MACROPHAGES AND LYMPHOCYTE RESPONSIVENESS TO MITOGEN

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Doctor of Philosophy

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Purified B-cells fail to proliferate in response to the strong thymus-independent (TI) antigen Lipopolysaccharide (LPS) in the absence of macrophages (Corbel and Melchers, 1983). The fact that macrophages, or factors derived from them are required is supported by the inability of marginal zone B-cells in infants to respond to highly virulent strains of bacteria such as *Neisseria meningitidis* and *Streptococcus pneumoniae* (Timens, 1989). This may be due to the lack of CD21 expression on B-cells in infants which could associate with its co-receptor (C3d) on adjacent macrophages. It is not clear whether cell surface contacts and/or soluble products are involved in lymphocyte-macrophage interactions in response to certain antigens. This thesis describes the importance of the macrophage in lymphocyte responses to T-dependent (TD) and TI antigens. The major findings of this thesis were as follows:

(1). Macrophages were essential for a full proliferative response to a range of T- and B-cell mitogens and TI-1 and TI-2 antigens, including Concanavalin A, LPS, Pokeweed mitogen (PWM), Dextran sulphate, Phytohaemagglutinin-P (PHA-P) and Poly[I][C].

(2). A ratio of 1 macrophage to 1000 lymphocytes was sufficient for the mitogens to exert their effects.

(3). The optimal conditions were established for the activation of an oxidative burst in cells of the monocyte/macrophage lineage as measured by luminometry. The order of ability was OpZ >PMA/Ionomycin >f-MLP >Con A >DS >PHA >Poly[I][C] >LPS >PWM. Responses were only substantial and protracted with OpZ and PMA. Peritoneal macrophages were the most responsive cells, whereas splenic and alveolar macrophages were significantly less active and no response could be elicited with Kupffer cells, thus demonstrating heterogeneity between macrophages.

(4). Activated macrophages that were then fixed with paraformaldehyde were unable to restore mitogenic responsiveness, even with a ratio of 1 macrophage to 5 lymphocytes.

(5). Although highly purified T- and B-cells could respond to mitogen provided live macrophages were present, maximum activation was only observed when all 3 cell types were present.

(6). Supernatants from purified macrophage cultures treated with a range of activators were able to partially restore lymphocyte responses to mitogen in macrophage-depleted splenocyte cultures, and purified T- and B-cell cultures. In fact supernatants from macrophages treated with LPS for only 30 minutes could restore responsiveness. Supernatants from OpZ treated macrophages were without effect.

(7). Macrophage supernatants could not induce proliferation in the absence of mitogen. They therefore provide a co-mitogenic signal required by lymphocytes in order to respond to mitogen.

(8). Macrophage product profiles revealed that LPS and Con A-treated macrophage supernatants showed elevated levels of IL-1β, TNF-α LTB_4 and TXB_2. These products were therefore good candidates as the co-mitogenic factor. The possible inhibitory factors secreted by OpZ-treated macrophages were PGE_2, IL-10 and NO.

(9). The removal of cytokines, eicosanoids and TNF-α from LPS-treated macrophage supernatants using Cycloheximide, Dexamethasone and an MMPI respectively, resulted in the inability of these supernatants to restore macrophage-depleted lymphocyte responses to mitogen.

(10). rIL-1β and rTNF-α are co-mitogenic factors, as macrophage-depleted lymphocytes incubated with rIL-1β and rTNF-α can respond to mitogen.

Macrophage supernatants, T-Independent antigens, Marginal zone, Lymphocyte proliferation, Luminometry

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ABBREVIATIONS

Ab	antibody
AFC	antibody forming cells
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP	acid phosphatase
ATP	adenosine triphosphate
B-CLL	B-cell lymphoblastoid leukaemia
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation
CO_2	carbon dioxide
Con A	concanavalin A
DAG	diacylglycerol
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DS	dextran sulphate
DTH	delayed type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
E-GF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FACScan	fluorescent activated cell scanner
FCS	foetal calf serum
FDC	follicular dendritic cell
F-GF	fibroblast growth factor
FITC	fluorescein iso-thiocyanate
f-MLP	f-Methionine-Leucine-Phenylalanine
FSC	forward scatter
GC	germinal centre
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
H_2O_2	hydrogen peroxide
HOCl	hypochlorite
[^{3}H]TdR	tritiated thymidine
Ia	I-region associated
ICAM-1	intercellular adhesion molecule-1
IDC	interdigitating cell
IFN	interferon
Ig	immunoglobulin

