lymph nodes of recipient mice 3 days after priming with OVA + LPS was 3-fold higher than with OVA alone. The synergistic effect of LPS on CD4 clonal expansion was mimicked by substituting for LPS with either IL-1 or TNF-α, but not IL-12. By contrast, IL-12 but not IL-1 enhanced $CD8^+$ T cell expansion *in vivo* (Curtsinger *et al.*, 1999). Therefore, LPS-inducible cytokines may be critical regulators of clonal expansion *in vivo*.

Lack of CD28 expression had a dramatic inhibitory effect on the accumulation of transferred transgenic $CD4^+$ T cells in the lymph nodes of recipient mice. Since LPS could not compensate for CD28 deficiency it was assumed that CD28 must be required for the adjuvanticity of LPS (Khoruts *et al.*, 1998). Indeed, CD28 offers an attractive mechanism for the effects of LPS on T cells. Since LPS induces the maturation of dendritic cells (DC) characterised by increased expression of B7, the ligand for CD28. Hence LPS maturation of DC may result in increased CD28 signalling in T cells. CD28 is not only important for the initial activation of T cells (see chapter 1.2B.) but also provides pro-survival

signals, e.g. upregulating the expression of molecules like Bcl-2 (Jenkins *et al.*, 2001)

Supporting this idea is a body of evidence suggesting that LPS can prevent the post-activation cull of T cells. 5 days post immunisation with OVA, virtually all transferred $CD4^+$ $KJ1\text{-}26^+$ T cells have been eliminated. However, elevated numbers of transgenic $CD4^+$ T cells were still detectable in the LN of BALB/c recipients that were given OVA + LPS or OVA + TNF-α (Pape *et al.*, 1997). Similarly, it has been reported that injection of LPS prior to the activation of T cells with SEB prevents superantigen-induced apoptosis (Vella *et al.*, 1995). Furthermore, recent experiments using whole body immunohistology to determine the migration of adoptively transferred $CD4^+$ transgenic T cells post-immunisation showed that LPS also maintains survival of activated T cells at effector sites. In these studies, injection of antigen into the recipient mice resulted in proliferation of transgenic T cells (OT-II) in the lymph node. This was followed by a mass exodus of antigen-specific T cells to peripheral tissues (lungs, liver, gut and salivary glands). Injection of antigen and LPS induced a comparable level of migration to non-lymphoid tissues. Where antigen alone had been given the redistributed T cells disappeared within 10 days of the primary immune response. Significantly, priming with both antigen and LPS resulted in the continued detection of T cells in secondary lymphoid and peripheral tissues 60 days post immunisation (Reinhardt *et al.*, 2001).

Such *in vivo* studies have also shown that LPS injection can affect T cell migration. Here T cells were observed to migrate from the T cell-rich paracortex

to the B cell-rich follicular regions of the lymph nodes when mice were immunised by injection of OVA and LPS but not OVA alone. Hence LPS stimulation facilitated supplying B cells with T cell help due to the spatial rearrangement of the two cell types within the lymph node.

Finally, LPS may modify the type of effector T cell response generated during an immune response. One property of LPS that may contribute to this, is its ability to stimulate the secretion of cytokines by dendritic cells. LPS-matured DC have been shown to induce the differentiation of T cells into potentially IFNγ-secreting cells. Thus, LPS may indirectly polarise the $CD4^+$ T cell response to a "T_H1" type of cytokine secretion upon stimulation with antigen (Whelan *et al.*, 2000). Furthermore, activation of $CD4^+$ T cells with antigen, in the absence of an inflammatory context, generated memory cells that were unable to produce effector cytokines upon secondary antigenic stimulation. This has been postulated to be attributable to the absence of the "differentiating factor" IL-12 (Jenkins *et al.*, 2001).

In summary, LPS has been proven not only to be a potent polyclonal activator of B cells but to stimulate T cells at many different levels. LPS can enhance or suppress multiple T cell effector functions depending on experimental conditions. Using adoptive transfer techniques LPS has been observed to affect the migration, location, clonal expansion, survival and cytokine secretion of antigen-stimulated T cells.

As mentioned previously, work carried out by Tough *et al.* showed that LPS *in vivo* selectively stimulated the proliferation of CD8 "memory" phenotype cells. However, the effects of LPS were not totally restricted to this population of T cells, since both naïve and memory CD4 and CD8 T cells altered CD69 expression after stimulation with LPS *in vivo*. Therefore the initial aim of this thesis was to further characterise the extent of T cell activation by LPS *in vivo*. Using T cell phenotype as a marker of LPS stimulation we also addressed the question of the cellular requirements for T cell activation. Finally, we have attempted to investigate whether the systemic administration of LPS *in vivo* affected the ability of T cells to respond to proliferative stimuli *in vitro*.

3.2. Results

3.2A. Examination of T cell phenotype after LPS injection into mice.

Initially, to investigate the effects of LPS on T cell activation status *in vivo*, we sacrificed mice 24 hours after injection of LPS (50μg) or PBS and examined T cell phenotype using a panel of monoclonal antibodies against integrins, cytokine receptors, adhesion and co-stimulatory molecules. For the majority of molecules included in our investigations (CD2, CD3, CD4, CD8, CD11a, CD11b, CD25, CD26, CD30, CD40, CD43, CD44, CD80, CD102, CD103, CD122, CD126, IL6R, FasL, CD40L, CTLA-4 and CDw132), expression on the T cell surface did not change after LPS injection. However LPS did significantly alter T cell surface expression levels of a number of other molecules. Up-regulation of molecules (fig. 3.01-3.) typically affected by LPS on T cells (Ly6C (Pihlgren *et*

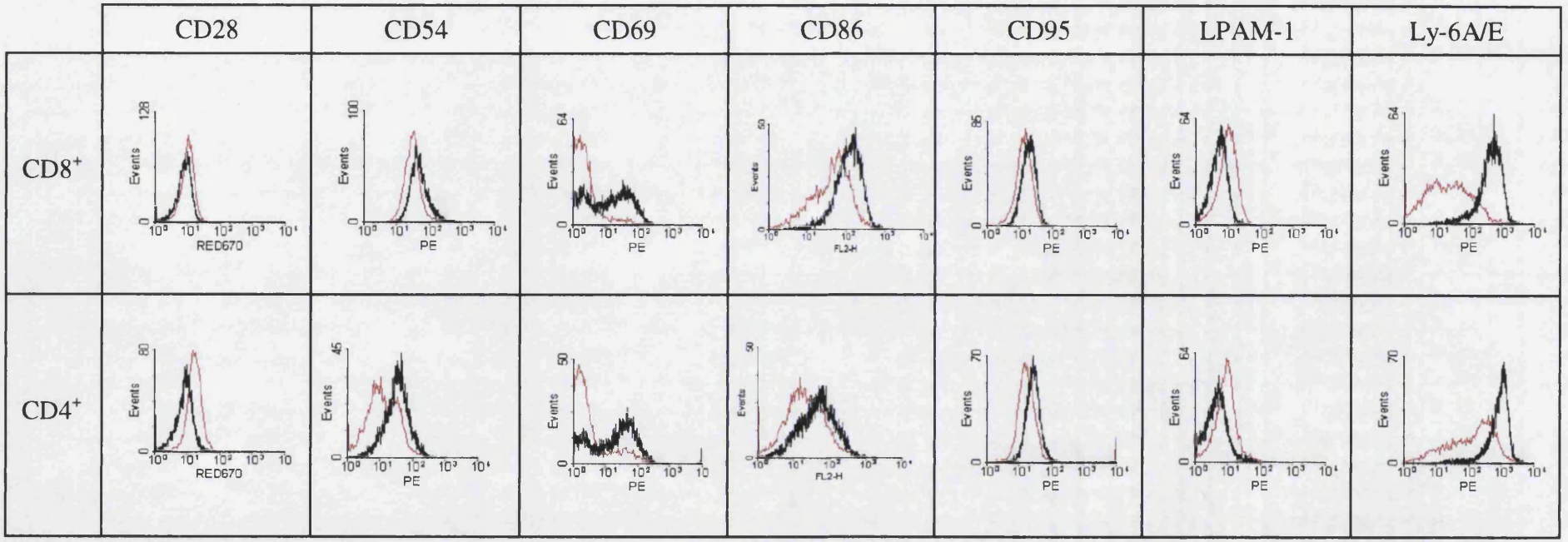


Figure 3.01. T cell phenotype following exposure to LPS for 24 hours *in vivo*.

C57BL/6 mice were injected i.v. with 50µg of LPS in 0.2ml PBS or 0.2ml PBS alone and sacrificed 24 hours later. Their lymph nodes were excised and the resulting lymphocyte suspensions were stained for either CD8-Cy5 or CD4-Cy5 and also for a number of T cell surface markers (biotinylated CD28 antibody detected by streptavidin-RED670 and PE conjugated antibodies: Ly-6A/E, CD54, CD69, CD95 and LPAM-1). T cells were visualised using the FACScalibur and dead cells were excluded from analysis as described previously (2.2.4). The single colour histogram overlays above were gated on either CD8 or CD4 positive lymphocytes. The red histograms represent surface marker expression levels on T cells derived from PBS-injected mice whilst the black histograms represent surface marker expression levels on T cells derived from LPS-injected mice (— = PBS, — = LPS).

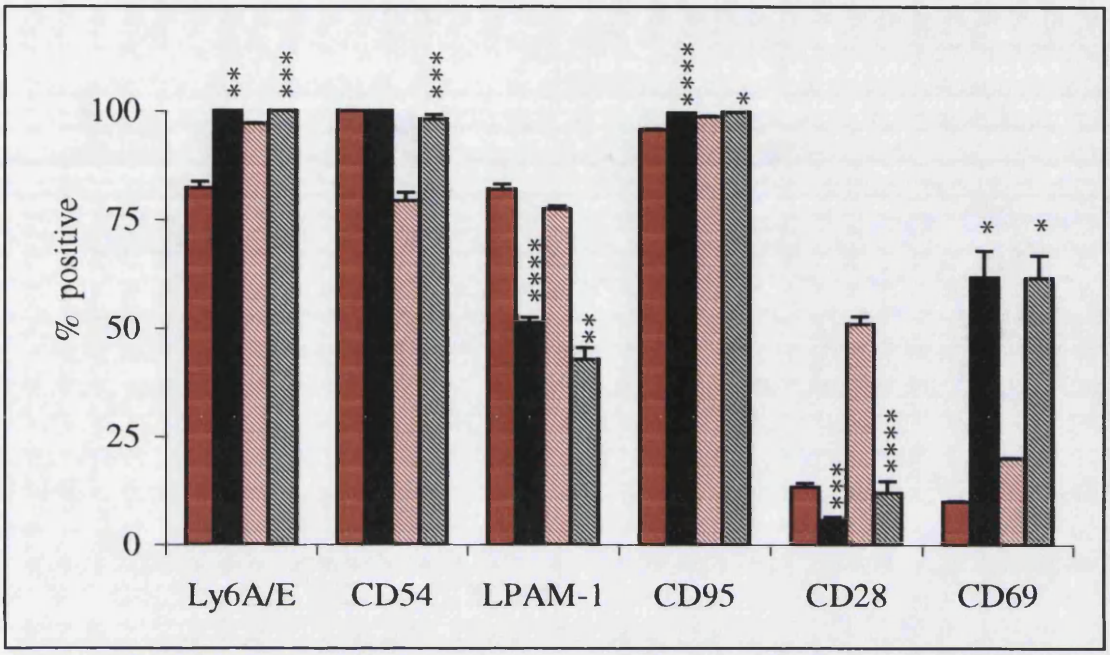


Figure 3.02. LPS induces multiple changes in T cell phenotype *in vivo*.

C57BL/6 mice were injected i.v. with 50µg of LPS in 0.2ml PBS or 0.2ml PBS alone and sacrificed 24 hours later. Their lymph nodes were excised and the resulting lymphocyte suspensions were stained for either CD8-Cy5 or CD4-Cy5 and also for a number of T cell surface markers (biotinylated CD28 antibody detected by streptavidin-RED670 and PE conjugated antibodies: Ly-6A/E, CD54, CD69, CD95 and LPAM-1). T cells were visualised using the FACScalibur and dead cells were excluded from analysis as described previously (2.2.4.).

Markers were set on single colour histograms using isotype controls or unconjugated second step fluorochromes. Data are mean ± s.e.m. of triplicates (■,CD8 PBS; ■, CD8 LPS; ▨, CD4 PBS; ▨, CD4 LPS). P values, 2-sample t-test (PBS v LPS); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

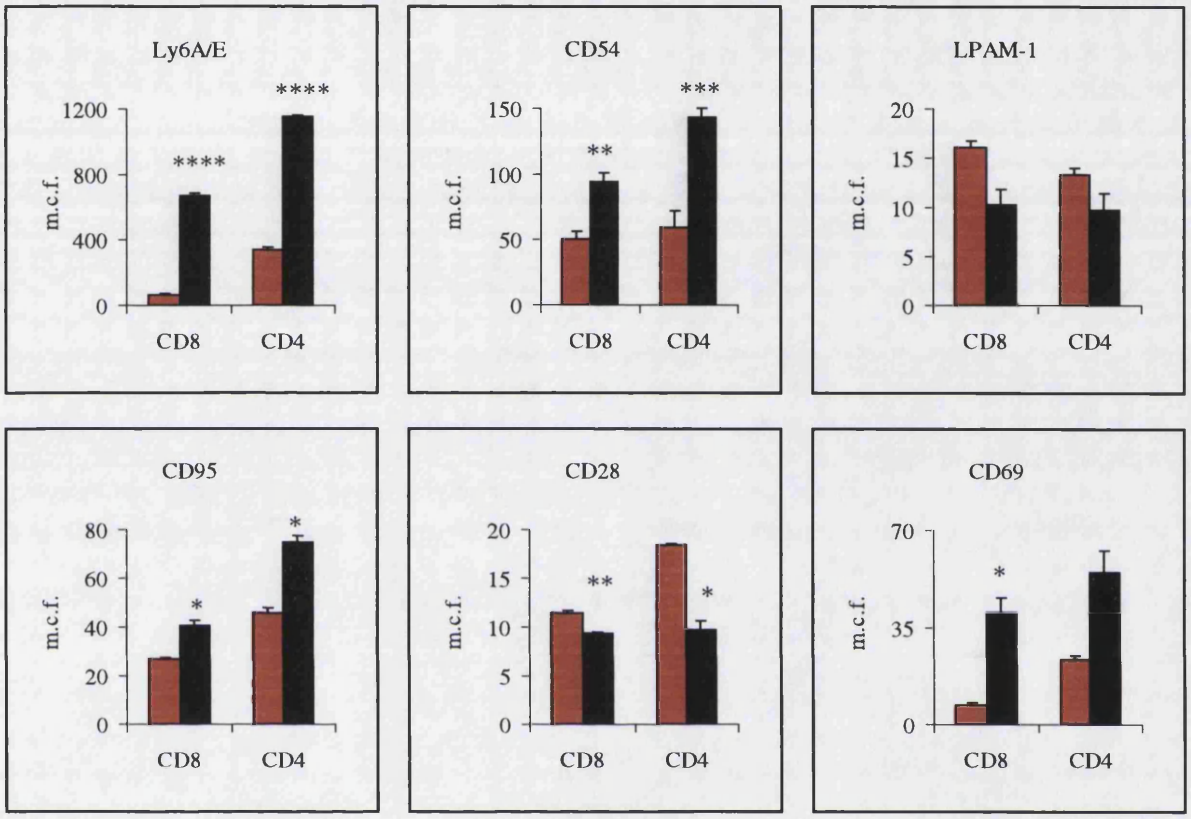


Figure 3.03. LPS upregulates the expression of a number of T cell surface molecules.

C57BL/6 mice were injected i.v. with 50µg of LPS in 0.2ml PBS or 0.2ml PBS alone and sacrificed 24 hours later. Their lymph nodes were excised and the resulting lymphocyte suspensions were stained for either CD8-Cy5 or CD4-Cy5 and also for a number of T cell surface markers (biotinylated CD28 antibody was detected by steptavidin-RED670, all other antibodies i.e. Ly-6A/E, CD54, CD69, CD95 and LPAM-1 were directly conjugated to PE). T cells were visualised using the FACScalibur and dead cells were excluded from analysis as described previously (2.2.4).

The average "mean channel fluorescence" (m.c.f.) of each T cell surface marker was calculated. Data are the mean ± s.e.m. of triplicates (□, PBS; ■, LPS), p values, 2-sample t-test (PBS v LPS); (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$).

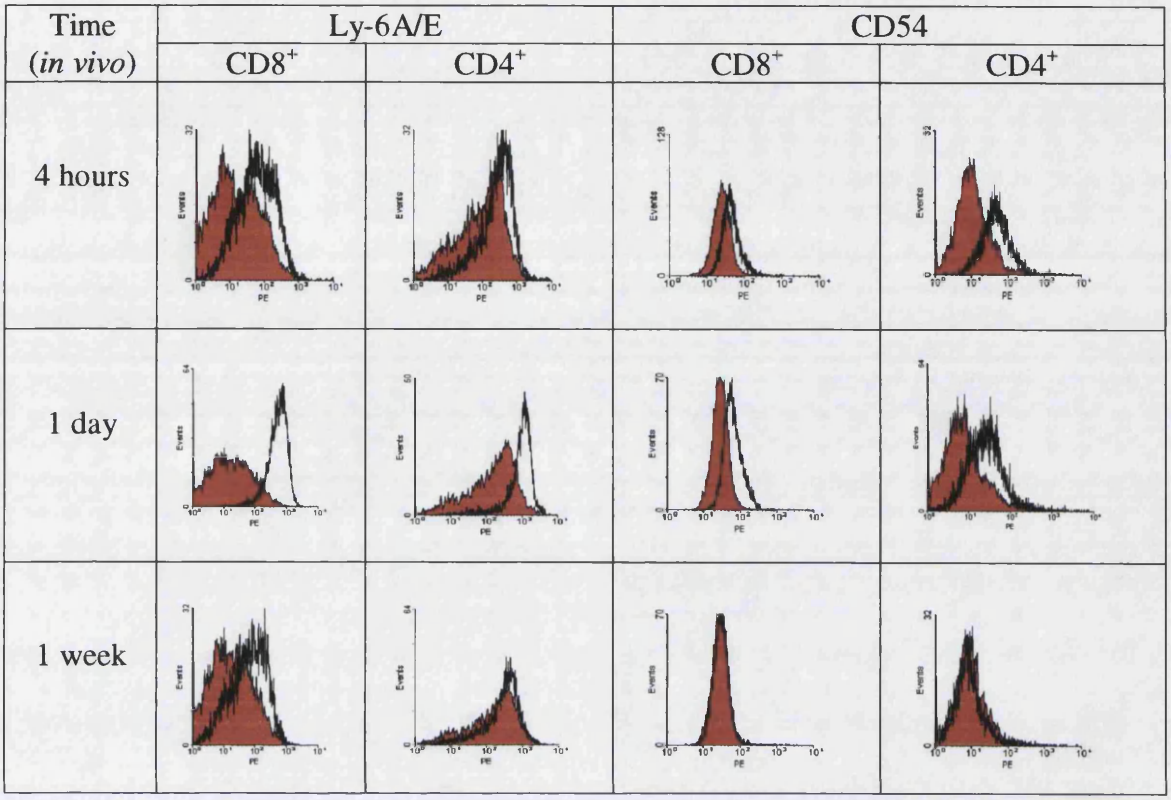
al., 1996), CD69 (Castro *et al.*, 1998)) were accompanied by phenotypic changes more commonly associated with responses to LPS by non-T cells (CD54 (Ohshima *et al.*, 1997), CD86 (Verhasselt *et al.*, 1997) and Ly-6A/E (Snapper *et al.*, 1991)). Surprisingly, CD95 expression was elevated whilst both CD28 and LPAM-1 surface expression was diminished 24 hours post LPS injection.

Phenotypic changes tended to take the form of shifts in expression intensity rather than a major increase in the percentage of surface marker-positive T cells (e.g. CD54). Therefore, in the majority of cases, mean channel fluorescence has been used as a sensitive indicator of altered expression levels.

LPS-induced changes in T cell phenotype occurred within 4 hours post injection and were maximal by 24 hours. The phenotypic alterations were transient, since by 1-week post injection all the surface markers investigated had returned or were returning to their background expression levels (fig. 3.04.). At 24 hours post-injection, the up-regulation of CD86, CD54 and Ly-6A/E expression by LPS followed a linear $\log_{10}$ dose response. Changes in T cell marker expression were observable with doses as low as 10ng (fig. 3.05.).

Phenotypic changes occurred amongst both "naïve" and "memory" T cell subsets as demonstrated by fig. 3.06. Thus, LPS affected the phenotype of both CD44lo (naïve-phenotype) and CD44hi (memory-phenotype) T cells *in vivo*. Although CD44 is commonly used as a marker of the activation status of T cells, $CD44^{lo}$ T cells may not be a totally naïve population due the possibility of $CD44^{hi}$ revertant cells (Tough and Sprent, 1995). To consider whether truly naïve T cells were

A.



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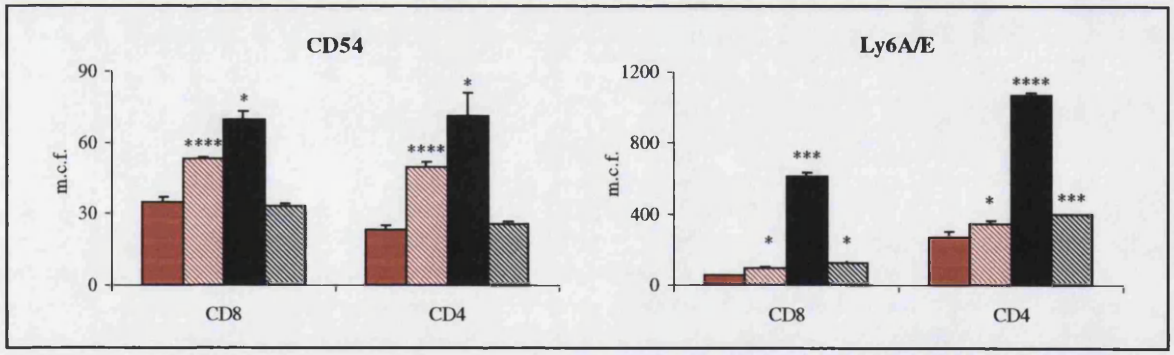


Figure 3.04. LPS-induced T cell phenotypic changes are both rapid and transient.

Mice were injected with either PBS or 50µg LPS i.v. They were sacrificed at 4 hours, 24 hours and 1 week post injection (p.i.). Total LNs were removed and the subsequent cell suspensions were stained with anti-CD4-Cy5 or anti-CD8-Cy5 and anti-Ly-6A/E-PE or anti-CD54-PE. **A.** Single colour histogram overlays were gated on $CD4^+$ or $CD8^+$ T cells. The red histograms and black overlays represent surface marker expression on T cells derived from PBS and LPS-injected mice (— = PBS, — = LPS). **B.** Data is the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of triplicates (■, PBS; ▨, 4 hours p.i.; ■, 24 hours p.i.; ▧, 1 week p.i.), p values, 2-sample t-test (PBS v LPS); (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$).

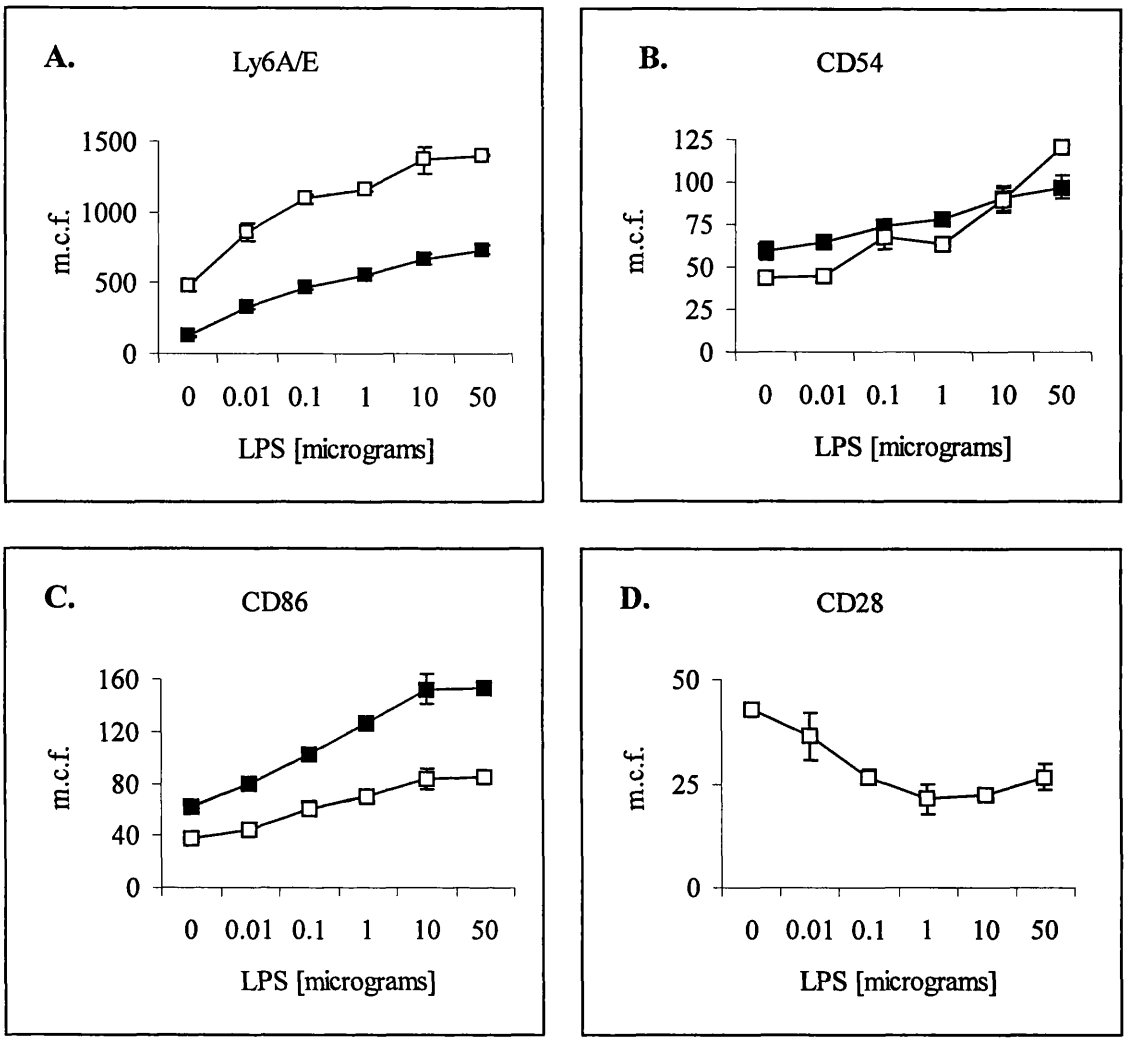
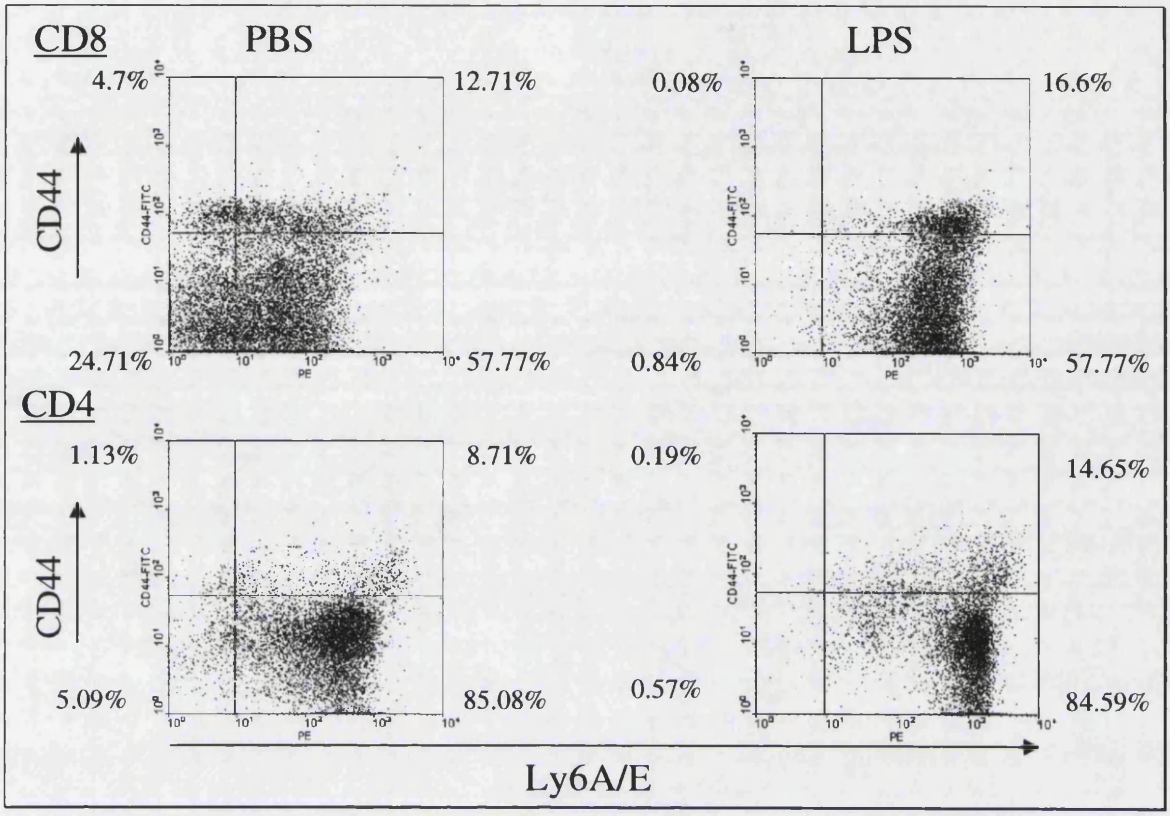


Figure 3.05. LPS induces multiple T cell phenotypic changes at low doses *in vivo*.

C57BL/6 mice were injected with either PBS (i.e. 0μg) or between 10ng and 50μg LPS i.v. All were sacrificed 24 hours later and their LNs were isolated for the preparation of lymphocyte suspensions. Cells were stained with anti-CD8-Cy5 or anti-CD4-Cy5 and also a number of PE conjugated antibodies to T cell surface markers (**A**, Ly-6A/E; **B**, CD54; **C**, CD86; **D**, CD28). Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of triplicates (■, CD8; □, CD4).

Ly-6A/E, CD54 and CD86 expression followed a $\log_{10}$ dose response to LPS (linear regression p values; Ly-6A/E, CD8 $p = 0.001$, CD4 < 0.005; CD54, CD8 $p = 0.001$, CD4 $p < 0.05$; CD86 CD8 and CD4 $p = 0.002$). The CD28 response curve did not fit a $\log_{10}$ response curve. However, CD28 down-regulation was statistically significant (2-sample t-test p values; 10ng, $p > 0.05$; 1μg and 50μg, $p < 0.05$; 0.1μg and 10μg, $p < 0.01$).

A.



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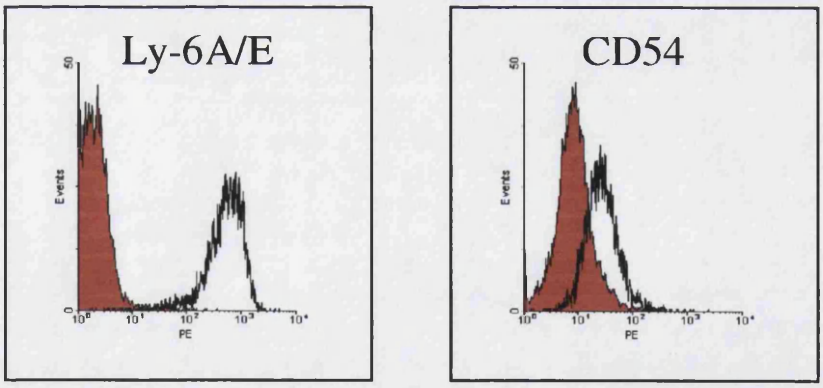


Figure 3.06. LPS upregulates surface markers on naïve and "memory" T cells.

A. C57BL6 or **B.** DO11.10 mice were injected i.v. with 10µg of LPS in 0.2ml PBS or 0.2ml PBS alone and sacrificed 24 hours later. Their lymph nodes were excised and the resulting lymphocyte suspensions were stained with conjugated antibodies. T cells were visualised using the FACScalibur and dead cells were excluded from analysis as described previously (2.2.4). **A.** Lymphocytes were stained with anti-Ly-6A/E-PE, anti-CD44-FITC and anti-CD8-Cy5 or anti-CD4-Cy5. Dot plots were generated by gating on $CD4^+$ or $CD8^+$ T cells. The quadrant was set using an isotype control. **B.** Lymphocyte preparations were stained with anti-CD4-Cy5, anti- KJ1-26-FITC and anti-Ly-6A/E-PE or anti-CD54-PE. The single colour histogram overlays above were gated on $CD4^+$ $KJ1\text{-}26^+$ lymphocytes. The red histograms and black overlays represent surface marker expression on truly naïve T cells derived from PBS and LPS-injected mice (— = PBS, — = LPS).

affected, we assessed the effects of LPS injection into CD4 TCR transgenic DO11.10 mice. In the absence of intentional immunisation with specific antigen (OVA), T cells in these mice expressing the transgenic receptor, which can be detected by the clonotypic antibody KJ1-26, are bone fide naïve cells. It should be noted that because these mice are on a BALB/c background, cells from control (PBS-injected) mice are essentially Ly-6A/E$^-$ (see p133). As shown in fig. 3.06B., transgenic T cells derived from these mice strongly up-regulated CD54 and Ly-6A/E surface expression after injection of LPS, demonstrating definitively that naïve T cells respond to LPS-injection by upregulating activation markers.

3.2B. Structural and signalling requirements for LPS-induced changes in T cell phenotype.

Lipopolysaccharide differs greatly in size and structure depending on which species of bacterium it is extracted from. For example, "smooth" LPS derived from strains of E. *coli* and S. *enteritidis* contain a highly immunogenic carbohydrate repeat motif called the O-antigen (Triantafilou *et al.*, 2000), which is absent in other strains of bacteria (e.g. Neissieria meningitidis (Jennings *et al.*, 1980)). This major difference in structure has been shown to be an important factor in determining the dependency of macrophages on CD14 to mediate responses to LPS (Gangloff *et al.*, 1999). Therefore it was necessary to test lipopolysaccharide extracted from different bacterial species for their ability to induce T cell phenotypic changes (fig. 3.07.).

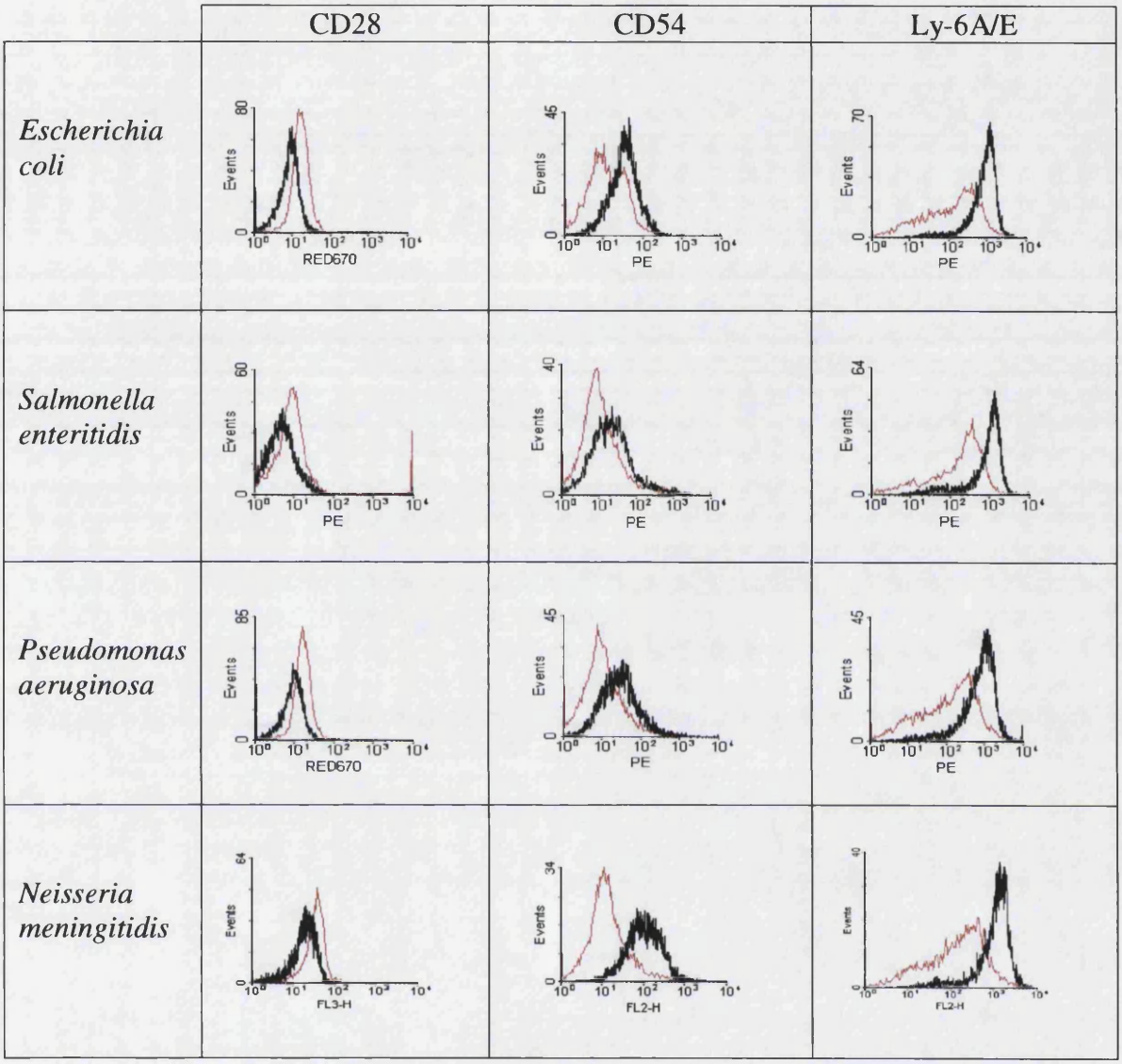


Figure 3.07. LPS extracted from different strains of gram-negative bacteria induce similar T cell phenotypic changes.

C57BL6 mice were injected i.v. with LPS extracted from various strains of gram negative bacteria. They were either injected with 50µg of LPS in 0.2ml PBS or 0.2ml PBS alone and sacrificed 24 hours later. Their lymph nodes were removed and the resulting lymphocyte suspensions were stained with anti-CD4-Cy5 and anti-Ly-6A/E-PE, anti-CD54-PE or anti-CD28-biotinylated (detected with streptavidin-RED670). T cells were visualised using the FACScalibur and dead cells were excluded from analysis as described previously (2.2.4). The single colour histogram overlays above were gated on $CD4^+$T cells. The red histograms and black overlays represent surface marker expression of $CD4^+$ T cells derived from PBS and LPS-injected mice (— = PBS, — = LPS).

The biological activity of LPS is determined by the lipid A structure (Schromm *et al.*, 2000). Hence, the four LPS molecules chosen for investigation were all different in their lipid A moieties. For example, N. meningitidis lipid A differs significantly from Escherichia coli lipid A by the nature and locations of fatty acids (Kulshin *et al.*, 1992). The presence of only five acyl residues in the major lipid A fraction has been suggested to account for the low endotoxic activity observed with P. aeruginosa lipopolysaccharide (Kulshin *et al.*, 1991). However, despite these structural differences, all four LPS molecules tested induced similar T cell phenotypic changes (fig. 3.07.). Of the four types of LPS examined, LPS extracted from N. meningitidis appeared to be the most potent modulator of T cell phenotype (see particularly changes in CD54 expression). This may not have been a difference in potency *per se* but a reflection of the small size of the N. menininginitidis LPS molecule compared to LPS derived from e.g. E. coli. Since doses were measured in micrograms rather than using molar quantities, differences in size may have been a significant factor. Thus, the proportion of lipid A units per μg may have been disproportionately higher in the N. meningitidis LPS solutions.

Injection of purified Monophosphoryl lipid A (MPLA) by itself, induced up-regulation of the same phenotypic markers on T cells as injection of LPS (fig. 3.08A.). However, the dose of MPLA required to alter Ly-6A/E expression was 10,000 fold higher than for LPS. The clear contrast in the activity of MPLA and LPS was likely due to the massive difference in the two structures. MPLA lacks the potentially stimulatory KDO and O-antigen carbohydrate structures. However, since MPLA is the biologically active portion of LPS (Zahringer *et al.*,

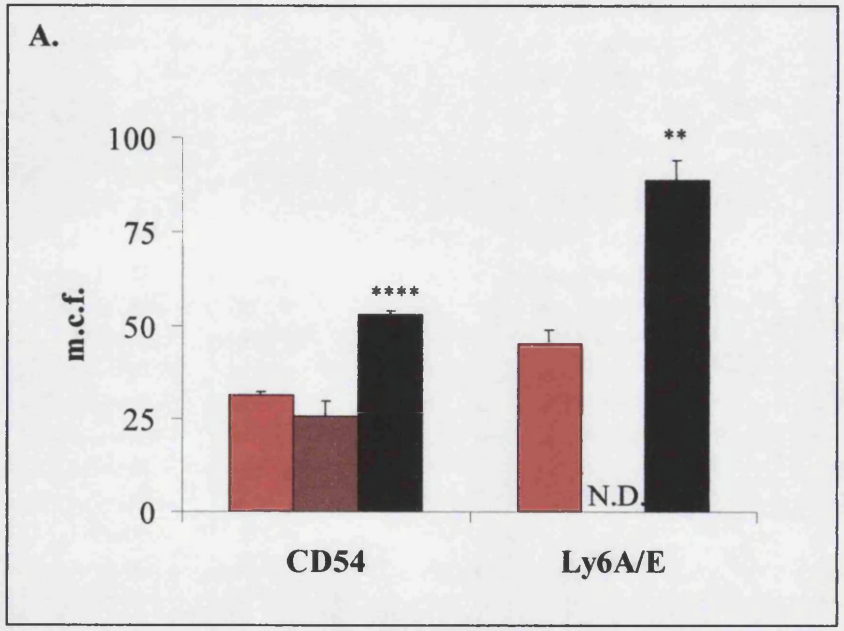
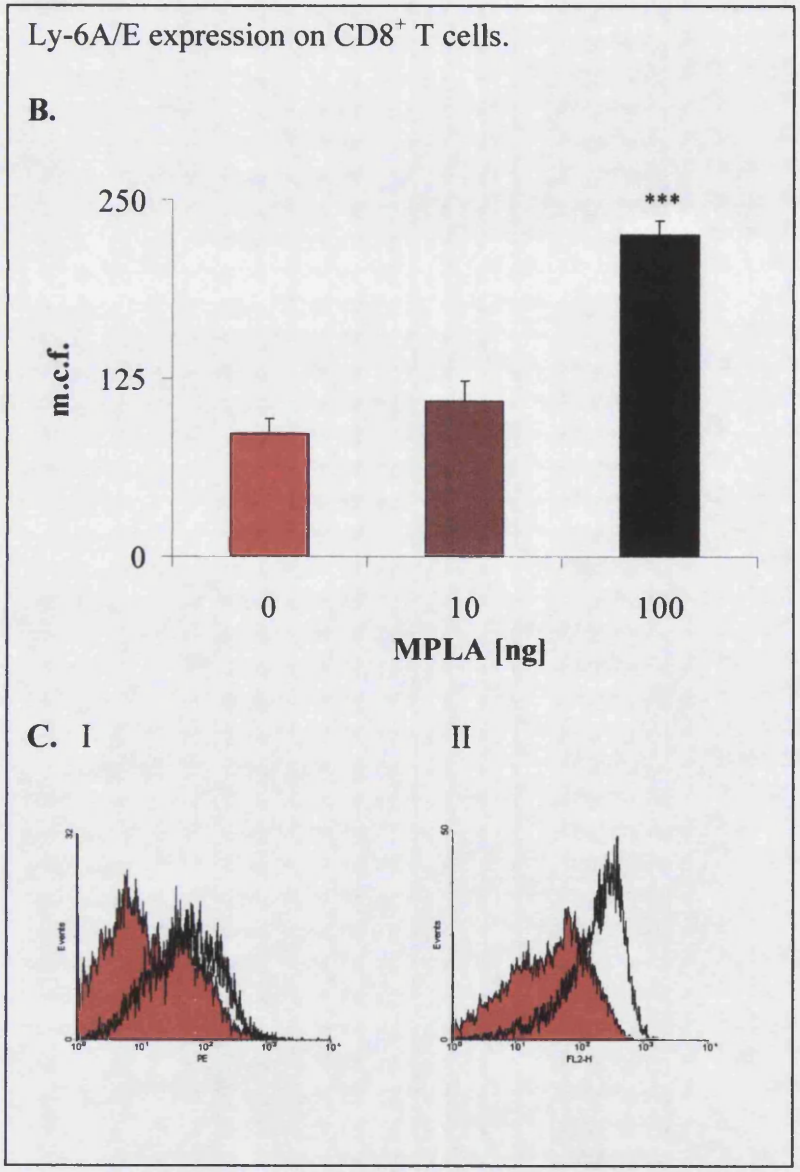


Figure 3.08. Monophosphoryl Lipid A (MPLA) induces T cell phenotypic changes *in vitro* and *in vivo*.