ABBREVIATIONS

IL	interleukin
IU	international unit
LFA	lymphocyte function associated antigen
LPS	lipopolysaccharide
LTB_4	leukotrieneB_4
MØ	macrophage
MAF	macrophage activation factor
M-CSF	macrophage colony stimulating factor
MFR	mannosyl-fucosyl receptor
MHC	major histocompatibility complex
MiniMACS	mini magnetic activated cell sorter
MMPI	matrix metalloproteinase inhibitor
MNC	mononuclear cell
MPO	myeloperoxidase
MZ	marginal zone
NH_2Cl	monochloramine
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
$O_2^{\cdot}$	singlet oxygen
$OH^{\cdot}$	hydroxyl ion
OpZ	opsonised zymosan A
PAF	platelet activation factor
PALS	periarteriolar lymphoid sheath
PB	polymyxin B
PBS	phosphate buffered saline
PFC	plaque forming cell
PGE_2	prostaglandinE_2
PHA-P	phytohaemagglutinin
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cell
Poly[I][C] or PIC	poly-inosinic poly-cityidylic acid phosphate
PPi	inorganic pyrophosphate
PTK	protein tyrosine kinase
PWM	pokeweed mitogen
RLU	relative light unit
RNA	ribonucleic acid

ABBREVIATIONS

R-PE	R-phycoerythrin
Rpm	revolutions per minute
SEM	standard error of the mean
SI	stimulation index
SRBC	sheep red blood cells
SSC	sideways scatter
TCR	T-cell receptor
TD	thymus-dependent
TGF	transforming growth factor
TI	thymus-independent
TIMP	tissue inhibitors of metalloproteinases
TNP	2,4,6-trinitrophenyl
TNF	tumour necrosis factor
TXB_2	thromboxaneB_2

1. INTRODUCTION

The requirement for the macrophage in lymphocyte responses to antigen or mitogen has not been conclusively researched. The aim of this study is to broaden the understanding of interactions between lymphocytes and macrophages. The focus of my research is to investigate the importance of the murine splenic macrophage and T-cell in immune responses by B-cells to T-independent (TI) antigens and the requirement for the macrophage in T-cell responses to mitogen. This introduction will provide an overview of current knowledge about the requirement for macrophages in various lymphocyte responses.

1.1 The Spleen

The spleen is an important secondary lymphoid organ involved in immune reactions against all types of antigens that appear in the circulation. Its importance is emphasised by the fact that it is quantitatively the main lymphoid organ involved in lymphocyte recirculation and splenectomised patients show marked susceptibility to certain infections (Cavill *et al*, 1996). Its complex anatomical organisation with several different compartments containing specialised cell types allows for all cell-cell interactions necessary for the generation of a humoral immune response. A description of the architecture of the spleen will form the basis of this section.

1.1.1 Structure and Function

Macroscopically, the spleen can be divided into red and white pulp. The red pulp contains mainly erythrocytes and some nucleated cells, such as lymphocytes, megakaryocytes and macrophages. The white pulp consists of three compartments, the periarteriolar lymphoid sheath (PALS), follicles and the marginal zone (MZ). The structure of the spleen is directly related to its vasculature. Blood enters at the hilus through the splenic artery which opens out into trabecular arteries. Small vessels leave the trabeculae, these are called central arterioles which gradually become surrounded by

lymphatic tissue; the PALS. The follicles are globular structures attached to these vessels and consist mainly of resting B-cells and follicular dendritic cells (FDC). The MZ surrounds the PALS and follicles and contains B-cells, macrophages and relatively few T-cells. Terminal arterioles open directly into the MZ sinus, where, due to the low sheer forces caused by reduced flow, the first cell-cell interactions between T-cells, B-cells and macrophages may occur.