A. Mice were injected with PBS, ■; 50µg MPLA, ■ and 100µg MPLA, ■. All were sacrificed 24 hours later and their LN excised and homogenised. **B.** Splenocytes were cultured overnight with PBS, 10ng MPLA or 100ng MPLA. Cells were stained with anti-CD8 Cy-5 and anti-CD54-PE or anti-Ly-6A/E-PE Data represents the average of the mean channel fluorescence (m.c.f.) ± s.e.m. of triplicates (**, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$). **C.** Histogram overlays of Ly6A/E expression on $CD8^+$ T cells treated with I, 100µg MPLA *in vivo* and II, 100ng MPLA *in vitro* (— = PBS, — = MPLA).

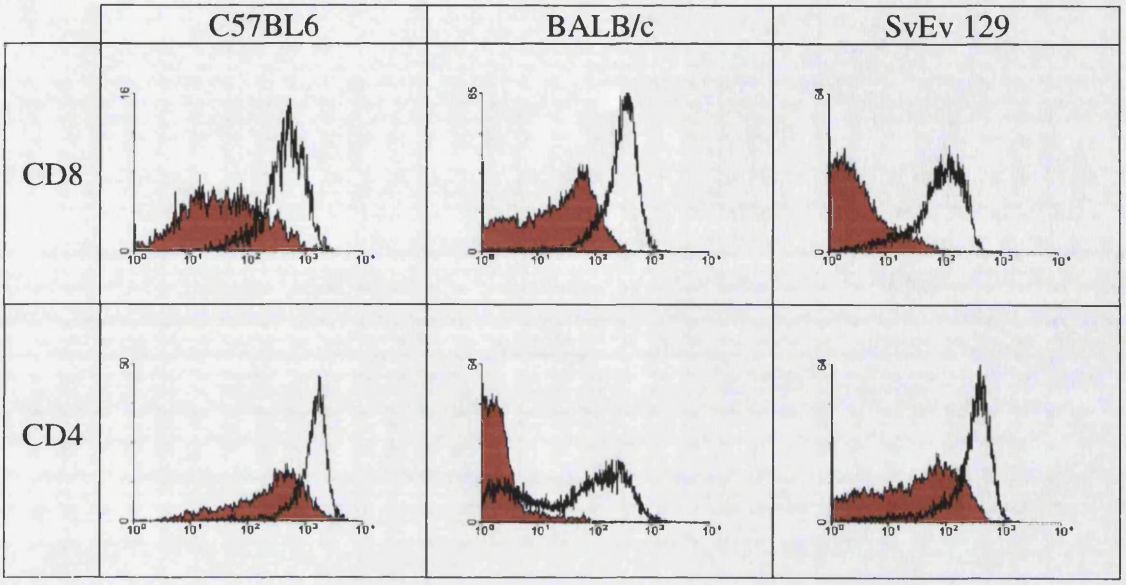


1994; Henderson *et al.*, 1996; Thieblemont *et al.*, 1998) it was surprising that concentrated MPLA was less potent than the whole LPS molecule.

C57BL6 mice were used for the majority of experiments. Therefore it was important to compare the effects of LPS on different mouse strains to eliminate strain-restricted observations from our investigations. One such change was up-regulation of CD95 expression, which was not observed on T cells derived from LPS-treated BALB/c mice. However, this situation was unique since all the other molecules investigated exhibited altered expression levels in response to LPS, regardless of mouse strain. Interestingly, the initial expression of Ly-6A/E was markedly different between strains. $CD4^+$ T cells derived from BALB/c mice and $CD8^+$ T cells derived from SvEv 129 mice initially expressed only very low levels of Ly-6A/E. Unlike in the other mouse strains, a proportion of BALB/c $CD4^+$ T cells remained Ly-6A/E^{lo} after LPS stimulation (fig. 3.09A.).

Phenotypic changes were also compared after injection of LPS into C3H/HeN versus C3H/HeJ mice. The latter mouse strain is deficient for Toll-like receptor 4 (TLR4) (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999; Qureshi *et al.*, 1999), an important signal-transducing component of the multimeric LPS-binding complex. In accord with defective TLR4 function, up-regulation of Ly-6A/E was markedly reduced upon LPS injection into C3H/HeJ compared to C3H/HeN mice (fig. 3.09B.). Unexpectedly, however, there was a small but statistically significant up-regulation of Ly-6A/E observed on $CD8^+$ T cells derived from LPS-injected C3H/HeJ mice. One possible explanation for this is that the injected LPS may contain impurities able to signal via a TLR-4 independent

A.



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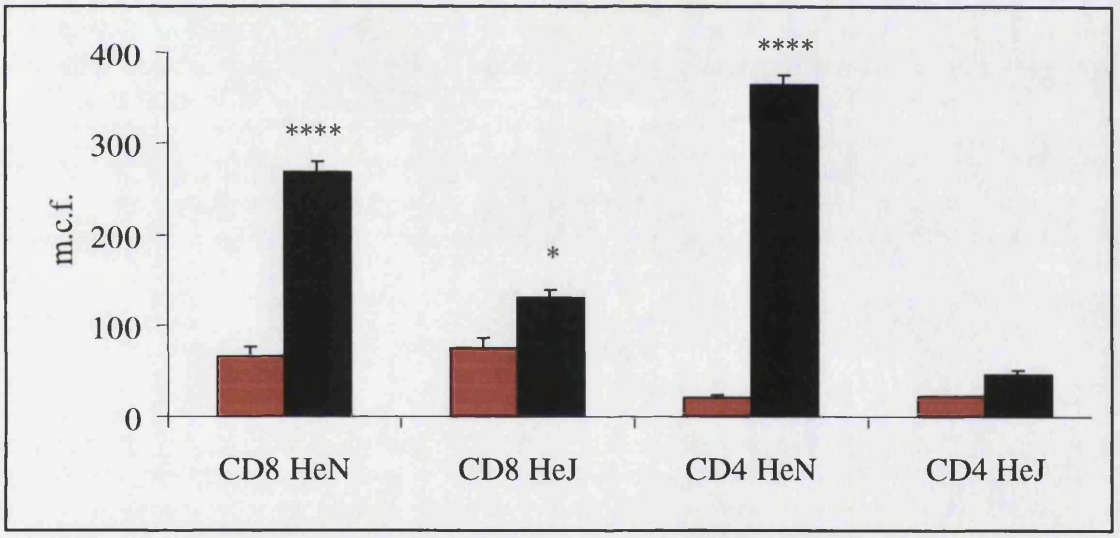


Figure 3.09. LPS induces similar T cell phenotypic changes in different mouse strains.

Mice were injected with PBS or LPS and sacrificed 24 hours later. LN were excised and cell suspensions prepared. Cells were stained for CD8 or CD4 and Ly-6A/E. $CD8^+$ or $CD4^+$ T cells were gated on for analysis. **A.** Histogram overlays representing the expression of Ly-6A/E on $CD8^+$ and $CD4^+$ T cells derived from PBS or LPS-treated C57BL6, BALB/c and SvEv 129 mice (— = PBS, — = 10μg LPS). **B.** Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of Ly-6A/E expression on $CD8^+$ or $CD4^+$ T cells derived from C3H/HeN and C3H/HeJ (TLR4 null mutants) PBS (▩) or LPS (■) treated mice. (****, $p < 0.001$; *, $p < 0.05$)

pathway. In this respect, Hirschfield *et. al.* showed that some commercially acquired preparations of LPS were able to signal via TLR2; an ability that was abrogated by the re-purification of the LPS. They postulated that this was due to the removal of contaminating microbial proteins (Hirschfeld *et al.*, 2000). However, this set of experiments was performed *in vitro* using cell lines expressing high levels of transfected TLR2. Whether low level contaminants in LPS could behave similarly *in vivo*, acting on cells expressing normal levels of TLR-2, is unclear. Whatever the mechanism, it is apparent that most of the effect of LPS on T cell phenotype is TLR4-dependent. In fact, the phenotype of both $CD4^+$ T cells and $CD19^+$ B cells was unchanged in C3H/HeJ mice injected with LPS (see chapter 5 for B cell data). It is also noteworthy to add that highly purified LPS prepared from N. meningitidis (a kind gift from S. Andersen, Jenner Institute) induced a comparable and perhaps more potent response than the commercially prepared "Sigma LPS" (fig. 3.07.).

3.2C. Cellular requirements for LPS-induced changes in T cell phenotype.

In addition to altering T cell phenotype *in vivo*, LPS was able to induce multiple phenotypic changes on T cells when added to splenocyte suspensions *in vitro*. These changes occurred at very low doses of LPS. In this regard, up-regulation of Ly-6A/E expression was particularly sensitive to LPS *in vitro*; the threshold for increased expression appeared to be 100pg (fig. 3.10A. and B.). MPLA was also able to induce phenotypic alterations when added to splenocytes *in vitro*, although, consistent with the *in vivo* data, a much higher dose of MPLA than LPS (1,000 fold) was required (fig. 3.08B.).

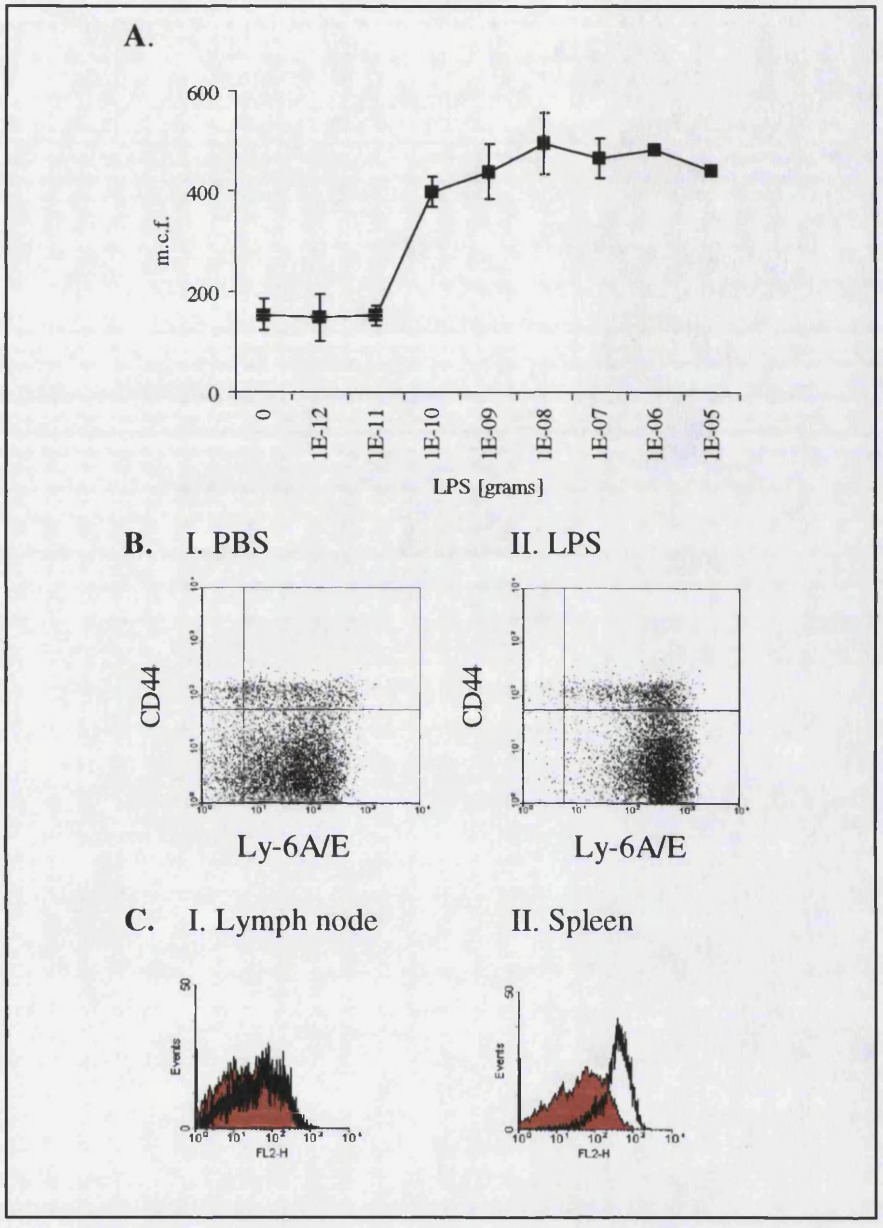


Figure 3.10. Ly-6A/E is upregulated by 0.1ng LPS *in vitro*.

PBS or LPS (10µg – 10ng) was added to splenocytes and cultured overnight. T cells were then stained with anti-CD8-Cy5, anti-CD44-FITC and anti-Ly-6A/E-PE. Ly-6A/E expression on PBS and LPS-treated T cells was analysed by gating on $CD8^+$ cells. **A.** Data is the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of triplicates (p values; 0.1ng and 0.1µg-10µg $p < 0.05$; 1ng and 10ng $p < 0.005$). **B.** Dotplots representing CD44 (y-axis) v Ly-6A/E (x-axis) expression on $CD8^+$ T cells that were treated with either I, PBS or II, 100pg LPS *in vitro*. **C.** Histogram overlay of Ly-6A/E expression on $CD8^+$ T cells (— = PBS, — = 1ng LPS) derived from I, LN ($p > 0.05$) or II, spleen ($p < 0.005$).

Interestingly, much less alteration of T cell surface marker expression was observed upon addition of LPS to LN preparations (fig. 3.10C.). This infers that there are differences between the LN and spleen that affect the response to LPS *in vitro*. This may have indicated that LN T cells were reduced in their responsiveness to the effects of LPS *in vitro*. However, a more likely possibility is that spleen preparations contain a higher proportion of "accessory" cells (macrophages, dendritic cells, B cells) that are able to mediate an *in vitro* response to LPS. Indeed, purification of splenic T cells rendered them unresponsive to LPS *in vitro* (fig. 3.11.). Not surprisingly, considering present dogma, it appears that LPS does not act directly on T cells *in vitro*. Therefore the cellular composition of tissue preparations will be important in determining the magnitude of a response to LPS *in vitro*.

This conclusion was re-inforced following experiments investigating the effect of LPS on T cell phenotype only 1 hour after injection. Here, mice were injected with PBS or LPS and culled just 1 hour later. No effect was detected when T cell phenotype was analysed directly *ex vivo*. LN and splenocyte suspensions derived from these mice were then placed into culture for 18 hours. After the culture period, marked up-regulation of Ly-6A/E was observed on T cells in splenocyte (fig. 3.12A. and fig. 3.13.) but not LN (fig. 3.13.) cell suspensions. T cell phenotype was subsequently shown to be altered even when an endotoxin inhibitor, polymyxin B sulphate, was added to the culture (fig. 3.12A.), ruling out the possibility that LPS carried-over into the medium of splenocyte suspension was responsible for the observed changes. Figure 3.12B. shows that the polymyxin B sulphate is functionally active as it was able to inhibit up-regulation

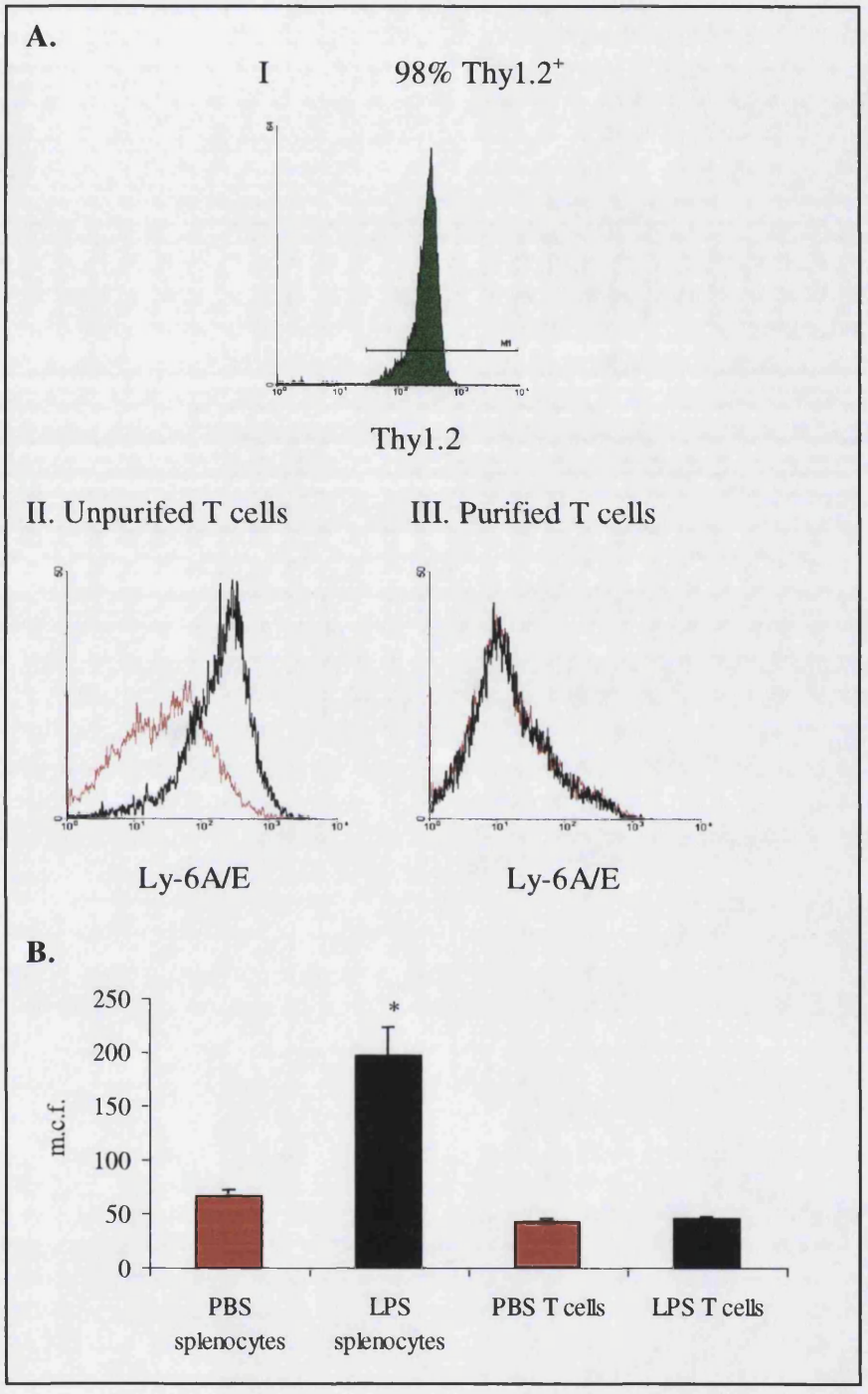
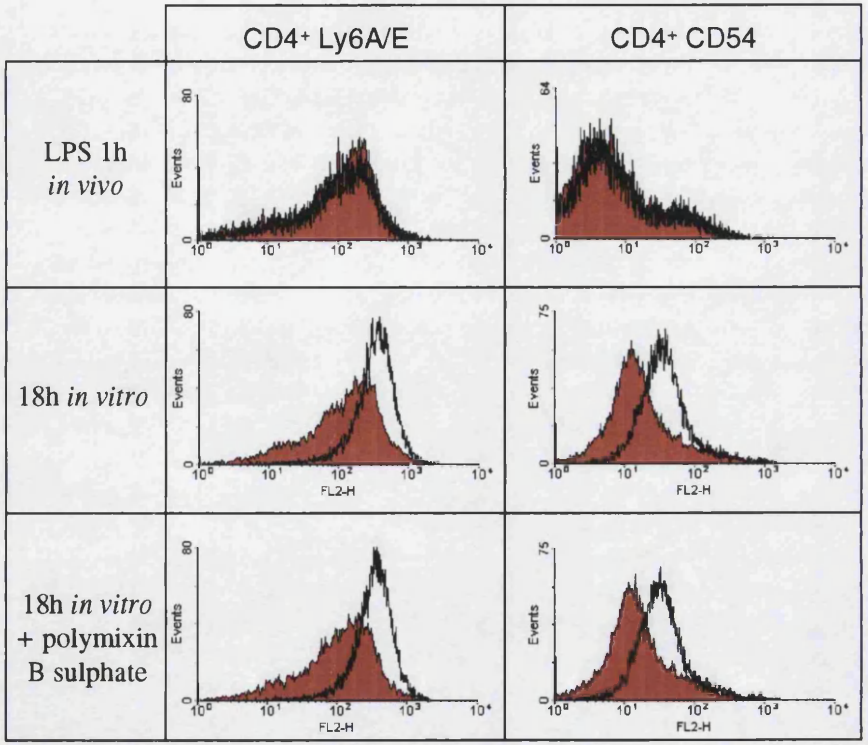
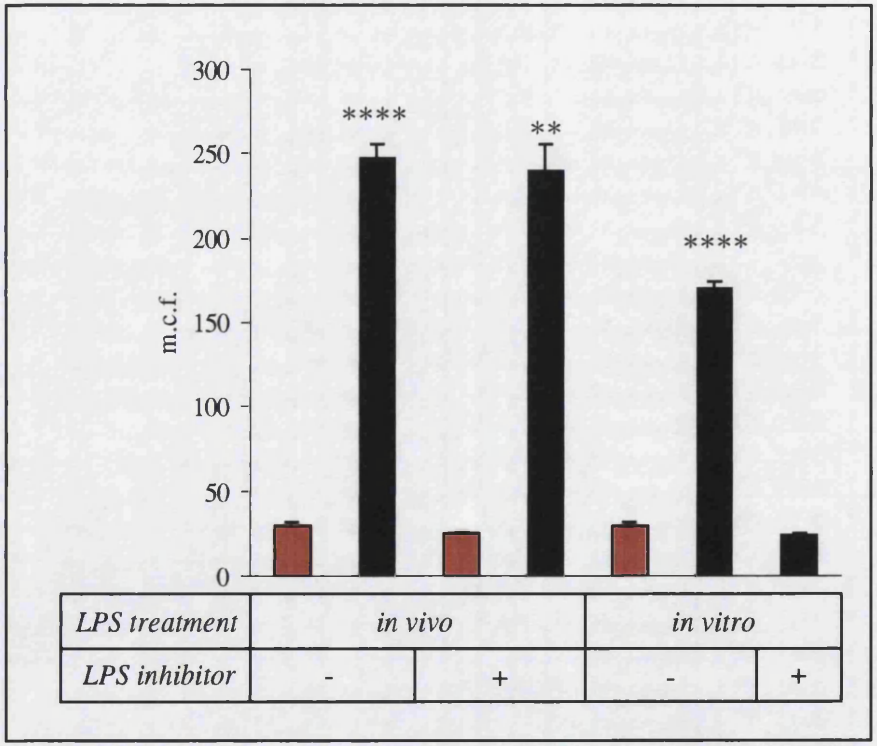


Figure 3.11. LPS does not act directly on T cells *in vitro*.

A. Splenocytes were stained with anti-Thy1.2-FITC and positively selected on the MoFlo. Sorted populations were > 95% pure (I, histogram of sorted Thy1.2$^+$ T cells.). The histogram overlays above represent the expression of Ly-6A/E on CD8 T cells and the effect of PBS (—) or 100ng LPS (—) *in vitro* on II, unpurified or III, purified T cells. **B.** CD8 T cells were gated for analysis and the mean channel fluorescence of Ly-6A/E surface expression was obtained. Data is the average m.c.f. ± s.e.m. (*, $p < 0.05$).

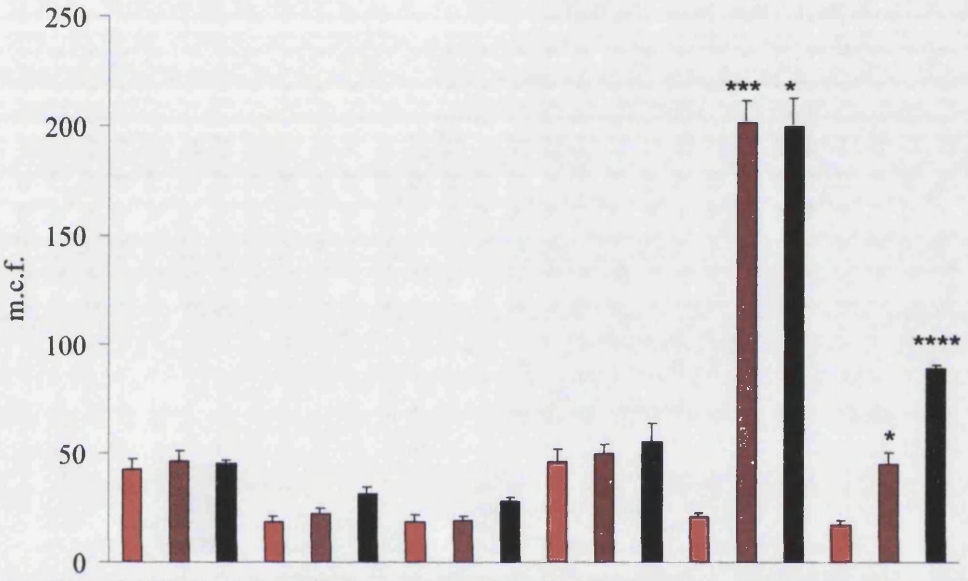
Figure 3.12. The effect of LPS 1 hr p.i.: T cell phenotype after overnight culture in the presence of an endotoxin inhibitor.





A. C57BL/6 mice were injected with either PBS or LPS and culled one hour later. Splenocyte suspensions were prepared and either stained directly *ex vivo* or placed in culture for 18 hours ± polymixin B sulphate (100U/ml). Cells were stained with anti-CD4-Cy5 and either anti-Ly-6A/E-PE or anti-CD54-PE. Histograms represent surface marker expression on PBS (■) or LPS (—) treated splenocytes.

B. Splenocytes derived from ■, PBS or ■, LPS (20ng) treated mice (1h p.i.) were placed in culture ± the endotoxin inhibitor, polymixin B sulphate (100U/ml). Normal splenocytes were also treated *in vitro* with PBS or 1μg LPS, ± inhibitor. 18 hours later the cells were stained with fluorescent antibodies. CD8 T cells were gated to analyse the m.c.f. of Ly-6A/E expression. Data represents the average m.c.f. of triplicates ± s.e.m. (**, $p < 0.01$; ****, $p < 0.001$).



Time (hrs p.i.)	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Tissue	LN									Spleen								
Cultured *in vitro*.	×	×	×	✓	✓	✓	✓	✓	✓	×	×	×	✓	✓	✓	✓	✓	✓
Purified T cells.	×	×	×	×	×	×	✓	✓	✓	×	×	×	×	×	×	✓	✓	✓

Figure 3.13. Non-T cells are not an absolute requirement for the upregulation of Ly-6A/E on T cells *in vitro* in response to brief exposure to LPS *in vivo*.

Mice were injected with PBS or LPS and culled 1 and 2 hours post injection (PBS or 0 hrs, ■; 1hr, ■; 2 hrs, ■). LN and spleens were collected and cell suspensions prepared. T cells were stained for ex vivo expression of Ly-6A/E. Total lymphocytes or purified T cells (> 95% purity) were placed in tissue culture conditions for 18 hours. CD8 T cells were stained for Ly-6A/E surface expression and the mean channel fluorescence determined. Data represents the average m.c.f. ± s.e.m. of triplicates (*, $p < 0.05$; ***, $p < 0.005$; ****, $p < 0.001$). Upregulation of Ly-6A/E on purified splenic T cells was linear with time after injection ($p < 0.001$).

of Ly-6A/E expression on T cells when 1μg LPS was added exogenously to spleen cells. Therefore, these results show that triggering of the cell type(s) responsible for the T cell responses occurs in the spleen but not LN within 1 hour of LPS injection.

A possible hypothesis to explain these results was that T cells stimulated by LPS *in vivo* within 1 hour required additional time *in vitro* to be able to visualise its effects (e.g. to synthesise and export proteins to the cell surface). A series of experimental conditions displayed in figure 3.13. showed that splenic T cells were "triggered" to up-regulate Ly-6A/E after only brief exposure to LPS *in vivo* in a time-dependent manner. Mice were injected with PBS or LPS and sacrificed 1 or 2 hours post injection. T cells were purified from LN or splenocyte suspensions derived from these mice and cultured *in vitro*. After 18 hours Ly-6A/E was up-regulated on T cells isolated from the spleens but not the LNs of LPS-treated mice. Statistical analysis showed that the progressive up-regulation of Ly-6A/E on splenic T cells from 0-2 hours post injection was highly significant ($p = 0.0005$). In contrast, the up-regulation of Ly-6A/E on T cells when total splenocytes were placed in culture was the same for both time points and far greater than the up-regulation observed when purified T cells were cultured. Thus whilst LPS-treatment for 1-2 hours *in vivo* was sufficient to induce T cells to up-regulate Ly-6A/E, a continuing contribution from other cell types (which were also triggered within 1 hour of injection) was required to provide optimum conditions *in vitro*.

Both purified and unpurified LN-derived T cells from LPS-treated mice did not significantly up-regulate Ly-6A/E when derived from mice 1-2 hours post-injection. One possibility was that this was due to the intravenous route for the administration of LPS. To address this question, mice were injected subcutaneously with LPS and culled 1 hour later. The purpose of the s.c. injection was to deliver a high dose of LPS directly to the draining LN. Cell preparations were prepared from the draining inguinal lymph nodes and cultured *in vitro* for 18 hours. Consistent with previous data, T cell phenotypic changes were not observed (data not shown). Therefore, the cellular composition of lymph node single cell suspensions may not be conducive for the stimulation of T cells to subsequently up-regulate Ly-6A/E *in vitro*.

In an attempt to understand which cell types were important for the up-regulation of Ly-6A/E on T cells *in vitro*, macrophages and dendritic cells were depleted from splenocyte suspensions by plastic adherence. T-depleted splenocytes from PBS or LPS-treated mice were co-cultured with purified LN T cells in transwell plates for 18 hours *in vitro*. By removing macrophages and dendritic cells, splenocytes derived from LPS-treated mice (1 hour post injection) were unable to significantly up-regulate Ly-6A/E expression on T cells (fig. 3.14.). Also of noteworthy attention was the fact that control splenocytes derived from LPS-injected mice were able to up-regulate Ly-6A/E on T cells in the absence of direct contact with T cells. This implicated soluble factors as important mediators of T cell phenotypic changes.

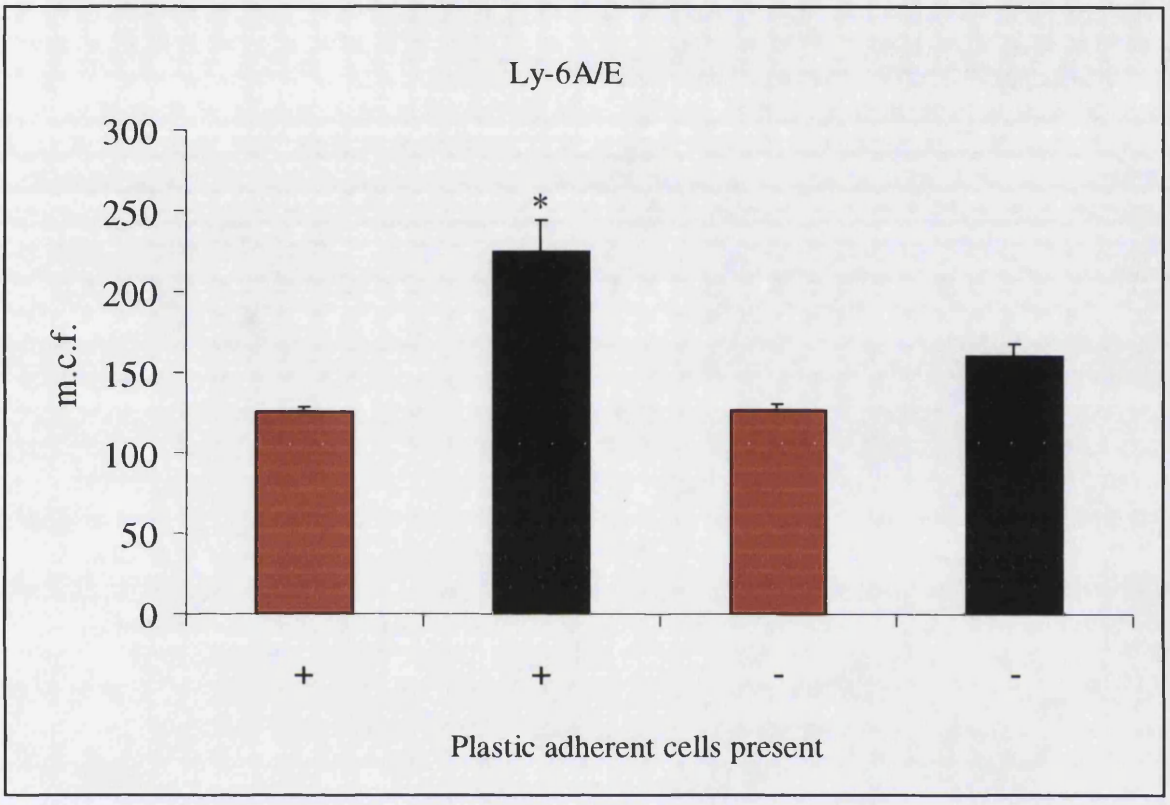


Figure 3.14. The removal of plastic adherent cells affects Ly-6A/E upregulation on $CD4^+$ T cells *in vitro*.

C57BL/6 mice were injected with PBS or LPS and culled one hour later. LN and spleen were removed and cell suspensions prepared. Splenocytes were either only depleted of T cells (+) or in addition depleted of macrophages and dendritic cells (-) by plastic adherence. Cells were plated into the lower chamber of a transwell whilst untreated purified T cells were placed in its upper chamber. Cells were cultured at 37°C for 18 hours and stained for CD4 and Ly-6A/E. $CD4^+$T cells were gated for analysis and the "mean channel fluorescence" (m.c.f.) of Ly-6A/E expression was determined. Data represents the average of triplicates ± s.e.m. (▩, PBS; ■, LPS; +, plastic adherent cells present; -, plastic adherent cells depleted). (PBS ≠ LPS; *, $p < 0.05$) Statistical analysis showed that the m.c.f. for LPS (+) and LPS(-) was significantly different ($p < 0.05$).

To test the validity of this assumption, the effect of soluble factors released from LPS-stimulated splenocytes was investigated. Splenocytes were isolated from mice 1 hour after injection of PBS or LPS. Subsequently. splenocyte supernatant was generated *in vitro* as described previously (2.2.9A.) and used to culture LN T cells derived from C57BL6 and C3H/HeJ mice. Supernatant from LPS-treated splenocytes strongly up-regulated Ly-6A/E expression on T cells (fig. 3.15.). Analysis of the cytokine content of the supernatant revealed that splenocytes derived from LPS-treated mice produced high levels of IL-12p40, TNF-α and IFNγ *in vitro* (fig. 3.16.). The supernatant was shown to exhibit anti-viral activity, conferring resistance to L929 cells from the cytopathic effect of Encephalomyocarditis virus (EMCV) indicating that IFN-I was also present. Supernatant harvested from control splenocytes was unable to protect L929 cells from being lysed by EMCV. The assay was used to titrate the anti-viral activity of the supernatant against known quantities of recombinant IFN-α as described previously (see 2.2.9C.). Supernatant from LPS-stimulated splenocytes (diluted 1:2) induced a virus-resistant state in L929 cells at equivalent levels to IFN-α concentrations of between 8 and 16 Units/ml (data not shown).

The detection of interferons in the splenocyte supernatant was of particular interest, since these cytokines have been shown to up-regulate Ly-6A/E (Snapper *et al.*, 1991). Further indirect evidence for the involvement of interferons in the induction of T cell phenotypic changes was provided by the fact that poly I:C; a potent inducer of interferons evoked similar phenotypic changes to LPS. Thus, CD54, Ly-6A/E and CD86 (amongst others) were all up-regulated by relatively low doses of poly I:C *in vivo* (fig. 3.17A.). Consistent with the previous data

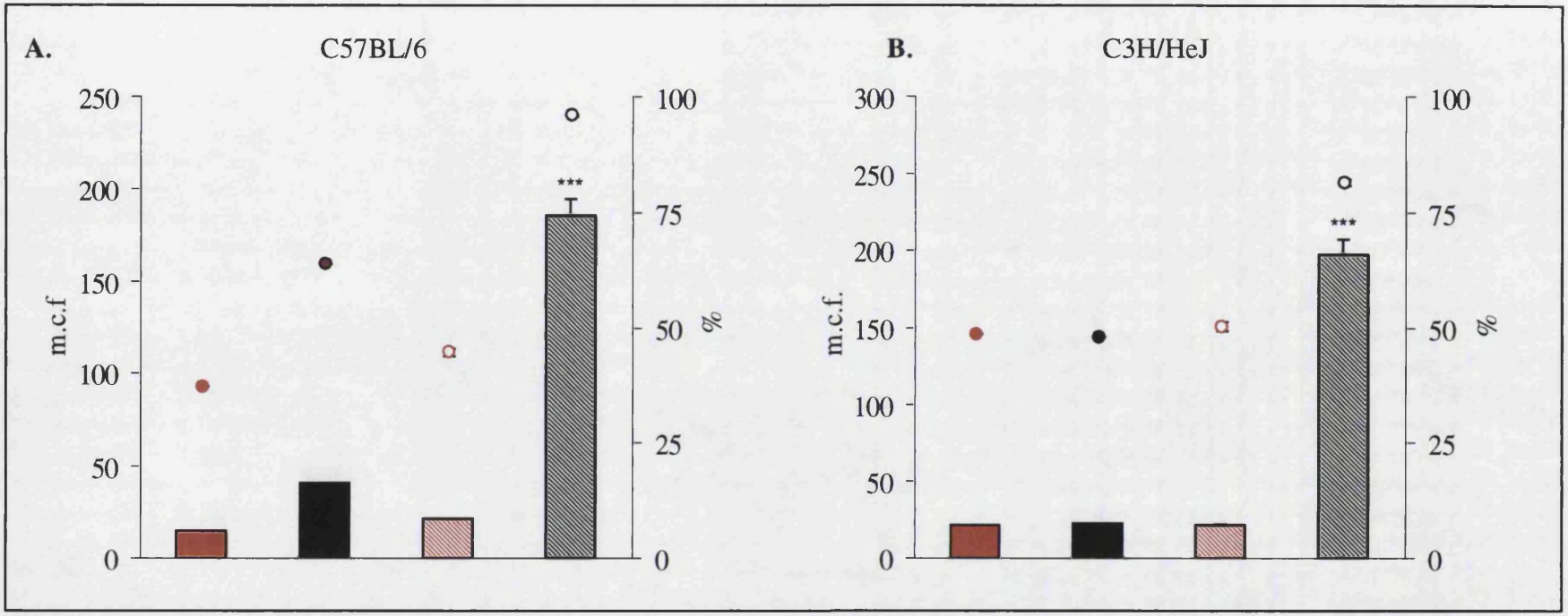


Figure 3.15. Supernatant from LPS-stimulated splenocytes upregulates Ly-6A/E expression on $CD8^+$ T cells.

Mice were injected with PBS or LPS and sacrificed 1 hour post injection. Spleens were removed, cell suspensions prepared and cultured at 37°C for 18 hours in the presence of polymixin B sulphate (100U/ml). The supernatants were harvested and filter sterilised. LN preparations from **A.** C57BL6 and **B.** C3H/HeJ mice were resuspended in supernatant and cultured at 37°C in vitro. Control LN cells were resuspended in medium ± 1μg LPS. After 18 hours the cultured LN cells were stained for CD8 and Ly-6A/E surface expression. $CD8^+$ T cells were gated on for analysis and the mean channel fluorescence (m.c.f.) and percentage of Ly-6A/E^+ T cells was obtained. Data represents the mean of triplicates ± s.e.m. (***, $p < 0.005$). (Bars = m.c.f.; ■, PBS; ■, LPS; ▨, supernatant harvested from PBS-stimulated splenocytes; ▨, supernatant harvested from LPS-stimulated splenocytes. Circles = % Ly-6A/E^+; ●, PBS; ●, LPS; ○, supernatant harvested from PBS-stimulated splenocytes; ○, supernatant harvested from LPS-stimulated splenocytes.)