In the mouse the PALS can be sub-divided into the inner PALS and outer PALS. The inner PALS consists mainly of resting T-cells and interdigitating cells (IDC) which are extremely potent accessory cells in the induction of T-dependent (TD) responses. The outer PALS is predominantly populated by B-cells, some T-cells and a special subset of macrophages. These macrophages are actually located in the MZ but have processes protruding into the outer PALS. In view of their staining with silver they were termed marginal metallophillic macrophages (Buiting *et al,* 1996). Antibody forming cells (AFC) are found mainly around terminal arterioles but also in high numbers in the outer PALS as a layer inside the ring of marginal metallophillic macrophages. Another macrophage subset can be found within the MZ, the MZ macrophage. These macrophages are Ia-negative, acid-phosphatase positive and have an extraordinary phagocytic capacity, combined with a strategic localisation with respect to the white pulp capillaries, allowing them to meet and ingest antigens effectively. These cells are recognised by their uptake of TI antigens, such as TNP-ficoll (Chused *et al,* 1976; Boswell *et al,* 1980) or particulate antigens, such as haptenated liposomes (Claassen *et al*, 1986). The MZ further consists of a particular population of B-cells that are IgM+ and IgD- (Tarahkovsky *et al*, 1994).

Primary follicles mainly consist of resting B-cells, easily identified by an anti-IgM antibody. Upon antigen stimulation secondary follicles are generated, these contain a germinal centre (GC). They consist of a follicle centre which contains blast-like activated B-cells and a follicle corona filled with resting B-cells. FDCs within the follicle form an intricate network, called the follicular web, where B-cells lie in close association with FDCs (Van Roojen, 1993). They are thought to present antigens in the

form of immune complexes (Crowley *et al,* 1990), which are retained on their surface by Fc receptors and C3 receptors for long periods of time. These complexes appear to be involved in B-cell memory, and recirculating memory B-cells preferentially migrate to GCs (Zhang *et al,* 1988).

The specialised arrangement of this organ enables it to rapidly remove antigens reaching it via the circulation. The antigens may have entered directly into blood vessels or indirectly through the lymphatic system which collects in the thoracic duct and drains into the left subclavian vein and thus into the blood circulation. Other important functions of the spleen are the production of large amounts of circulating antibody, the production of memory B-cells after immunisation and the ability to elicit a fast secondary immune response.

1.1.2 The Marginal Zone

As discussed previously, the MZ is a layer surrounding the PALS and B-cell follicles. It is predominantly composed of intermediate-sized lymphocytes. Its presence was first described in 1929 by MacNeal who recognised its importance in phagocytosis. There is substantial inter-species variation in this zone (Claassen *et al*, 1991). The basic structure of the MZ consists of a meshwork of reticular cells. The opening of a part of the arterial blood supply into wider spaces right at the border of the MZ and the white pulp creates a sinus resulting in blood flow with lower resistance. This gives cells already in the MZ the opportunity to react with incoming cells and antigens. It is in the MZ that specialised interactions occur between lymphocytes and macrophages (Van den Eertwegh, 1992) and hence is an area of particular interest in these studies.

1.1.2.1 Marginal Zone B-lymphocytes

Due to the structure of the MZ, a number of blood borne lymphocytes are continuously present in this area on their way to either the white or red pulp. However most of the lymphocyte population found here are static high density B-cells. In fact, the MZ in the

spleen is the only B-cell-dependent area in the body where B-cells are not organised into follicles. These B-cells unless stimulated are small resting B-cells with low recirculating capacity. They show a markedly reduced expression of IgD on their surface, this, together with their size and Interleukin (IL)-2 receptor expression is indicative of a state of partial activation, no doubt related to their specialised function in combating blood borne infection (Maclennan and Liu, 1991). There is another population of B-cells which reside where IgM+ and IgD- cells are found and these are CD5+ B-cells which form a distinct lineage (Tarakhovsky *et al,* 1994), they are therefore largely restricted to the MZ.