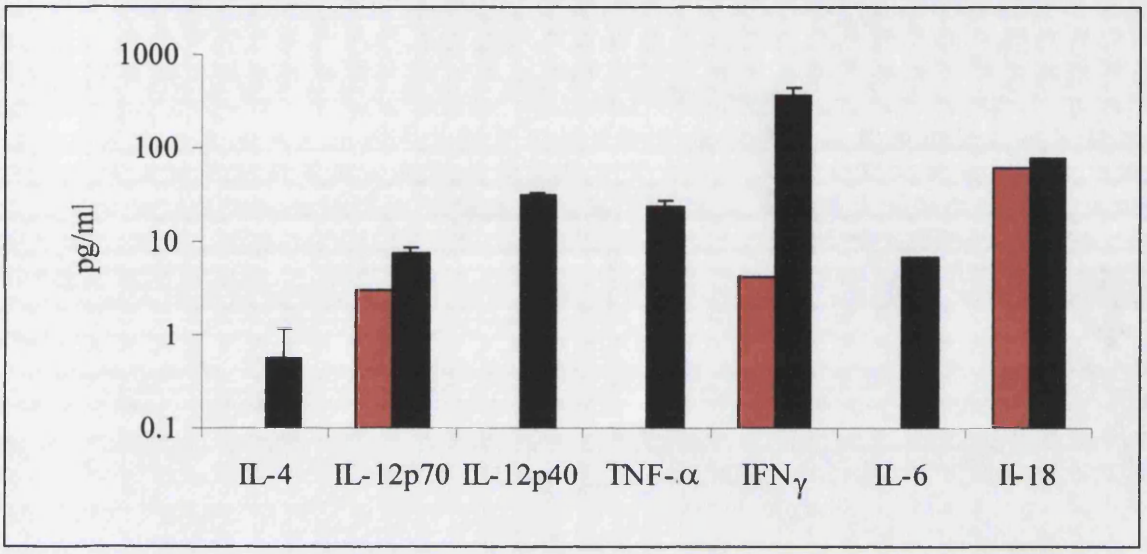
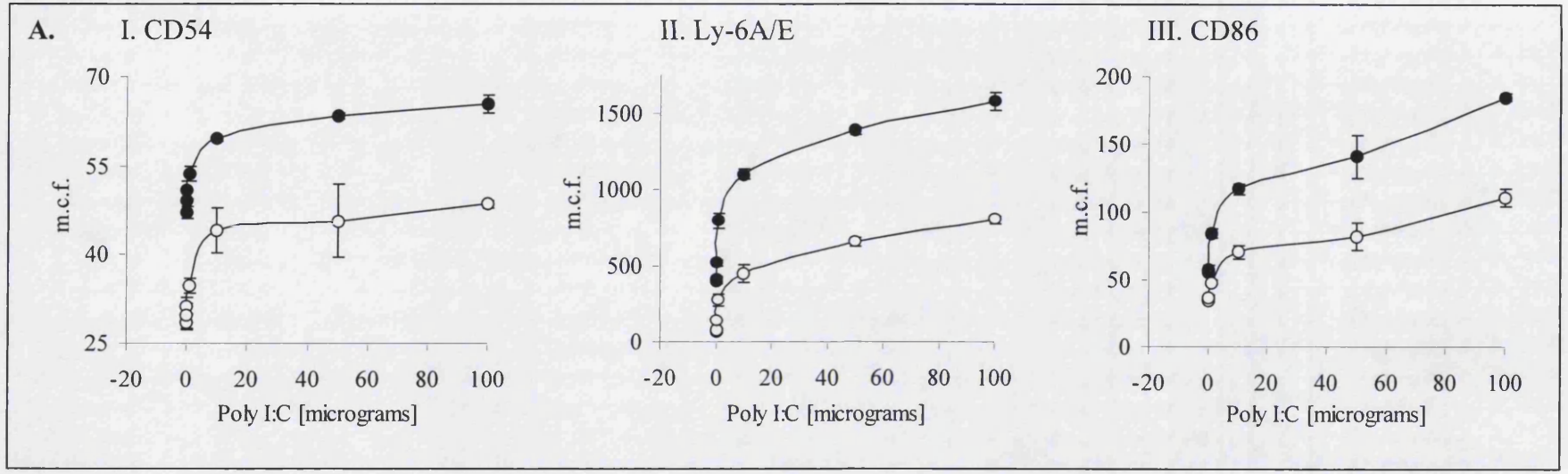


Figure 3.16. Analysis of the cytokine content of LPS-stimulated splenocyte supernatant.

Mice were injected with PBS(■) or LPS (■) and sacrificed one hour later. Their spleens were removed and the resulting cell suspensions were cultured at 37°C in the presence of polymixin B sulphate. After 18 hours the supernatant was harvested and the cytokine content analysed by ELISA. Data represents the mean of triplicates ± s.e.m.



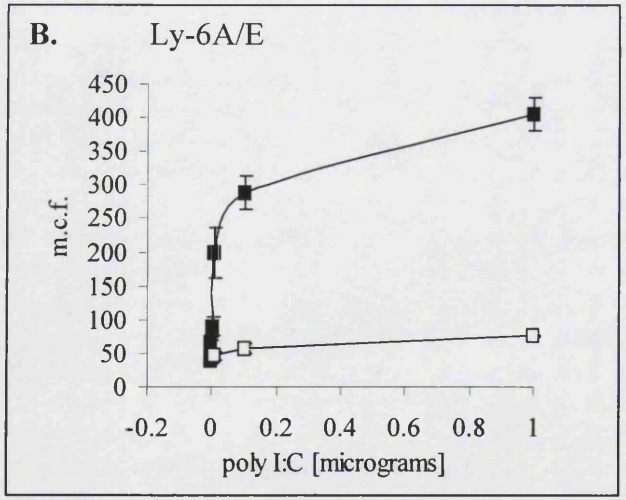


Figure 3.17. The effect of Poly I:C on T cell phenotype *in vivo* and *in vitro*.

A. C57BL/6 mice were injected with PBS or Poly I:C (0.01μg-100μg in 0.2ml PBS) and culled 24 hours later. LN were removed and cell suspensions were prepared. Cells were stained for CD4 or CD8 and T cell surface molecules (I, CD54; II, Ly-6A/E; III, CD86). Either $CD4^+$ or $CD8^+$ T cells (●, CD4, ○, CD8) were gated for analysis.. Data represents the average "mean channel fluorescence" (m.c.f.) of triplicates ± s.e.m. **B.** PBS or Poly I:C (0.1ng/ml - 1μg/ml) was added to LN (□) or splenocyte (■) cell suspensions which were cultured for 18 hours in vitro. T cell phenotype was then analysed by staining for CD8 and Ly-6A/E expression. $CD8^+$ T cells were gated for analysis and the m.c.f. of Ly-6A/E expression obtained. Data represents the average m.c.f. of triplicates ± s.e.m. (Log_{10} linear regression, ■, $p < 0.001$; □, $p > 0.05$)

using LPS, splenocytes reacted to poly I:C *in vitro* by inducing multiple T cell phenotypic changes. Yet within these parameters, LN preparations appeared to be comparably unresponsive (fig. 3.17B.), suggesting that similar accessory cell populations were required for T cell responsiveness to LPS and poly I:C.

To test more directly the role of IFNs, we utilised mouse strains lacking functional IFN receptors. Initially, IFNγR -/- and wild type splenocytes were cultured with PBS and LPS for 18 hours *in vitro*. The subsequent splenic T cell phenotype in terms of Ly-6A/E and CD54 expression is shown in figure 3.18A. Interestingly, basal expression of Ly-6A/E and CD54 was lower on T cells from IFN-γR KO mice, implying a role for IFN-γ in defining T cell phenotype under "resting" conditions. The up-regulation of these two molecules in response to LPS was diminished but not abrogated by the absence of IFNγR. Therefore, although IFNγ may play a role in the alteration of T cell phenotype, IFN-γ-independent mechanisms were also operative.

By contrast, IFN-α/β appeared to be an absolute requirement for the up-regulation of Ly-6A/E, at least in response to low doses of LPS *in vivo* (fig. 3.18B.). T cells from IFN-α/β receptor knockout mice had lower basal expression of Ly-6A/E, suggesting that IFN-α/β, like IFNγ, affects T cell phenotype under resting conditions. Importantly, injection of 10ng LPS into IFN-α/βR KO mice did not induce any up-regulation of Ly-6A/E, whilst only a minimal response was observed at the higher dose of 10μg.

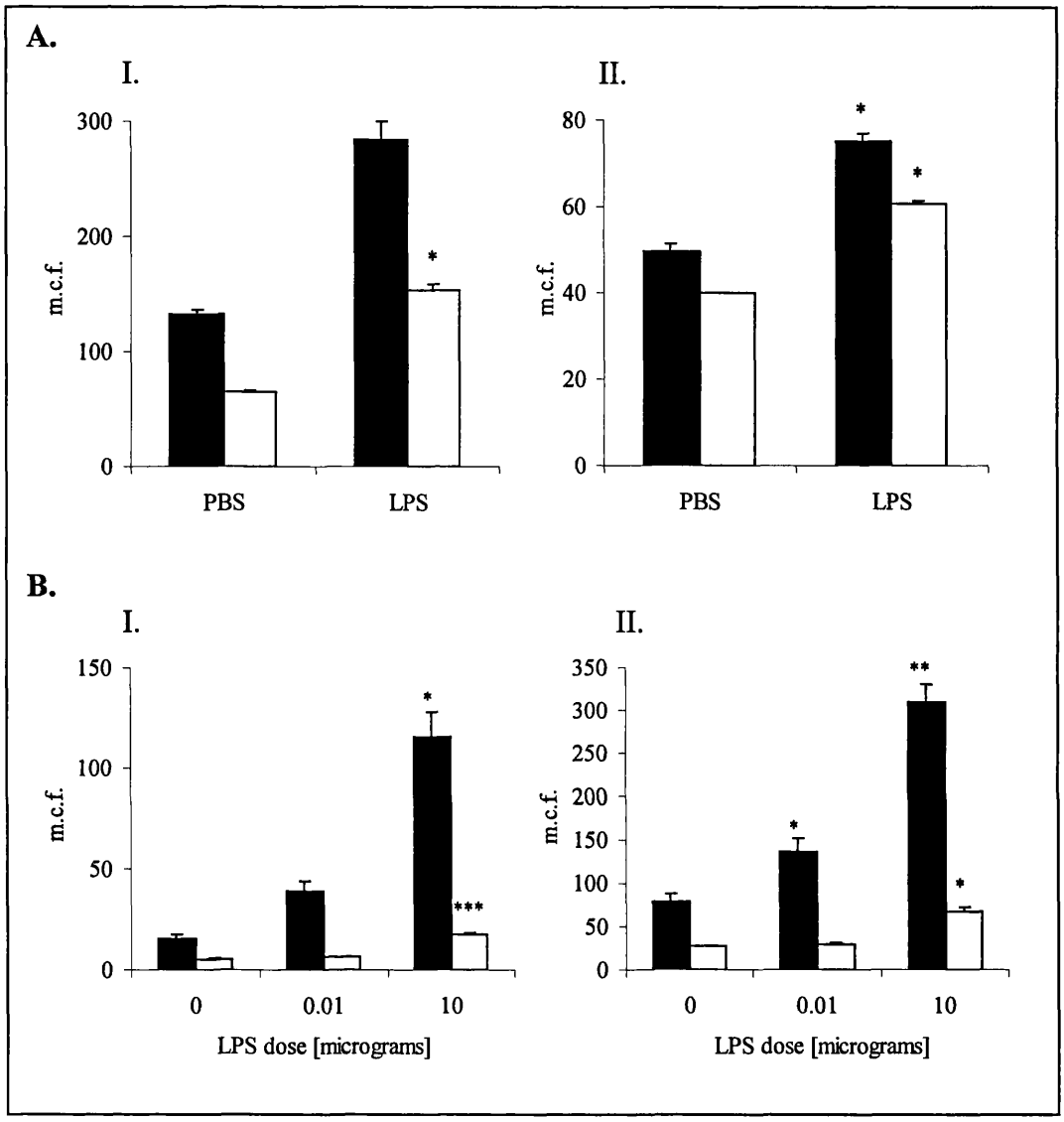


Figure 3.18. The effect of LPS in interferon receptor knockout mice.

A. Splenocyte suspensions were prepared from wild type SvEv 129 (■) and IFNγR -/- (□) mice. Cells were cultured for 18 hours at 37°C with either PBS or 100ng LPS, after which they were stained for CD4 and I, Ly6A/E or II, CD54. Surface marker expression was analysed by gating on $CD4^+$ T cells and obtaining the mean channel fluorescence (m.c.f.) of either Ly6A/E or CD54. Data represents the m.c.f. of duplicates ± s.e.m. (PBS ≠ LPS; *, $p < 0.05$). **B.** Wild type SvEv 129 (■) and type I IFNR -/- (□) mice were injected with PBS or LPS and sacrificed 24 hours later. LN were removed and the resulting cell suspensions stained for CD4 or CD8 and Ly6A/E. Ly6A/E expression was analysed by gating on either I, $CD8^+$ or II, $CD4^+$ T cells and obtaining the mean channel fluorescence (m.c.f.). Data represents the average m.c.f. triplicates ± s.e.m. (PBS ≠ LPS; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

In view of the fact that some IFN-α/β-independent up-regulation of Ly-6A/E was observed at the higher dose of LPS, we also examined the effects on T cells of other cytokines found in the supernatant of splenocytes from LPS-injected mice. Figure 3.19A. shows the capacity of IL-12, IL-15, IL-18, IFNγ and IFN α/β to up-regulate CD54 and Ly-6A/E expression on T cells *in vitro*. Purification of T cells prior to cytokine treatment did not appear to affect the magnitude of Ly-6A/E up-regulation (fig. 3.19B), inferring that these cytokines may act directly on T cells *in vitro*. The ability of IL-12, -15 and –18 to up-regulate CD54 and Ly-6A/E suggests that they may participate in the LPS-induced phenotypic changes. Here, however, the profound reduction in the response in IFN-α/βR KO needs to be considered. One explanation is that the cytokine concentrations used in this *in vitro* experiment are higher than those produced in response to LPS injection (see fig. 3.16). However, it should be borne in mind that local cytokine concentrations could reach very high levels *in vivo*. Thus, an alternative explanation is that production of many of the cytokines is in turn partially dependent on IFN-α/β; there is some evidence that this is indeed the case for IL-15 (Mattei *et al.*, 2001).

3.2 D Examination of LPS effects on T cell responsiveness *in vitro.*

The marked alterations in T cell phenotype, occurring in response to LPS-induced cytokines, raised the question of whether this "partial activation" influenced the ability of T cells to respond to other stimuli. We investigated this possibility by examining T cell responses in a number of *in vitro* assays.

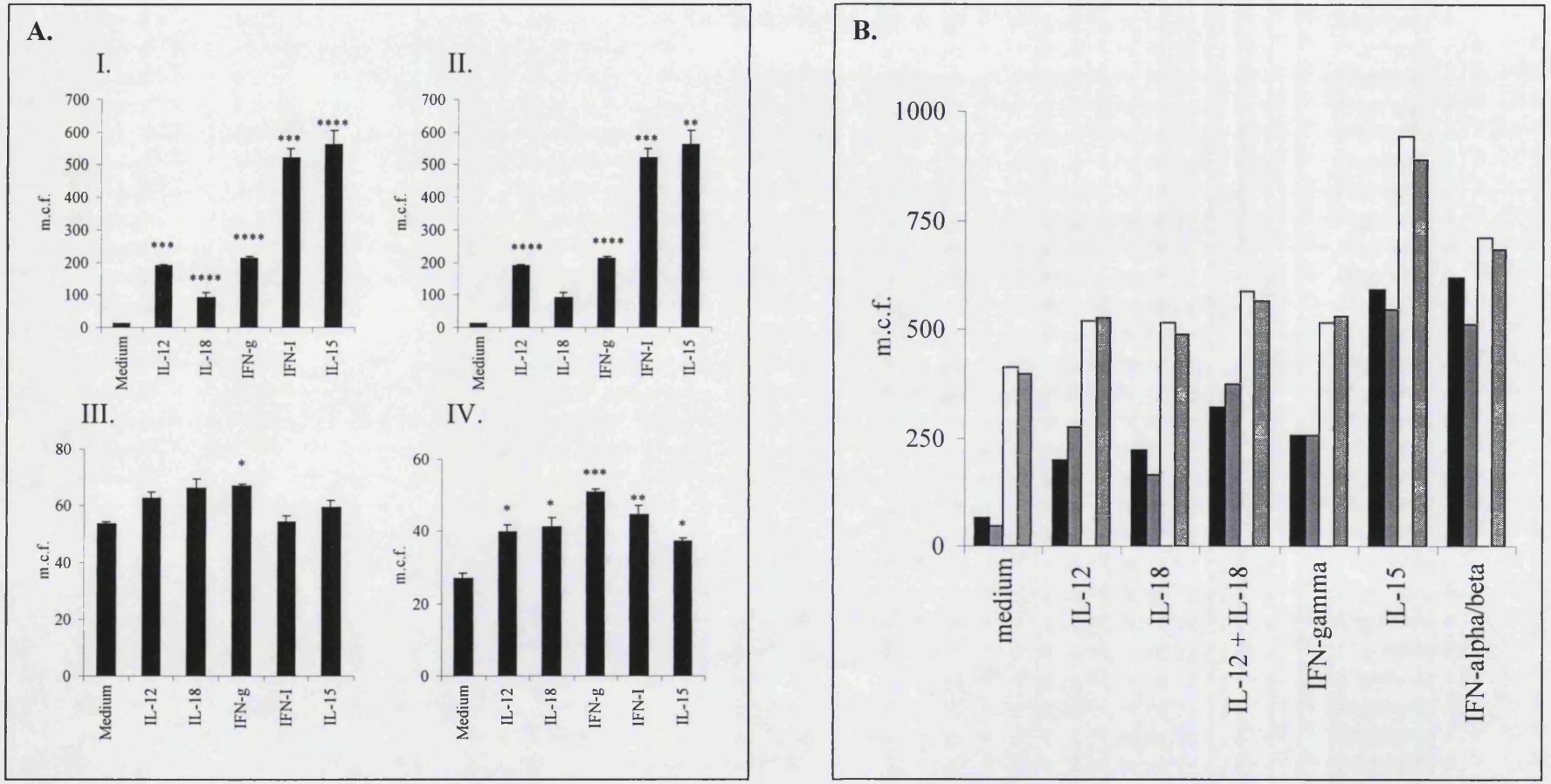


Figure 3.19. The effect of recombinant cytokines on T cell phenotype *in vitro*.

Splenocytes, LN cells and purified T cells from spleens and LN were cultured in medium ± recombinant cytokines ($2x10^4$ U/ml IFN-I, 250ng/ml IL-15, 100ng/ml IL-12, IL-18 or IFNγ) at 37°C for 48 hours. Cells were then stained for CD4 or CD8 and CD54 or Ly-6A/E. **A.** CD8 or CD4 T cells were gated and the mean channel fluorescence of either CD54 or Ly-6A/E expression obtained. Data represents the average m.c.f. of triplicates ± s.e.m. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$). (I. & III. LN cells; II. & IV. splenocytes; I. & II. $CD8^+$ T cell Ly-6A/E expression; III. & IV. $CD4^+$ T cell CD54 expression.) **B.** $CD8^+$ T cell Ly-6A/E expression (■, LN; ▩, LN T cells; □, splenocytes; ▩, splenocyte T cells).

Initially, we compared the ability of T cells from control versus LPS-injected mice to respond in a mixed lymphocyte reaction (MLR), using allogeneic stimulator cells. Unfortunately, observing definitive results in these assays proved to be more difficult than initially envisaged. In particular, interesting but conflicting observations were frequently obtained and reproducibility was a recurrent obstacle. This may have been exacerbated by the use of two different methods to prepare cell suspensions, either tissue disruption using a homogeniser or tissue digestion using collagenase/DNase (see material and methods 2.2.3.). Enzyme digestion of lymphoid organs yielded higher cell viability than tissue disruption using a homogeniser. Consequently, the magnitude of the control response *in vitro* may have reflected the cell viability of both the responders and the stimulators. Therefore, in order to discuss these results clearly, the experiments have been separated into two groups depending on the technique used for generating cell suspensions.

Mice were injected with PBS or LPS and sacrificed 1, 6 and 24 hours post injection. Spleen cells from BALB/c mice used as stimulators whereas LN cells from B6 mice were used as responders. In experiments using a homogeniser to generate cell suspensions, LN responders derived from LPS-treated mice displayed a bi-phasic pattern in their relative responsiveness to allo-antigen. Responders derived from LPS-injected mice culled 1 hour p.i. were hyper-proliferative but by 24 hours p.i. they were hypo-proliferative (fig. 3.20A,C.). At 6 hours p.i. there was no difference between responders derived from PBS or LPS-injected mice (fig. 3.20B.). Unfortunately the control response for the 1 hour time point experiments was consistently poor, whilst the "LPS-enhanced"

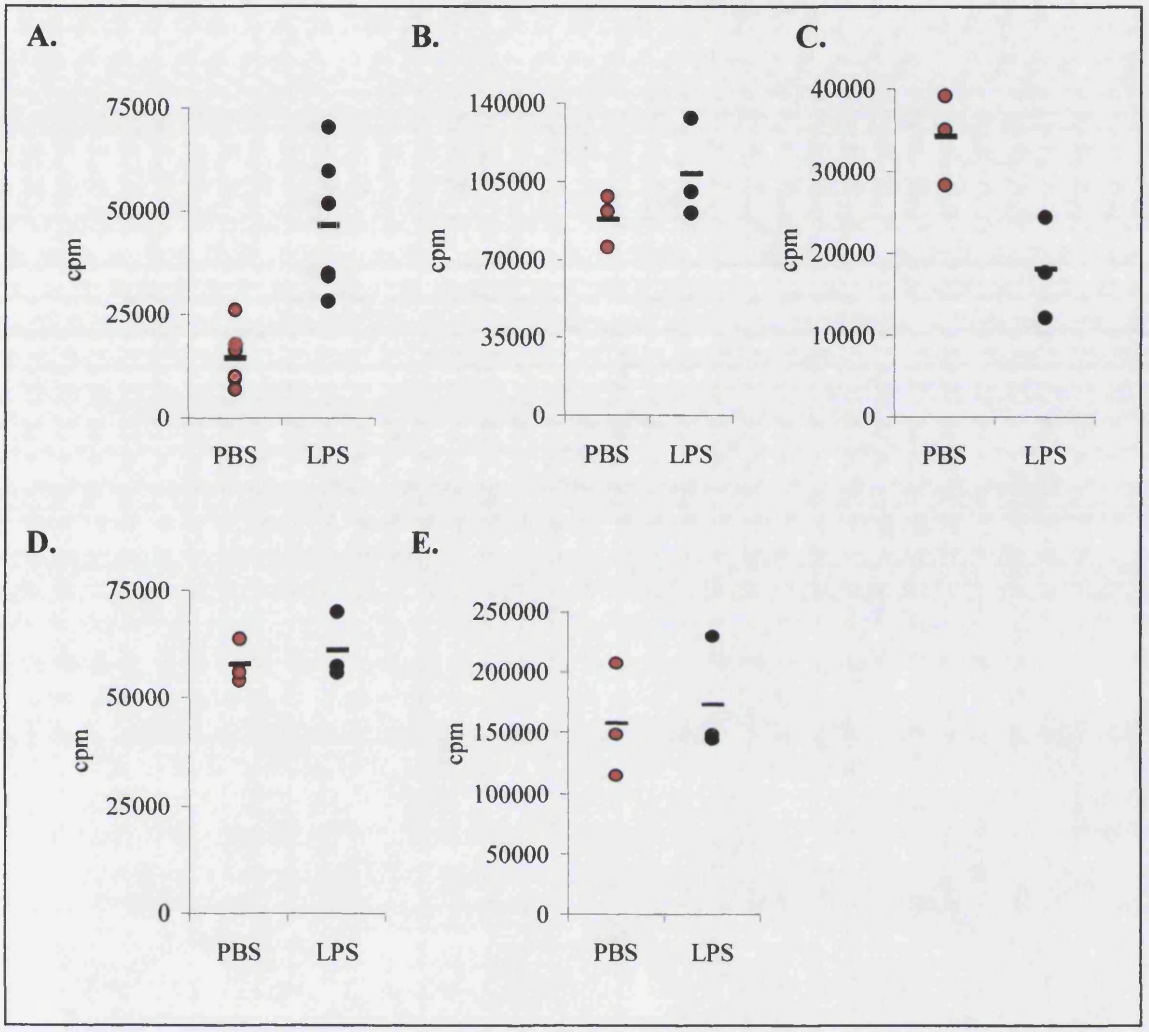


Figure 3.20. The *in vitro* response to allo-antigen after the administration of LPS *in vivo*.

B6 mice were injected with PBS or LPS and sacrificed 1 (**A** and **D**), 6 (**B**) and 24 (**C** and **E**) hours later. LN were removed and single cell suspensions prepared by either homogenising (**A-C**) or the collagenase technique (**D** and **E**) described in 2.2.2. 1.5 x 10^5 LN cells were co-cultured with 5 x 10^5 irradiated BALB/c splenocytes. Assays were pulsed with tritiated thymidine (2.2.8) at the peak of the response; after 96 hours (**A-B** and **D-E**) and 120 hours (**C** only) in culture. Proliferation was quantified 18 hours later using a scintillation counter. ●, PBS; ●, LPS; —, mean proliferation. Statistical analysis was performed using a 2-sample t-test (p values: A, $p < 0.001$; B, $p > 0.05$; C, $p < 0.05$; D, $p > 0.05$; E, $p > 0.05$).

response may be viewed as substandard. However, the augmented proliferation was consistently statistically significant (4/4). In Figure 3.20A., the difference in activity between the two responder populations was highly significant ($p < 0.0005$). It should be noted that the reduction in proliferation of responders obtained from mice 24 hours p.i. (fig. 3.20C.) was only statistically significant when cells were pulsed with ^{3}H-thymidine after 120 hours in culture. At 96 hours, the proliferation of responders from PBS and LPS-treated mice was identical (fig. 3.21. I.).

Experiments using responders prepared from LN using the collagenase/DNase technique yielded different results. Thus, no difference in proliferation was observed between responders derived from PBS or LPS-treated mice that had been culled 1 hours post injection (fig. 3.20D.). Furthermore, at the peak of the mixed leukocyte reaction, there was no difference in the responder activity of lymphocytes derived from PBS or LPS-treated mice 24 hours post injection (fig. 3.20E.). However, the kinetics of the MLR may have differed between the two responder populations, with lymphocytes derived from LPS-treated mice displaying a more prolonged peak response (fig. 3.21. II.). Overall, it is difficult to make any clear conclusions on the responsiveness to antigen of T cells from LPS-treated mice based on these MLR data.

The variability of observations may have been in part due to the fact that the MLR was a fairly crude system for investigating the response of T cells to antigen *in vitro* in as much as the antigenic specificities of the responding T cells are unknown. Therefore, a second approach was taken using antigen-specific

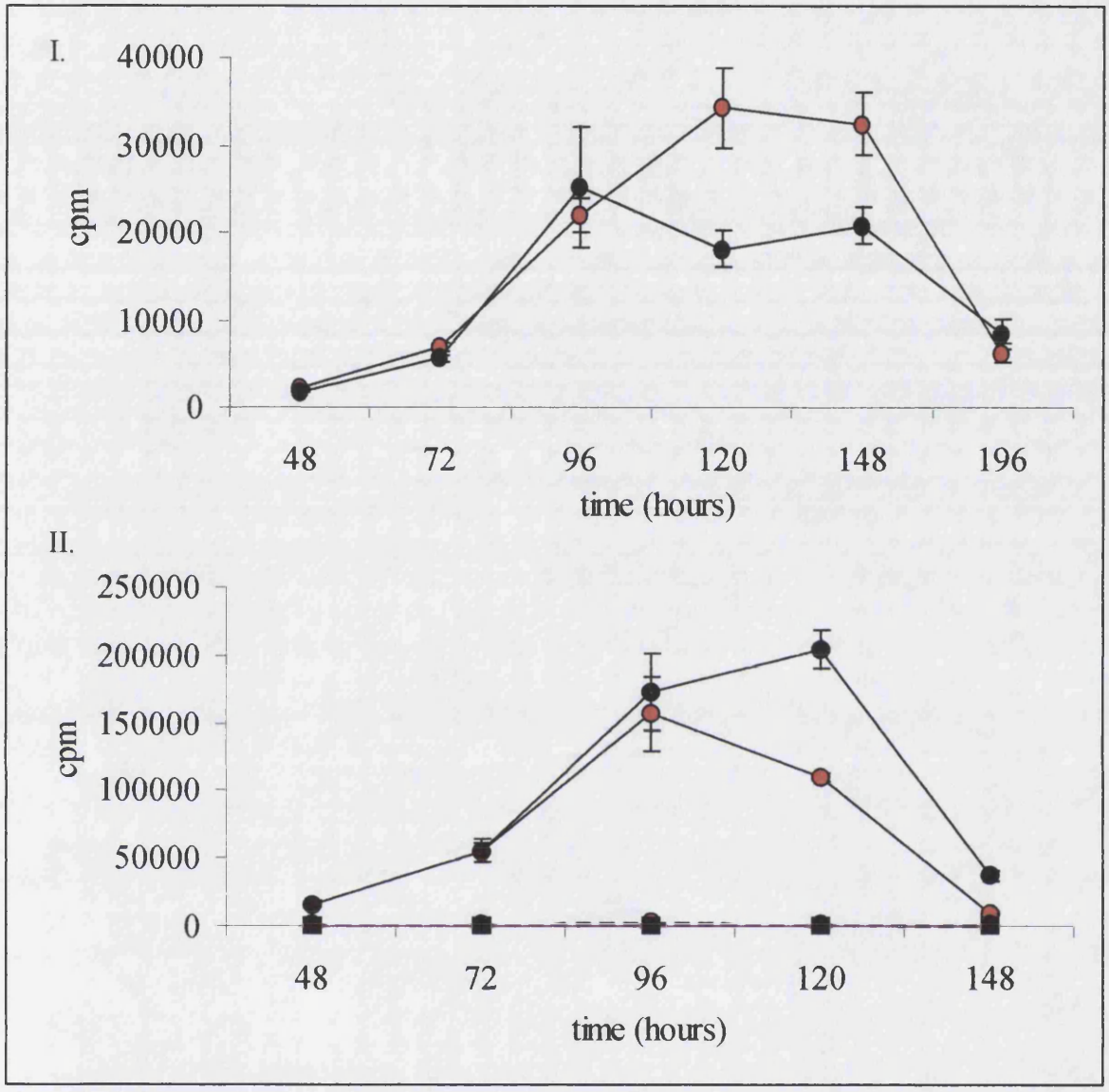


Figure 3.21. The effect of LPS 24 hours post injection on T cell responsiveness to allo-antigen *in vitro*.

Mice were injected with PBS or LPS and sacrificed 24 hours later. LN were removed and single cell suspensions prepared by either I, homogenising or II, by enzyme digestion (2.2.3.). 1.5 x 10^5 LN cells were co-cultured with 5 x 10^5 irradiated BALB/c splenocytes. Assays were pulsed with tritiated thymidine (2.2.8) between 48 and 148 hours in culture. Plates were frozen 18 hours later and proliferation was subsequently determined using a scintillation counter. Data represents the mean proliferation of triplicate responder populations; allogeneic response - ●, PBS; ●, LPS; syngeneic response – ○, PBS; ○, LPS (■, irradiated responders + irradiated stimulators). Statistical analysis was performed using a 2-sample t-test for the differences observed after 120 hours in culture. (p values: I, $p < 0.05$; II, $p > 0.005$).

TCR transgenic mice. DO11.10 mice, in which $CD4^+$ T cells expressing a transgenic TCR respond to an ovalbumin peptide in association with $I\text{-}A^d$, were injected with PBS or LPS and sacrificed 1, 6 and 24 hours post injection. LN cells were placed *in vitro* with BALB/c stimulators and various doses of peptide (see 2.2.8B.). Prior exposure to LPS *in vivo* did not alter the $CD4^+$ T cell proliferative response to peptide stimulation *in vitro* (fig. 3.22. and data not shown). This was true when cell suspensions were made by either method.

Similar results were obtained using the 2C $CD8^+$ TCR transgenic system. 2C mice, in which $CD8^+$ T cells expressing a transgenic TCR recognise the synthetic peptide SIYRYYGL presented on $H\text{-}2K^b$, were injected with PBS or LPS and sacrificed 24 hours later. LN cells were placed *in vitro* with C57BL6 stimulators and various doses of peptide (see 2.2.8C.). As with the CD4 transgenic system, the $CD8^+$ antigen-specific response to 2C peptide *in vitro* was the same regardless of previous treatment *in vivo* (fig. 3.23).

Taken together, the experiments utilising TCR transgenic mice suggested that, at least under the conditions employed in these assays, stimulation with LPS *in vivo* did not confer an altered ability to respond to specific antigen *in vitro*. Further evidence that the ability of T cells to respond through their TCR was not intrinsically altered came from studies measuring T cell proliferation in response to cross-linking anti-CD3ε antibodies. As with transgenic T cell responses to peptide antigens, no significant differences in the ability of $CD8^+$ or $CD4^+$ T cells derived from LPS-injected mice to respond to CD3ε cross-linking were observed (fig. 3.24).

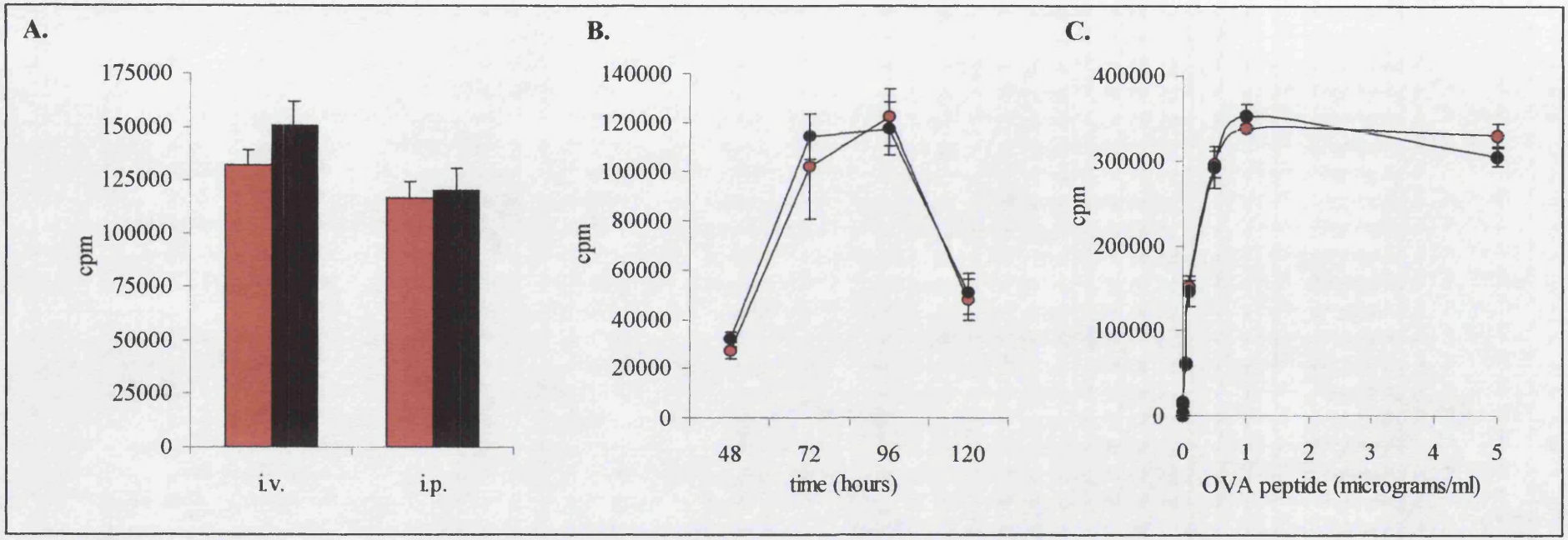


Figure 3.22. LPS *in vivo* does not alter the CD4 proliferative response to specific antigen *in vitro*.

A. DO11.10 mice were injected with PBS or LPS i.p. and sacrificed 6 hours later. LN were removed and homogenised to create single cell suspensions. 1 x 10^5 LN cells were cultured with 5 x 10^5 irradiated BALB/c splenocytes in the presence of OVA peptide (0.05μM). Proliferation was determined by pulsing with tritiated thymidine after 96 hours in culture and measured 18 hours later by using a β-scintilation counter. Data represents the mean of triplicate mice ± s.e.m (□, PBS; ■, LPS). **B-C.** DO11.10 mice were injected with PBS or LPS i.v. and sacrificed 24 hours later. LN were removed and homogenised (B) or digested with enzymes (C) to create single cell suspensions. 1 x 10^5 LN cells were cultured with 5 x 10^5 irradiated BALB/c splenocytes in the presence of OVA peptide (B, 0.5μM). Proliferation was determined by pulsing with tritiated thymidine (C, after 96 hours in culture) and measured 18 hours later by using a β-scintillation counter. Data represents the mean of triplicate mice ± s.e.m (○, PBS; ●, LPS).

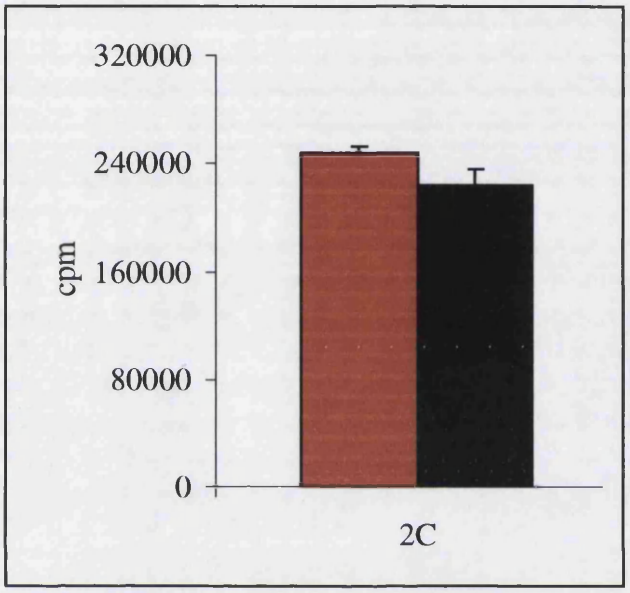


Figure 3.23. LPS *in vivo* does not alter the CD8 proliferative response to specific antigen *in vitro.*

2C mice were injected with PBS or LPS i.v. and sacrificed 24 hours later. 1.5 x10^5 $CD8^+$ T cells (purified from LN) were cultured with 5 x 10^5 irradiated C57BL6 in the presence of 2C peptide (10ng/ml). Proliferation was determined by pulsing with tritiated thymidine after 96 hours in culture and measured by β-scintillation 18 hours later. Data represents the mean of triplicate mice ± s.e.m. (■, PBS; ■, LPS).

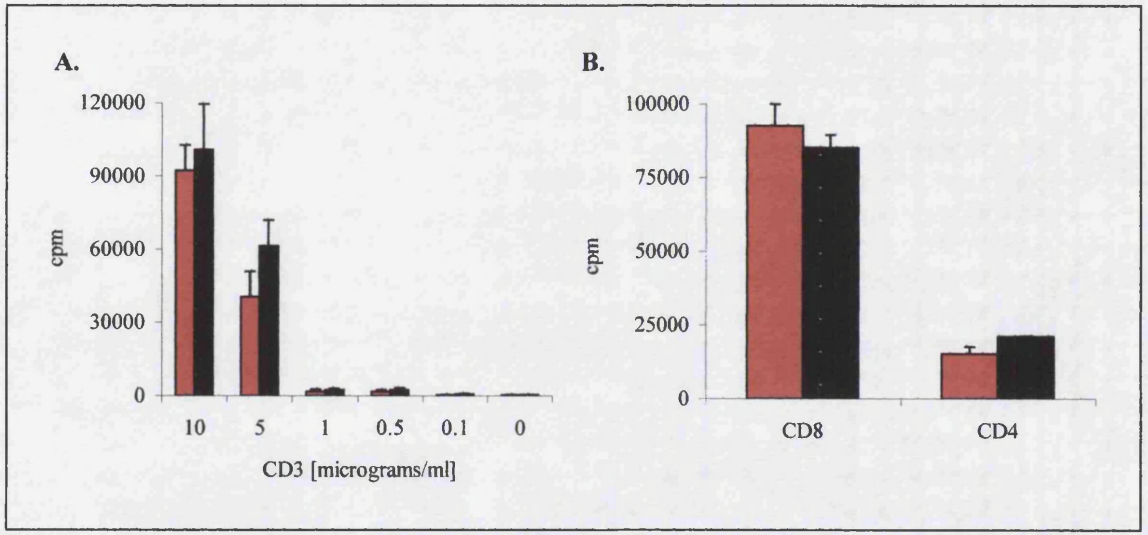


Figure 3.24. LPS 24 hours *in vivo* does not alter the anti-CD3 proliferative response.

Mice were injected with PBS or LPS i.v. and sacrificed 24 hours later. **A.** 1 x 10^5 LN cells were incubated in plates pre-coated with various amounts of anti-CD3ε antibody (see 2.2.8). **B.** 1 x10^5 sorted $CD8^+$ or $CD4^+$ T cells were incubated in plates pre-coated with 5μg/ml anti-CD3ε antibody. Both assays were pulsed with tritiated thymidine after 72 hours in culture. Proliferation was measured 18 hours later by β-scintillation. Data represents mean proliferation ± s.e.m. (■, PBS; ■, LPS).

By contrast, purified T cells derived from LPS-treated mice proliferated far greater than T cells from control mice (fig. 3.25) when stimulated by cross-linking Ly-6A/E *in vitro*. This observation was repeatable using either a low dose of LPS (10ng) or different mouse strains (e.g. B6 and BALB/c). A preliminary experiment suggested that T cell stimulation by anti-Ly-6A/E was dependent on the presence of lipid rafts. Pre-incubation with methyl-β-cyclodextran (m.c.d.) (a compound that can disrupt the association of proteins with lipid rafts by extracting cholesterol) prior to cross-linking Ly-6A/E *in vitro* appeared to have a dramatic effect on subsequent proliferation *in vitro*. However, the apparent decrease in proliferation due to m.c.d. treatment was not highly statistically significant ($p = 0.08$). Also, a mere 25% of cells were retrievable after only a 30-minute incubation with the raft inhibitor. Importantly we have not answered the question whether the inhibitory effect of m.c.d. on Ly-6A/E mediated proliferation was mediated by the dissociation of this protein from the lipid rafts. Therefore it is not possible to attach too much significance to this observation at the present time. A thorough investigation of this question is beyond the scope of this thesis and may be carried out by others in the group.

The final assay used to assess T cell responsiveness was to measure proliferative responses to the plant lectin, Concanavalin A (Con A). This T cell mitogen can activate T cells by binding to the TCR and MHC independently of the MHC peptide groove. In figure 3.26A. LN cells (but not splenocytes) derived from mice treated with LPS 24 hours before (N.B. but not 1 hour, data not shown) were much more sensitive to Con A stimulation than their control counterparts. This phenomenon did not appear to be restricted to a particular T cell subset,

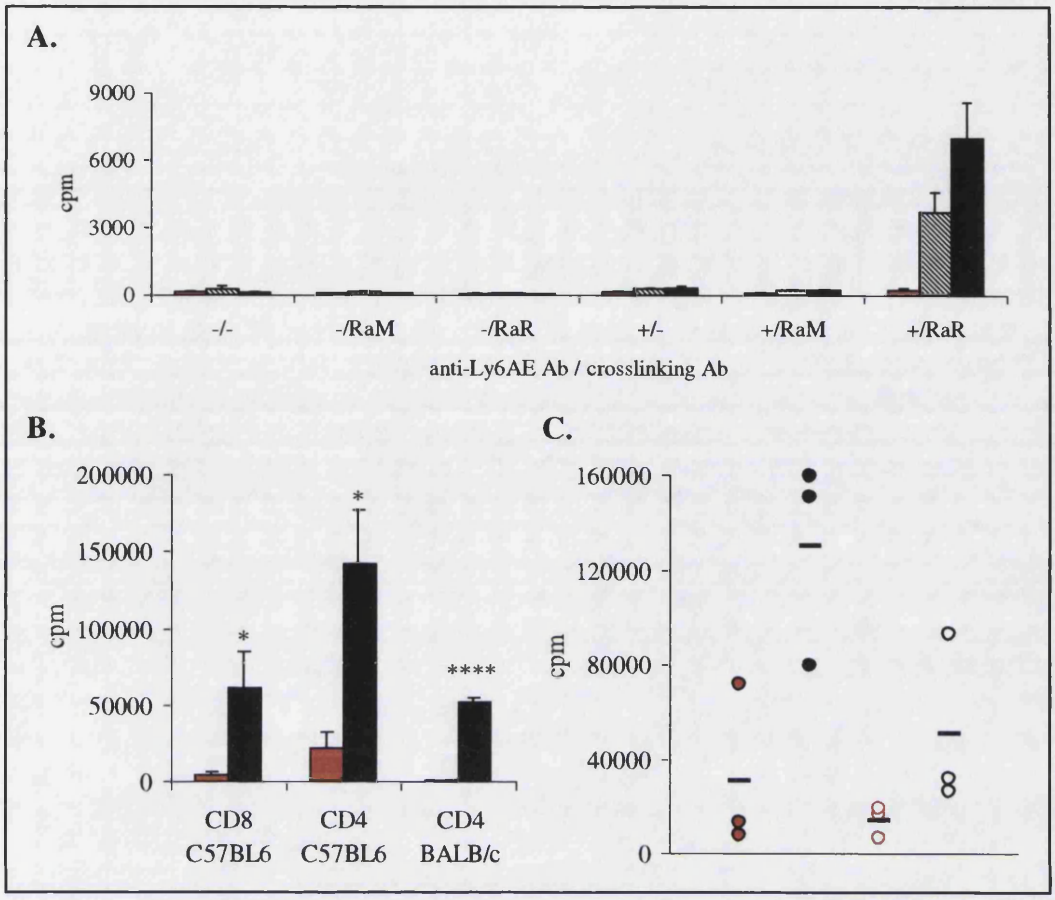


Figure 3.25. Ly-6A/E mediated T cell proliferation *in vitro* is enhanced by LPS *in vivo*.