1.1.2.2 Marginal Zone Macrophages

As mentioned previously several types of macrophage(s) reside in the MZ, each with a defined location and set of characteristics. The metallophillic macrophages are found at the outer border of the MZ and have processes protruding into the outer PALS. Although these macrophages possess acid phosphatase activity they do not phagocytose well (Eikelenboom *et al*, 1978). Their function was assessed using a monoclonal antibody, called MOMA-1 which specifically reacts with these macrophages (Kraal and Janse, 1986). Multiple injections of the antibody in mice led to a drastic reduction in the number of cells but not a complete elimination. When these animals were then challenged with various antigens, it was found that responses to thymus-dependent antigens and thymus-independent type 2 antigens were impaired (Kraal and Janse, 1986). It was later discovered using a monoclonal antibody called SER-4, that these cells express receptors for sialic acid-containing glycoconjugates, which is indicative of a role in the interaction with selectin-bearing lymphocytes, giving strong evidence for contact mediated lymphocyte-macrophage interactions (Crocker *et al*, 1988).

The mononuclear phagocyte which was first recognised in the MZ, was the MZ macrophage by Humphrey (1981), who showed that injected neutral polysaccharides were selectively trapped and retained by these large phagocytic cells. Later, a monoclonal antibody (ERTR-9) became available that exclusively recognised this macrophage population in the murine spleen (Dijkstra *et al,* 1985). The function of the

MZ macrophage is related to its extraordinary phagocytic capacity. The selective retention of neutral polysaccharides on these cells has led to the assumption that they play a role in the immune response to such antigens (Delemarre *et al,* 1990), and their role in the response to TI antigens will be discussed in more detail in section 1.4.2. Another possible function of the MZ macrophage is the recruitment and/or retention of MZ B-cells. Elimination of splenic macrophages did reduce the number of (incoming) B-cells and this effect was only complete when this specific macrophage population disappeared (Kraal *et al,* 1989). No definite conclusions can be drawn on the mechanisms that play a role in this interaction between MZ macrophage and MZ B-cell populations.

1.1.2.3 The Function of the Marginal Zone

The MZ is especially important in the migration of lymphocytes within the spleen. In particular, the MZ macrophage is important for optimal retention and localisation of B-cells (Jaffe *et al*, 1975). There is compelling evidence that the presence of the spleen is essential for dealing with TI antigens and a role for the cells of the MZ in particular has been suggested (Chused *et al,* 1976; Boswell *et al,* 1980). The two most important functions of the MZ are therefore the regulation of lymphocyte trafficking and the ability to cope with a diverse range of antigens, in particular TI antigens.

1.1.3 The Importance of the Spleen

The spleen plays a major role in the protection against bacterial infections. This can be attributed to its enormous phagocytic capacity and the production of specific antibody. The spleen is especially important in the protection against virulent bacteria with polysaccharide capsules such as *Streptococcus pneumoniae* or *Haemophilus influenzae*, as can be seen from the often overwhelming infections with these organisms in post-splenectomy patients (Gray *et al,* 1985; Cavill *et al,* 1996). This can be remedied by immunisation protocols before splenectomy, or only a partial splenectomy being undertaken (Zimran *et al,* 1995), but this is not possible in all cases. The immune

response to polysaccharide antigen is almost completely defective in infancy, this has been attributed to the absence of CD21 expression on the B-cells of the MZ (Timens *et al,* 1987; Timens *et al,* 1989; Snapper *et al,* 1997).

1.1.4 TD versus TI Antigens

The spleen is primarily involved in immune reactions against antigens present in the circulation. Furthermore, they can be characterised as thymus-dependent (TD) or thymus-independent (TI) antigens. Different antigens may require different lymphoid and non-lymphoid cells for their processing and presentation. Properties of antigens determine the type of immune response they elicit. Protein antigens elicit both humoral and cell-mediated immune responses for which they require involvment of T-cells, B-cells and accessory cells. Some antigens such as polysaccharides, induce humoral immune responses without the absolute need for T-cells, and hence have been termed thymus-independent antigens, in contrast to TD antigens which have an obligate dependence on T-cell help (see Table 1.1 for properties, classification and examples).

Table 1.1 TD versus TI Antigens. Adapted from Abbas, A.K., Lichtman, A.H., Pober, J.S. 1991. Cellular and Molecular Immunology, pp 199. Wonsiewicz, M.J. (ed). W.B. Saunders Company.