A. Mice were injected with PBS (■), 10ng (▨)or 10µg (■) LPS i.v. and sacrificed 24 hours later. $CD8^+$ T cells were purified (by negative selection) from LN and incubated on ice for 30 minutes with (+) or without (-) anti-Ly-6A/E antibody, clone D7 (10µl/10^6 cells). 2 x 10^5 cells were cultured in the presence of PMA (5ng/ml) ± a cross-linking (RaR, rabbit anti-rat IgG) or control (RaM, rabbit anti-mouse IgG) antibody diluted 1:100. Data represents the mean of triplicate mice ± s.e.m. Both 2-sample t-tests and linear regression show that the dose response to LPS is statistically significant ($p < 0.05$). **B.** C57BL6 and BALB/c mice were injected with either PBS (■) or LPS (■) i.v. and sacrificed 24 hours later. $CD8^+$ and $CD4^+$ T cells were purified from C57BL6 LN whilst $CD4^+$ T cells were purified from BALB/c LN. In vitro treatment was identical to the procedure described above, however only values ≥ 2000 cpm have been displayed. Data represents the mean of triplicate mice ± s.e.m (p values; *, $p < 0.05$; ****, $p < 0.001$). **C.** Mice were injected with PBS (●) or LPS (●) i.v. and sacrificed 24 hours later. $CD8^+$ T cells were purified from LN and incubated with PBS + 5% FCS (closed circles) or 10mM methyl-β-cyclodextran (open circles) for 30 minutes at 37°C. Subsequent treatment *in vitro* was identical to the previous experiments.

All the assays were pulsed with tritiated thymidine after 48 hours of culture. Proliferation was measured 18 hours later as described previously (2.2.8)

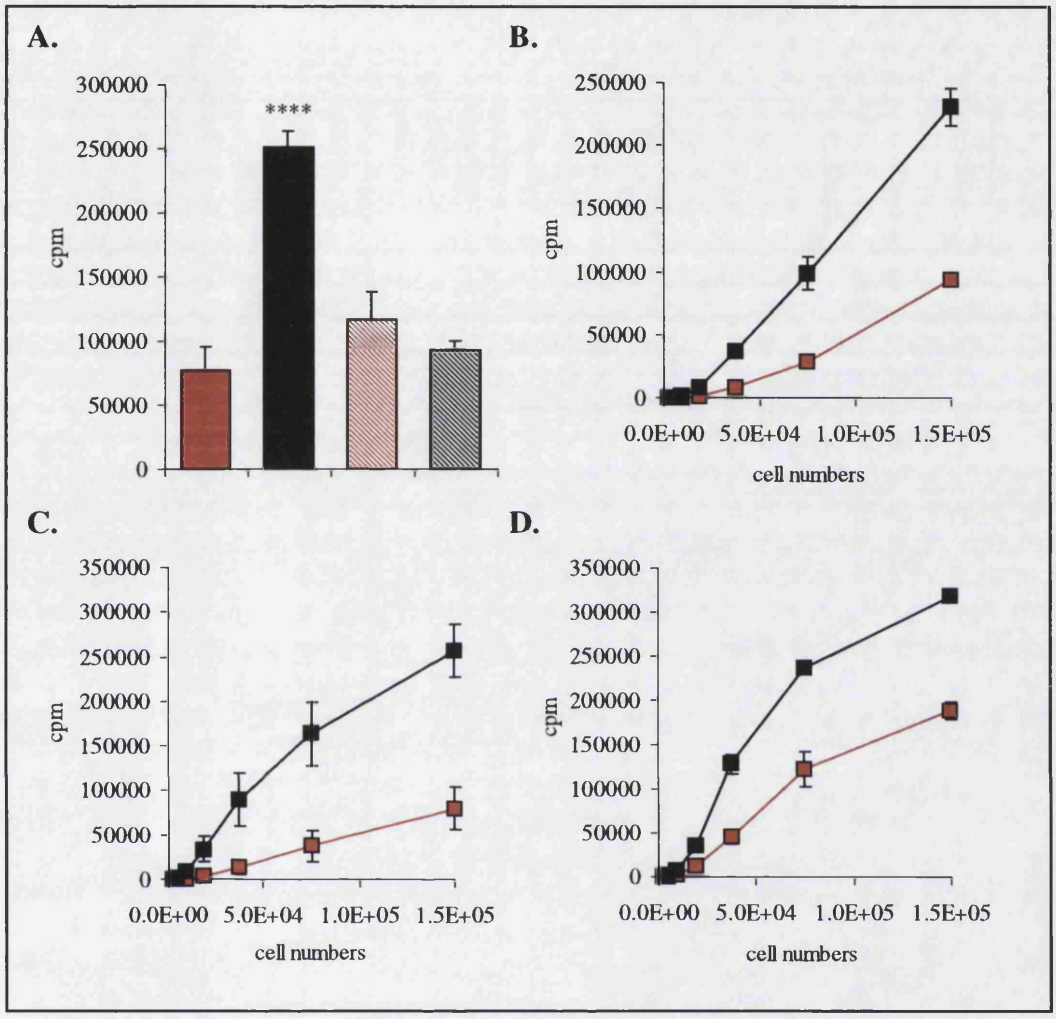


Figure 3.26. LPS 24 hrs *in vivo* enhances the T cell mitogenic proliferative response *in vitro*.

A. Mice were injected with PBS (red) or LPS (black) and sacrificed 24 hours later. LN (filled bars) and spleen (striped bars) were removed and cell suspensions prepared. 1.5 x 10^5 cells per well were cultured with Con A (1µg/ml). **B.** Mice were injected with PBS (red) or LPS (black) and sacrificed 24 hours later. 1.5 x 10^5 LN cells (per well) were serially diluted (1:2) and cultured with 1 µg/ml Con A. Prior to culture $CD4^+$ (**C**) and $CD8^+$ (**D**) T cells were removed from LN preparations. **A-D.** Proliferation was assessed 48 hours later by the addition of tritiated thymidine (see 2.2.8). Data represents the mean of triplicate mice ± s.e.m. (p values; ****, $p \leq 0.001$). Statistical analysis was performed for the highest cell concentrations **B.** $p = 0.001$, **C.** $p = 0.01$, **D.** $p = 0.0001$ and also the lowest cell concentration at which $p < 0.05$ was determined, **B.** 1.88 x 10^4 cells, **C.** 7.5 x 10^4 cells and **D.** 2.34 x 10^3 cells.

since enhanced responses were observed using either CD4-depleted or CD8-depleted responder populations from LPS-treated mice (fig. 3.26C and D.).

Since the Con A response of T cells is dependent on accessory cells, the enhanced proliferation could have been due to alterations in either T cells or APCs. To clarify in which population the alteration lay pure LN T cells derived from PBS or LPS-treated mice were mixed with T-depleted LN cells, also derived from PBS or LPS-treated mice. Thus the four different permutations were 1. control T cells and non-T cells (P:P), 2. LPS-treated T cells and control non-T cells (L:P), 3. control T-cells and LPS-treated non-T cells (P:L) and finally 4. LPS-treated T cells and non-T cells (L:L). There was no difference between the proliferation of groups 1 and 2, however groups 3 and 4 proliferated much more than groups 1 and 2 (N.B. neither purified T cells nor T-depleted cells alone responded to Con A, data not shown). Therefore it would appear that LPS was acting on non-T cell types rather than intrinsically altering the T cells' capacity to respond to Con A (fig. 3.27.). The identity of which cell types may be responsible for augmenting the Con A response will be discussed in the succeeding chapters.

3.3. Summary

In our studies LPS has been observed to induce multiple T cell phenotypic changes *in vivo* and *in vitro*, as summarised in Table 1. Altered surface marker expression was both rapid and transient and observed even when using low doses of LPS. LPS could not act directly on purified T cells *in vitro*. Studies

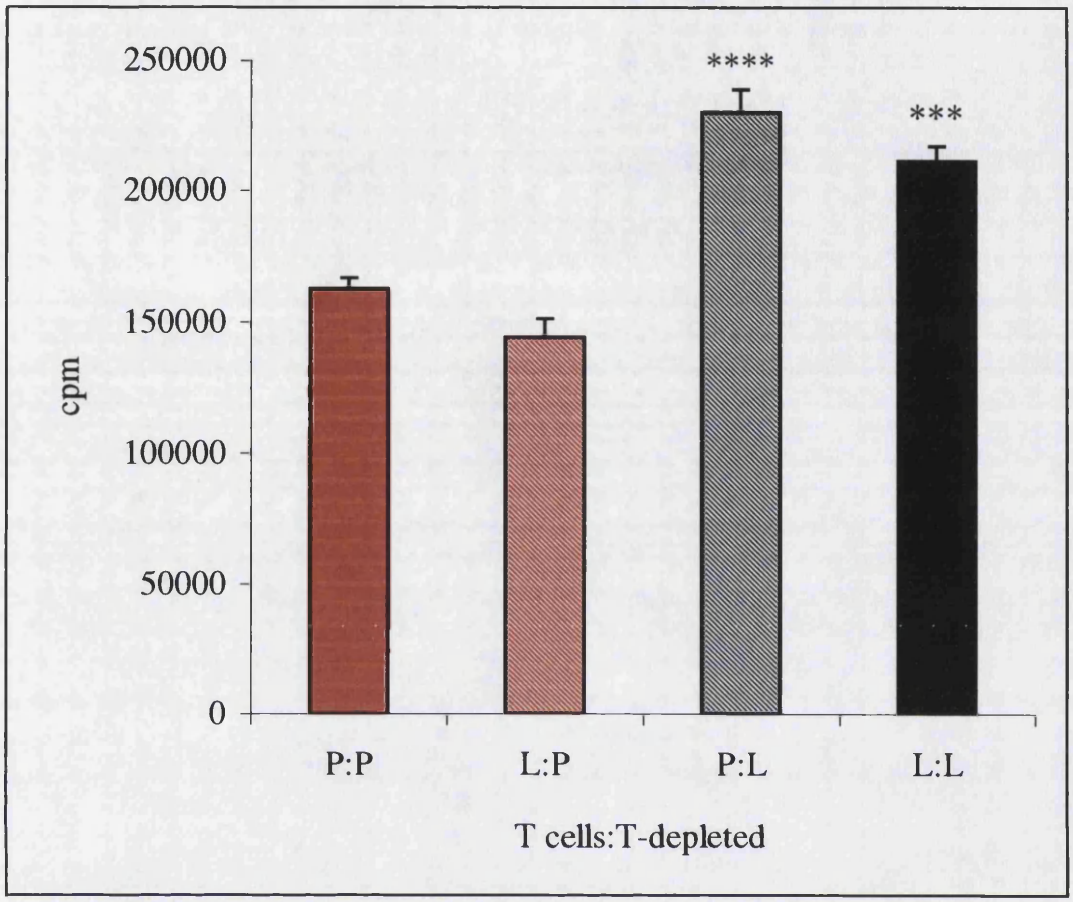


Figure 3.27. LPS *in vivo* does not intrinsically alter the responsiveness of T cells to Con A *in vitro*.

Mice were injected with PBS or LPS i.v. and sacrificed 24 hours later. Pure T cells and T-depleted LN cell suspensions were prepared. 7.5 x 10^4 T cells were mixed with 7.5 x 10^4 T-depleted LN cells to create four different populations (■, T cells and T-depleted cells derived from PBS-injected mice; ▨, T cells derived from LPS-injected mice + T-depleted cells from PBS-injected mice; ▨, T cells derived from PBS-injected mice + T-depleted cells from LPS-injected mice; ■, T cells and T-depleted cells derived from LPS-injected mice.). Mixed cells were cultured with Con A (1μg/ml) for 48 hours before being pulsed with tritiated thymidine. Proliferation was measured 18 hours later as previously described (2.2.8). Data represents the mean of triplicate mice ± s.e.m. (p values; ***, $p < 0.005$; ***, $p < 0.001$).

examining the response *ex vivo* to LPS 1 hour *in vivo* revealed the involvement of non-T cells in modulating T cell phenotype. Cytokines, in particular, seemed to be potent regulators of T cell marker expression when added to T cells *in vitro* directly or as an active component of LPS-stimulated splenocyte supernatant. The importance of cytokines in determining both the resting and LPS-induced T cell phenotype was demonstrated using IFNγ and IFNα/β receptor knockout mice.

Table 3.1. Summary of the T cell phenotypic changes induced by LPS.
Symbols (+) and (-) indicate an increase or decrease, respectively, in surface expression. The symbol (=) means that LPS did not alter the expression level of this molecule on T cells.

		CD69	CD54	CD95	CD86	Ly6C	Ly-6A/E	LPAM-1	CD28
CD8	*in vivo*	++	+	+	++	+	++++	-	=/-
	In vitro	=	+	n.d.	+	+	++++	=	=
CD4	*in vivo*	++	++	+	++	+	++	-	--
	In vitro	=	++	n.d.	+	+	++	=	=

Overall, there seemed to be little difference in the response to specific or allo-antigen by T cells from PBS or LPS-treated mice. At particular time points *in vivo*, LPS did appear to affect the capacity of T cells to respond to allo-antigen. However, the interpretation of these observations was complicated by the use of different techniques to prepare cell suspensions, which often yielded conflicting data.

Whilst LPS *in vivo* appeared not to affect proliferation due to CD3ε cross-linking of T cells, this was not the case when cross-linking Ly-6A/E. Unstimulated T cells only proliferated at very low levels in response to Ly-6A/E cross-linking. However, T cells derived from LPS-treated mice responded several thousand-fold higher than control T cells.

The activation status of T cells, post LPS injection, was further assessed by investigating the mitogenic response to Concanavalin A (Con A). T cells from LPS-treated mice proliferated to Con A several times more than T cells from PBS-treated mice. Experiments showed that LPS was not exerting its effects directly on T cells but rather at the non-T cell level.

3.4. Discussion

We have shown that LPS induces multiple T cell phenotypic changes either after injection into mice or following treatment of splenocytes *in vitro*. However, it should be noted that the LPS preparation used for the *in vivo* studies was able to stimulate $CD8^+$ T cells in TLR-4 deficient mice (C3H/HeJ), albeit with greatly reduced activity. This may have been due to minor contamination in our LPS solution. An alternative viewpoint may be argued that TLR-4 deficient mice may not be as unresponsive to LPS as previously thought. For example, clearance of *E. coli* was 10 times more efficient in B10ScN TLR-4 deficient mice than in the normal B10SnJ (Haziot *et al.*, 2001). Furthermore, the question of whether TLR-2 is required for LPS-signalling is far from resolved. Recently,

Leptospiral LPS has been reported to utilise TLR-2 and not TLR-4 (Werts *et al.*, 2001). Since the LPS preparations used in that study were devoid of LPS-associated proteins, the possibility remains that responses to LPS are not exclusively TLR-4-dependent. In addition, it is conceivable that TLR4-independent mechanisms may exist for low level LPS responses, possibly utilising one of the many newly discovered TLRs. Regardless of the mechanism involved in the minimal response observed in C3H/HeJ mice, it was clear that both the commercially prepared *E.coli* LPS and the laboratory purified *N. meningitidis* LPS solutions potently stimulated murine T cells to alter phenotype in normal mice, implicating TLR4 in this process. By contrast, we were unable to observe similar T cell phenotypic changes when stimulating human peripheral blood lymphocytes with LPS *in vitro* (data not shown).

This is of interest in view of data by Juffermans *et. al.* (2000) showing that injection of LPS i.v. into humans stimulated T cells to increase surface expression of chemokine receptors CXCR4 and CCR5. This difference between *in vitro* and *in vivo* effects of LPS on human T cells are reminiscent of our observations on mouse LN T cells. Thus, despite marked changes in T cell phenotype on LN T cells 24 hours after injection of LPS, treatment of LN T cells with LPS *in vitro* had minimal effects. The implication is that human peripheral blood, like murine LN, is relatively deficient in "accessory" cells that mediate the LPS-induced phenotypic alterations in T cells. Another similarity between our observations in mice and those seen by Juffermans *et. al.* in humans is the kinetics of the phenotypic changes. Both species responded to LPS by rapidly but transiently altering T cell phenotype. Within the parameters of T cell

phenotype, both species exhibited a minimum lag phase of 2 hours. Possibly this indicates a common characteristic of cells from both species for a minimum amount of time to observe changes in surface marker expression.

Indeed, exposure to LPS *in vivo* for 2 hours was not of sufficient duration to detect a response directly *ex vivo*. Subsequent culture of purified T cells resulted in the emergence of a phenotypic response. This indicated that brief LPS-stimulation *in vivo* could condition T cells to subsequently alter phenotype. For example, Ly-6A/E up-regulation may have been triggered *in vivo* but not observed *ex vivo*. Alternatively, T cells may have been stimulated to differentiate into IFNγ-secreting cells, which then exerted paracrine activity *in vitro*.

It seems unlikely that LPS was acting directly on T cells *in vivo*. Nevertheless, LPS-reactive T cells have been previously described (Vogel *et al.*, 1983) and more recently, it has been discovered that T cells express TLR-2 and TLR-4 at the mRNA level (Matsuguchi *et al.*, 2000). In contrast, it has yet to be reported that T cells express CD14. Therefore, LPS was probably exerting its effects on T cells *in vivo* and *in vitro* by an indirect pathway. Our *in vitro* data showing that LPS failed to induce phenotypic activation of purified T cells supports this idea.

Conversion of T cells to an "LPS-stimulated" phenotype *in vitro* was greatly enhanced by the presence of additional cell types that had been stimulated by LPS *in vivo*. Certainly the secretion of cytokines by macrophages and dendritic cells appeared to be an obvious candidate pathway since depletion of these cell

types from splenocytes prior to culture removed T cell-stimulatory activity from the resultant supernatant. Interferons in particular were potent substitutes for LPS *in vitro* and were important for defining the phenotype of both resting and stimulated T cells.

We demonstrated that LPS stimulated both CD4 and CD8 T cells to lose expression of CD28. Whilst the decreased surface expression of CD28 had little effect on the co-stimulatory activity of this molecule (data not shown) recent literature indicates a possible correlation between CD28 downregulation and an increased susceptibility to undergo apoptosis. Walker *et. al.* observed that Jurkat cells that are induced to down-regulate CD28 by CD95-ligation were consequently pre-disposed to undergoing apoptosis (Walker *et al.*, 1998). It has also been reported that IFN-I and IL-15 induced human $CD8^+$ T cells to lose CD28 expression. This was accompanied by an increased sensitivity to activation induced death (Borthwick *et al.*, 2000). Possibly these cytokines induced CD8 T cells to differentiate to a terminal effector phase since perforin expression appears to be more restricted to the CD28 negative $CD8^+$ T cell subset. Similarly, increased CD95 expression may have increased the susceptibility of T cells to apoptosis (Colamussi *et al.*, 2001). Taken together these observations raise the possibility that LPS induces T cells into a semi-active status, poised to respond to antigen but also destined for clearance at the end of the immune response.

LPS also induced both CD4 and CD8 T cells to become LPAM-1 negative. While the functional significance of this was not investigated, other investigators

have demonstrated that blocking LPAM-1 with antibodies to the α_4-integrin chain prevented the trafficking of $CD4^+$ T cells to peripheral tissues. Moreover, inhibition of LPAM-1 binding to its natural ligand (expressed on HEV) resulted in the accumulation of naïve $CD4^+$ T cells and an increased magnitude in the secondary response (as measured by IL-2 secretion) in the spleen (Bradley *et al.*, 1998). These results imply that LPS-induced downregulation of LPAM-1 might alter T cell homing, perhaps altering cell distribution to optimise the possibility of contact with antigen and antigen presenting cells.

For the majority of experiments, LPS was given to mice in the absence of antigen. Therefore, T cells receiving signal 2 in the absence of signal 1 may be alerted to the prospect of antigen stimulation and induced to reside in areas of antigen presentation (such as the lymph node and spleen). Therefore, the "semi-activated" T cells may remain in the secondary lymphoid organs until they are able to respond to antigen or the LPS-induced inflammatory conditions subside. Hence, using this conceptual model, a context for the loss of LPAM-1 expression in response to LPS may be envisaged.

Perhaps the most difficult phenotypic change to interpret was the dramatic increase in Ly-6A/E expression. Whilst the literature describing this molecule is extensive, the role of Ly-6A/E in T cell activation has yet to be fully discerned (see chapter 1.2C.). More recently there has been a growing consensus that GPI-linked molecules found within lipid rafts are involved in T cell activation via the "immunological synapse" (Horejsi *et al.*, 1999; Moran and Miceli, 1998).

Therefore, the expression of very high levels of Ly-6A/E on the surface of T cells could alter the composition of these signalling domains.

Consistent with the idea that Ly-6A/E may interact with signalling molecules on T cells; we confirmed that cross-linking multiple Ly-6A/E molecules in the presence of PMA results in T cell proliferation. Significantly, prior exposure to LPS *in vivo* greatly enhanced T cell activation via Ly-6A/E. We also investigated the possibility that activating T cells via Ly-6A/E was dependent on the association of this molecule with lipid rafts. Incubation with the raft disrupter methyl-β-cyclodextrin decreased the subsequent Ly-6A/E-mediated T cell activation *in vitro*, providing some evidence that this was the case. Further experiments are required to verify the specificity of methyl-β-cyclodextrin.

Another possible consequence of increased Ly-6A/E expression may be increased adhesive properties of T cells. In this respect, T cells overexpressing Ly-6A.2 homotypically aggregated when cultured *in vitro*, suggesting that Ly-6A.2 participates in cell-cell adhesion (English *et al.*, 2000). Such an adhesion role for Ly-6A/E might fit with other phenotypic changes that were observed. In particular, CD86 (B7-2) and CD54 (ICAM-1) are also both known to participate in T cell adhesion, although they are usually perceived as ligands for receptors expressed on T cells. Therefore, increased surface expression of Ly-6A/E, CD86 and CD54 may act together to enhance T cell-T cell adhesion. Indeed, B7 has been reported as an important molecule for T cell homotypic interactions (Wyss-Coray *et al.*, 1993).

Published studies support an important role for CD54 in LPS effects *in vivo*. For example, knockout mice have been used to show that CD54 deficiency results in resistance to the lethal effects of high does of LPS, indicating a link between CD54 and generating an inflammatory response to LPS *in vivo*. Furthermore, experiments with these mice demonstrated that CD54 was required for optimal antigen-specific T cell response, such as mixed lymphocyte reactions (MLR) (Sharpe, 1995). Whether expression of CD54 on T cells, APCs or both was important was not investigated.

Interpretation of our MLR data was complicated by conflicting results. When control T cells responded well to allo-antigen, responder cells from LPS-treated mice either responded equally well or worse than controls. By contrast, sub-optimal MLRs were enhanced by previous exposure of the responder cells to LPS *in vivo*. A similar situation has been described by Spear *et. al.* (1986) investigating human lymphocyte cultures. They observed that LPS enhanced only low cytotoxic responses while having little effect on naturally high responses. It has also been reported that addition of LPS *in vitro* enhances "weak" murine MLRs (Forbes *et al.*, 1975). Perhaps under limiting conditions, increased surface marker expression (e.g. CD54) on T cells due to LPS *in vivo* enhanced their ability to respond to allo-antigen *in vitro*. Alternatively perhaps the conflicting observations reflect the precarious balance between activation and apoptosis of LPS-stimulated T cells.

The opposing data is more likely a product of using two different methods for the preparation of cell suspensions from lymphoid tissue (as discussed previously).

Indeed, the confusing results illustrated clearly the limitations of using *in vitro* assays to investigate the *in vivo* response of T cells to LPS. Both techniques used for the preparations of lymphocyte suspensions by necessity involved the disruption of lymphoid tissue and hence the destruction of the spatial relationship between T cells and APC. Furthermore, the inflammatory signals generated *in vivo* cannot be truly replicated *in vitro*. Therefore removing LPS-stimulated T cells from the *in vivo* inflammatory context may restrict observations of functional differences *in vitro*.

Nevertheless, LPS stimulation *in vivo* consistently had a dramatic effect on the T cell mitogenic response *in vitro*. LPS has previously been described as enhancing T cell responses to Concanavalin A (Con A) *in vitro* (Forbes *et al.*, 1975), although the identity of the cells responsible remained undefined. We report here that injection of LPS does not alter the intrinsic responsiveness of T cells to Con A. Rather, LPS exerts its effect on other cell types, possibly by increasing the co-stimulatory capacity of B cells and dendritic cells. The subsequent chapters will discuss how these cell types respond to LPS and their role in T cell activation.

Chapter 4: The phenotypic and functional alteration of dendritic cells by LPS.

4.1 Introduction

Dendritic cells (DC) undergo a series of changes upon encountering infection-associated stimuli, characterised by activation, migration, maturation and finally, apoptosis (see chapter 1.3). The following paragraphs detail how LPS modulates this process.

LPS is thought to activate dendritic cells via a receptor complex including CD14 and TLR-4. DC are also believed to self-regulate LPS receptor expression since CD14 expression is up-regulated in response to LPS, thus inducing a positive feedback loop (Mahnke *et al.*, 1997). CD14 has also been reported to be involved in the recognition of LPS by human peripheral blood (PB) dendritic cells *in vitro*. However, since dendritic cells derived from PB do not express CD14 on their membrane they appear to be able to compensate for this by utilising soluble CD14 present in serum (Verhasselt *et al.*, 1997).

Upon LPS-recognition, DC rapidly respond by down-modulating their intrinsic migratory ability and becoming strongly adherent. Thus during this initial phase of activation, DC seem to be maximising antigen uptake by stopping at the site of inflammation and simultaneously increasing endocytic activity (Granucci *et al.*, 1999). This has been demonstrated *in vitro* by stimulating a growth-factor dependent DC line (D1) with LPS and analysing the "bimodal" behaviour LPS-

induced maturation. For example, D1 cells up-regulated CCR1 chemokines (i.e. MIP-1α and MIP-2) mRNA levels within 30 minutes of LPS treatment *in vitro*. Thus, if dendritic cells respond similarly *in vivo*, LPS may indirectly amplify the innate response by inducing the recruitment of additional DC, macrophages and neutrophils to the site of inflammation (Foti *et al.*, 1999). However, within 3-4 hours *in vitro*, the effects of LPS on DC were reversed. D1 cells downmodulated CCR1 mRNA levels and antigen uptake activity and showed recovery of migratory activity (Foti *et al.*, 1999; Granucci *et al.*, 1999).

In vivo evidence also suggests that LPS may promote DC migration and the accumulation of inflammatory cells *in vivo*. LPS injected i.v. induced massive loss (> 95%) of murine MHC class II^+ leukocytes from the heart and kidney but a concomitant increase in macrophages and neutrophils. DC migration from the peripheral tissues appeared to involve inflammatory cytokines since administration of polyclonal TNF-α antiserum before the injection of LPS prevented DC loss. Systemic administration of recombinant TNF-α and IL-1α also induced the migration of cardiac and renal DC (Roake *et al.*, 1995).

Furthermore, *in vivo* studies imply that migratory DC stimulated by LPS (or LPS-induced cytokines) relocate to lymphoid tissues such as the spleen and lymph nodes. Using mAbs to processed hen egg lysozyme (HEL), Sousa *et al.* were able to monitor the localisation of antigen-bearing DC in the spleen. They found that the systemic administration of HEL preparations containing LPS contaminants induced the accumulation of antigen-bearing DC within the periarteriolar lymphoid sheaths (PALS). In the absence of LPS-responsiveness

(i.e. in C3H/HeJ primed mice), no such accumulation of DC was observed (Sousa and Germain, 1999).

Experiments by MacPherson *et al* provide compelling evidence that endotoxin promotes the trafficking of DC from peripheral tissues to lymph nodes via afferent lymphatics. Briefly, normal and mesenteric-lymphadenectomised (MLNX) rats were cannulated and injected i.v. with LPS. The DC content within the lymph collected from MLNX rats rose 8-15 fold within 10-15 hours after LPS injection. However, efferent mesenteric lymph collected from normal rats over a 48 hour period contained only a low frequency of DC even after LPS-injection. Therefore, LPS appeared to be stimulating DC release from the periphery which were then retained within the mesenteric lymph node. Co-injection of LPS and anti-TNF-α mAb cancelled the effects of LPS (MacPherson *et al.*, 1995). Intradermal administration of TNF-α by itself has been demonstrated to provoke Langerhans cells to redistribute to the lymph nodes (Cumberbatch *et al.*, 1992). Hence TNF-α may act indirectly or directly to induce DC migration.

Accompanying LPS or cytokine-induced DC migration is the reversal of "immature" characteristics and the adoption of a "mature" phenotype. Evidence in support of this idea is the down-regulation of macropinocytosis and the increase in surface expression of co-stimulatory molecules such as CD40, CD54 and B7 on human PBMC derived DC cultured with either LPS or TNF-α *in vitro* (Sallusto *et al.*, 1995). LPS has also been reported not only to dramatically augment MHC class I and II expression, but also to increase the half-life of MHC

class II molecules thus stabilising peptide-MHC complexes (Rescigno *et al.*, 1999). Peptide loading onto MHC class II molecules may also be enhanced by LPS, resulting in more efficient antigen presentation thus amplifying the adaptive immune response (Manickasingham and Sousa, 2000).

One possible mechanism for the regulation of the adaptive immune response by mature DC is the production of T and B cell stimulatory cytokines such as IL-12 and IFNs. For example, human monocyte-derived $CD83^-$ $CD1a^+$ DC transiently secrete IL-12 in response to LPS but quickly become "exhausted" *in vitro*. Indeed, DC in their "final" stage of LPS-induced maturation are poor IFNγ responders and IL-12 inducers (Kalinski *et al.*, 1999). IL-12 in particular appears to have an influential role in determining T helper cell polarisation. Consequently the kinetics of cytokine production by DC *in vitro* may have a profound effect on the $CD4^+$ T cell cytokine response to antigen. To demonstrate this Langenkamp *et al.* cultured "immature" monocyte-derived DC with LPS for 8 or 48 hours *in vitro*, after which they were pulsed with antigen and cultured with autologous $CD4^+$ T cells. DC that had been pre-treated with LPS for only 8 hours actively secreted large amounts of IL-12 and preferentially stimulated the production of IFN-γ secreting cells. Conversely, DC that had been pre-treated for the longer period of 48 hours produced comparably little IL-12 and selectively promoted the production of IL-4. Thus, mature DC secreting IL-12 generated a primarily T_H1 driven response, whilst "exhausted" DC generated a T_H2-type response (Langenkamp *et al.*, 2000).

Finally, DC homeostasis is thought to be restored by apoptosis. De Smedt *et al.* (1996) observed that 48 hours after injection of LPS into BALB/c mice, DC numbers were dramatically decreased within the spleen. Whilst this was initially interpreted as reflecting the migration of DC from the spleen, subsequent immunohistology of spleen sections revealed that LPS significantly increased the number of $TUNEL^+$ (apoptotic) $CD11c^+$ cells. Thus, LPS stimulation alone was sufficient to induce DC to undergo programmed cell death. TCR-transgenic mice were injected with LPS with or without antigen to assess the effect of activated T cells on DC survival. In the presence of antigen and LPS the apoptosis of DC was delayed (but not prevented) by at least 8 hours compared to LPS alone (De Smedt *et al.*, 1998). Thus, it appears that the death sentence is inevitable if homeostasis is to be resumed.

In conclusion, LPS may induce different functional states of maturation. Initially, it seems that DC are alerted by LPS to cease movement through peripheral tissues, recruit innate cells and ingest antigen. This phase is only brief since DC rapidly lose their phagocytic activity and resume their migratory behaviour. The outcome is a mass exodus of DC from peripheral tissues to the T cell areas of lymphoid tissues. Meanwhile, DC acquire the characteristics of full maturity: high level expression of co-stimulatory molecules and potent antigen presentation that are important factors for the stimulation of naïve T cells. Thus, LPS-stimulation of such an essential cell type has important consequences on both the innate and adaptive immune system. Hence, the termination of activated DC is a necessary regulatory final phase of the inflammatory response to LPS.

The aim of our experiments was to investigate the effect of LPS on antigen presenting cells *in vivo*, specifically its effect on the activation and maturation of dendritic cells. Our data also indirectly addressed how LPS *in vivo* may have affected two other phases of the DC life cycle: migration and apoptosis.

4.2 Results

4.2A. The effect of LPS on the stimulatory capacity of antigen-presenting cells.

Our initial approach to investigate the effects of LPS *in vivo* on APC was to assess the stimulatory capacity of allogeneic splenocytes in MLRs. BALB/c mice were injected with either PBS or LPS and sacrificed at various time-points from 30 minutes to 24 hours post injection. The splenocyte suspensions derived from these mice were irradiated and used to stimulate the proliferation of C57/BL6 lymph node responder cells *in vitro*. Priming with LPS *in vivo* appeared to have a bi-phasic effect on the potency of splenocyte stimulators *in vitro* (fig. 4.01.).

Spleen cells obtained from mice only 30 minutes after injection of LPS had an enhanced ability to stimulate an MLR *in vitro*. Similarly, a longer period of LPS-stimulation *in vivo* (6 hours) also enhanced the subsequent allogeneic response *in vitro*. However, splenocytes derived from mice treated with LPS 24 hours earlier were poor stimulators compared to controls.

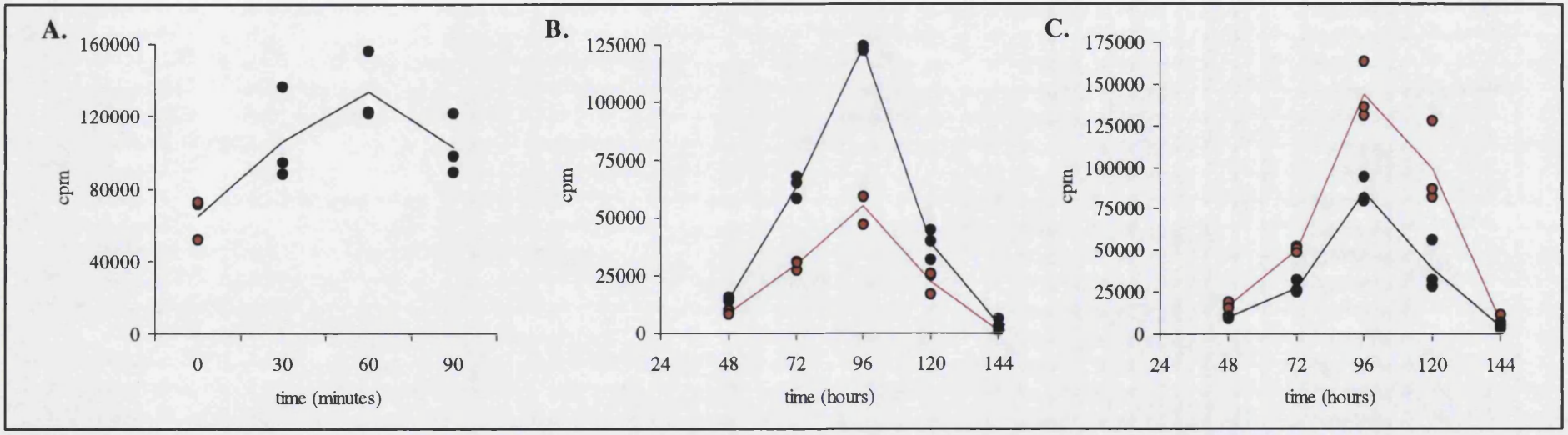


Figure 4.01. The effects of LPS *in vivo* on splenocyte stimulator function *in vitro* is bi-phasic.

BALB/c mice were injected i.v. with PBS or LPS and sacrificed **A.** between 30 and 90 minutes later, **B.** 6 hours later or **C.** 24 hours later. Spleens were excised and single cell suspensions prepared. 5 x 10^5 irradiated (3000 RAD) splenocytes were cultured with 1.5 x 10^5 C57BL/6 LN responder cells. **A.** Assays were pulsed with tritiated thymidine (2.2.8) after 96 hours in culture. **B.** & **C.** Assays were pulsed with tritiated thymidine every 24 hours (between 48 and 144 hours in culture) and the plates were frozen 18 hours later. Upon thawing, proliferation was determined using a scintillation counter. Circles (●, PBS; ●, LPS) show proliferation data (cpm) derived from triplicate cultures of individual stimulator populations while lines show mean values for groups of 3 stimulator populations (—, PBS; — , LPS) and their corresponding mean counts per minute (cpm), —, PBS; —, LPS. Statistical analysis (2-sample t-test) was performed on the values from assays pulsed after 96 hours in culture: **A.** 30', $p > 0.05$; 60', $p < 0.01$; 90', $p < 0.05$; **B.** $p < 0.001$; **C.** $p < 0.01$.

Similar trends were reflected in experiments using the antigen-specific DO11.10 system. BALB/c mice injected with PBS or LPS were sacrificed either 6 or 24 hours later. Splenocytes derived from these mice were pulsed with $OVA_{323-329}$ peptide and cultured with DO11.10 LN responder cells. Stimulator function was dramatically lower for splenocytes derived from mice injected with LPS 24 hours previously compared to control stimulators (fig. 4.02C.). Splenocytes from mice treated with LPS 6 hours earlier were also relatively poor stimulators of the $CD4^+$ T cell antigen-specific response (fig. 4.02B.). However using a lower dose of peptide reversed this observation. Under these conditions, LPS administered i.v. or i.p. 6 hours before sacrifice enhanced the stimulatory capacity of splenocytes by 3 to 5-fold respectively (fig. 4.02A.). The implication was that splenocytes from LPS-treated mice were more efficient at activating T cells under conditions when the available antigen was limiting.

In summary, LPS stimulation *in vivo* altered both the response to allo-antigen and the $CD4^+$ T cell antigen-specific response *in vitro*. The kinetics of the effect of LPS *in vivo* could be separated into two phases: an initial "adjuvant" effect followed by a severe loss of stimulator function.

4.2B. The effect of LPS on dendritic cell phenotype.

Since DC have been reported to be the principal stimulators of MLRs in mice (Steinman *et al.*, 1983), we investigated whether changes in the stimulatory activity of spleen cell populations induced by LPS reflected alterations in DC. Initially, the expression of co-stimulatory molecules on DC was analysed at

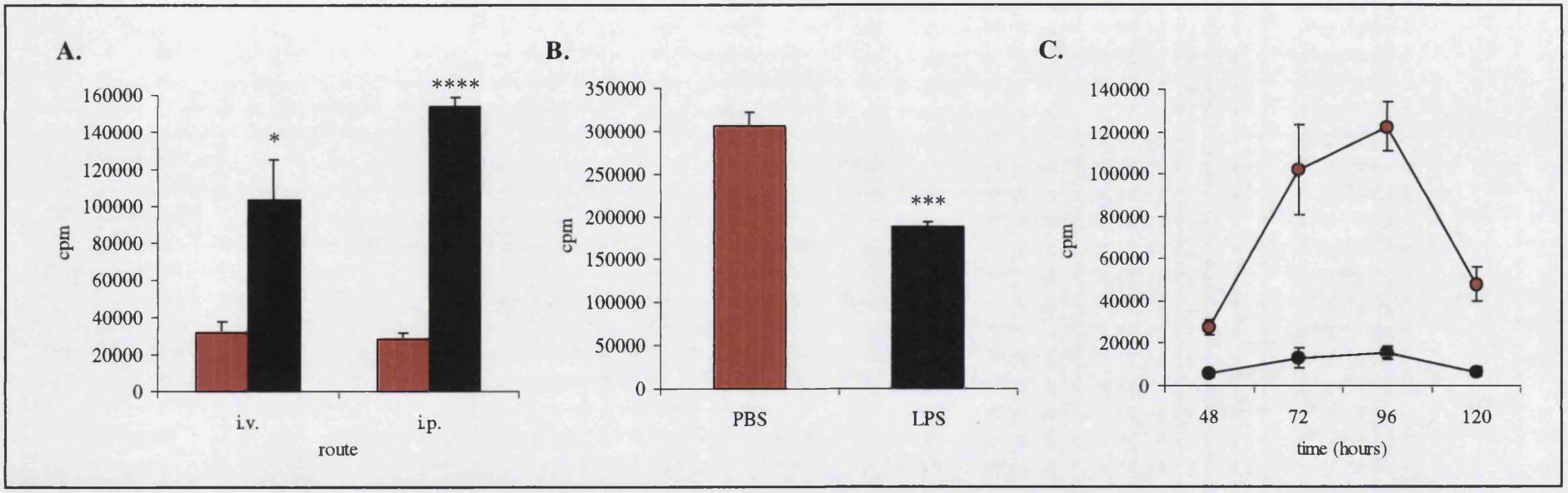


Figure 4.02. LPS *in vivo* affects the capacity of splenocytes to stimulate antigen-specific responses *in vitro*.

BALB/c mice were injected i.v. (**A-C.**) or i.p (**A.** only) with PBS or LPS and sacrificed **A-B.** 6 hours later or **C.** 24 hours later. Spleens were excised and single cell suspensions prepared. 5 x 10^5 irradiated (3000 RAD) splenocytes were cultured with 1.5 x 10^5 DO11.10 LN responder cells in the presence of $OVA_{323-329}$ peptide (**A.** 0.05μM/ml, **B.** & **C.** 20μM/ml). The assays were pulsed with tritiated thymidine (2.2.8) after **A.** 96 hours, **B.** 72 hours, & **C.** between 48 to 120 hours in culture. Plates were harvested 18 hours later and proliferation was subsequently determined using a β-scintillation counter. Data represents the mean proliferation of triplicate stimulator populations; ●, PBS; ●, LPS. Statistical analysis (2-sample t-test) was performed for peak values: **A- B.** *,$p < 0.05$; ***, $p < 0.005$; ****, $p < 0.001$; **C.** $p < 0.001$.

different time points post LPS-injection. However, the large reduction in splenocyte stimulator function 24 hours post injection did not correlate phenotypically with the activation status of DC. On the contrary, cell surface molecules typically associated with the maturation of DC and the acquisition of enhanced APC function were significantly up-regulated by LPS *in vivo* (fig. 4.03.). For example, CD54, CD40 CD86, CD80, MHC class I and MHC class II surface expression were markedly increased following 24 hours after injection of LPS. In addition expression levels of Ly-6A/E, CD95 and CD69 were also elevated.

Furthermore, changes in DC phenotype by LPS *in vivo* were not bi-phasic (fig. 4.04A.). LPS-induced phenotypic changes were detectable as early as 6 hours post injection and were maintained up to and including 24 hours post injection. The expression of the majority of surface markers investigated gradually increased over 24 hours (e.g. CD95 and Ly-6A/E) although there were some exceptions (e.g. CD40 and CD69).

LPS also induced a reduction in the percentage of $CD11c^+$ DC scoring positive for CD8 or CD4 expression (fig. 4.03.). For $CD4^+$ DC, this apparent reduction may have resulted from down-regulation of CD4 expression. In contrast, the majority of DC expressing even low levels of CD8 seemed to have disappeared. CD11c was in itself down-regulated both in terms of percentage and the intensity of surface expression. Low doses of LPS were able to induce multiple phenotypic changes including the down-regulation of CD11c, CD8 and CD4 expression (fig. 4.04B.). In contrast to the effects of LPS *in vivo*, however, a

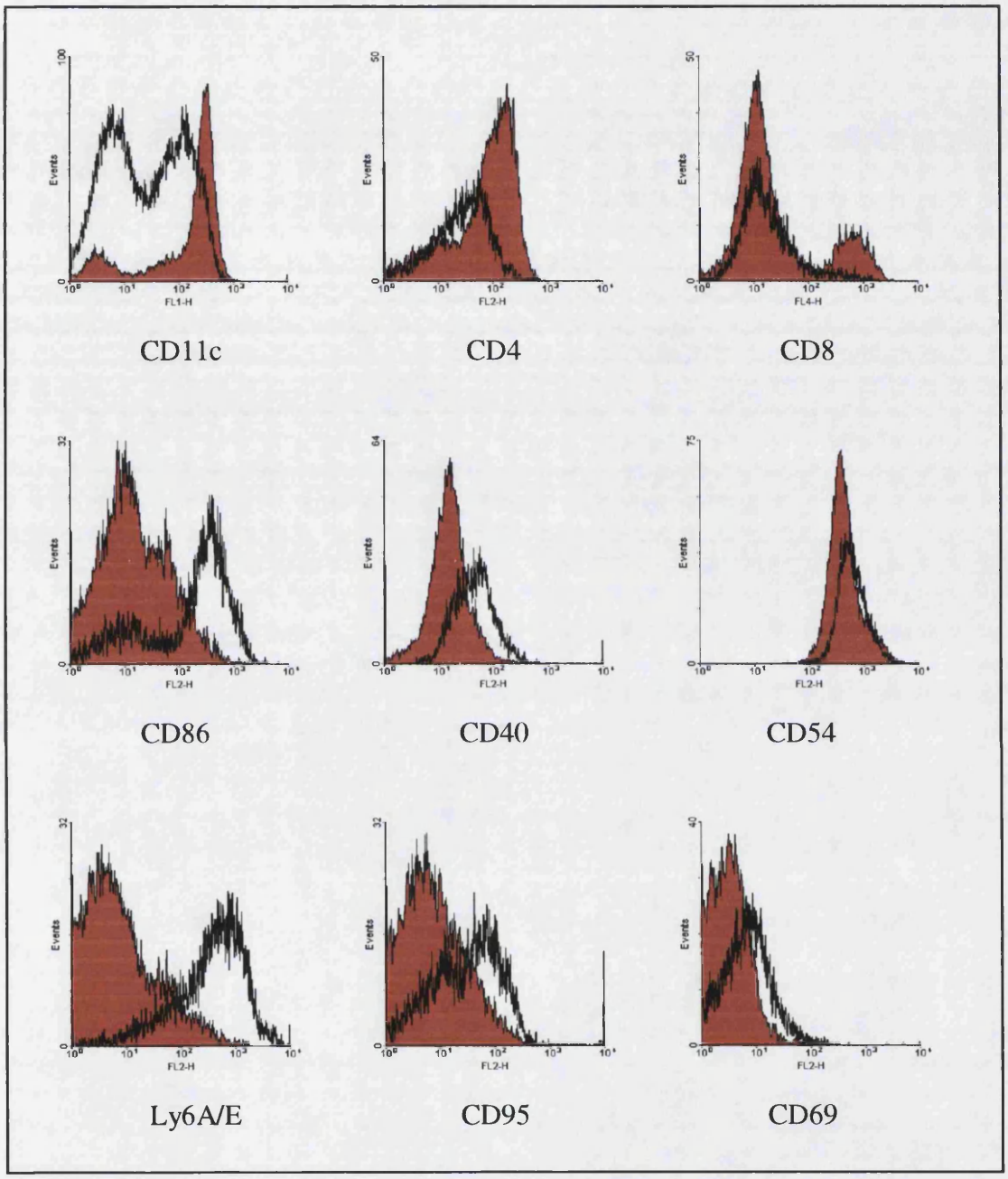


Figure 4.03. LPS induces multiple phenotypic changes on $CD11c^+$ dendritic cells.

BALB/c mice were injected with PBS or LPS and sacrificed 24 hours later. Splenocyte suspensions were enriched for DC (2.2.7) prior to staining with antibodies (anti-CD11c-FITC, anti-CD8-Cy5, anti-CD4-Cy5, anti-CD86-PE, anti-CD40-PE, anti-CD54-PE, anti-Ly6A/E-PE, anti-CD95-PE and anti-CD69-PE). Cells were visualised by flow cytometry. Histograms represent either the surface expression of CD11c on the DC-enriched cell populations (upper left) or the expression of molecules on $CD11c^+$-gated dendritic cells derived from PBS (■) or LPS (—) injected mice.

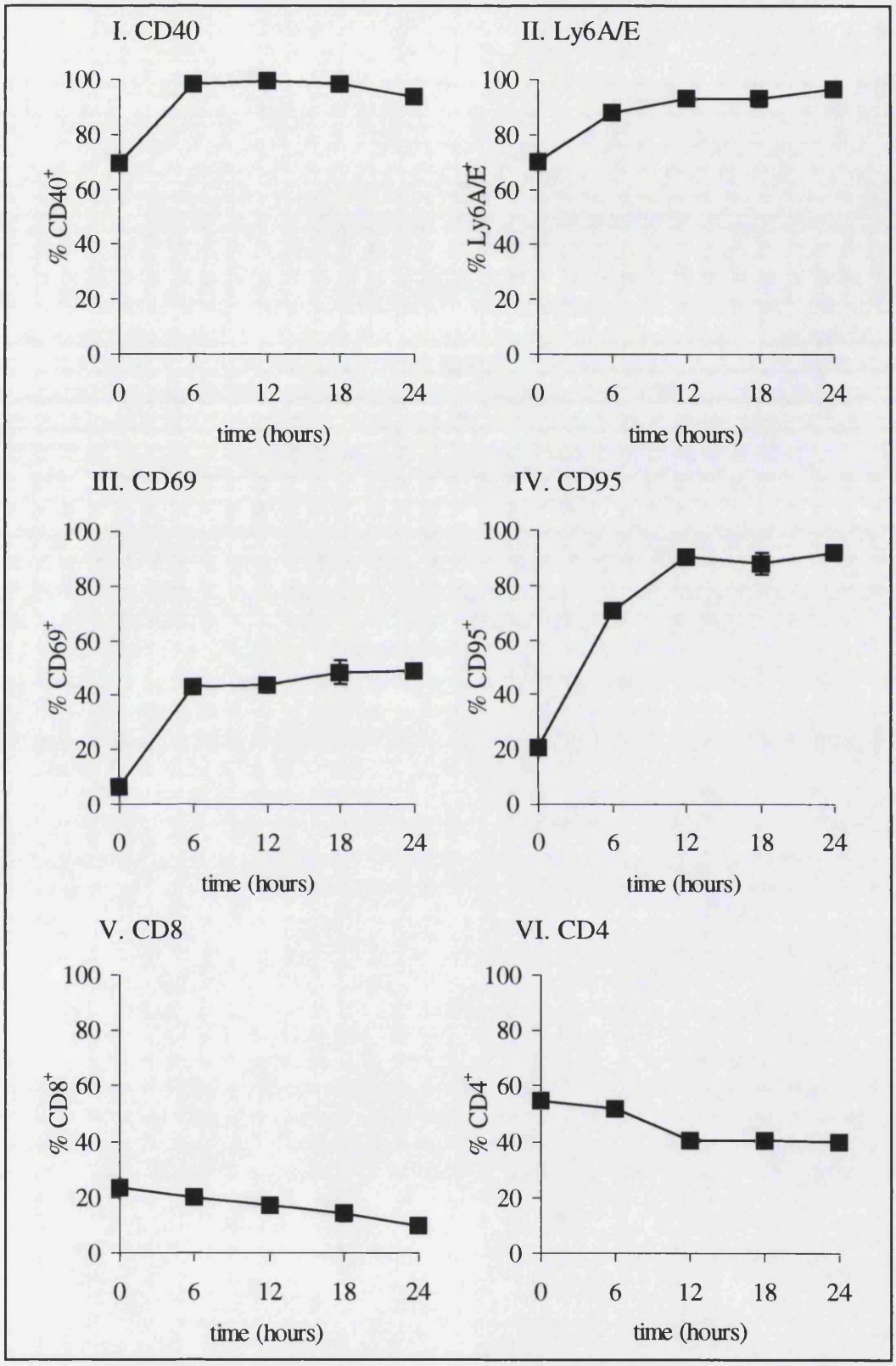


Figure 4.04A. LPS induces phenotypic changes on CD11c$^+$ dendritic cells between 6 and 24 hours post injection.

Mice were injected with PBS (i.e. 0) or LPS and sacrificed between 6 and 24 hours later. Splenocyte suspensions were enriched for DC (2.2.7) prior to staining with antibodies. Cells were visualised by flow cytometry and data was analysed by gating on CD11c$^+$ cells. Data represents the average percentage of triplicates ± s.e.m. Statistical analysis was performed using 2-sample t-tests (earliest time point at which $p < 0.05$; V. 12 hours, I-III. & VI. 6 hours).

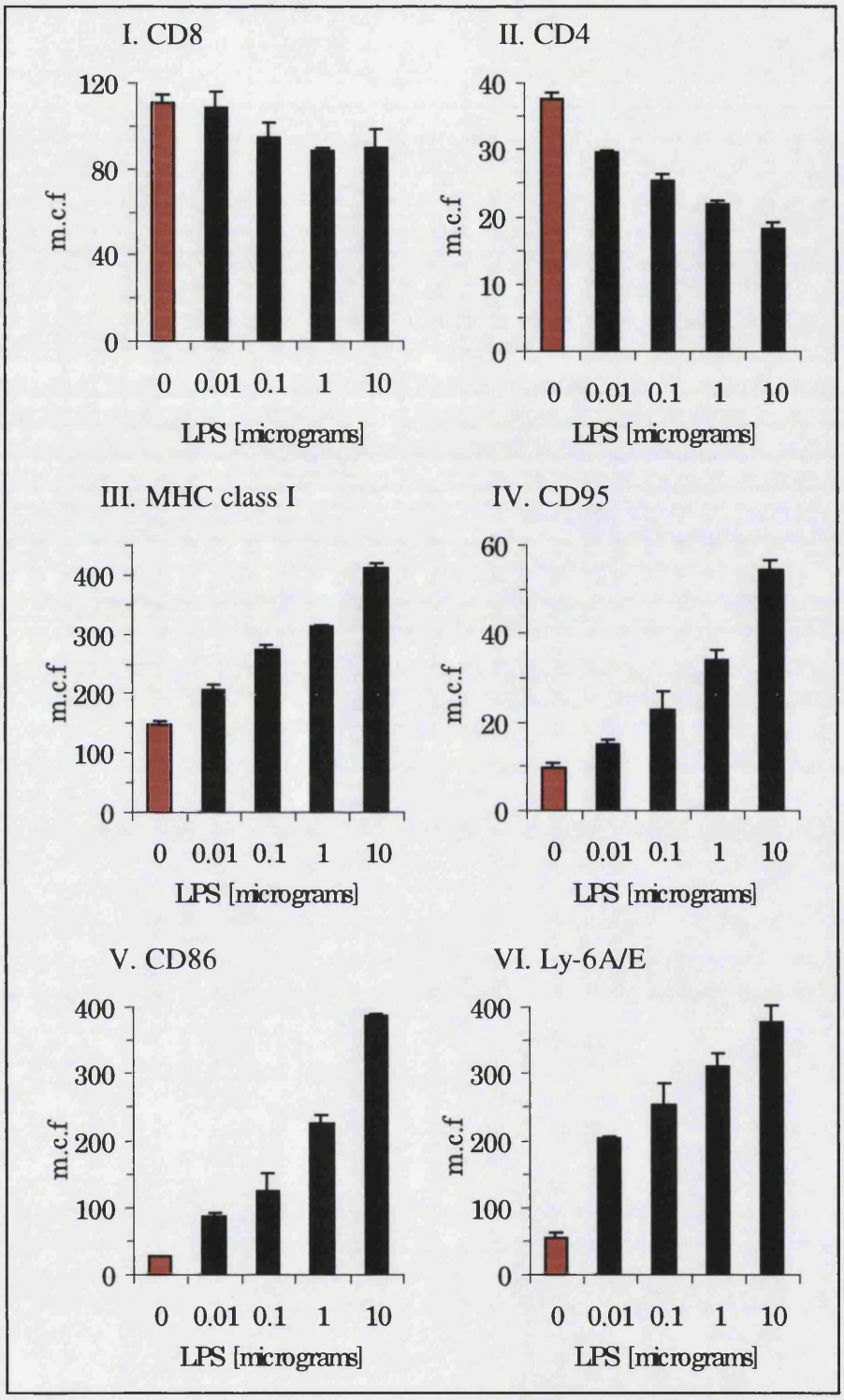


Figure 4.04B. Phenotypic changes on CD11c$^+$ dendritic cells are induced by low doses of LPS.

Mice were injected with either 0 μg LPS (i.e. PBS ■) or between 10ng and 10μg LPS (■) and sacrificed 24 hours later. Splenocyte suspensions were enriched for DC (2.2.7) prior to staining with antibodies. Cells were visualised by flow cytometry and data was analysed by gating on CD11c$^+$ cells. Data represents the average "mean channel fluorescence" (m.c.f.) of triplicate mice ± s.e.m. Statistical analysis was performed using linear regression (m.c.f. v $\log_{10}$LPS; III. $p < 0.005$; I. - II. & IV - VI. $p < 0.001$).

high concentration of LPS added directly to purified DC *in vitro* did not affect CD11c expression. The same was true for CD8, CD4 and Ly-6A/E expression on $CD11c^+$ DC (fig. 4.05.). These observations imply that LPS was not inducing these phenotypic changes *in vivo* by acting directly on mature DC.

4.2C. The effect of LPS on DC secretion of cytokines.

To determine whether LPS treatment *in vivo* affected the secretion of cytokines by DC, splenic $CD11c^+$ DC were purified at different times after LPS injection, placed in culture overnight and secreted cytokines assayed by ELISA. We chose to study only a small selection of cytokines, including IL-12, IL-18, TNF-α and IFN-γ, since LPS was expected to stimulate the secretion of cytokines involved in polarising T_H1 responses. Consistent with this, *in vivo* exposure to LPS for only 1 hour was sufficient to increase the secretion of IL-12p40, although not IL-18, by purified DC cultured for 18 hours *in vitro*. Splenic $CD11c^+$ DC derived from LPS-treated mice also secreted 3.5-fold more IFNγ and 3.5-fold less TNF-α than DC derived from PBS-treated mice (fig. 4.06B.). The reduction in TNF-α secretion by DC from LPS-treated mice was surprising, given that LPS is known to induce the production of this cytokine *in vivo* (Engelberts *et al.*, 1991). Furthermore, when purified DC were treated with LPS *in vitro*, these cells secreted increased amounts of TNF-α (fig. 4.06C.)

In considering how to explain the discrepancy between the *in vivo* and *in vitro* effects of LPS on TNF-α-secretion, we explored the possibility that *in vivo* phenotypic changes could influence subsequent cytokine secretion, since these

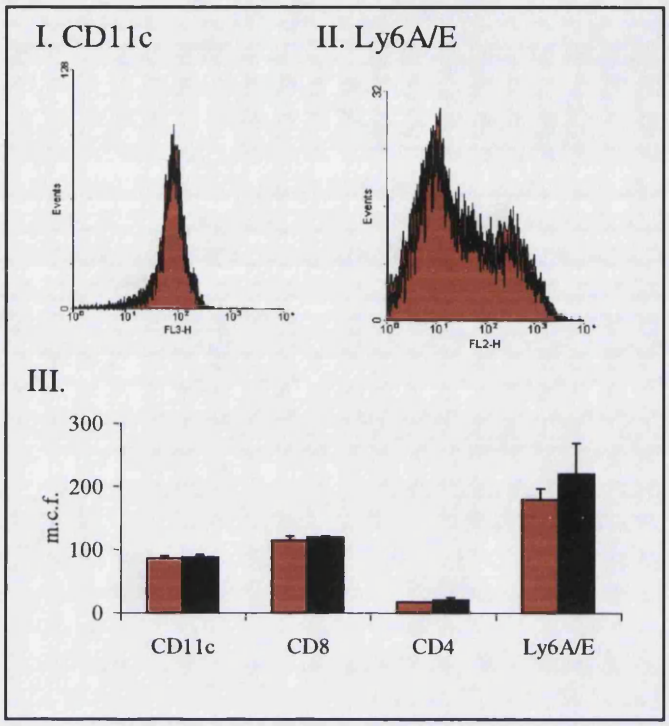


Figure 4.05. LPS does not induce phenotypic changes on purified DC *in vitro*.

Splenic DC were prepared as described previously (2.2.7C). 1.5 x 10^6 DC were cultured with PBS, or LPS (10µg/ml) for 24 hours. DC were harvested and stained for anti-CD11c, anti-Ly-6A/E, anti-CD8 and anti-CD4. Cells were visualised by flow cytometry. I. Histogram overlay of CD11c expression on PBS (■) or LPS (—) treated splenic DC. II. Histogram overlay of Ly6A/E expression on $CD11c^+$ gated on PBS (■) or LPS (—) treated splenic DC. III. DC cell surface marker expression. Data represents the average mean channel fluorescence (m.c.f.) of triplicates ± s.e.m. CD8, CD4 and Ly-6A/E m.c.f. was determined by gating on $CD11c^+$ cells treated with either PBS (■) or LPS (■).

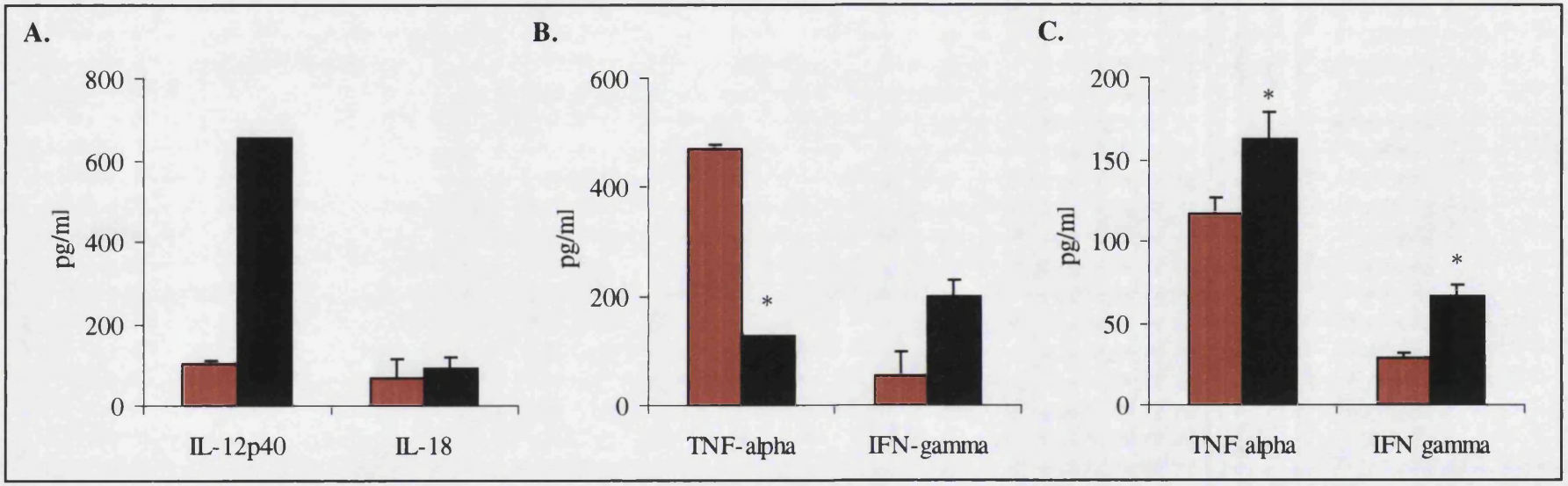


Figure 4.06. The effect of LPS on the secretion of cytokines by $CD11c^+$ dendritic cells.

Mice were injected with PBS or LPS and sacrificed **A.** 1 hour and **B.** 6 hours later. Splenic $CD11^+$ dendritic cells were prepared as described previously (2.2.7C) and cultured overnight at a concentration of 1×10^6 cells/ml. **C.** Splenic DC were prepared from uninjected mice and incubated for 24 hours with either PBS or 10µg LPS. **A-C.** DC supernatant was harvested and the cytokine content was analysed by ELISA. Data represents the mean of duplicates ± s.e.m. (■, PBS; ■, LPS; *, $p < 0.05$).

changes did not occur after *in vitro* treatment with LPS. Specifically, we tested the effects of cross-linking Ly-6A/E on the surface of DC. As shown in fig. 4.07., cross-linking of Ly-6A/E reduced secretion of TNF-α by DC from both control and LPS-treated mice. These results are consistent with the idea that up-regulation of Ly-6A/E can lead to down-regulation of TNF-α production, perhaps through its interaction with a natural ligand(s).

The secretion of cytokines by DC after a very brief exposure to LPS *in vivo* raised the possibility that these cells could potentially be responsible for the T cell-stimulatory activity present in the supernatant of spleen cells from mice injected 1 hour before with LPS. Indeed, supernatant harvested from DC derived from LPS-treated mice potently up-regulated surface markers on purified T cells *in vitro* (fig. 4.08.). Interestingly, control supernatant generated by DC from PBS-treated mice was also able to weakly stimulate T cells to alter their phenotype. Thus, manipulation alone *in vitro* was sufficient to induce partial activation of dendritic cells.

4.2D. The effect of LPS *in vivo* on the stimulatory capacity of purified DC *in vitro.*

We have reported that exposure to LPS *in vivo* for 24 hours led to loss of stimulator function. However, phenotypic analysis of DC at this time point showed that they were highly activated by LPS stimulation. Up-regulation of co-stimulatory molecules and MHC class I and MHC class II by LPS should theoretically confer an increased capacity to stimulate immune responses *in vitro.*

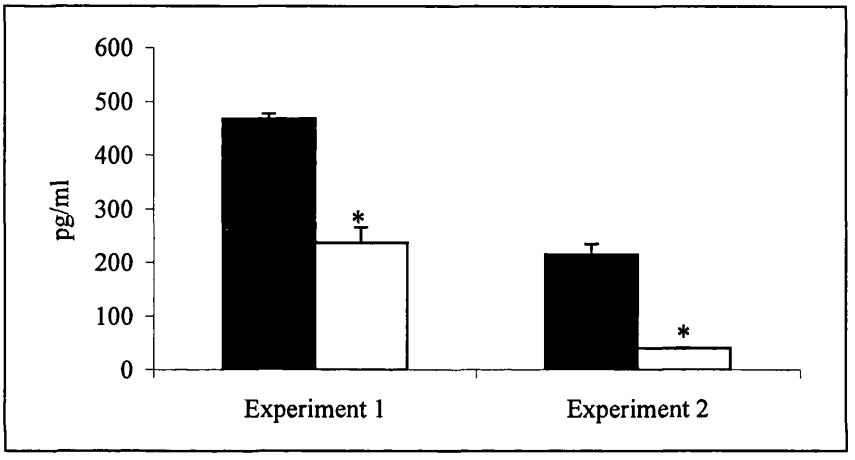


Figure 4.07. The effect of Ly6A/E cross-linking on TNF-α secretion by DC *in vitro*.

Splenic DC were prepared as described previously (see 2.2.7C) and incubated on ice for 30 minutes with PBS, ■ or anti-Ly6A/E mAb, □. 2 x 10^5 $CD11c^+$ DC per well were cultured in the presence of PMA (5ng/ml). Supernatant was harvested 48 hours later and the TNF-α content was determined by ELISA. Data represents the mean of duplicates ± s.e.m. Statistical analysis was performed using 2-sample t-tests individual experiments (p values: *, $p \leq 0.05$) and a paired t-test to assess the statistical significance of data combined from both experiments ($p = 0.002$).

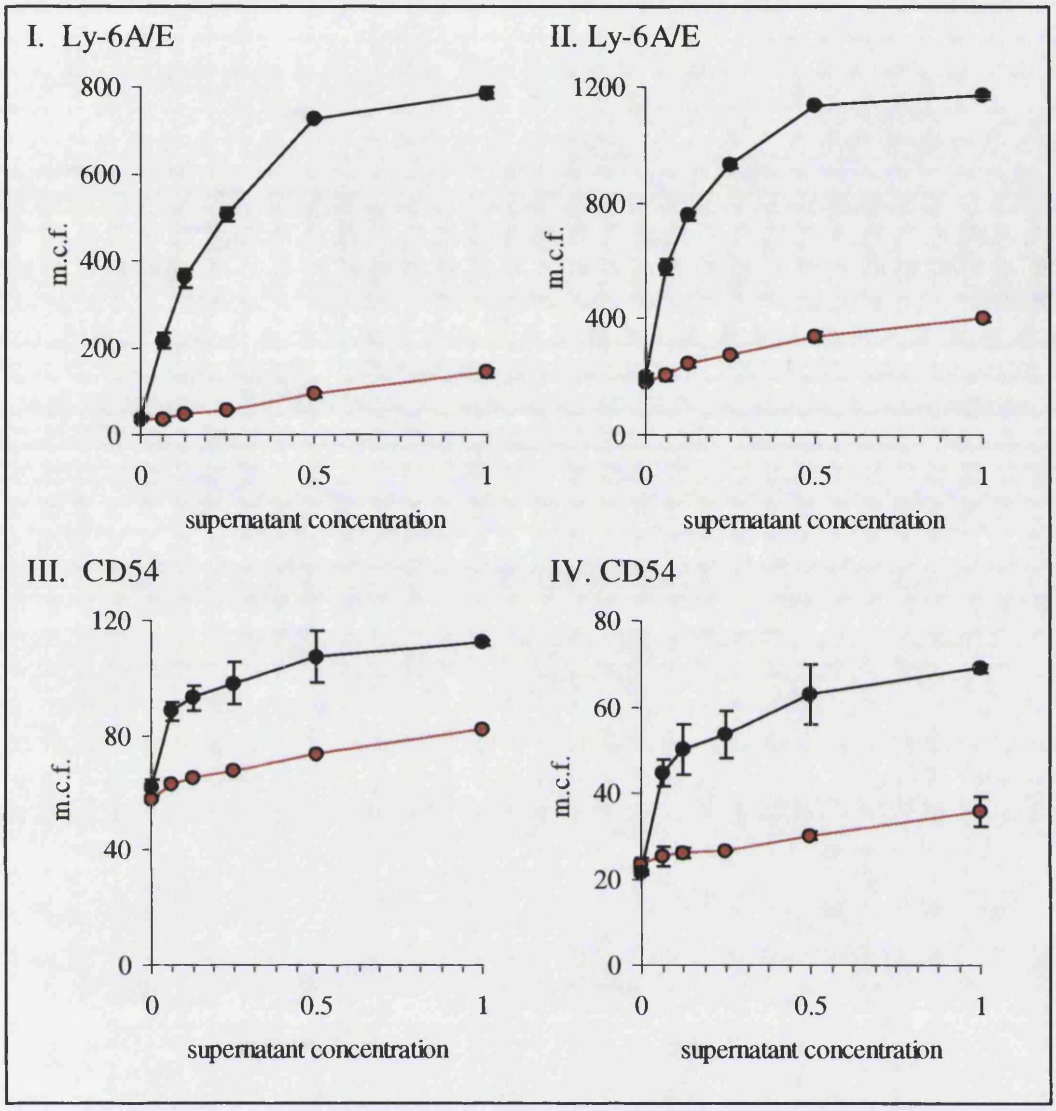


Figure 4.08. The effect of DC supernatant on T cell phenotype *in vitro*.

Mice were injected with either PBS or LPS and sacrificed 1 hour later. Splenic DC were prepared as described previously (2.2.7C) and cultured *in vitro* for 24 hours at a concentration of 1 x 10^6 cells/ml. Purified T cells were then resuspended, at a concentration of 1 x 10^6 cells/ml, in harvested neat (i.e. $^1/_1$) or diluted ($^1/_2$–$^1/_{16}$) DC culture supernatant. Following a 24-hour culture period *in vitro,* T cells were harvested and stained for CD8 or CD4 and CD54 or Ly6A/E. Cells were visualised by flow cytometry. Data represents the surface expression levels of Ly-6A/E (I. & II.) and CD54 (III. & IV.) on $CD8^+$-gated (I. & III.) and $CD4^+$-gated (II. & IV.) T cells. Each value represents the mean of duplicates ± s.e.m. (●, PBS; ●, LPS). Where the supernatant concentration = 0, ● = PBS and ● = 1μg LPS added to purified T cells *in vitro*. Statistical analysis was performed using 2-sample t-tests.

p values	I	II	III	IV
0 ≠ PBS	< 0.01	< 0.005	< 0.005	> 0.05
0 ≠ LPS	< 0.001	< 0.001	< 0.001	< 0.001
PBS ≠ LPS	< 0.001	< 0.001	< 0.005	< 0.05

To test this assumption splenic DC were purified from PBS or LPS-treated mice (culled 24 hours p.i.) and used to stimulate allogeneic responses *in vitro*. The proliferative responses generated by DC from PBS or LPS-treated mice were of equal magnitude (fig. 4.09.). Therefore even though DC from LPS-treated mice possessed a more activated phenotype than control DC, their ability to stimulate an allogeneic response remained unaffected.

The effect of LPS on T cell stimulatory ability was also examined using antigen-specific, TCR transgenic T cells as responders. DC were cultured with syngeneic transgenic responder cells in the presence of specific peptide. To examine the response of $CD8^+$ T cells, DC derived from C57BL6 mice, injected 12 hours before with PBS or LPS, were cultured with 2C LN $CD8^+$ T cells expressing the transgenic peptide-specific TCR. DC from both PBS and LPS injected mice induced comparable levels of $CD8^+$ T cell proliferation in response to SIYR peptide (fig. 4.10C.). The capacity of DC to stimulate antigen-specific $CD4^+$ T cell responses was evaluated using the DO11.10 system. BALB/c mice were injected with PBS or LPS and sacrificed 1 hour or 12 hours post injection. Splenic DC derived from these mice were incubated with DO11.10 transgenic $CD4^+$ T cells in the presence of peptide ($OVA_{323-329}$). Here, the results differed depending on the time point at which the DC were purified. DC isolated from mice injected 1 hour before with LPS appeared to be slightly better stimulators of $CD4^+$ T cells than control DC, although the differences were not statistically significant (fig. 4.10.). In contrast, DC from mice injected 12 hours previously with LPS were much less efficient than control DC in stimulating proliferation of DO11.10 $CD4^+$ T cells. Taken together, therefore, the results show that despite

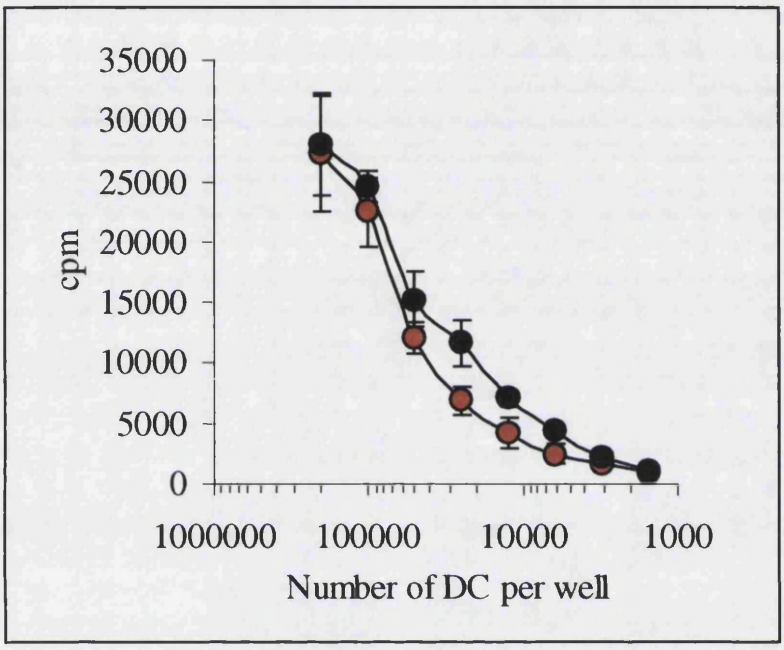


Figure 4.09. LPS stimulation *in vivo* does not significantly affect the capacity of DC to stimulate allogeneic responses *in vitro*.

C57BL/6 mice were injected i.v. with PBS or LPS and sacrificed 24 hours later. Splenic DC were prepared as described previously (2.2.7C). Between 1.562 x 10^3 and 2 x 10^5 DC per well were cultured with 1.5 x 10^5 BALB/c responders. The assay was pulsed with tritiated thymidine 72 hours later. . Plates were harvested 18 hours later and proliferation was subsequently determined using a β-scintillation counter. Data represents the mean proliferation of triplicate stimulator populations ± s.e.m.; ●, PBS; ●, LPS.

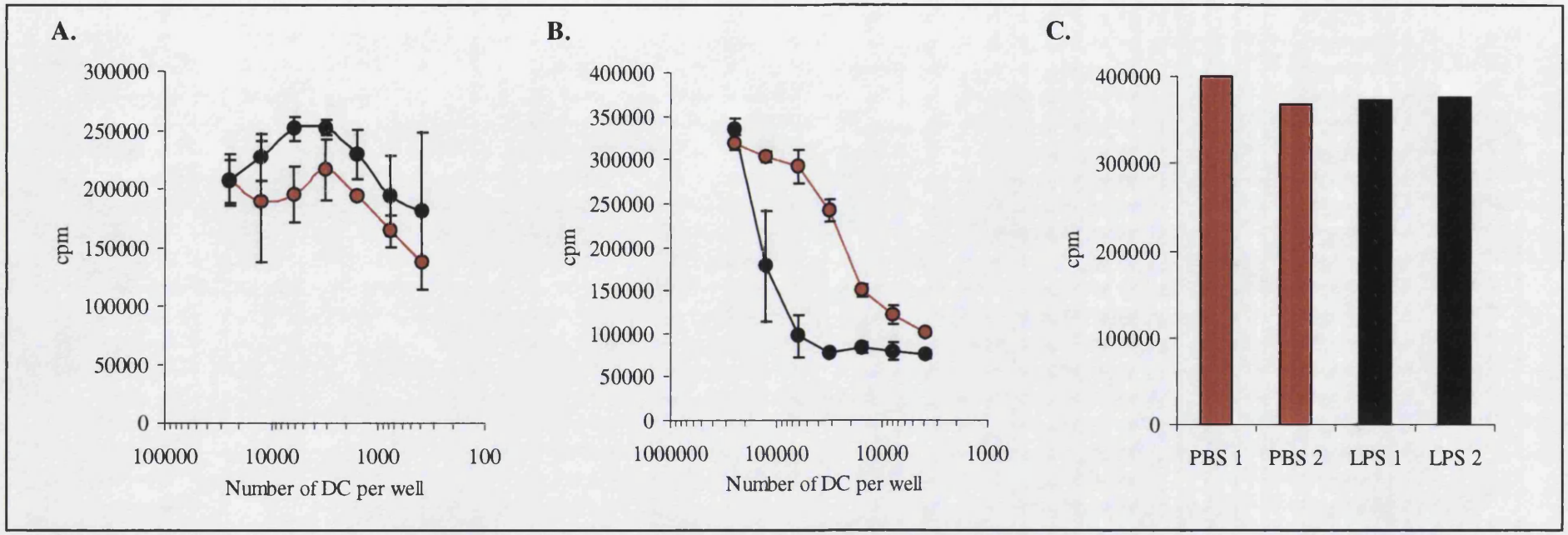


Figure 4.10. LPS *in vivo* does not enhance the capacity of DC to stimulate antigen-specific responses *in vitro*.

BALB/c mice were injected with PBS or LPS and sacrificed **A.** 1 hour and **B.** 12 hours later. Splenic DC were prepared as described previously (2.2.7C) and cultured with 1.5 x 10^5 DO11.10 LN responders in the presence of $OVA_{323-329}$ peptide (0.05µg/ml). The cultures were pulsed with tritiated thymidine after 96 hours and proliferation was determined 18 hours later (see 2.2.8). Data represents the mean of duplicates ± s.e.m. (●, PBS; ●, LPS). Statistical analysis was performed using a 2-sample t-test. **A.** The difference between the two means was not significantly different. **B.** The difference between the two populations was statistically significant at DC concentrations 6,250 ($p < 0.05$), 3,130 ($p < 0.01$) and 1,560 cells per well ($p < 0.05$). **C.** C57BL/6 mice were injected with PBS or LPS and sacrificed 12 hours later. Splenic DC were prepared as described previously (2.2.7C) and cultured with 1.5 x 10^5 2C LN responders in the presence of 2C (SIYR) peptide (10ng/ml). The assay was pulsed with tritiated thymidine after 96 hours in culture and proliferation was determined 18 hours later (see 2.2.8). Data represents the mean of triplicate wells (■, PBS; ■, LPS).

expressing an activated phenotype, DC from LPS-treated mice do not exhibit an enhanced ability to stimulate T cell proliferation on a per cell basis.

4.2E. LPS induced changes in DC numbers in lymphoid organs *in vivo*.

Thus when DC numbers were equal, LPS treatment *in vivo* was either inhibitory or had no effect on subsequent responses to antigen *in vitro*. Therefore, the effects of LPS observed on whole splenocyte stimulators may have been attributable to differences in DC numbers within the splenocyte suspensions. As described previously, LPS down-regulated $CD11c^+$ expression on low density enriched cell suspensions. The percentage of these cells expressing CD11c was also diminished. However, the percentage of control cells expressing CD11c following density centrifugation can vary significantly between experiments. For that reason, CD11c expression was analysed for spleen and lymph node tissue preparations without prior enrichment for low-density cells.

C57BL/6 mice were injected with PBS (time point 0) or LPS and sacrificed between 6 and 72 hours later (fig. 4.11.). Over this time-period, the percentage of $CD11c^+$ DC in splenocyte suspensions followed a bi-phasic pattern. Between 6 hours and 12 hours post-injection both the percentage of $CD11c^+$ DC and relative cell numbers increased significantly. This effect was short-lived as by 24 hours post injection the percentage of $CD11c^+$ cells had fallen to below starting levels. The percentage of $CD11c^+$ cells appeared to be returning to normal levels by 72 hours (fig. 4.11. II.).

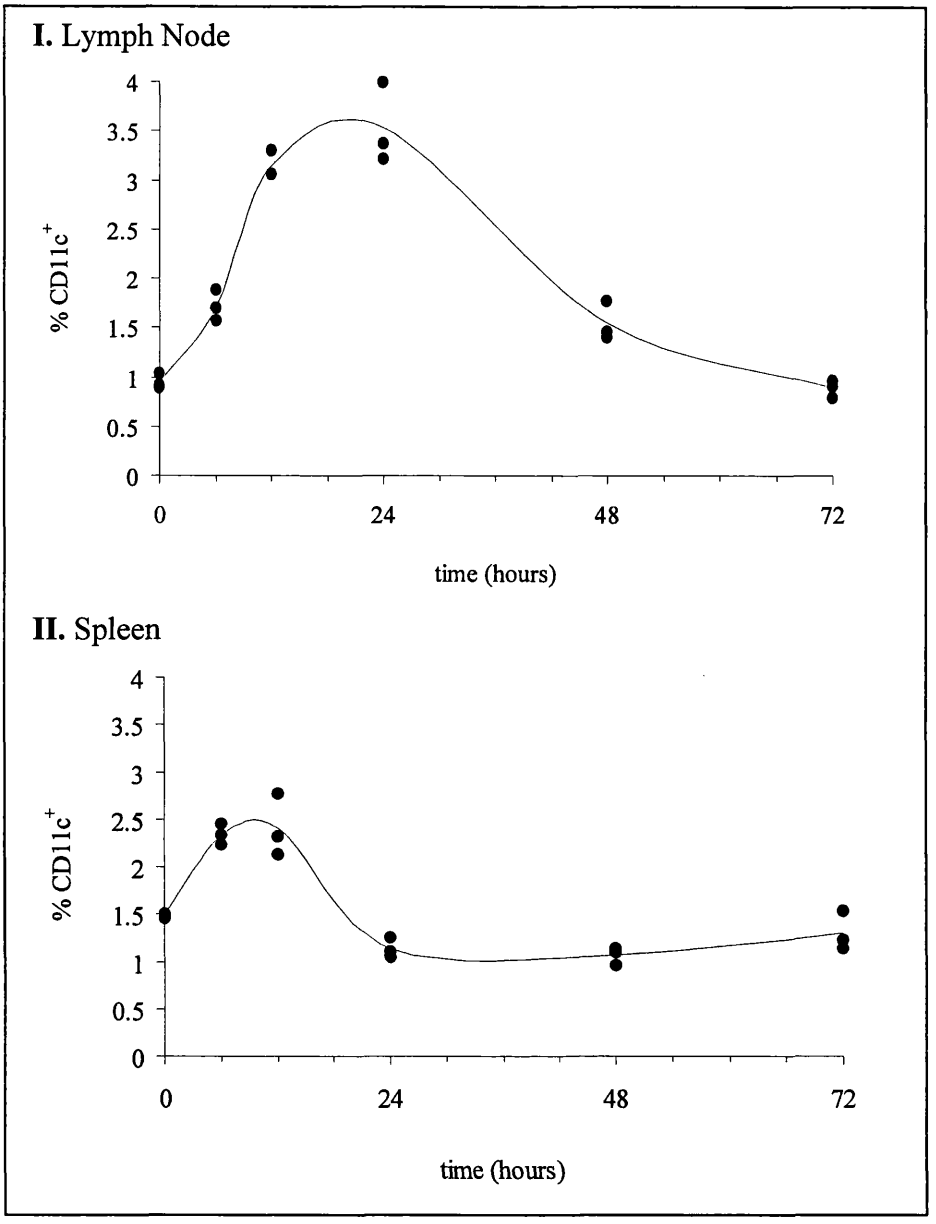


Figure 4.11. The LPS-induced increase in the proportion of $CD11c^+$ DC within the LN and spleen is both rapid and transient.

Mice were injected with PBS (time point zero) or LPS i.v. and sacrificed 6, 12, 24, 48 and 72 hours later. **I.** LN and **II.** spleen were removed and single cell suspensions were prepared using enzyme digestion (see 2.2.3). Cells were stained with anti-CD11c-FITC and visualised on the FACScalibur. Data from both the FL1 and FL2 channels were stored. Data represents the percentage of cells that were $CD11c^+$ but did not auto-fluoresce in FL2. Statistical analysis was performed using 2-sample t-tests. LN p values: 6, 12 and 24 hours, $p < 0.001$; 48 hours, $p < 0.01$; 72 hours, $p > 0.05$. Spleen p values: 6 hours, $p < 0.001$; 12 hours, $p < 0.01$; 24 and 48 hours, $p < 0.005$; 72 hours, $p > 0.05$.

A similar transient increase in $CD11c^+$ DC was also observed in lymph nodes. However, there were distinct differences in the data obtained from the two types of lymphoid organs. Firstly after 12 hours LPS stimulation, the magnitude of increase in $\%CD11c^+$ DC in the LN was 2-fold greater than the increase observed in the spleen (fig. 4.11 I.). Secondly, the increase in % $CD11c^+$ in the LN was not followed by a subsequent decrease but instead a more gradual return to starting levels. Finally, the kinetics of the response to LPS in the LN was slower than in the spleen.