Properties	T-dependent	T-independent 1	T-independent 2A
Prototypes	proteins	lipopolysaccharide	polymeric antigen
Antibody response in athymic mice	No	Yes (IgM and IgG)	Yes
Ab response in T-cell depleted cultures	No	Yes (IgM and IgG)	No (or reduced)
Isotype switching	Yes	No	No (usually)
Affinity maturation	Yes	No	No
Form memory Cells	Yes	No	No
Polyclonal B-cell activation	No	Yes	No
Induction of DTH	Yes	No	No
Example	Ovalbumin	LPS, dextran sulphate	TNP-dextran, poly[I][C]

In early studies classification of TI antigens was evaluated by the ability of antigens to elicit antibody responses in T-cell-deficient nude mice (Mosier and Subbarao, 1982). In general, TI antigens have repeating determinants that can be recognised by antibody receptors on B-cells. Thus, not only synthetic compounds such as hapten-coupled acrylamides or polyvinyl pyrolidone, but also liposomes behave as TI antigens. These antigenic epitopes are usually present in very small numbers on proteins with the exception of malarial circumsporozoite proteins (Schofield and Uadia, 1990).

TI antigens are not degraded readily and thus are retained in the body for very long periods. Most TI antigens induce primarily IgM and some IgG production. They do not induce memory responses, with some exceptions. Thus, Colle *et al,* 1983, reported that TNP-LPS can induce memory in certain strains of mice, e.g. C57BL/6, but these memory responses are short-lived in comparison to protein antigen. Immune responses to TI antigens differ from TD antigens in that they do not exhibit affinity maturation

which is probably related to their inability to induce strong secondary responses. TI antigens can be converted to TD antigens by coupling them to protein carriers. TI antigens have been subdivided into three types based upon their differential ability to induce antibody responses in neonates and CBA/N (xid) mice, which have an X-linked immune deficiency. They are classified into TI-1, TI-2A and TI-2B.

1.1.4.1 TI-1 and TI-2 Antigens

Antigens such as TNP coupled to LPS or Brucella abortus (BA), which can induce excellent antibody responses in neonates and CBA/N mice, belong to the TI-1 antigens classification. Whereas antigens such as TNP-dextran, pneumococcal polysaccharides and TNP-dextran which induce good immune responses in adults but not in the young, nor in CBA/N mice have been classified as Type-2. Another often noted difference between TI-1 and TI-2 antigens is their ability to induce polyclonal B-cell activation, TI-1 antigens can but, TI-2 antigens cannot with the exception of poly[I][C] (poly-inosinic poly-cityidylic acid phosphate) and Levan (Abbas *et al*, 1991).

The differential ability of TI-1 and TI-2 antigens to stimulate B-cells from CBA/N and neonatal mice, as well as the differences in their capacity to polyclonally activate B-cells, suggests two distinct subsets of B-cells may be involved in TI-1 and TI-2 responses. If so, the TI-2 responding subset should be absent in neonatal and CBA/N mice, consistent with this is the fact that B-cells from neonates and CBA/N mice share several surface phenotypes, such as IgM+ IgD- expression (Zitron *et al*, 1977; Subbarao *et al*, 1979). The B-cells from CBA/N mice do not express Lyb3, 5 and 7, whereas a subset of the B-cells from normal adults express these. Depletion of Lyb5+ B-cells from normal adult mice eliminates immune responses to TI-2 antigens but not TI-1. The lack of ability of neonates and CBA/N mice to respond to TI-2 antigens is therefore due to a lack of Lyb5+ cells, because supplementation with normal adult splenic accessory cells does not restore the response to TI-2 antigens (Mosier *et al*, 1976; Quintans, J., 1979). Lyb5+ and not Lyb5- B-cells exhibit MHC-restricted interactions with accesssory cells in their response to TNP-Ficoll (Cambier *et al*, 1982).

The lack of Lyb5+ B-cells in these mice appears to be because they are unable to develop without a functional thymus (Hayakawa *et al*, 1988). An interesting finding is that T-cell or accessory cell derived factors are able to render CBA/N B-cells responsive to TI-2 antigens whereas there is no such response with neonates (Mond *et al*, 1989). The TI-2 antigens are subdivided into TI-2A and TI-2B, to highlight their ability to activate different T-cell subsets, the table below demonstrates this.