It was possible that the difference in kinetics between LN and spleen was due to the route of administration of LPS. We investigated this possibility with regard to the increase in $CD11c^+$ cells observed in LN 24 hours p.i. by comparing the effects of i.v. versus s.c. LPS injection. Thus, mice were injected intravenously or subcutaneously with LPS. After 24-hours "total" LN (see 2.2.2.) was removed from i.v.-injected mice but only the draining inguinal lymph nodes were removed from s.c.-injected mice. The percentage of $CD11c^+$ DC was increased by LPS injected either i.v. or s.c., indicating that this phenomenon was not restricted to one particular route (fig. 4.12.).

The phenotype of $CD11c^+$ DC in lymph nodes was also analysed. It was found that they did not express the macrophage surface markers CD14 or F4/80 but the vast majority of $CD11c^+$ DC did express CD11b (a subunit of Mac-1) suggesting that they were of a myeloid lineage (Henri *et al.*, 2001; Pulendran *et al.*, 1999). LN DC from LPS-injected mice expressed high levels of CD54, Ly-6A/E, CD95, CD40 and CD86 (data not shown).

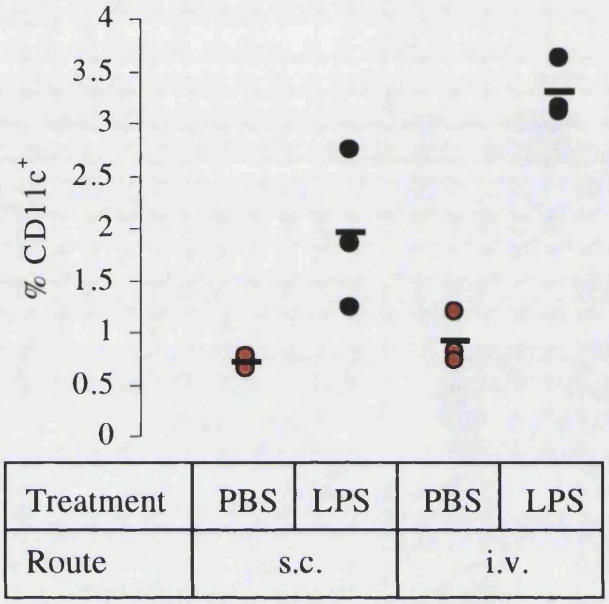


Figure 4.12. The proportion of $CD11c^+$ DC within the lymph node increases 24 hours after LPS injection.

Mice were injected with PBS, ● or LPS, ●, i.v. or s.c. and sacrificed 24 hours later. LN and spleen were removed and single cell suspensions were prepared using enzyme digestion (see 2.2.3.). Cells were stained with anti-CD11c-FITC and visualised by flow cytometry. Data from both the FL1 and FL2 channels were stored. Data represents the percentage of cells that were $CD11c^+$ but did not auto-fluoresce within FL2. Statistical analysis was performed using ANOVA (p values: LN, s.c. $p = 0.03$, i.v. $p = 0.0006$).

The distinct kinetics of DC appearance in LN versus spleen raised the question of whether LPS-induced DC migration was responsible for the enhaced response to Con A observed following LPS-injection (see 3.2D.). This seemed worth considering since hyper-responsiveness to Con A *in vitro* after LPS stimulation *in vivo* was only observed for LN preparations and not splenocyte suspensions. To address the role of DC in this phenomenon, DC were depleted from LN cell suspensions prior to Con A stimulation. Depletion of $CD11c^+$ DC (2.2.7D.) from LN cell suspensions from control mice completely abrogated proliferation to Con A. However, proliferation was still observed in DC-depleted LN samples derived from LPS-treated mice. This suggested that DC were the main APC responsible for "co-stimulating" the T cell response to Con A in resting LN cells, but upon LPS-stimulation other cell types became involved (fig. 4.13.).

4.3 Summary

We have observed that LPS *in vivo* has various effects on stimulator function *in vitro*. Brief exposure to LPS *in vivo* appeared to enhance both allogeneic and antigen specific responses *in vitro*, whilst longer periods of *in vivo* stimulation inhibited subsequent *in vitro* proliferative responses. We investigated the possibility that this bi-phasic pattern of stimulation was reflected by DC phenotype. Indeed, stimulation with LPS for 6 hours *in vivo* was a sufficient period of time for DC to acquire an activated phenotype. However, DC from mice treated with LPS for 24 hours also appeared to be highly activated.

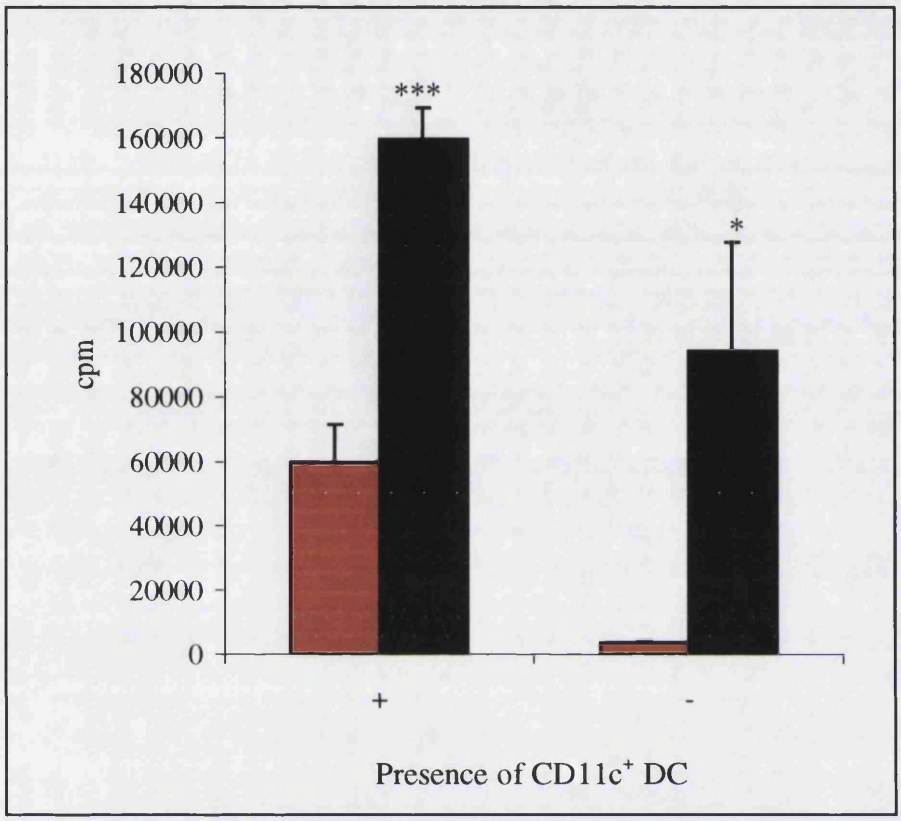


Figure 4.13. $CD11c^+$ dendritic cells are required for the T cell mitogenic response to Con A.

C57BL/6 mice were injected with PBS or LPS and sacrificed 24 hours later. LN were excised and cell suspensions were prepared by enzyme digestion. 1.5×10^5 cells were cultured with Con A (1μg/ml) in the presence (+) or absence (-) of $CD11c^+$ DC. The assay was pulsed with tritiated thymidine after 48 hours in culture and proliferation was determined 18 hours later (2.2.8.). Data represents the mean of triplicate mice ± s.e.m. (■, PBS; ■, LPS). Statistical analysis was performed using 2-sample t-tests (*, $p < 0.05$; ***, $p < 0.005$).

Furthermore, purified DC from both PBS and LPS-treated mice stimulated allogeneic and antigen-specific responses with equal efficiency (with the exception of fig. 4.10B.), suggesting that DC numbers rather than activation status may be an important factor in the altered stimulatory capacity of total splenocytes. Reinforcing this idea was the observation that the proportion of DC within the LN and spleen was altered after stimulation with LPS *in vivo*.

4.4 Discussion

We have reported that LPS induced a number of phenotypic changes on DC *in vivo*. Amongst the molecules affected by LPS treatment *in vivo* were lineage-related markers, such as CD11c, CD11b, CD4 and CD8. The function of the T cell markers CD8 and CD4 on DC is still unclear. However they have been reported to define three DC subtypes of separate lineages: $CD4^-CD8\alpha^+$, $CD4^+CD8\alpha^-$, $CD4^-CD8\alpha^-$ (Kamath *et al.*, 2000). Therefore, it was interesting to observe that the systemic administration of LPS appeared to increase the proportion of $CD4^-CD8\alpha^-$ at the expense of $CD4^-CD8\alpha^+$ and $CD4^+CD8\alpha^-$ DC. A possible explanation could be that LPS induced DC to revert to a double negative phenotype. However, since CD4 and CD8 expression on DC was unaffected by LPS *in vitro*, the effects observed *in vivo* may be because of selective loss of particular DC subsets rather than phenotypic conversion.

Since $CD8\alpha^+$ and $CD8\alpha^-$ $CD11c^+$ DC have been shown to have contrasting properties, it is interesting to speculate about the possible consequences of LPS induced loss of $CD8\alpha^+$ $CD11c^+$ DC *in vivo*. For example, $CD8\alpha^+$ DC, often

referred to as "lymphoid DC", have been demonstrated to be the major source of IL-12 *in vivo*. Hence $CD8\alpha^+$ DC preferentially elicit a T_H1- type response *in vivo*. By contrast $CD8\alpha^-$ DC, termed "myeloid", specifically promotes the differentiation of T_H2-type responses (Maldonado-Lopez *et al.*, 1999). Thus, LPS administration *in vivo* may unbalance the $CD4^+$ T cell cytokine response to antigen in the spleen by removing a particular DC subset. Furthermore, myeloid DC have been reported to express CD11b. Thus, whilst LPS induced DC loss in the spleen there was a simultaneous increase in the number of myeloid DC within the LN. This may further polarise a subsequent T cell response towards a T_H2 pattern of cytokine production.

Other groups have reported that lymphoid DC may have a T cell regulatory function. $CD8\alpha^+$ DC express high levels of "self-peptides" implying a role in T cell tolerance. Furthermore, $CD8\alpha^+$ DC have also been demonstrated to kill activated T cells via CD95 (Fas) engagement (Kronin *et al.*, 1997; Kronin *et al.*, 2000). Therefore, selective loss of lymphoid DC by LPS could conceivably remove negative regulatory mechanisms *in vivo* thus allowing T cells to respond more vigorously to later challenge with antigen.

The magnitude of the phenotypic response to LPS by the three DC subsets investigated was unequal. $CD8\alpha^+$ and $CD4^+$ $CD11c^+$ DC up-regulated their surface markers significantly more than $CD8\alpha^-$ $CD4^+$ $CD11c^+$ DC 24 hours after LPS injection (data not shown). However, LPS up-regulated the expression of the same surface molecules on DC regardless of subset. These can be divided into those thought to be important in co-stimulation (CD40, CD86) and adhesion

(CD54), those normally associated with apoptosis (CD95) and those that are known only as markers of activation (Ly-6A/E, CD69).

B7 molecules, such as CD86, are widely regarded as important molecules required for the initiation of adaptive immune responses. The expression of B7 on a cell that lacks other co-stimulatory molecules can convert a non-functional APC into a functional one. Interruption of the B7/CD28 pathway leads to an inhibition of T cell responses and under certain conditions, either antigen-specific hypo-responsiveness or anergy (reviewed in Lenschow *et al.*, 1996). Therefore, upregulation of CD86 expression following LPS injection may have important implications for up-regulation of the immune response.

Similarly, an increased CD40 expression on DC may also conceivably amplify a T or B cell response. B cell activation appears to be particularly dependent on CD40 expression since absence of its functional ligand (either in CD40L knockout mice or immunodeficient humans) prevents isotype switching and germinal centre formation (Xu *et al.*, 1994). CD40 ligation is also important for T cell response, since interruption of CD40:CD40L DC:T cell interactions *in vitro* results in reduced T cell proliferation. CD40 ligation induces DC to release inflammatory cytokines (e.g. IL-12 and TNF-α) and up-regulate its own surface expression of B7 molecules and CD54.

There is also evidence supporting a co-stimulatory function for CD54 (ICAM-1) (Simon *et al.*, 1993). Thus, CD54/LFA-1 interactions have been shown to be

important for the generation of both allogeneic and mitogenic response by DC (Rescigno *et al.*, 1999; de Fougerolles and Springer, 1992).

The consequences of CD95 upregulation on DC may be complex. CD95 is usually associated with apoptosis. However, recently it has been reported that ligation of this molecule on DC may promote alternative responses. For example, CD95 cross-linking on DC has been described to have the potential to enhance TNF-α production. There is also evidence that CD95 is involved in the interaction of DC with T cells, resulting in IFNγ production. Furthermore, ligation increased MHC class II, B7 and CD40 surface expression on bone marrow-derived murine DC (Rescigno *et al.*, 2000). Taken together, these experiments suggest that CD95 may have some co-stimulatory capacity on dendritic cells.

Thus, enhanced expression of adhesion molecules like CD54 and CD95 and also "classical" co-stimulatory molecules such as CD40 and B7 may account for our observations that LPS injection enhanced the stimulator activity of splenocytes derived from mice culled at early time points post injection. However, the kinetics of the up-regulation of co-stimulatory molecules on splenic DC by LPS *in vivo* did not correlate with splenocyte stimulation of T cell allo-responses. Although DC, the principal stimulators of MLRs (Steinman *et al.*, 1983), expressed several co-stimulatory molecules at significantly higher levels than control DC 24 hours post LPS-injection, this did not appear to confer increased allo-stimulatory function on purified DC, as assayed *in vitro*. Possibly, increased co-stimulator function may not be able to compensate for other regulatory factors

present *in vitro*. For example, Kamath et. al. have demonstrated that LPS injection shortened DC lifespan *in vivo*. Thus, it is conceivable that despite enhanced surface marker expression, DC from LPS-treated mice were poor stimulators because of a reduced lifespan *in vitro*. In addition, DC markers may exert dual functions during the overall response to LPS. For example, CD54 and CD95 have both been suggested to be involved in the targeted apoptosis of DC via interactions with activated T cells (Matsue *et al.*, 1999)

However, CD95 has been reported not to be involved in provoking splenic DC to undergo apoptosis after systemic administration of LPS (De Smedt *et al.*, 1998). This may not be altogether surprising since LPS-matured DC have consistently been shown not to be sensitive to CD95-induced apoptosis (Ashnay *et al.*, 1999) (Rescigno *et al.*, 2000). Interestingly De Smedt *et. al.* suggested that poor stimulator function induced by LPS *in vivo* may be in part due to defective B7 function. The addition of anti-CD28 antibody to MLRs restored normal stimulator activity to splenocytes derived from LPS-injected mice (De Smedt *et al.*, 1996).

It also appeared that the effects of LPS *in vivo* might not have been due to a direct effect on DC. For example, the surface expression of Ly-6A/E was significantly increased by low doses of LPS *in vivo* but remained unchanged by high doses *in vitro*. MHC class I expression has also been shown not to be altered by LPS *in vitro* (Roake *et al.*, 1995). Nevertheless, DC were not completely unresponsive to LPS *in vitro* since TNF-α and IFNγ secretion was enhanced, albeit only marginally; control DC also secreted TNF-α suggesting

possible stimulation due to manipulation *in vitro*. This suggests that other cell types are required to manifest a phenotypic response to LPS *in vitro* and thus it is likely that LPS-induced cytokines produced by non-DC are involved *in vivo*.

Curiously, purified DC derived from mice injected with LPS secreted less TNF-α than control DC. This may in part be due to the 12-hour time point chosen for investigation. Lanzavecchia et. al. have shown that at least *in vitro*, DC cytokine secretion profiles after LPS stimulation varies greatly with time. TNF-α in particular was demonstrated to have very rapid kinetics. mRNA levels were elevated within 30 minutes of LPS-stimulation and peaked by 3 hours. Furthermore, TNF-α mRNA production was arrested after only 12 hours *in vitro* (Langenkamp *et al.*, 2000). Therefore, it is possible that the DC derived from LPS-stimulated mice were "exhausted" in terms of TNF-α production compared to their control counterparts.

Alternatively, DC may be interacting homotypically *in vitro*, suppressing the release or production of TNF-α. In this regard, Ly-6A/E cross-linking of purified DC also significantly reduced the TNF-α content within the supernatant. It has been previously shown that Ly-6A/E aggregates with a putative ligand *in vitro* (English *et al.*, 2000). Therefore, it is conceivable that the augmented expression of Ly-6A/E on DC-derived from LPS-stimulated mice may be responsible for lowering TNF-α secretion *in vitro*. This hypothesis may also account for the apparent disparity between the TNF-α data generated by stimulating DC with LPS *in vivo* and *in vitro*, since Ly-6A/E expression was not up-regulated on DC treated with LPS *in vitro*.

However, it was clear that only brief LPS stimulation (i.e. 1 hour) *in vivo* was sufficient to stimulate IL-12 production by DC *in vitro*. Furthermore, supernatant harvested from these DC cultured *in vitro* potently stimulated T cell phenotypic changes *in vitro*. At early time points after LPS injection (e.g. 1 hour p.i.), the proportion of $CD11c^+$ DC within the spleen was the same as controls but stimulator function was significantly enhanced. Therefore, in the absence of differences in DC numbers within splenocyte populations (at least directly *ex vivo*), cytokine production may have been an important factor in determining stimulator function *in vitro*. It would be interesting to test this idea by investigating the effect of DC supernatant on T cell responses *in vitro*.

At time points greater than 1 hour *in vivo*, our data revealed that LPS induced a bi-phasic flux in the overall percentage of $CD11c^+$ DC within the spleen over a 3-day period. The kinetics of DC numbers within the spleen closely correlated with the conditioning of splenocyte stimulator function *in vitro* by LPS administration *in vivo*. LPS also increased the proportion of $CD11c^+$ DC within lymph nodes, albeit with delayed kinetics compared to the spleen. Changes in the proportion and number of $CD11c^+$ DC may be interpreted in a number of ways.

1. LPS *in vivo* induced the migration of peripheral DC to lymphoid tissues. Certainly, as discussed previously (see 4.1) there is sufficient published data to suggest this as a likely possibility. In particular, *in vivo* work by MacPherson *et al* (1995) has shown that LPS given parentally to rats, induced the migration of

mucosal DC from the lamina propria to the mesenteric lymph nodes. Endotoxin-induced migration of intestinal DC to the MLN was demonstrated to be dependent on TNF-α. Whilst DC from LPS-treated mice secreted less TNF-α *in vitro* than control DC, overall TNF-α secretion by total splenocytes derived from LPS-injected mice was markedly elevated from background concentrations. Thus, TNF-α may be a putative stimulus for DC accumulation within LNs in our murine model.

2. LPS stimulated the differentiation of resident $CD11c^-$ precursors into $CD11c^+$ DC or recruited precursors to the lymph nodes upon which they expressed $CD11c^+$. Indeed, LPS-induced cytokines (e.g. TNF-α and IL-1β) have been demonstrated to induce the loss of CD14 expression on blood monocyte precursors and concomitantly acquire the surface expression of CD11c (Pickl *et al.*, 1996; Yamada and Katz, 1999).

3. LPS increased turnover of resident DC. Experiments using bromodeoxyuridine (BrdU) to measure the turnover of DC *in vivo* have shown that LPS increased BrdU incorporation of splenic DC within 24 hours (Kamath *et al.*, 2000).

Whatever the mechanism, the 3.5-fold increase in the percentage of $CD11c^+$ DC within the LN 24 hours post LPS-injection coincided with a large increase in the proliferative response of LN cell suspensions to Con A *in vitro*. The importance of dendritic cells in the response to Con A *in vitro* was confirmed by the dramatic loss of proliferation due to the depletion of $CD11c^+$ DC from LN

suspensions. LN cells derived from LPS-treated mice were able to respond to Con A even in the absence of DC. This suggests that LPS activated previously uninvolved cell types to become competent stimulators of a mitogenic response.

Similarly, monophosphoryl lipid A (a derivative of LPS) has been shown to induce B cells and macrophages to stimulate MLRs when previously only DC were able to activate naïve T cells (De Becker *et al.*, 2000). Therefore, both the enhanced Con A response seen in LN and the hyper-stimulatory activity of splenocytes derived from LPS-treated mice, may not simply reflect an increase in the proportion of DC within the cell suspensions. The relative contribution of other "non-professional" APC *in vitro*, such as macrophages and B cells, may have been dramatically altered by LPS stimulation *in vivo*.

In conclusion, LPS administrated systemically *in vivo* affected the proliferative responses of LN and splenocyte suspensions *in vitro*. Augmentation and inhibition of *in vitro* responses appeared to correlate with the proportion of DC within the lymphoid tissue preparations. The significance of DC "accumulation" within lymphoid tissues was not tested directly *in vivo*. However, the recruitment of professional APC to the "central" immune system may conceivably be an important mechanism for the adjuvant activity of LPS *in vivo*.

Chapter 5: The phenotypic and functional alteration of B cells by LPS *in vivo.*

5.1. Introduction

The biological effects of LPS on murine B cells may be described as pleiotropic since LPS can act as a mitogen, a non-specific activator, an antigen or an adjuvant. Recent discoveries into the mode of LPS recognition by B cells may help to explain these different effects. B cells express LPS receptors of differing affinity: high-affinity membrane bound immunoglobulin receptors and low-affinity toll-like receptors: TLR-4 and RP105 (a novel B cell TLR (Ogata *et al.*, 2000)). Murine B cells do not normally express CD14 on the surface of their cell membrane, although human B cell lines have been reported to possess membrane bound CD14. Therefore, it would appear that for the murine system at least, the TLR signalling module expressed by B cells is sufficient for activation via LPS (reviewed in Moller, 1999). Soluble CD14 has also been reported to possess B cell stimulatory properties, however this was demonstrated to be independent of LPS binding (Filipp *et al.*, 2001; Kimura *et al.*, 2000).

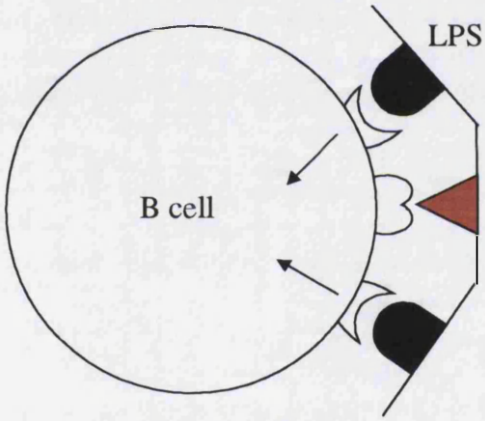
The schematic diagram on page 2 summarises the different receptor requirements for polyclonal and antigen specific activation. Large quantities of LPS are able to activate B cells independently of their Ig receptor specificity. Pathogen associated molecular patterns (PAMPs) within the LPS molecules bind to the genome encoded pattern-recognition receptors (PRRs) e.g. TLR4. However, B cells expressing surface Ig that are specific for immunogenic epitopes can bind

the LPS molecule with high affinity. In this regard, very low concentrations of LPS (as few as 10 molecules (Rudbach, 1970)) can activate LPS-specific B cells. Nevertheless, as illustrated in the diagram, TLR4 is likely to also be involved in the antigen-specific response to LPS, since C3H/HeJ mice are unable to produce polyclonal antibodies *in vitro* or specific anti-LPS antibodies *in vivo* (Moller, 1999).

A schematic diagram illustrating B cell activation by LPS.

A. Polyclonal activation

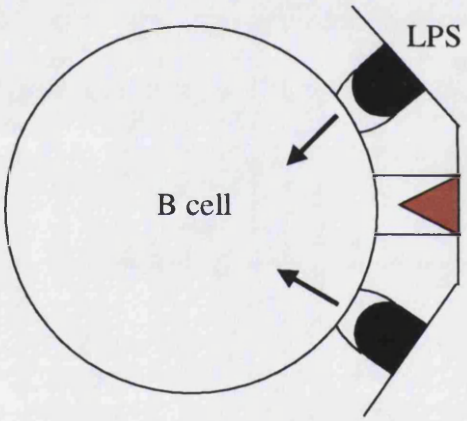
PAMPs (black) binds
low affinity LPS receptors



No binding to Ig receptors

B. Specific activation

PAMPs (black) binds to
low affinity LPS receptors



Antigenic determinant (red) binds
to high affinity Ig receptors

(Adapted from Moller, 1999)

B cell polyclonal activation also differs from antigen-specific activation in terms of the requirement for T cell help. LPS has been grouped with a diverse range of molecules called thymus-independent (TI) antigens. They all share the ability to stimulate the production of polyclonal antibodies by B cells in the absence of T cells. LPS alone stimulates purified B cells to proliferate and secrete large

amounts of IgM, moderate quantities of IgG2b and IgG3 and detectable levels of IgG1 antibodies. Optimal production of IgM, IgG2b and IgG3 appears to be partly dependent on the autocrine action of TGF-β (Snapper *et al.*, 1993). These antibodies were found to bind not just exogenous model antigens such as sheep red blood cells (SRBC) but also "self" antigens e.g. erythrocytes, albumin and even lymphocytes (Moller, 1999).

Despite the conventional classification of LPS as "T-independent", the antigen-specific B cell response to LPS *in vivo* requires regulation by activated T cells. Mice given a single immunogenic dose of LPS stimulate an oscillatory pattern of antibody production that is not observed in T cell deficient nu/nu mice. This has been interpreted to suggest that T cells are required for the initiation, amplification and suppression of the anti-LPS response (Hiernaux *et al.*, 1982; Elkins *et al.*, 1987).

Furthermore, through the secretion of cytokines, T cells are able to regulate the selection of isotypes generated by LPS-activated B cells. Three cytokines in particular are pivotal regulators of isotype switching: IL-4, IFN-γ and TGF-β. *In vitro* these three cytokines can positively regulate all 6 non-IgM non-IgD murine Ig isotypes: IL-4 stimulates polyclonally activated B cells to produce murine IgG1 and IgE, IFN-γ induces IgG2a and TGF-β promotes isotype switching to IgG2b and IgA. Experiments attempting to discern the mode of action of these cytokines suggest that they are "switch factors" rather than growth factors that selectively stimulate particular isotype-committed B cells. For example, germ-line γ1 and ε-chain mRNA transcripts can be isolated from B cells cultured *in*

vitro in the presence of LPS and IL-4. Moreover, prior incubation of resting IgG^- B cells with IL-4 allows them to make a predominantly IgG1 response upon subsequent stimulation with LPS alone. Similarly, TGF-β in the presence of LPS can induce surface IgA^- B cells to synthesise α-chain mRNA, express surface IgA and secrete this isotype. By contrast, IL-5 appears to promote IgA secretion mainly by further activating B cells that already express surface IgA (reviewed in Finkelman *et al.*, 1990; Snapper and Mond, 1993).

The nature of IFN-γ isotype switching activity remains unclear. IFN-γ may be a true "switch factor" or simply augment IgG2a secretion (Bossie and Vitetta, 1991; Snapper and Mond, 1993). The profound antagonism of this T_H1 cytokine on IL-4-mediated IgG1 and IgE production however, is well documented (Finkelman *et al.*, 1990; Finkelmann *et al.*, 1988; Metzger *et al.*, 1997; Hasbold *et al.*, 1999). Likewise, the T_H2 cytokine IL-4 antagonizes IFN-γ-mediated IgG2a secretion (Finkelman *et al.*, 1990).

Thus, these three T cell-derived cytokines are able to determine the polarisation of the antibody response i.e. T_H1 (IgG2a) versus T_H2 (IgG1 and IgE). However, production of these cytokines is not exclusive to T cells. Hence other cell types, such as NK cells have been shown to be an important source of IFN-γ necessary for IgG2a class switching by LPS-treated splenocytes (Snapper and Mond, 1993).

Furthermore, non-T cells may also be important for the production of other cytokines that are able to direct the polyclonal B cell response. For example, IL-

12, produced by macrophages and dendritic cells, can activate $CD4^+$ T helper type I and natural killer cells to secrete large amounts of IFN-γ (Metzger *et al.*, 1997). IFN-α, produced by numerous cell types, has also been demonstrated to possess similar properties to IFN-γ with regards to IgG2a secretion and the antagonistic effect on B cells cultured with LPS and IL-4 (Finkelman *et al.*, 1991).

Investigations into the polyclonal response of B cells to LPS have proven to be a very useful simple *in vitro* system. However, polyclonal antibody production in the absence of antigen *in vivo* does not occur at any appreciable level (Armerding and Katz, 1973). Nevertheless, LPS has potent adjuvant properties on antibody production *in vivo*. Enhancement of an antibody response to protein antigens by purified LPS was first demonstrated by Johnson *et al* as early as 1956 (Johnson *et al.*, 1956). Rabbits injected intravenously three times with ovalbumin and 5μg LPS over a 9 day period, increased the antibody response 20-fold compared to ovalbumin alone. Similarly, subsequent murine experiments using a synthetic polypeptide antigen showed that the *in vivo* administration of LPS with the antigen enhanced the primary IgM and secondary IgG response (Ness *et al.*, 1976). Furthermore, the antibody response to SRBC in spleen cell-injected irradiated mice was significantly enhanced when the donor cells had been pre-treated with LPS for only 30 minutes *in vitro* (Nakano *et al.*, 1973).

The T cell requirement for this potentiation of B cell responses has been investigated *in vivo*. Allison and Davies (Allison and Davies, 1971) used thymectomised mice to show that LPS adjuvanticity required the presence of T

cells. Moreover, removal of T cells from antigen-pulsed splenocytes prior to adoptive transfer prevented LPS enhancing the secondary response upon subsequent challenge *in vivo* (Hamaoka and Katz, 1973).

Recent work by Le Bon *et al.* (2001) using type I interferon, an LPS-induced cytokine, implies a role for dendritic cells in mediating the effect of adjuvants on the humoral response. Co-injection of antigen and IFN-α enhanced antibody responses to a similar extent to complete Freunds adjuvant (CFA). Utilising a system where only DC were able to respond to IFN-I *in vivo*, they demonstrated that antigen-pulsed DC were sufficient for mediating the IFN-I-induced potentiation of antibody production.

Considering the changes in T cell phenotype 24 hours after injection of LPS, T cell sensitivity to Con A and the proportion of $CD11c^+$ DC within lymph nodes, reported in chapters 3 and 4, our aims were three fold: I, to analyse the effect of LPS on B cell activation status; II, to investigate the role of B cells from LPS-treated mice as APCs for Con A stimulated T cells; III, to examine the effect of prior LPS stimulation on the humoral response to a model antigen *in vivo.*

5.2 Results

5.2A. The effect of LPS and LPS-induced cytokines on B cell phenotype.

Previously, Tough *et al.* confirmed that LPS activated B cells by monitoring turnover *in vivo.* Mice were continuously given bromodeoxyuridine (BrdU) in

their drinking water for 3 days, after which turnover of $B220^+$ B cells was measured by detecting BrdU incorporation (see 2.2.5.). Not surprisingly, LPS injection provoked proliferation of B cells, particularly in the spleen. Furthermore, LPS *in vivo* was also shown to significantly increase the expression of CD69 on $B220^+$ B cells (Tough *et al.*, 1997). Following on from these experiments, we have shown that CD69 was a marker of B cell activation since proliferation was restricted to $CD69^+$ B cells (fig. 5.01.).

Thus, we began our investigation of the effect of LPS on B cells by studying possible changes in surface marker expression. A panel of monoclonal antibodies was used to characterise both "resting" and LPS-activated B cell phenotype (fig. 5.02.). A number of molecules were either not expressed on B cells (e.g. fasL, CD27 and CD80), or were expressed on resting B cells but remained unaffected by LPS-stimulation *in vivo* (e.g. LPAM-1, CD62L, MHC class II, CD19, CD5 and CD24). Curiously, both CD23 and CD40 were downregulated by LPS stimulation *in vivo*. However, the surface expression of CD86, CD54, LFA-1, CD69 and Ly-6A/E was significantly increased on B cells from mice culled 24 hours after LPS-injection. Ly-6A/E expression was not up-regulated on B cells in mice lacking functional TLR4. B cells from C3H/HeJ mice (possessing a point mutation in the *Tlr4* gene (Poltorak *et al.*, 1998)) injected with LPS did not alter their surface expression of Ly-6A/E, although those B cells derived from the closely related C3H/HeN strain, were able to increase Ly-6A/E expression (fig. 5.03.).

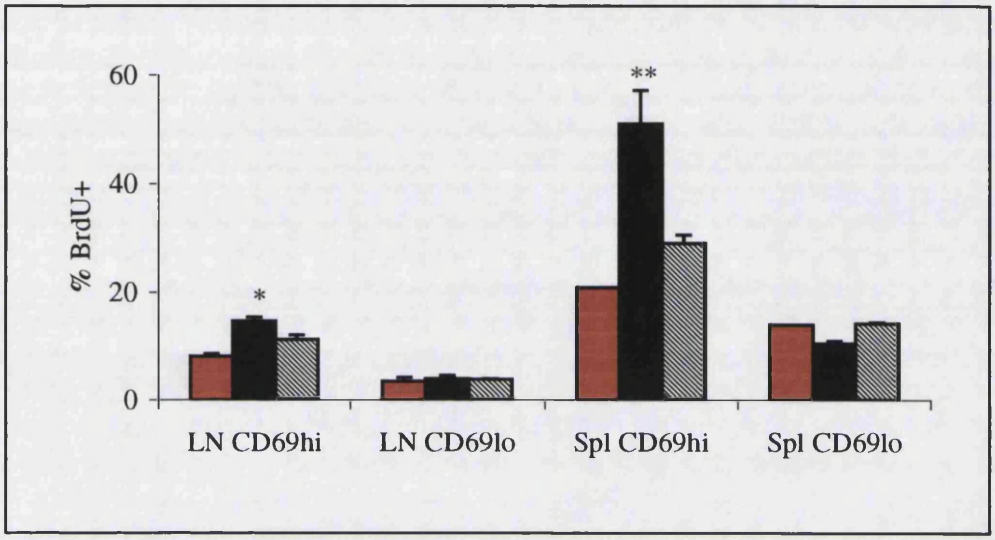


Figure 5.01. CD69 is a marker of B cell activation by LPS *in vivo*.

C57BL/6 mice were injected with PBS (▢), 50µg LPS (■) or 50µg MPLA (▨) i.v. Mice were continuously administered the thymidine analogue, 5 - bromo-2-deoxyuridine (BrdU) in their drinking water (0.8mg/ml) until they were sacrificed, 72 hours post injection. LNs and spleens were excised and single cell suspensions prepared (2.2.3.). Cells were first surface stained for B220 and CD69 (2.2.4.) and then stained intracellularly for BrdU incorporation (2.2.5.). Cells were visualised by flow cytometry. $B220^{+}$ B cells were gated for the purpose of analysis. $CD69^{hi}$ and $CD69^{lo/-}$ cells were further gated to distinguish the BrdU incorporation within the two subsets. Data represents the mean percentage BrdU incorporated ± s.e.m by a particular gated B cell subset. Statistical analysis was performed using ANOVA: *, $p < 0.05$; **, $p < 0.01$.

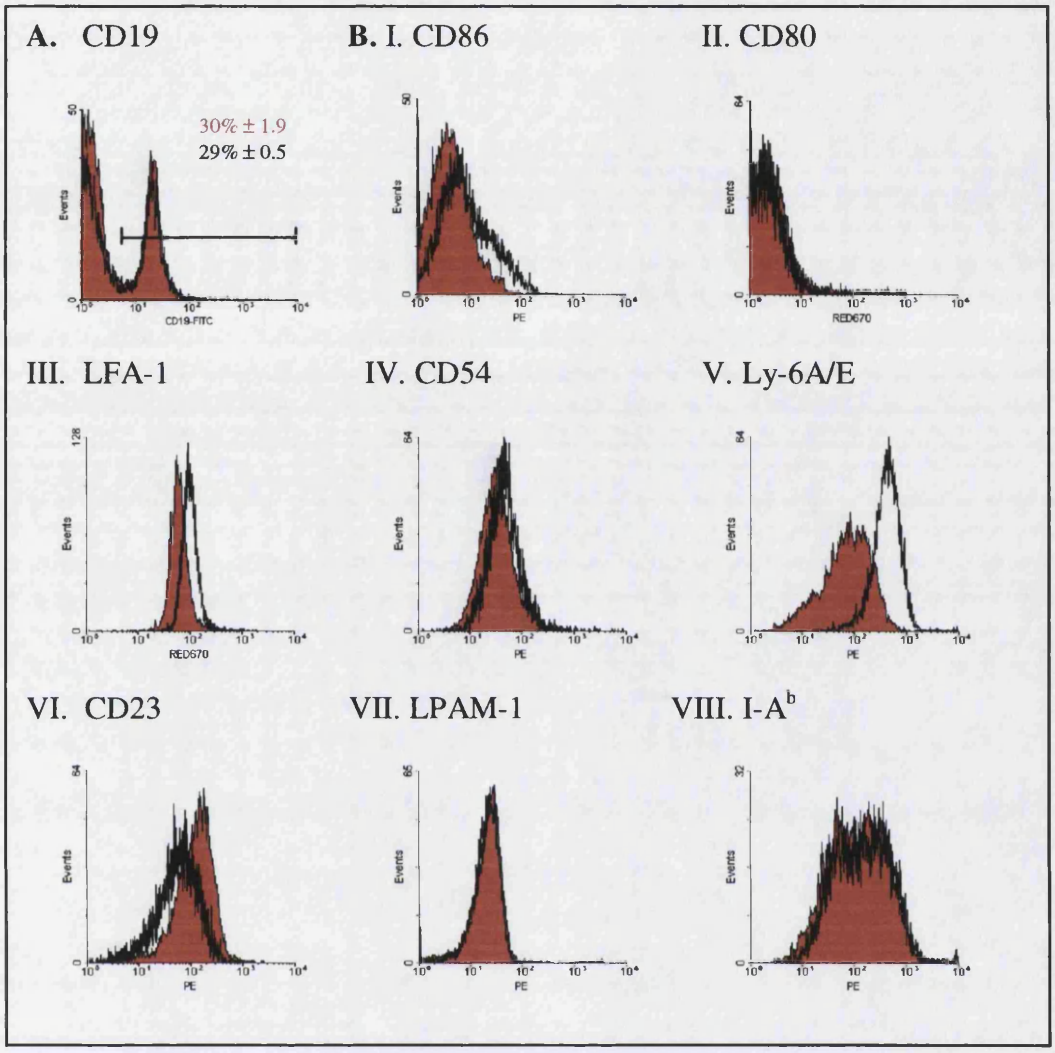


Figure 5.02. The effect of LPS on B cell phenotype *in vivo*.

C57BL/6 mice were injected with PBS or LPS i.v. and culled 24 hours later. LN were excised and single cell suspensions prepared. **A.** B cells were detected by staining for CD19. Histogram overlay represents the expression of CD19 on lymphocytes derived from PBS (■) or LPS (—) treated mice. Data represents the mean % $CD19^+$ B cells ± s.e.m. of lymphocytes from **PBS** or **LPS**-injected mice. **B.** Cells were stained with anti-CD19-FITC and either PE-conjugated (CD86, CD54, Ly-6A/E, CD23, LPAM-1 and $I\text{-}A^b$) or biotinylated (CD80 and LFA-1) antibodies (which were detected by a second incubation with streptavidin-RED670). $CD19^+$ B cells were gated on for analysis. Histogram overlays represent the expression of various surface molecules on $CD19^+$ B cells from PBS (■) or LPS (—) treated mice.

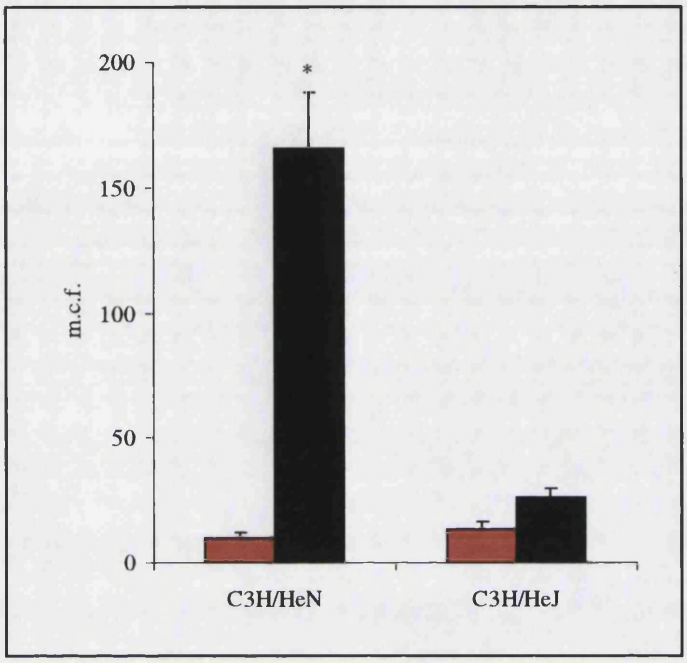


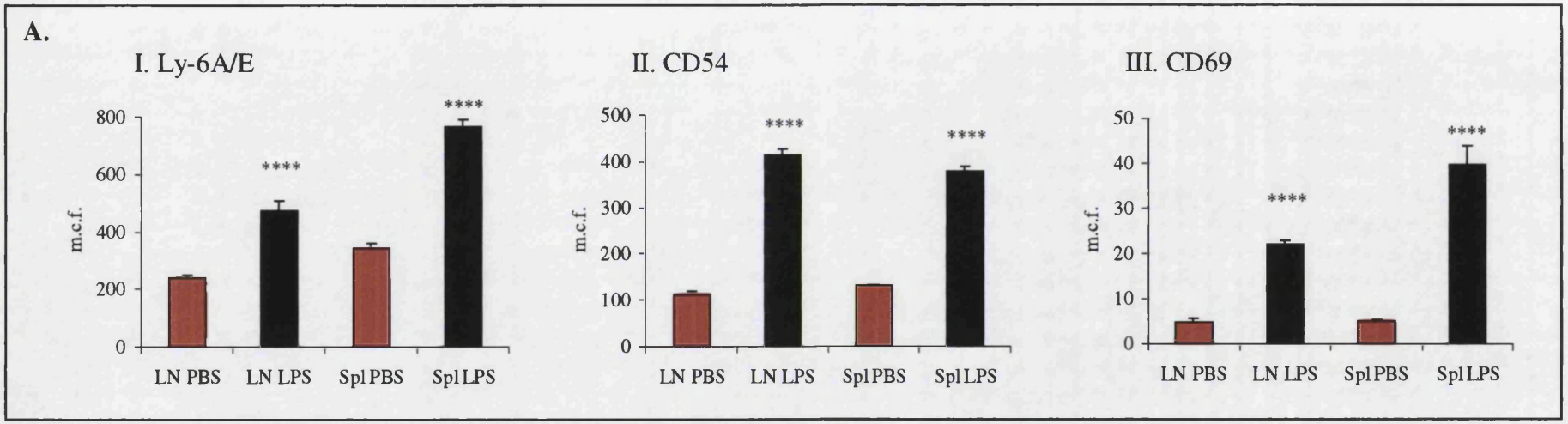
Figure 5.03. LPS does not significantly upregulate Ly-6A/E on $CD19^+$ B cells in mice that lack TLR4.

C3H/HeN (wild type) and C3H/HeJ (TLR4 mutants) mice were injected with PBS or LPS and sacrificed 24 hours later. LN were excised and cell suspensions prepared. Cells were stained for CD19 and Ly6A/E. $CD19^+$ cells were gated on for analysis. Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of Ly6A/E expression on $CD19^+$ B cells derived from PBS (□) or LPS (■) treated mice (*, $p < 0.05$; no asterisk, $p > 0.05$).

LPS also stimulated multiple B cell phenotypic changes when added to LN or spleen cell suspensions *in vitro* (fig. 5.04A.). In contrast to LPS-induced changes in T cell phenotype, alterations in B cell phenotype also occurred when LPS was added to purified B cells *in vitro* (fig. 5.04B.). Thus, unlike T cells, LPS can act directly on B cells to modify phenotype.

Despite the ability of LPS to activate B cells directly, it remained possible that LPS-induced cytokines may also play a role, given that many of the same phenotypic changes were evident on T and B cells. The involvement of LPS-induced cytokines in the alteration of B cell phenotype was initially investigated by using splenocyte supernatant. Mice were injected with PBS or LPS and sacrificed 1 hour later. Spleens were excised, single cell suspensions prepared and cultured *in vitro* for 18 hours. Supernatants from cultured splenocytes were added to purified B cells *in vitro* and the phenotype of the cells analysed 24 hours later. Ly-6A/E was significantly up-regulated by supernatant from LPS-stimulated splenocytes (fig. 5.05.). Control supernatant also provoked increased expression of Ly-6A/E. However, supernatant from control splenocytes only increased Ly-6A/E expression to levels comparable to supernatant from activated splenocytes that had been diluted 10-fold. CD54 expression on B cells was unaltered by supernatant from both control and LPS-stimulated splenocytes.

Since we have previously shown that splenocytes derived from LPS-injected mice produce both IFNγ and IFN-α/β, we investigated the role of interferons in the upregulation of Ly-6A/E and CD54. Initially, this was done by injecting poly I:C, a potent inducer of interferons, into normal C57BL/6. Consistent with a role



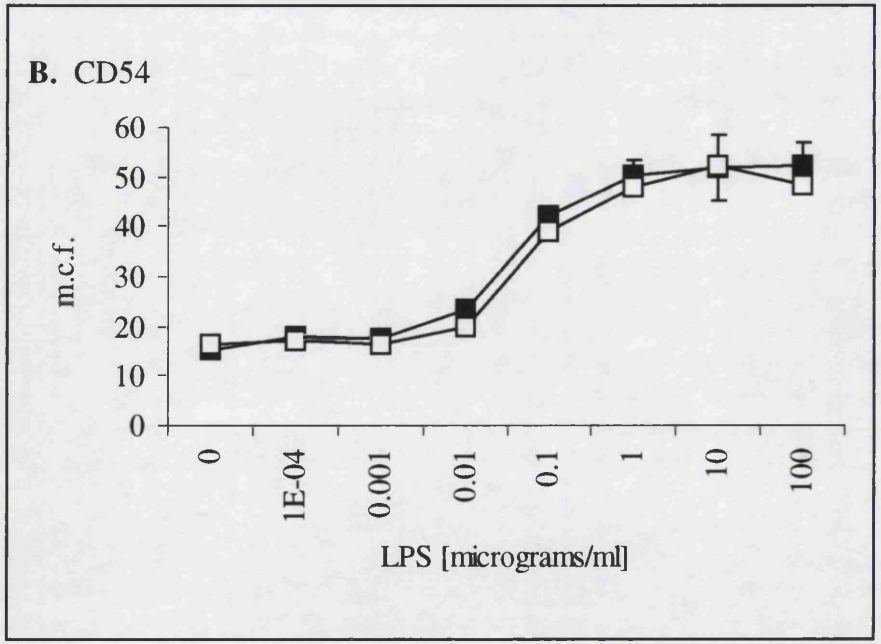


Figure 5.04. The effect of LPS on B cell phenotype *in vitro*.

LN and spleen were excised from un-injected mice and lymphocyte suspensions were prepared. **A.** Cells were incubated with PBS or 1µg LPS for 18 hours *in vitro*, after which they were stained for CD19-FITC and Ly6A/E-PE, CD54-PE or CD69-PE. $CD19^+$ cells were gated on for analysis. Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of surface markers on $CD19^+$ B cells treated with either PBS (□) or LPS (■) (****, $p \leq 0.001$). **B.** LN cells (■) or purified B cells (□) were incubated with various doses of LPS (0.1ng-100µg) *in vitro*. 18 hours later, cells were stained for CD19-FITC and CD54-Cy5. $CD19^+$ cells were gated on for analysis. Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of CD54 expression on $CD19^+$ B cells. Statistical analysis was carried out using 2-sample t-tests. LN cells, 10µg $p < 0.01$, 0.01µg and 100µg $p < 0.005$, 0.1µg and 1µg $p < 0.001$. Purified B cells, 0.01µg $p < 0.05$, 0.1-100µg $p < 0.001$.

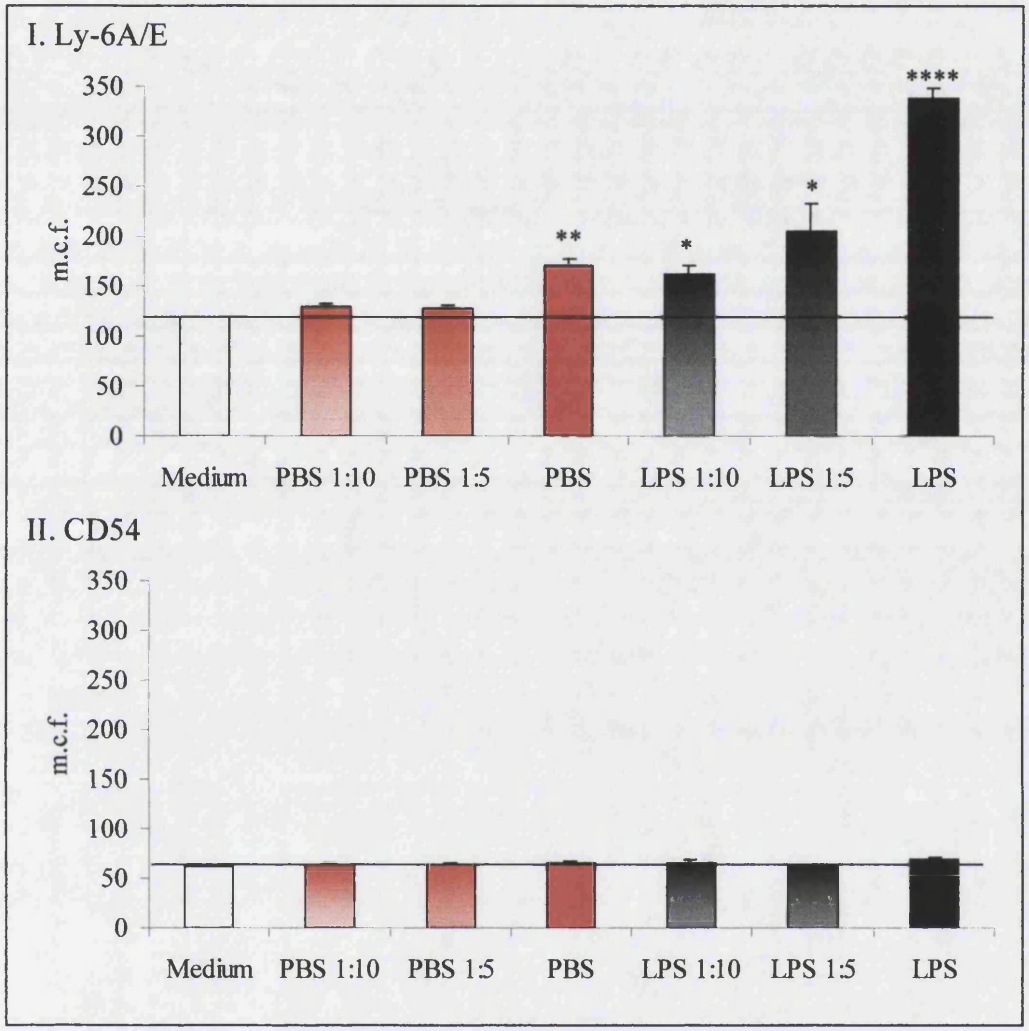


Figure 5.05. The effect of splenocyte supernatant on B cell phenotype *in vitro*.

C57BL/6 mice were injected with PBS or LPS and sacrificed 1 hour later. Spleens were excised and single cell suspensions prepared. Splenocytes were cultured *in vitro* in the presence of the endotoxin inhibitor polymixin B sulphate (100U/ml). After 18 hours, the supernatant was harvested and cells were removed by syringe filtration. Purified B cells were resuspended in neat or diluted supernatant and cultured for 24 hours *in vitro*. Cells were then removed and stained for CD19 and either I. Ly-6A/E or II. CD54. $CD19^+$ B cells were gated for analysis. Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of surface marker expression on $CD19^+$ B cells treated with splenocyte supernatant (PBS (□) or LPS (■)). (*, $p < 0.05$, **, $p < 0.01$, ****, $p < 0.001$)

for interferons in LPS-induced changes in B cell phenotype, many of the same changes occurred after poly I:C injection, including upregulation of CD86 and Ly-6A/E and downregulation of CD23. Notably, however, injection of poly I:C did not result in up-regulation of LFA-1 or CD54, implying that surface expression of these molecules was not regulated by interferons (fig. 5.06.). In agreement with this, recombinant murine IFN-α/β added directly to purified B cells *in vitro* dramatically affected Ly-6A/E expression but had no effect on CD54 (fig. 5.07.).

Thus, Ly-6A/E expression (but not CD54) was highly sensitive to both IFN-α/β and IFN-inducing agents. The dependency of Ly-6A/E on IFN-α/β for determining its expression levels on B cells was further clarified by using mice lacking a functional receptor for IFN-α/β. 129 SvEv mice and IFN-α/βR-/- mice (on a 129 background) were injected with PBS or LPS (10ng or 10μg) and culled after 24 hours. Ly-6A/E expression was remarkably reduced even on resting B cells that were derived from mice that were unresponsive to IFN-α/β (fig. 5.08.). Furthermore, resistance to the action of IFN-α/β completely prevented the upregulation of Ly-6A/E on B cells by LPS *in vivo*, even at high doses. In this regard B cells differed from T cells, since 10μg of LPS (but not 10ng) was sufficient stimulation to induce modest upregulation of Ly-6A/E on T cells from mice lacking the receptor for IFN-α/β (see fig. 3.18.).

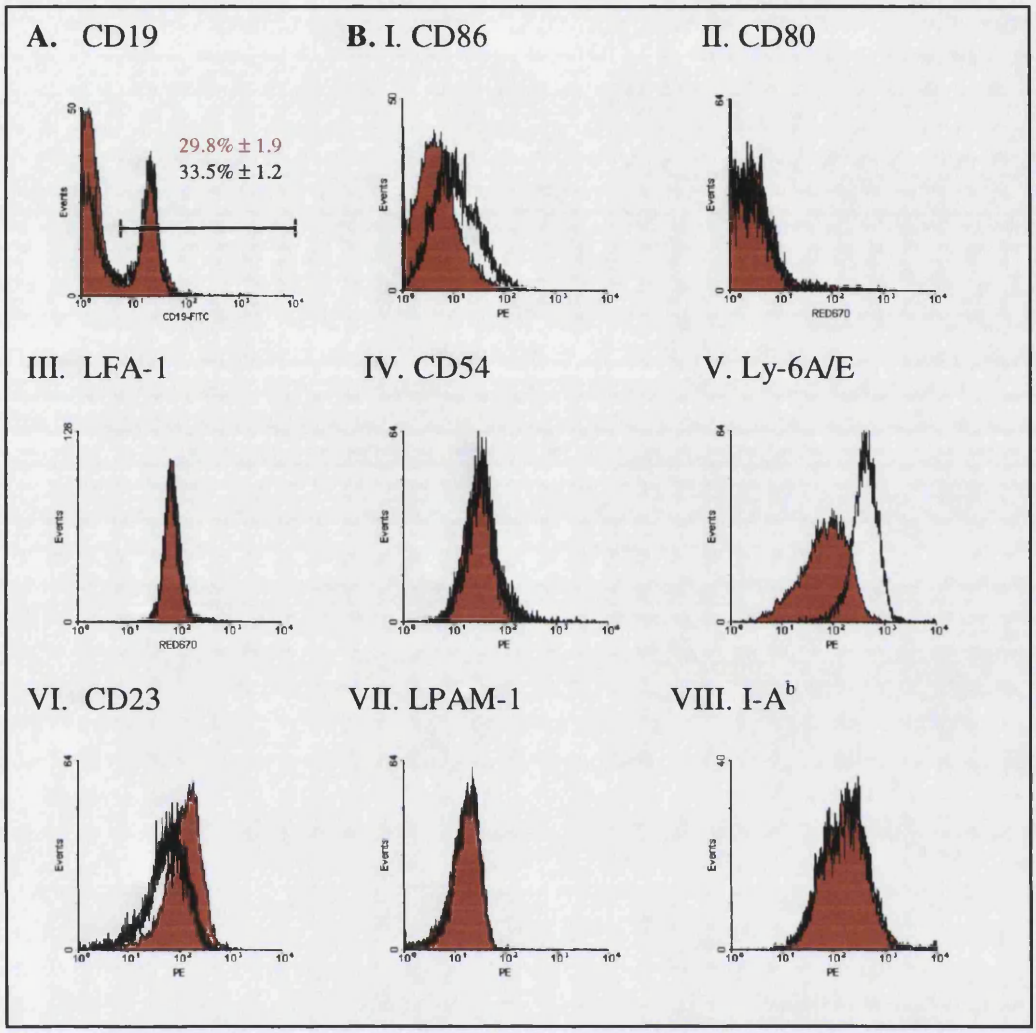


Figure 5.06. The effect of Poly I:C on B cell phenotype *in vivo*.

C57BL/6 mice were injected with PBS or Poly I:C i.v. and culled 24 hours later. LN were excised and single cell suspensions prepared. **A.** B cells were detected by staining for CD19. Histogram overlay represents the expression of CD19 on lymphocytes derived from PBS (■) or Poly I:C (—) treated mice. Data represents the mean % $CD19^+$ B cells ± s.e.m. of lymphocytes from **PBS** or **Poly I:C**-injected mice. **B.** Cells were stained with anti-CD19-FITC and either PE-conjugated (CD86, CD54, Ly-6A/E, CD23, LPAM-1 and I-A^b) or biotinylated (CD80 and LFA-1) antibodies (which were detected by a second incubation with streptavidin-RED670). $CD19^+$ B cells were gated on for analysis. Histogram overlays represent the expression of various surface molecules on $CD19^+$ B cells from PBS (■) or Poly I:C (—) treated mice.

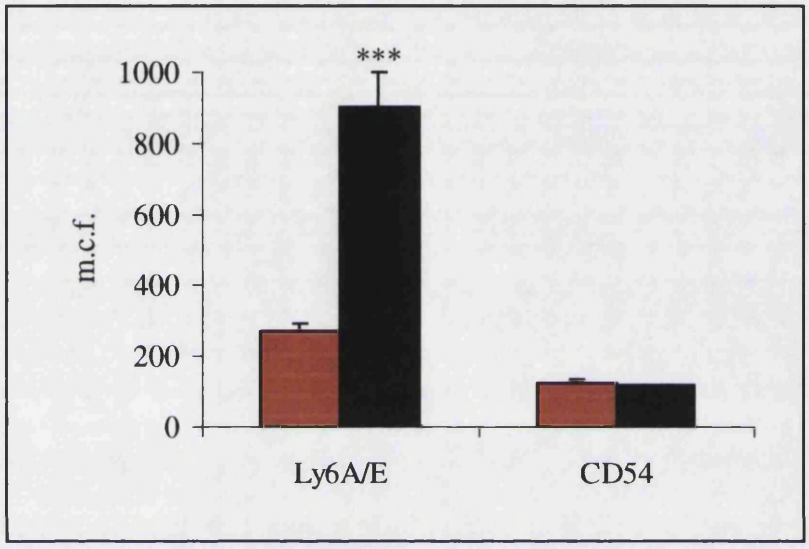


Figure 5.07. The effect of IFN-α/β on B cell phenotype *in vitro*.

LN were excised from un-injected mice and single cell suspensions were prepared. B cells were purified by negative depletion and incubated with 2000Units of IFN-α/β for 18 hours. The cells were then stained for CD19 and either Ly6-A/E or CD54. $CD19^+$ B cells were gated for analysis. Data represents the average "mean channel fluorescence" (m.c.f.) ± s.e.m. of surface marker expression on $CD19^+$ B cells, PBS (▭) or IFN-α/β (■). (***, $p < 0.005$)

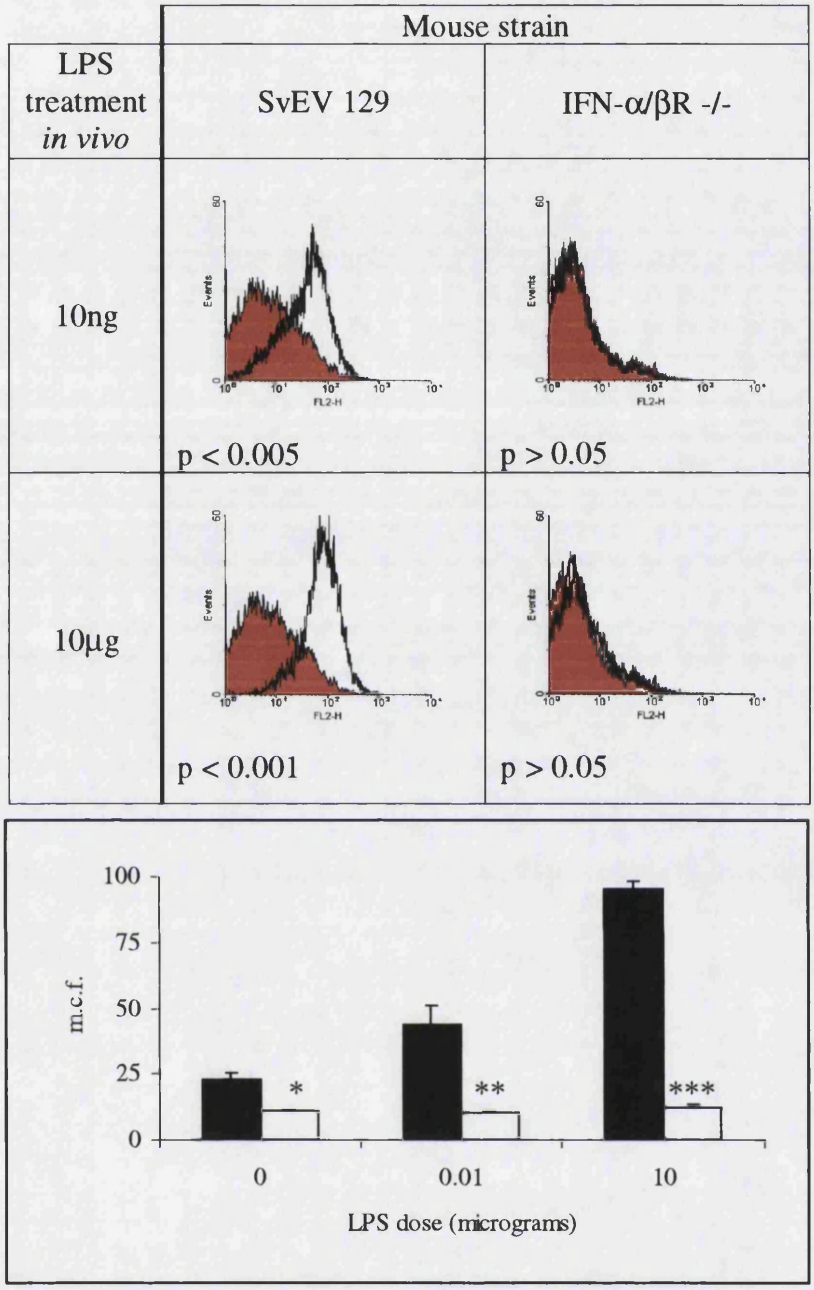


Figure 5.08. The effect of LPS on Ly6A/E expression on B cells in the absence of IFN-α/βR.

Wild type SvEv 129 (■) and type I IFNR -/- (□) mice were injected with PBS or LPS and sacrificed 24 hours later. LN were removed and the resulting cell suspensions stained for CD19 and Ly6A/E. Ly6A/E expression was analysed by gating on $CD19^+$ B cells and obtaining the mean channel fluorescence (m.c.f.). **A.** Histogram overlays represent Ly6A/E expression on $CD19^+$ B cells derived from PBS (■) or LPS (■) treated mice. The statistical significance of the effect of LPS on Ly-6A/E expression was calculated using 2-sample t-tests. **B.** Data represents the average "mean channel flourescence" of Ly-6A/E expression on $CD19^+$ B cells. The statisitcal significance of IFN-I non-responsiveness was determined using 2-sample t-tests. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

5.2B. The effect of LPS on B cell function.

Since LPS increased the expression of molecules such as CD86 and CD54, we examined the relative importance of B cells as co-stimulators of polyclonal T cell activation. As previously described, LN cells derived from mice injected 24 hours before with LPS exhibited an enhanced ability to proliferate to Con A *in vitro*. However, LPS appeared to be exerting its effect via non-T cells. Furthermore, depletion of $CD11c^+$ DC from the LN cell suspensions abrogated the Con A response of LN cells from control but not LPS-treated mice (see fig. 4.03. and fig. 5.09.). Thus, increased co-stimulation by LPS-activated B cells may conceivably have been a contributory factor. To assess this possibility, we initially compared the proliferative response to Con A of B-depleted LN cells from PBS- versus LPS-injected mice (fig. 5.09.). In fact, removal of B cells from LN cell suspensions resulted in a greater proliferative response by cells from both control and LPS-treated mice. Moreover, in the absence of B cells, LN cells from LPS-treated mice still proliferated more than cells from PBS-treated mice, indicating that the increased response was not solely due to the presence of activated B cells in the LN from LPS-injected mice. In contrast, when both B cells and $CD11c^+$ DC were depleted from the cell suspensions, the Con A response of LN cells from both PBS and LPS-treated mice was almost completely eliminated. These observations have several implications regarding T cell responses to Con A: (1) DC are the most efficient APC for providing co-stimulatory signals. (2) LPS-injection enhances the ability of DC to act as accessory cells. (3) B cells can also act as APC, and their ability to do so is augmented following LPS injection.

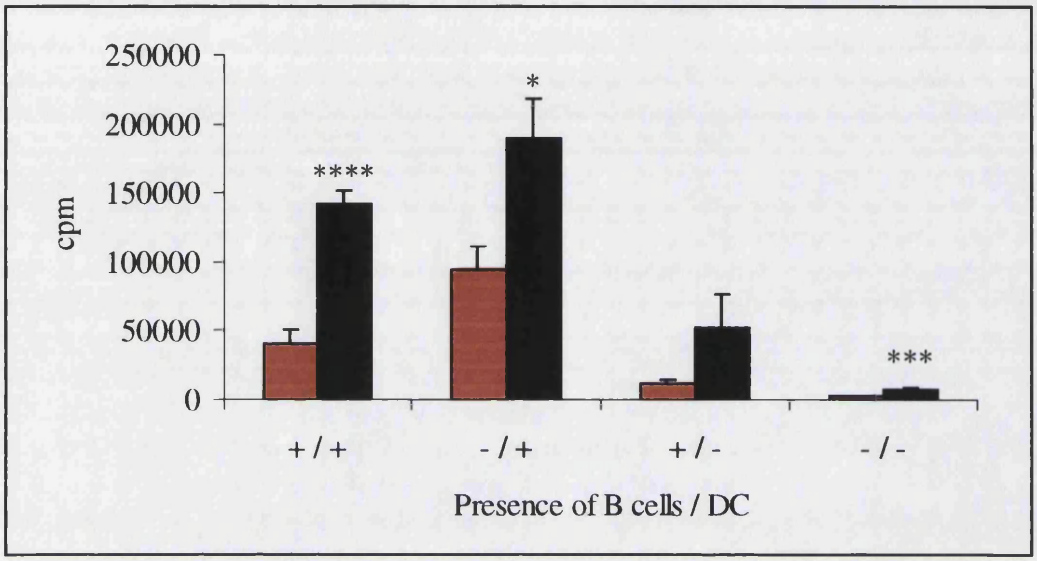


Figure 5.09. B cells are not absolutely required for the proliferative response to Con A *in vitro*.

C57BL/6 mice were injected with PBS or LPS and culled 24 hours later. LN were excised and single cell suspensions prepared by collagenase/DNase digestion. 1.5 x 10^6 "total" LN cells (+/+), B cell-depleted LN cells (-/+), DC-depleted LN cells (+/-) and B cell & DC-depleted LN cells (-/-) were seeded per well and cultured with 1μg/ml Con A. The assays were pulsed with tritiated thymidine after 48 hours in culture. Proliferation was determined 18 hours later by β-scintillation. Data represents the mean cpm of 6 samples ± s.e.m., PBS (■), LPS (■). (*, $p < 0.05$; ***, $p < 0.005$; ****, $p < 0.001$)

In addition to studying the effect of LPS *in vivo* on the ability of B cells to act as APC, we also investigated whether LPS-injection altered the subsequent response of B cells to activating stimuli. Two methods of *in vitro* B cell activation were used: I, LPS and II, cross-linking surface IgM. Figure 5.10A. clearly shows that prior exposure to LPS *in vivo* neither enhances nor inhibits the subsequent proliferative response to LPS *in vitro*. The effect of LPS *in vivo* on cross-linking surface IgM was less clear. When anti-IgM was added to total LN cells, lymphocytes derived from LPS-injected mice proliferated more than LN cells from PBS-injected mice (fig. 5.10B. I.). However, when the same dose of anti-IgM antibodies was added to purified B cells at high numbers per well (1.5 x 10^5), there was no observable difference in the proliferation of cells from LPS- versus PBS-injected mice (fig. 5.10B. II.). Further complicating interpretation was the observation that at sub-optimal concentrations of purified B cells (9.3 x 10^3 – 7.5 x 10^4 per well), proliferation was significantly higher for B cells derived from LPS-treated mice than B cells derived from control mice (fig. 5.10B. III.).

Taken together, these experiments suggest that B cells from LPS-treated mice may be hyper-sensitive to stimulation via the B cell receptor (BCR). This raised the question of whether the antibody response to antigen *in vivo* may be altered by prior exposure to LPS. To examine this question mice were injected with PBS or LPS i.v. and 24 hours later challenged s.c. with a soluble protein antigen (CGG) in the absence of adjuvant. Immunisation with CGG in the absence of adjuvant is known to induce a low but detectable antibody response, which is restricted to antibodies of the IgG1 subclass; addition of adjuvants can enhance

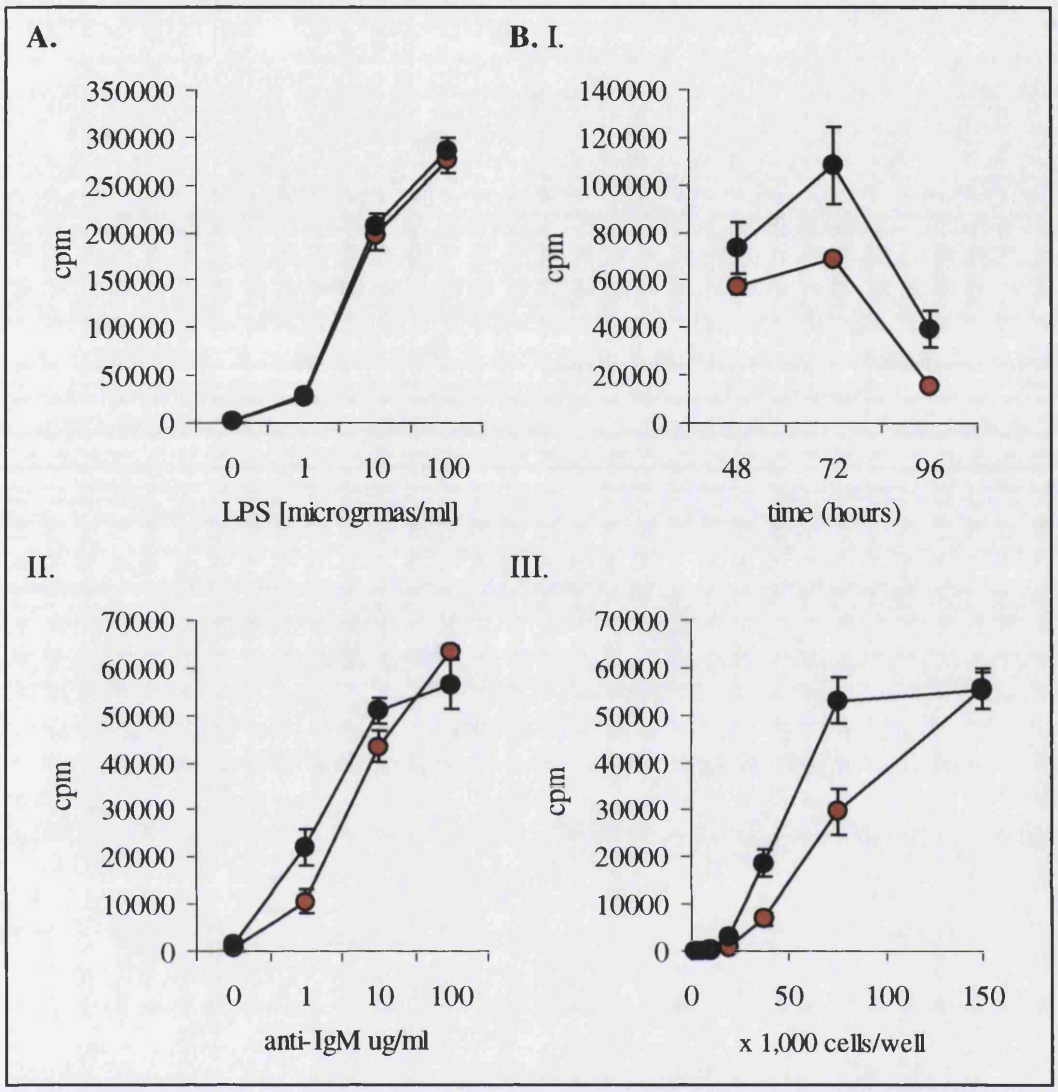


Figure 5.10. The effect of LPS *in vivo* on B cell activation *in vitro*.

C57BL/6 mice were injected with PBS, ● or LPS, ● and sacrificed 24 hours later. LN were excised and single cell suspensions were prepared. **A.** 1.5 x 10^5 cells per well were incubated with various doses of LPS *in vitro*. After 48 hours, the assay was pulsed with tritiated thymidine and frozen 18 hours later. **B.** I. 1.5 x 10^5 cells per well were incubated with 10µg/ml soluble anti-IgM cross-linking antibody. The assays were pulsed with tritiated thymidine after 48, 72 and 96 hours in culture. Plates were frozen 18 hours after pulsing. II.-III. B cells were purified from LN excised from PBS or LPS-injected mice. II. 1.5 x 10^5 B cells were cultured with various amounts of soluble IgM cross-linking antibody. III. Various concentrations of purified B cells were incubated with 10µg/ml anti-IgM cross-linking antibody. After 72 hours, both assays were pulsed with tritiated thymidine and frozen 18 hours later. **A-B.** Plates were thawed and proliferation was determined using β-scintillation. Data represents the mean cpm of triplicates ± s.e.m. **B.** Statistical analysis was performed using 2-sample t-tests. I. 72 and 96 hours, $p < 0.05$; II. 1-100µg/ml, $p > 0.05$; III. 7.5 x 10^4 cells – 9.375 x 10^3, $p < 0.05$.

the IgG1 response and promote switching to other isotypes (Le Bon *et al.*, 2001). Sera were collected 10 days after immunisation and the antibody response was determined by ELISA. In control mice, a significant anti-CGG IgG1 response was elicited (fig. 5.11.). In contrast, antibody titres were dramatically reduced in mice that had been injected 24 hours before with LPS; no Ig isotypes other than IgG1 were detected in these mice, indicating that the reduction in antibody titres was not due to induction of class switching (data not shown). Therefore, exposure to LPS 24 hours before immunisation inhibited rather than enhanced the humoral response.

5.3. Summary

LPS induced multiple B cell phenotypic changes *in vivo*. However, changes in phenotype were limited compared to the effect of LPS on T cell phenotype. Furthermore, not all phenotypic changes induced by LPS were observed after stimulation with poly I:C *in vivo* or with either IFN-containing splenocyte supernatant or recombinant IFN-α/β *in vitro*.

The effect of LPS on B cell function was less clear. Certainly, LPS *in vivo* did not induce a refractory period of non-responsiveness to LPS *in vitro*. Exposure to LPS for 24 hours *in vivo* appeared to affect the sensitivity of B cells to activation via the BCR. Nevertheless, the same period of LPS stimulation *in vivo* had a profound suppressive effect on the antibody response *in vivo* to a model antigen.

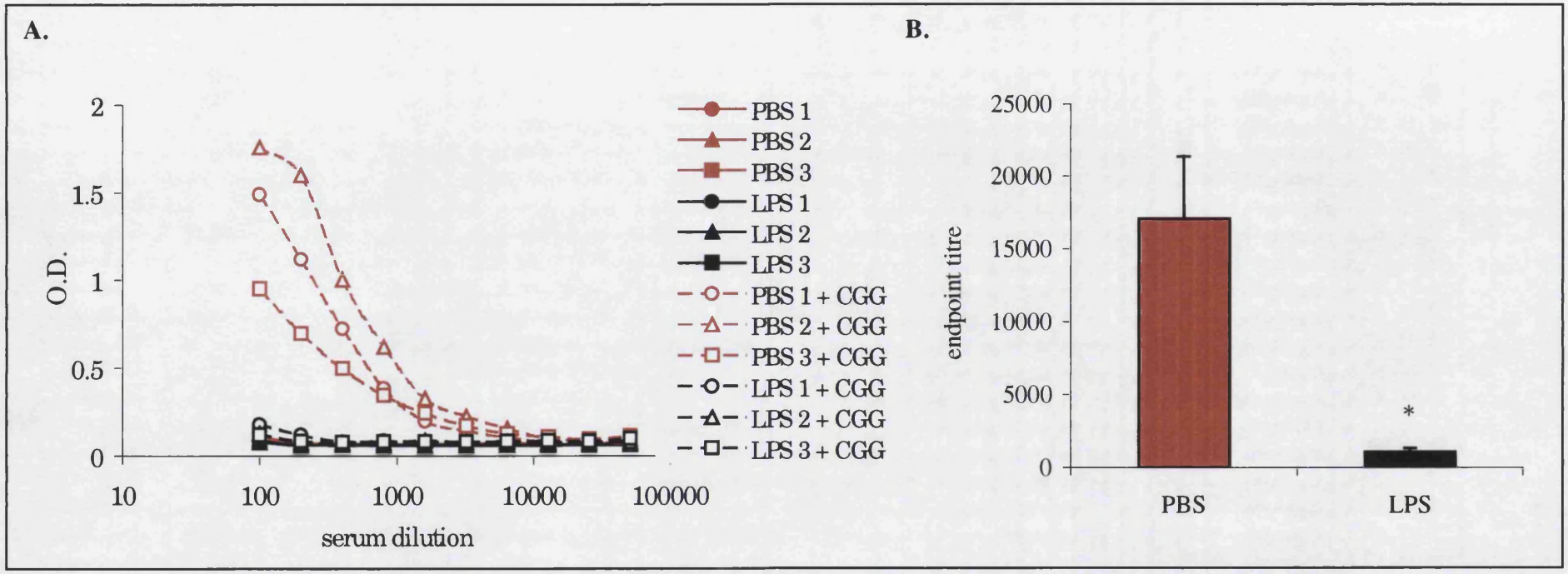


Figure 5.11. The effect of LPS on IgG1 antibody production to CGG *in vivo*.

C57BL/6 mice were injected with either PBS or LPS i.v. After 24 hours the mice were primed with 100µg chicken gammaglobulin (CGG) s.c. The mice were sacrificed 10 days post-immunisation and bled to collect serum samples. Antigen-specific antibody titres were determined by ELISA (see 2.2.10B.). No significant amount of antibody was detected in serum samples from mice that received PBS or LPS alone and sacrificed 11 days later. Furthermore, IgG1 was the only IgG isotype secreted in detectable quantities. **A.** Data represents the reciprocal serial dilution of each serum sample (red, PBS-treated mice; black, LPS-treated mice; open symbols and dotted line, CGG-primed mice) versus optical density. **B.** Results are expressed as the mean reciprocal endpoint titre of triplicate serum samples (± s.e.m.) from CGG-primed mice injected with either PBS (■) or LPS (■) (*, $p < 0.05$). Endpoint values were calculated using Excel (see 2.2.10B.). O.D. values from serum samples from mice injected with PBS only were used to compensate for background.

5.4. Discussion

We have reported that LPS injection induced B cells to alter surface expression of molecules associated with activation (Ly-6A/E and CD69), co-stimulation (CD86 and CD40) and adhesion (CD11a and CD54). The effect of LPS on B cell Ly-6A/E expression was shown to be LPS-specific, since B cells isolated from LPS-treated C3H/HeJ mice (lacking functional TLR4 (Poltorak *et al.*, 1998)) were unable to significantly up-regulate this activation marker. Similar to the results discussed in chapter 3, B cell phenotypic changes could be induced by LPS *in vitro*. However, in marked contrast to T cells, LPS appeared to be able to act directly on B cells, since purified B cells were able to up-regulate the expression of their cell surface markers after treatment with LPS *in vitro*. Furthermore, whilst Ly-6A/E expression on B cells showed similar sensitivity to poly I:C *in vivo* and IFN-α/β *in vitro* compared to T cells, CD54 B cell expression remained unaltered by these agents.

Perhaps of noteworthy attention are the subtle differences in the effect of LPS on splenic and LN B cell phenotype. Similar to the situation reported for T cells, LN cells appear to be much less responsive than splenocytes, in terms of the B cell phenotypic response to LPS *in vitro*. However, LPS induced equal levels of CD54 expression on B cells regardless of the tissue of origin. Therefore, it may be the case that up-regulation of CD54 on B cells is a consequence of direct stimulation with LPS, whilst the up-regulation of molecules such as Ly-6A/E and CD69 are dependent on cytokines released by non-T or B cells.

LPS-induced Ly-6A/E upregulation was similar to that observed on T cells and $CD11c^+$ DC, in as much as it was by far the most dramatic of all the phenotypic changes detected. Ly-6A/E has been reported to be associated with B cell activation. However, no physiological role for this molecule has been identified. $Ly\text{-}6A^{-/-}$ B lymphocytes (from Ly-6A null mice) appeared to respond in a relatively normal manner *in vitro*. Nevertheless, the absence of Ly-6A was observed to negatively affect antibody titres to KLH *in vivo* suggesting a possible involvement in B cell activation (Stanford *et al.*, 1997). Although the putative ligand is not known, Ly6A/E has been documented to be involved in B cell signal transduction. Engagement by cross-linking antibodies results in Ca^{2+} translocation (Snapper *et al.*, 1991) and proliferation *in vitro*, if in the presence of IL-4 and IFN-γ (Codias and Malek, 1990).

In many ways CD69 is similar to Ly-6A/E in terms of our present understanding of its biological properties. Like Ly-6A/E, its putative ligand remains unknown and thus cross-linking antibodies are the only current means of directly activating the molecule *in vitro*. Cross-linking in the presence of a PKC activator results in Ca^{2+} translocation and proliferation (reviewed in Testi *et al.*, 1994). Thus, it is assumed that CD69 has a physiological role in B cell activation, although $CD69^{-/-}$ mice exhibit only a very mild phenotype. $CD69^{-/-}$ B cells proliferated normally to LPS *in vitro* and only subtle effects on B cell development and antibody synthesis were observed (Lauzurica *et al.*, 2000).

Whilst in the absence of further data, we can only assume that Ly-6A/E and CD69 function via adhesion of their respective ligands, a role for LFA-1 and

CD54 in B cell adhesion is well recognised. For example, antibodies to LFA-1 or CD54 can inhibit T cell:B cell conjugates. Furthermore, co-culture of antigen-specific B cells and T cells in the presence of antigen, induces the clustering of LFA-1 (but not CD54) on B cells and CD54 (but not LFA-1) on T cells at the area of cell contact (Sanders and Vitetta, 1991).

LFA-1 has also been reported to be important for the formation of B cell:DC clusters. Rat DC and B cells were observed to form clusters within 1 hour of incubation *in vitro* and this could be inhibited by anti-LFA-1 but not anti-CD54 antibodies. Similar to our observation with murine B cells, LPS injected into rats "partially activated" splenic B cells since CD54, B7 and LFA-1 expression levels were all increased. However, LFA-1 expression did not correlate with B cell:DC cluster formation since splenic B cells activated by LPS *in vitro* or *in vivo* were unable to adhere to DC (Kushnir *et al.*, 1998).

By contrast, CD54 expression has been correlated with the co-stimulatory activity of B cells. Peripheral blood human B cells were sorted into $CD54^{lo}$ and $CD54^{hi}$ populations and used to co-stimulate T cell proliferation to Con A. B cells that expressed high levels of surface CD54 co-stimulated significantly higher proliferation than B cells with low levels of CD54 (Dennig *et al.*, 1994).

Considering that we have observed modest upregulation in CD54 and CD86 (or B7-2) after LPS stimulation *in vivo*, it seemed reasonable to investigate the co-stimulatory contribution of B cells from LPS-treated mice to the Con A response *in vitro*. However, the removal of B cells from LN cell suspensions actually

enhanced the Con A response of cells isolated from either control (PBS) or LPS-injected mice. B cell depletion may have simply enriched for T cells and dendritic cells, thus increasing the ratio of responder cells to "professional" APCs (i.e. dendritic cells). In agreement with this idea was the observation that removal of both B cells and DC had a significantly more dramatic negative effect on the Con A response of LN from LPS-treated mice than just DC depletion. Therefore, B cells from LPS-treated mice do appear to have increased co-stimulatory function, but this subtle difference was only evident in the absence of more "potent" APC.

Whilst B cells from LPS-treated mice may not have been critical for augmenting the T cell mitogenic response, T cells or APCs from LPS-treated mice perhaps may have been important for enhancing the B cell mitogenic response. LN cell suspensions derived from LPS-treated mice proliferated at significantly higher levels to soluble anti-IgM antibodies than LN cells from control mice. One possible mechanism that could account for this observation may be the release of cytokines from LPS-activated cells that coactivate B cells. For example, human IL-15 has been demonstrated to co-stimulate purified B cells to proliferate to immobilised anti-IgM antibody approximately 5-fold greater than antibody alone (Armitage *et al.*, 1995). This observation may help to explain why in our murine system, purification of LN B cells appeared to abrogate the enhancing effect of LPS *in vivo*, at least at optimal cell concentrations. Perhaps removal of APCs (e.g. macrophages and DC) during B cell purification also removed a major source of IL-15 required for optimal proliferation (Mattei *et al.*, 2001).

Alternatively, B cells from LPS-treated mice may be intrinsically altered, thus becoming hyper-responsive to activation via their Ig receptors. The heightened sensitivity to anti-IgM of purified B cells from LPS-injected mice when cultured at sub-optimal cell concentrations *in vitro* suggests this as a possibility.

Thus, LPS stimulation *in vivo* appeared to induce a "partially activated" B cell phenotype and increased B cell responsiveness to activation via the BCR. Therefore, it was of obvious interest to examine the effect of LPS injection on a subsequent humoral response to immunisation. Using chicken gammaglobulin (CGG) as a model antigen, it was found that serum samples collected from CGG-primed mice that had also been injected with LPS i.v. 24 hours prior to immunisation contained dramatically lower levels of anti-CGG IgG1 antibodies than sera from mice injected with PBS and then CGG.

There are several possibilities why LPS stimulation before challenge with an antigen suppressed the antibody response rather than providing an "adjuvant" effect. Downmodulation could act at the level of I, the effector B cell; II, the T helper cell or III, the professional APC.

With regard to the former, there is indeed some evidence that LPS can have direct, negative effects on B cell function. For example, induction of B cell apoptosis has been implicated as a mechanism by which LPS administered to SRBC-primed mice diminished the secondary response *in vitro*. Here, histological staining revealed an increase in fragmented DNA within the Ig^+ splenocytes located in lymphoid follicles after LPS injection into SRBC-primed

mice (Yokochi *et al.*, 1996). Furthermore, LPS activation of B cells *in vitro* can lead to apoptosis in an LFA-1/CD54 dependent manner (Wang and Lenardo, 1997). Thus, it seems theoretically possible that LPS induced B cell apoptosis *in vivo* that subsequently led to a reduced anti-CGG response. However, in our experiments the percentage of B cells within the spleen remained the same after LPS stimulation *in vivo*. Therefore, B cell numbers appeared to be unaffected by LPS *in vivo*.

Other B cell properties may have been altered by LPS treatment *in vivo*. For example, as mentioned previously, B cell:DC clustering has been reported to be downregulated by the systemic administration of LPS (Kushnir *et al.*, 1998). In addition, we observed downregulation of CD40 on B cells which may have affected interactions with DC or T cells. CD40 is key B cell activation molecule whose ligation is required for the production of non-IgM isotypes (Gray *et al.*, 1994; Xu *et al.*, 1994). Therefore, downregulation of CD40 may have induced only minimal levels of isotype switching resulting in depressed levels of IgG1.

Alternatively, isotype switching may have been modulated by IFNγ produced by LPS-activated NK cells or T cells, since this cytokine potently suppresses IgG1 antibody production (Bossie and Vitetta, 1991; Finkelmann *et al.*, 1988) (see 4.1). However reduced IgG1 synthesis by IFNγ is usually accompanied by enhanced IgG2a secretion (Snapper *et al.*, 1988). Furthermore, high IgM titres would probably have been detected in LPS-treated mice if decreased isotype switching was responsible for lower IgG1 production. Since no other Ig isotypes (including IgM) were detected in the sera from CGG-primed mice, IFNγ or

reduced CD40 expression, are less than likely candidates for the suppression of IgG1 synthesis in our experiments.

A more plausible explanation for the reduced antibody response may derive from our previous observations on alterations in DC 24 hours post-LPS-injection. As shown in chapter 4 (section 4.2E.), there is a substantial increase in the proportion and number of myeloid $CD11b^+$ $CD11c^+$ DC within the lymph nodes at this time point. At face value, this might lead one to predict that an enhanced humoral response should be elicited in these mice upon s.c. immunisation. In favour of this idea, $CD8^-$ $CD11c^+$ murine myeloid DC have been reported to preferentially stimulate $CD4^+$ T cells to secrete a T_H2 cytokine profile (Maldonado-Lopez *et al.*, 1999). Furthermore, selective expansion of $CD11b^+$ $CD11c^+$ myeloid DC *in vivo* by GM-CSF injection, prior to immunisation with OVA, resulted in a marked enhancement in the IgG1 response (Pulendran *et al.*, 1999).

However, maturation of DC by LPS *in vivo* may be the very mechanism that so dramatically dampened down the B cell response to CGG. Mature DC are potent APCs but are poor at endocytosis and processing of antigen (Winzler *et al.*, 1997; Granucci *et al.*, 1999). Thus, DC in LPS-treated mice may have been too mature to take up CGG and present processed antigen to T cells. Therefore, poor activation of $CD4^+$ T cells by DC may have compromised the T cell "help" required for isotype switching and B cell differentiation to plasma cells.

In addition, the role of DC in the humoral response is not solely confined to the activation of T cells. DC have also been reported to retain antigen in its native form *in vivo* and to release it for recognition by B cells (reviewed in MacPherson *et al.*, 1999). Experiments investigating the effects of LPS on DC *in vivo* (reported in chapter 4) implied that 24 hours p.i., LPS-stimulated DC may have been in a "final" terminal phase of maturation. Therefore, if the LPS-stimulated DC are in a terminal maturation state at the time of immunisation, the corresponding humoral response may be negatively affected due to I, reduced presentation of native antigen to B cells and/or II, poor presentation of processed antigen to $CD4^+$ T cells.

In conclusion, LPS induced "partial activation" of B cells *in vivo* in terms of their phenotype and responsiveness to non-specific activation *in vitro*. In these respects, LPS-activation of B cells *in vivo* was similar to that of T cells. However, production of specific antibody was markedly reduced upon subsequent challenge with antigen *in vivo*. This likely reflects the complex cellular requirements for initiation and regulation of the humoral response that may have been disrupted by LPS stimulation *in vivo*.

Chapter 6: Discussion

6.1. Précis

The adjuvant properties of lipopolysaccharide (LPS) have long been recognised (Johnson *et al.*, 1956), however, the mechanism by which LPS enhances adaptive immune response remains undefined. Therefore, our aim was to assess whether the immunostimulatory activity of LPS was associated with effects on both naïve lymphocytes and antigen presenting cells. The main focus of our investigations was to examine the possibility that the stimulatory potential of LPS was, at least in part, due to the alteration of cell adhesion and co-stimulatory molecules on naïve lymphocytes or antigen presenting cells (APC), altering their ability to respond to, or present specific antigen respectively.

In the previous chapters we have stated that following injection of LPS, naïve T and B cells and antigen presenting cells (DC) increase the expression of a number of cell surface activation markers, including Ly-6A/E, CD54, and CD86. T cell changes in phenotype were shown to be transient and appeared to be induced indirectly via the secretion of cytokines by LPS-activated DC. While we have no clear answers as to how these phenotypic changes pertain to the adjuvanticity of LPS, the results themselves raise a number of questions and point to possible areas of future research, which are summarised below.

6.2. DCs are induced to migrate to LNs following LPS-injection and stimulate phenotypic activation of T-lymphocytes through secretion of cytokines.

In chapters 3 and 5 we have reported that LPS induces multiple phenotypic changes on T and B cells *in vivo*. The *in vivo* events required for triggering the changes in T cell phenotype were shown to occur just 1 hour after LPS-injection. T cells purified from the spleens of LPS-treated mice (culled 1 hour post-injection) up-regulated the expression of surface activation markers upon subsequent culture *in vitro*. Since addition of LPS to purified T cells *in vitro* did not induce phenotypic changes, we may assume that LPS does not act directly on T cells *in vivo*. Therefore, the likely conclusion is that 1 hour *in vivo* was sufficient time for LPS to activate "accessory" cell-mediated stimulation of T cells.

However, in these experiments the magnitude of LPS-induced T cell phenotypic changes could be increased by either (1) increasing the length of exposure to LPS *in vivo* or (2) culturing total splenocytes. This suggests that (1) 1 hour following injection of LPS is sufficient (but not optimal) for triggering T cell phenotypic changes *in vivo* and (2) that other cell types are required for optimal T cell "phenotypic" activation. Addition of an endotoxin inhibitor to splenocyte cultures ruled out the possibility that the phenotypic changes were due to contamination of LPS *in vitro*. Furthermore, culturing purified T cells and T-depleted splenocytes in transwells revealed that cell-cell contact was not an absolute requirement for the induction of T cell phenotypic changes, thus implying that soluble factors may be involved.

Curiously, LN T cells exhibited significantly less dramatic modulation of T cell phenotype than splenocytes either when placed in culture 1 hour after LPS injection or when treated with LPS *in vitro*. In contrast, approximately the same level of upregulation of T cell phenotypic markers was observed on splenic and LN T cells 24 hours after LPS injection. Two possible explanations may explain this disparity: (1) following LPS injection, the spleen releases circulating soluble factors that stimulate T cells in peripheral lymphoid organs or (2) the accessory cell composition in the LN becomes altered 24 hours after LPS injection.

Currently we have no evidence to support or disprove the former hypothesis. However, we have observed that following LPS injection, the proportion of $CD11c^+$ DC in the LN is significantly increased. Therefore, we propose that upon LPS injection, $CD11c^+$ DC migrate to the LN and stimulate upregulation of the surface expression of T cell activation-associated molecules. Whilst we have no direct evidence to corroborate our theory *in vivo*, indirect evidence supports the notion that cytokine secreting DC are responsible for stimulating T cell phenotypic changes in the LN following LPS-injection. This comes from our studies of purified splenic DC, which were isolated from LPS-injected mice and placed in culture for 18 hours. Cytokine analysis of the resulting supernatant revealed that LPS-stimulation *in vivo* induced DC to secrete large amounts of IL-12p40 and IFN-γ. Furthermore, supernatant from LPS-activated DC potently stimulated the upregulation of cell surface markers on T-lymphocytes *in vitro*.

In vitro data implicates type I IFN as a putative "effector" cytokine since recombinant IFN-α/β may directly stimulate purified T cells to alter their phenotype *in vitro*. *In vivo* data using mice deficient for IFN-α/β also supports the idea that IFN-I may be required for mediating the T cell phenotypic response to LPS. Since DC have been demonstrated to be major producers of type I IFN following activation with LPS (Cella *et al.*, 1999; Rescigno *et al.*, 1999) it seems reasonable to postulate that upon migration to the LNs, LPS-activated DC produce IFN-α/β, which stimulates the phenotypic activation of T cells. However, within this framework hypothesis, several questions remain unanswered:

(1) Is the increase in the proportion of $CD11c^+$ DC in the LN following LPS injection generated by (a) DC migration, (b) maturation of resident precursor DC, (c) recruitment of circulating precursors or (d) all of the above? Evidence from published literature suggests that an inflammatory stimulus such as LPS is sufficient to induce migration of DC from peripheral tissues to draining LN (Roake *et al.*, 1995; MacPherson *et al.*, 1995). Data from experiments with human circulating monocytes have demonstrated that inflammation within LNs actively recruits cells with massive IFN-α/β producing capabilities.

(2) Do LN DC from LPS-treated mice produce IFN-α/β and if so, is this the only DC-produced cytokine involved in mediating T cell phenotypic changes? Considering that a number of recombinant cytokines were able to stimulate T

cells when added exogenously to *in vitro* cultures, IFN-α may not be the only pathway for LPS-induced alterations in T cell phenotype.

(3) Are DC the only cytokine-secreting cell type to stimulate T cell phenotypic activation after LPS injection? Macrophages may be another likely candidate for the production of IFN-α in response to LPS stimulation (Ohmori and Hamilton, 2001). However, previous attempts at the isolation of $CD14^+$ macrophages from LN and splenocyte preparations have proved difficult. Therefore, an alternative method for addressing this question may be to deplete of DC from LN and splenocyte suspensions derived from LPS-injected mice prior to culture, and assess whether the supernatant from these cells can stimulate T cell phenotypic activation.

6.3. LPS induces the up-regulation of Ly-6A/E expression on lymphocytes and DC *in vivo*.

Of all the activation markers investigated, the expression of Ly-6A/E on T cells, B cells and DC was by far the most sensitive to LPS treatment *in vitro* and *in vivo*. Ly-6E was the first member of the Ly-6 superfamily (Ly-6SF) to be characterised at the genetic level. A large multi-gene complex was subsequently identified on mouse chromosome 15 and proposed to include five loci A, B, C, D, E, existing in two allelic forms, with Ly-6.1 alleles usually expressed at lower levels than Ly-6.2 alleles. However, Ly-6A and Ly-6E were shown to be two allelic forms of the same molecule with the monoclonal antibody D7 recognising both alloantigens: Ly-6A.2 and Ly-6E.1 (Ortega *et al.*, 1986). Furthermore,

advances in bioinformatics and genomics have dramatically expanded the Ly-6SF in both mice and humans. Examples of recently discovered murine Ly-6 molecules are Ly-6F (Fleming *et al.*, 1993), Ly-6G (or the granulocyte marker Gr-1, (Fleming *et al.*, 1993)), Ly-6H (Apostolopoulos *et al.*, 1999) and Ly-6I (Pflugh *et al.*, 2000). Currently the human Ly-6SF consists of CD59, Ly-6H, RIG-E (the human homologue of thymic shared antigen-1), Thymocyte B cell antigen (ThB) or E48 (the human homologue of Ly-6D). In addition, the Ly-6 antigens have been reported to be homologous to a functionally diverse group of proteins such as snake venom α-neurotoxin and the urokinase plasminogen receptor.

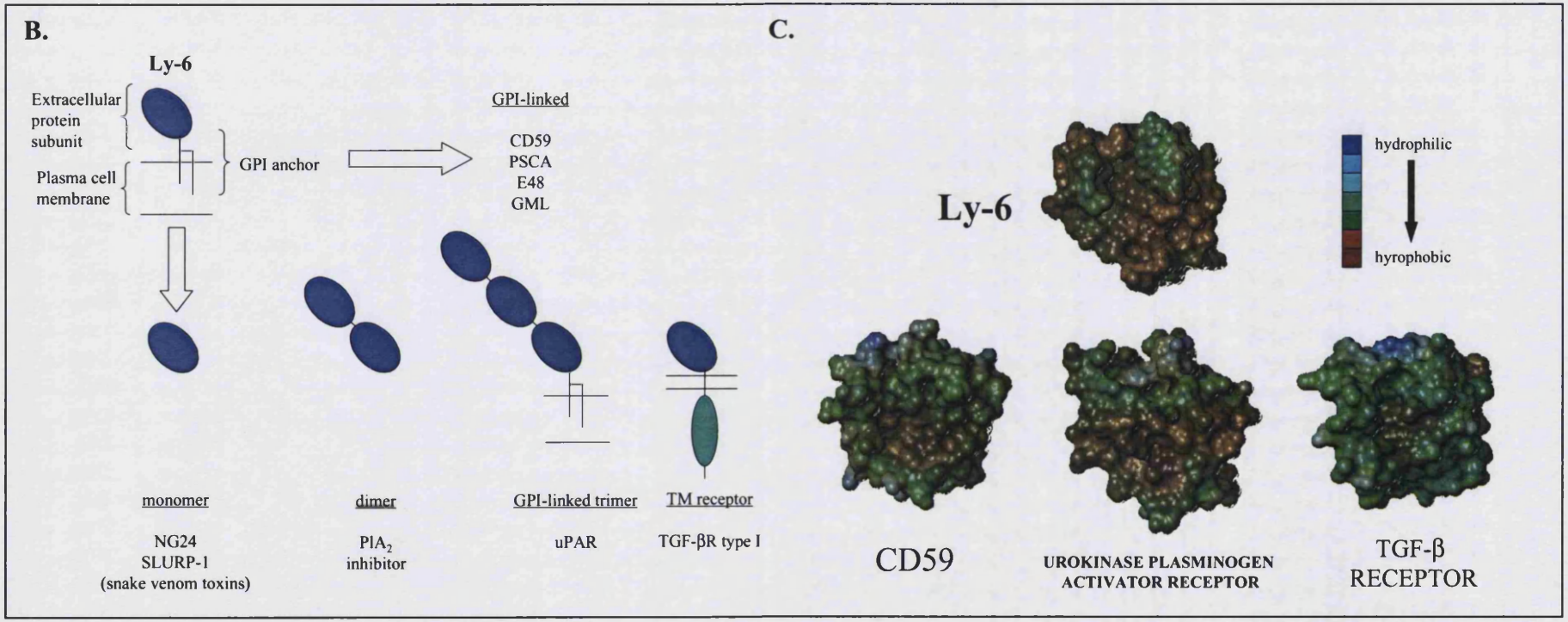
Currently, the physiological ligand and precise function of Ly-6A/E is not known. Therefore, we decided to ask Darren Flowers to use bioinformatic analytical techniques to try and predict putative functions and protein ligands for Ly-6A/E. However, the molecules with sequence similarity to Ly-6A/E were so functionally diverse (see table 6.1. and figure 6.1B.) that we were unable to produce any clear-cut conclusions with regards to predicting a putative function. Nevertheless, the results were still interesting and may suggest possible avenues for future investigations.

Table 6.1. Molecules bearing sequence similarity to Ly-6A/E. Relative sequence similarity is ranked, with the molecules bearing the greatest similarity to Ly-6A/E at the top of the table.

Sequence similarity	**Name**	**Function**
1	Ly-6C	Putative role in T cell activation and effector function (Johnson *et al.*, 1993)
2	Ly-6I	Unknown, distribution similar to Ly-6C and Ly-6A/E, implying possible role in redundancy in Ly-6C$^{-/-}$ and Ly-6A$^{-/-}$ mice (Pflugh *et al.*, 2000).
3	Ly-6B	Unknown
4	Ly-6F	Unknown
5	U-PAR (CD87)	Binds a proteolytic ligand, u-PA and appears to be involved in chemotaxis (Blasi, 1997)
6	Snake venom toxins	Bind to nicotinic acetylcholine receptor (nAchR) on post-synaptic membranes of skeletal muscles to block neuromuscular transmission (Chang *et al.*, 1999).
7	Ly-6H	Unknown, however expressed in central nervous and immune system (Apostolopoulos *et al.*, 1999)
8	Prostate stem cell antigen (PSCA)	Unknown function. However, expression correlates with prostate cancer and has been postulated to possess anti-apoptotic properties (Reiter *et al.*, 1998)
9	Phospholipase A2 inhibitor	Snake neurotoxin inhibitor (Ohkura *et al.*, 1994)
10	TSA-1	Appears to function in signal transduction and cell activation (Classon and Coverdale, 1994).

Sequence similarity	Name	Function
11	Ly-6G	Unknown, expression restricted to granulocytes (Fleming *et al.*, 1993).
12	CD59	Inhibits complement-mediated cytolysis by interfering with C9 membrane insertion and polymerisation (Yu *et al.*, 1997).
13	ThB/E48/Ly-6D	Cell-cell adhesion (Brakenhoff *et al.*, 1995)
14	TGF-βR type I	Transmembrane serine/threonin kinase involved in cell growth, apoptosis, cell differentiation, and extracellular matrix (ECM) synthesis (Choi, 2000; ten Dijke *et al.*, 1996).
15	NG24	Unknown. An unannotated open reading frame from the mouse major histocompatibility locus class III region (D. Flowers, personal communication).
17	Secreted Ly-6/uPAR-related protein 1 (SLURP-1)	Encoded by ARS (component B)-81/s locus, mutations of which causes Mal de Meleda (MDM); a rare autosomal recessive skin disorder (Fischer *et al.*, 2001).
18	GPI anchored molecules like protein (GML)	Putative pro-apoptotic role (Ueda *et al.*, 1999).
20	Xenopus transforming growth factor beta-related receptor type I	Directs mesodermalized tissue into muscle or notochord (Mahony and Gurdon, 1995).

Figure 6.1 Ly-6A/E and its relationship to other molecules. **A.** Ly-6A/E was modelled by D. Flower (Briefly, protein models were built using a program modeller (Sali, 1993 #583). Characteristic to the Ly-6SF, Ly-6A/E is a small cysteine-rich protein with a three-finger β-sheet and an N-terminal GPI-anchor motif. **B.** The protein sequence similarity of Ly-6A/E homologues were obtained by D. Flower using database searching strategies based on BLAST (Altschul, 1997 #580). Ly-6A/E appears to be homologous to a wide diversity of other proteins that may be grouped by their structure into (I) GPI-membrane anchored monomers, (II) soluble monomers, (III) soluble dimers, (IV) GPI-anchored trimer, (V) transmembranous monomer. **C.** A comparison of the hydrophobicity of Ly-6A/E, CD59, u-PAR and TGR-β receptor type I. (N.B. The structures of representative Ly6 homologues were predicted by D. Flower based on the published crystal structures of CD59 (Fletcher, 1994) available in the RCSB protein data bank (Berman, Acids Res 2000).



Three molecules with known ligands and immunological function were selected for the comparison with Ly-6A/E. The hydrophobicity of the four molecules was predicted by D. Flower and are presented in figure 6.1C. Ly-6-A/E appeared to be by far the most hydrophobic, and the most similar to u-PAR compared to CD59 and TGF-βR.

u-PAR is an interesting molecule with regards to its structure and biological function since it has a number of "chemokine-like" activities, including cell adhesion, chemotaxis and receptor clustering. Collectively they facilitate cell recruitment and infiltration as demonstrated by the lack of T cell and macrophage recruitment in uPA-deficient mice. Furthermore, cell migration has also been reported to be impaired in patients suffering from the immunodeficiency PNH. Interestingly, GPI-linked and soluble u-PAR have both been shown to be capable of inducing signal transduction, suggesting a situation comparable with CD14 where association with a TM adaptor protein is required for signalling (Blasi, 1997).

As mentioned in figure 6.1C., u-PAR possess multiple Ly-6-like domains. The linker region between two of these domains has been shown to possess chemotactic properties upon receptor-ligand binding. Thus, similar "chemotactic epitopes" are unlikely to be present in Ly-6A/E. However, the N-terminal Ly-6-like domain of u-PAR appears to bind the epidermal growth factor domain of u-PA, a situation comparable to the ligands for CD59 and Ly-6D (Apostolopoulos *et al.*, 2000). Therefore, members of the Ly-6SF may conceivably share a common binding region for ligands bearing EGF-like domains.

In the previous results chapters we have reported the dramatic upregulation of Ly-6A/E on T cells, B cells and dendritic cells after LPS injection. Whilst the properties of Ly-6A/E on the former two cell types have been investigated for many years, expression on dendritic cells has only recently been acknowledged (Izon *et al.*, 1996). Therefore, our observed modulation of DC cytokine production by Ly-6A/E stimulation *in vitro* may represent a significant and novel role for Ly-6A/E in the regulation of DCs. On a more cautious note, the physiological relevance of Ly-6A/E cross-linking may be questionable, particularly since PMA was added to the *in vitro* stimulation assays. Certainly, these experiments need to be repeated with the addition of F_C blocker and in the absence of PMA.

Nevertheless, the function of Ly-6A/E on dendritic cells is certainly an area worthy of future investigation. Given that (1) DC "accumulate" in LNs 24 hours following LPS-injection, (2) Ly-6A/E expression is up-regulated on LPS-activated DC and (3) Ly-6A/E is homologous to u-PAR, it may be interesting to speculate the involvement of Ly-6A/E in cell migration. To test this theory, it may prove informative to block Ly-6A/E, via the systemic administration of a Ly-6A/E F_C fusion protein, and then examine the proportion of $CD11c^+$ DC present in the LN following LPS injection.

6.4. The effect of LPS *in vivo* on APC function.

As mentioned in the previous sections, we have reported that 24 hours after intravenous injection of LPS, $CD11c^+$ DC accumulate within LNs. At the same time point we also observed disappearance of $CD11c^+$ DC from spleens taken from LPS-treated mice. This correlated with a decreased T cell stimulatory capacity of allogeneic splenocytes derived from LPS-injected mice compared to stimulators from control mice. By contrast, the co-stimulatory ability of APCs within cell suspensions prepared from the LNs of LPS-injected mice was dramatically augmented for the T cell proliferative response to Con A. The APCs responsible for mediating the LPS-induced enhancement of the Con A response were identified as B cells and $CD11c^+$ DC.

Therefore, considering that the co-stimulation of T cells by DC and B cells is critical for B cell activation and isotype switching (see 1.4B.), we decided to investigate the antibody response to a model soluble antigen *in vivo* following injection with LPS. Mice were injected i.v. with either LPS or PBS and 24 hours later primed with CGG s.c., thus preferentially directing the immunogen to the draining lymph nodes rather than the spleen. The resulting data clearly demonstrated that prior systemic stimulation with LPS profoundly depressed circulating levels of antigen-specific IgG1. Initially this effect of LPS appeared to be counter-intuitive when taking into account the other effects of LPS-injection we have observed; specifically (1) the accumulation of activated DC in peripheral lymph nodes and (2) the heightened responsiveness of B cells derived from LPS-injected mice to BCR stimulation *in vitro*.

However, upon further reflection the activation status of DC and B cells in LPS-injected mice may in fact explain the inhibitory activity of LPS on antibody production. Thus, following LPS stimulation *in vivo*, B cells that are hyper-responsive to BCR stimulation may disrupt the efficiency of the GC reaction to CGG, since lower-affinity B cells may possibly be recruited but not positively selected. Alternatively, increased reactivity to BCR stimulation may simply induce B cell AICD as a means of peripheral tolerance.

In addition, inhibition of the antibody response may be due to the terminal maturation of DC by LPS. This may result in a reduced DC lifespan (Kamath *et al.*, 2000), in which case these cells may not be able to present antigen for a sufficient duration to induce T cell activation (Lanzavecchia and Sallusto, 2001; Viola and Lanzavecchia, 1996). Furthermore, these mature DC, despite expressing high levels of co-stimulatory activity, may be relatively inefficient at antigen capture and processing (Banchereau *et al.*, 2000). Finally, as discussed above, one explanation for the increase in $CD11c^+$ DC in the LN is that LPS has induced the maturation of DC in peripheral sites and their subsequent migration into LN. Therefore, it is possible that peripheral tissues such as the skin are relatively depleted of immature DC 24 hours following LPS injection (Roake *et al.*, 1995). This, combined with the lack of immature DC in the LN, may lead to inefficient uptake of antigen after s.c. injection and ultimately poor T and B cell priming. Further characterisation of the DC present in LN and skin 24 hours after LPS injection will be required to address these issues.

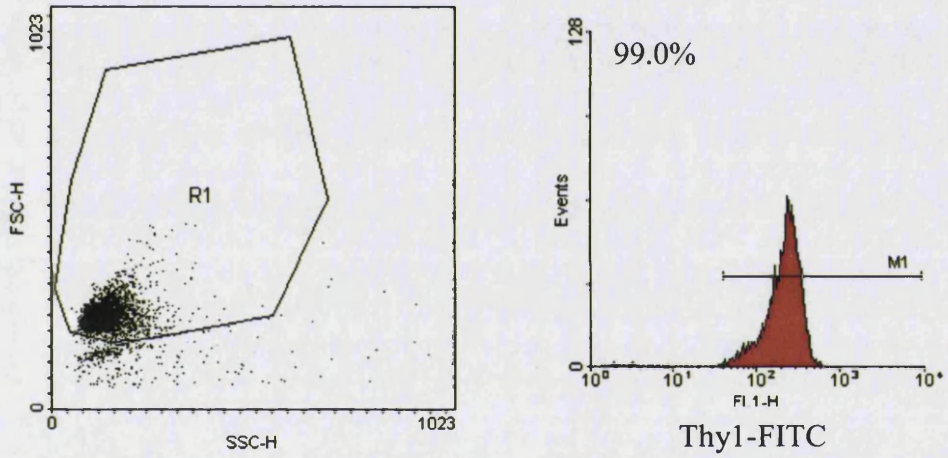
In conclusion, we have shown that the systemic administration of LPS affects lymphocyte phenotype, proliferation and in the case of B cells, effector function. However, the main cell type involved in all of these responses to LPS appears to be the dendritic cell. Therefore, we propose that activation of dendritic cells by PAMPs such as LPS may be important in determining the activation status of the adaptive immune system and for the instruction of lymphocyte responses to antigen *in vivo*.

Appendices

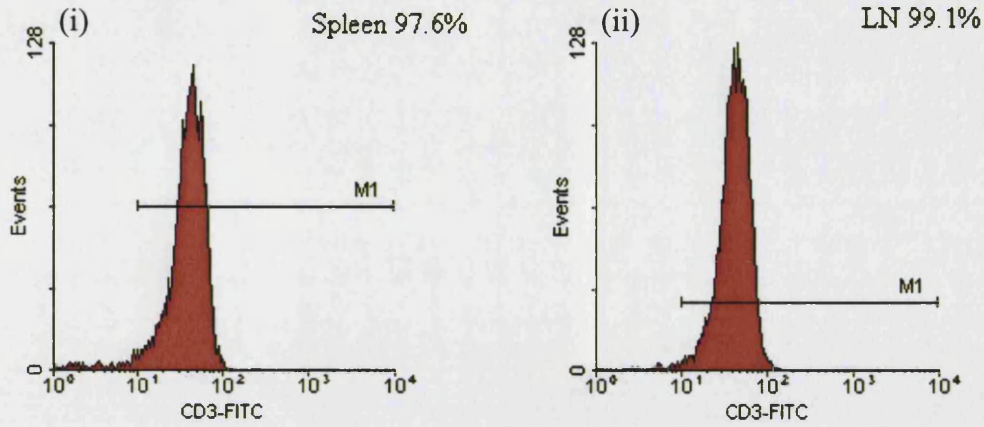
Appendix 1: FACS profiles illustrating the purity and nature of positively or negatively selected cells.

A. Purification of T cells by positive or negative selection.

I. Positive selection via fluorescence activated cell sorting. LN cells were stained with a fluorescent antibody specific for a T cell surface molecule and purified on the MoFlo. The histogram below represents the percentage of cells within the region R1 that expressed the T cell surface marker Thy1.2.

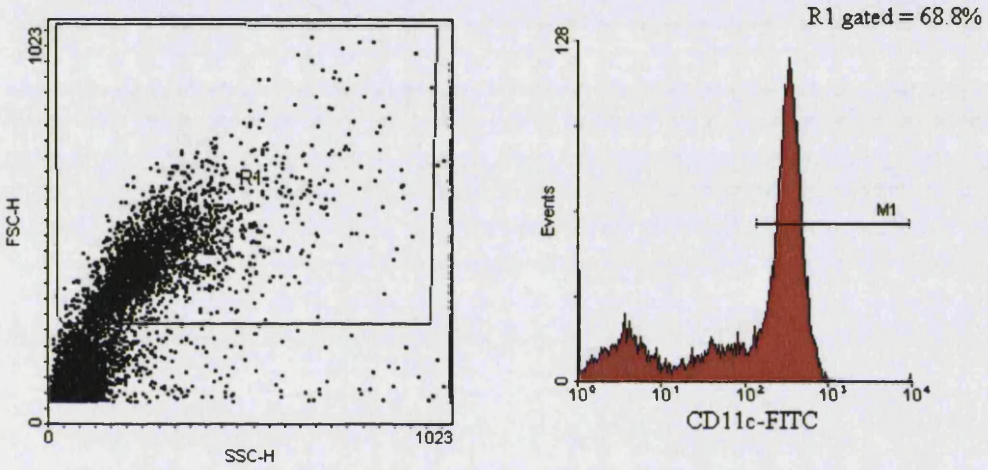


II. Negative selection using Dynal beads. Dynal beads were attached to non-T cells using lineage specific antibodies and depleted from single cell suspensions to produce a population of pure T cells (see 2.2.7.A.). The histograms below represent the percentage of (i) splenic and (ii) LN T cells (gated as above) that expressed the T cell surface marker CD3 following negative selection.

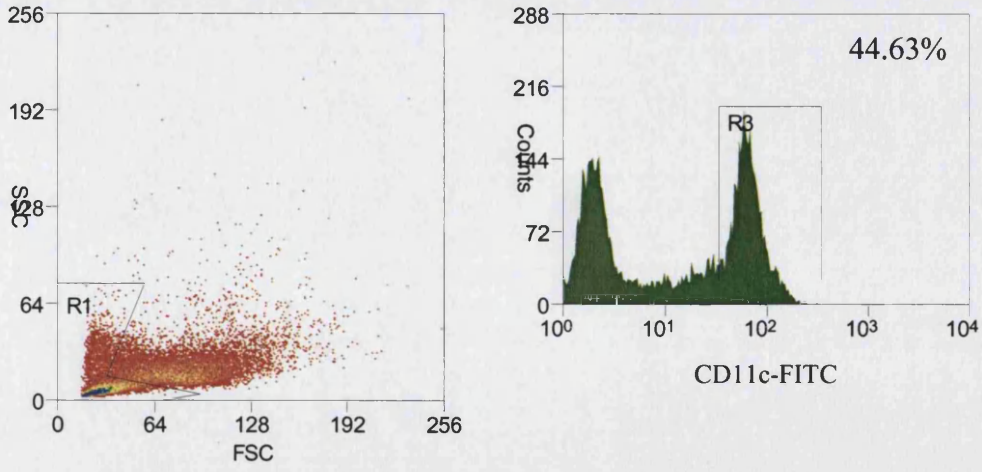


B. Purification and depletion of dendritic cells.

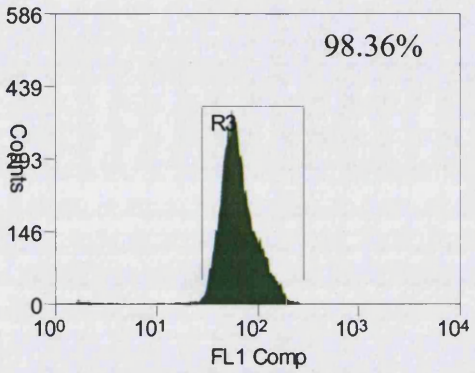
I. Enrichment of dendritic cells following density centrifugation. Low-density cells were enriched from splenocyte single cell suspensions using nycodenz density gradients (see 2.2.7. C.). $CD11c^+$ dendritic cells were enriched from 1-2% of splenocytes (see Fig 4.11.) to between 15% and 70%. The histogram below represents the percentage of R1-gated cells that express CD11c. The level of enrichment and yields following density centrifugation varied from 15-70% due to inconsistencies in the preparation of Nycodenz solutions.



II. Purification of $CD11c^+$ dendritic cells. Dendritic cells were first enriched by density centrifugation and labelled with a fluorescent anti-CD11c antibody. $CD11c^+$ DC were then positively selected using the MoFlo cell sorter (see 2.2.7. C.). The data below illustrates the selection criteria for unsorted nycodenz-enriched splenocytes. Viable cells (i.e. those not within the parameters of region R1) that fluoresced within gate R3 were positively selected.



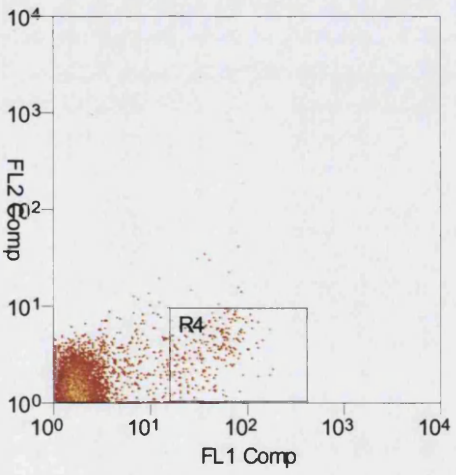
The histogram below represents a population of $CD11c^+$ cells sorted on the MoFlo. This method of DC purification routinely achieved purity exceeding 95%.



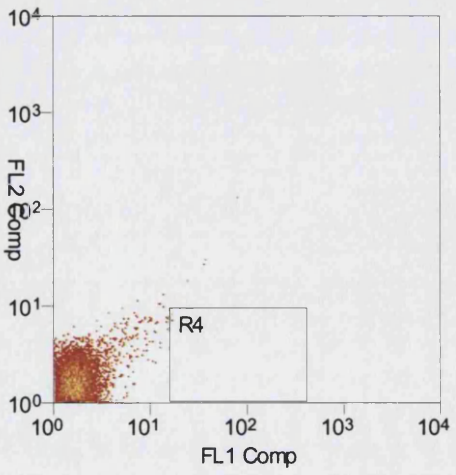
III. The depletion of $CD11c^+$ dendritic cells.

Single cell suspensions were labelled with a fluorescent anti-CD11c antibody. $CD11c^+$ DC were then depleted using the MoFlo. Viable cells (i.e. those not within the parameters of region R1 (see B II.)) that fluoresced within region R4 were depleted by the MoFlo and discarded leaving DC-depleted LN cells. The dot plots below represent the population of $CD11c^+$ FITC-labelled cells present in (i) unsorted (2.45%) and (ii) sorted cell suspensions (0.04%). Auto-fluorescent cells that fluoresced in both FL1 and FL2 channels were not excluded during the sorting process (see 2.2.7. C.)

(i) unsorted

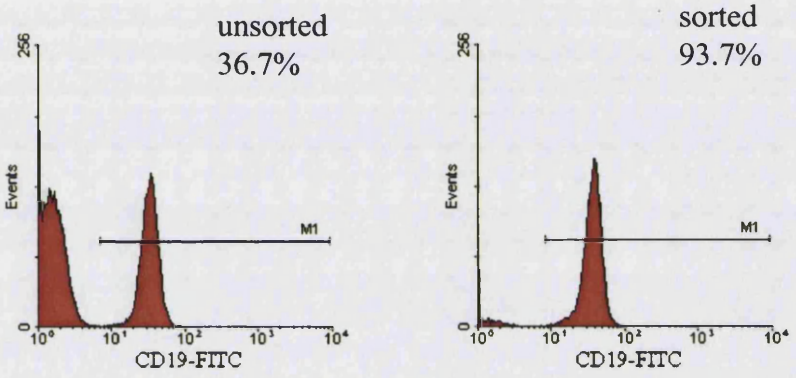


(ii) sorted



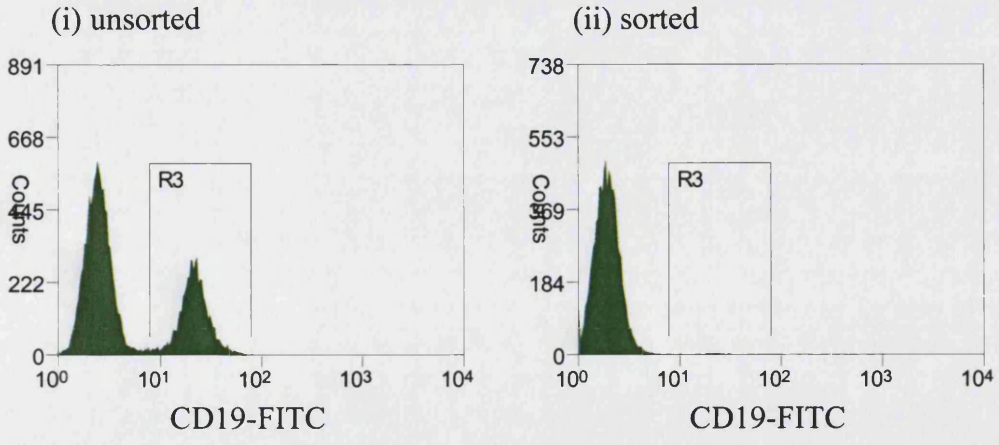
C. B cell purification and depletion.

I. Purification of B cells by negative selection using Dynal beads. Non-B cells were labelled with dynal beads and removed from LN cell suspensions (see 2.2.7. B.). The negatively selected B cells were typically ~ 95% pure. The histograms below represent the proportion of $CD19^+$ B cells in (i) unpurified and (ii) purified LN single cell suspensions.



II. B cell depletion via fluorescence activated cell sorting.

Single cell suspensions were labelled with a fluorescent anti-CD19 antibody. $CD19^+$ B cells were then depleted using the MoFlo. Viable cells (i.e. those not within the parameters of region R1 (see B II.)) that fluoresced within gate R3 were depleted by the MoFlo and discarded leaving B cell-depleted LN cells. The dot plots below represent the population of CD19-FITC labelled cells present in (i) unsorted (33.47%) and (ii) sorted cell suspensions (0.74%).



Appendix 2: A summary of the purification techniques used for experiments included in this thesis.

Figure	**Page**	**Population**	**Selection Technique**	**Method Reference**
3.11. LPS does not act directly on T cells *in vitro*.	138	Purified Thy1.1$^+$ T cells	Positive selection by fluorescence activated cell sorting.	2.2.7. A.
3.13. Non-T cells are not an absolute requirement for the upregulation of Ly-6A/E on T cells *in vitro* in response to breif exposure to LPS *in vivo*.	140	Purified T cells	Negative selection of T cells using Dynal beads.	2.2.7. A.
3.14. The removal of plastic adherent cells affect Ly-6A/E upregulation on CD4$^+$ T cells *in vitro*.	143	T-depleted splenocytes	Splenocytes were T-depleted using dynal beads. Additionally, some splenocyte populations were also depleted of macrophages and dendritic cells via plastic adherence.	2.2.7. A. 2.2.6. E.
3.19. The effect of recombinant cytokines on T cell phenotype *in vitro*.	151	Purified T cells	T cells were negatively selected via complement mediated lysis of non-T cells.	2.2.6. D.
3.24. LPS 24 hours *in vivo* does not alter the anti-CD3 proliferative response.	159	Purified CD8$^+$ & CD4$^+$ T cell subsets	T cell subsets were negatively selected using dynal beads.	2.2.7. A.

3.25. Ly-6A/E mediated T cell proliferation *in vitro* is enhanced by LPS *in vivo*.	161	Purified $CD8^+$ & $CD4^+$ T cell subsets	T cell subsets were negatively selected using dynal beads.	2.2.7. A.
3.26. LPS 24 hours *in vivo* enhances the T cell mitogenic proliferative response *in vitro*.	162	$CD8^+$- or $CD4^+$-depletion	Either $CD4^+$ or $CD8^+$ T cells were depleted from LN cell suspensions using dynal beads.	2.2.7. A.
3.27. LPS *in vivo* does not intrinsically alter the responsiveness of T cells to Con A *in vitro*.	164	Purified T cells T-depleted LN	T cells were either depleted or purified from LN cell suspensions using dynal beads.	2.2.7. A.
4.05. LPS does not induce phenotypic changes on purified DC *in vitro*.	188	Purified DC	$CD11c^+$ dendritic cells were positively selected via fluorescent activated cell sorting.	2.2.7. C.
4.06. The effect of LPS on the secretion of cytokines by $CD11c^+$ dendritic cells.	189	Purified DC	$CD11c^+$ dendritic cells were positively selected via fluorescent activated cell sorting.	2.2.7. C.
4.07. The effect of Ly-6A/E cross-linking on TNF-α secretion by DC *in vitro*.	191	Purified DC	$CD11c^+$ dendritic cells were positively selected via fluorescent activated cell sorting.	2.2.7. C.
4.08. The effect of DC supernatant on T cell phenotype *in vitro*.	192	Purified DC Purified T cells	$CD11c^+$ dendritic cells were positively selected via MACS beads. T cells negatively selected using dynal beads.	2.2.7. C. 2.2.7. A.
4.09. LPS stimulation *in vivo* does not significantly affect the capacity of DC to stimulate allogeneic	194	Purified DC	$CD11c^+$ dendritic cells were positively selected via fluorescent activated cell sorting.	2.2.7. C.

responses *in vitro*.				
4.10. LPS *in vivo* does not enhance the capacity of DC to stimulate atigen-specific responses *in vitro*.	195	Purified DC	$CD11c^+$ dendritic cells were positively selected via fluorescent activated cell sorting.	2.2.7. C.
4.13. $CD11c^+$ dendritic cells are required for the T cell mitogenic response to Con A.	201	DC-depleted LN suspensions	$CD11c^+$ DC were depleted from LN suspensions using the MoFlo.	2.2.7. D.
5.04. The effect of LPS on B cell phenotype *in vitro*.	222	Purified B cells	B cells were negatively selected from LN cell suspensions using dynal beads.	2.2.7. B.
5.05. The effect of splenocyte supernatant on B cell phenotype *in vitro*.	223	Purified B cells	B cells were negatively selected from LN cell suspensions using dynal beads.	2.2.7. B.
5.07. The effect of IFN-α/β on B cell phenotype *in vitro*.	226	Purified B cells	B cells were negatively selected from LN cell suspensions using dynal beads.	2.2.7. B.
5.09. B cells are not absolutely required for the proliferative response to Con A *in vitro*.	229	B cell depleted LN cells	B cells were depleted from LN cell suspensions using the MoFlo	2.2.7. B.
5.10. The effect of LPS *in vivo* on B cell activation *in vitro*.	231	Purified B cells	B cells were negatively selected from LN cell suspensions using dynal beads.	2.2.7. B.

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