Table 1.2 TI-2A versus TI-2B. (adapted from Bondada, S. and Garg, M. Ch14, pp347. In, Handbook of B- and T-lymphocytes. 1994. Snow, E.C. (ed). Academic Press, Inc.).

Properties	**T-independent 2A**	**T-independent 2B**
Response in aged mice	++	- to +
Mitogenicity	-	+/-
Polyclonal B-cell activation	-	+/-
High responder strains	Most	Balb/c, CBA
Low responder strains	CBA/N	Most in-bred strains
T suppressor cells	-	+++
T amplifier cells	-	+++
T contrasuppressor cells	-	+++
Idiotypic specific Th cells	-	+++
Example	TNP-Ficoll	capsular polysaccharides

1.2 B-Lymphocytes

1.2.1 Humoral Immunity

There are two complementary systems of humoral immunity; natural immunity and acquired immunity. Natural immunity has elements in common with the immune system of lower vertebrates, namely a genetically programmed expansion of a set of B-cell clones mediated by the stimulation of those B-cells specific for a particular immunoglobulin (Ig) antigen. The antibodies produced by these clones are transcribed

from gene sequences in the germ-line configuration and little modification of these antibodies occurs over the course of the response. The repertoire of these antibodies appear(s) to be best adapted for the most common pathogen(s), although it does not account for genetic variability of pathogen. This group of B-cells includes the CD5+ subset found in the MZ of the spleen (Hayakawa *et al,* 1988).Acquired immunity also results in the production of antibodies to protein antigens, but it differs to natural immunity in that it depends upon collaboration between T-cells and B-cells. This strategy seems to be used in late primary and early secondary responses. The antigen is endocytosed and presented as peptide fragments in association with MHC Class II molecules. CD4 positive T-cells with a specific T-cell receptor (TCR/CD3) for this complex will preferentially help those B-cells capable of generating the highest concentration of peptide MHC Class II complexes, this depends on the affinity of the B-cell surface Ig (sIg) for the antigen. This, together with interactions between the costimulatory molecule B7 on B-cells with CD28 on the T helper (Th) cells (Linsley *et al*, 1991) and ICAM-1 expressed on T-cells with LFA-1 on B-cells (Wawryk *et al*, 1989), leads to the class restricted, antigen-specific activation of Th cells. These activated T-cells express gp39 (CD40L) and secrete cytokines, which enable them to regulate B-cell differentiation (Lederman *et al*, 1992). The binding of gp39 to its receptor CD40 on B-cells results in the initial activation of B-cells (Armitage *et al*, 1992), while Th cell derived cytokines, such as IL-2, IL-4, IL-5, IL-6 and IFN-γ regulate proliferation and differentiation of B-cells (Hodgkin *et al*, 1991; Noelle *et al*, 1991).

The only apparent prior constraint on antibody responses is that the B-cell repertoire is devoid (by anergy or deletion) of receptors for self antigens, and that T-cells with high affinity receptors for self peptides are tolerant, which results in the lack of self-reactive B-cells. This concept is currently under question by Matzinger who suggests that it is not the recognition of non-self which mounts an immune response, but the presentation of a danger signal by professional antigen presenting cells, called sentinels. These sentinels detect tissue distress and relay these signals (Bonney and Matzinger, 1996; Fuchs and Matzinger, 1996).

Once B-cells are stimulated by antigen and have the appropriate help their antibody genes then freely mutate, as many as one half of the B-cell progeny may express antibodies with modified sequences. Selection results in further proliferation of B-cells expressing higher affinity sIg, increasing the chances of continuation of help. As a consequence, during an immune response to TD antigens, the mean affinity of antibody increases in parallel with the accumulation of somatic mutations in antibody genes, this is the phenomenon of affinity maturation.

1.2.1.1 Mitogens

Mitogens are agents which are able to induce proliferation in a high percentage of T- and B-cells. Unlike immunogens, which only activate lymphocytes bearing specific receptors, mitogens stimulate many clones of T- and B-cells irrespective of their antigen specificity. Because of this ability, mitogens are known as polyclonal activators. A variety of diverse agents function as mitogens. A number of common mitogens are proteins called lectins that are derived from plants and bind to sugar residues. Lectins recognise different glycoproteins on the surface of various cells, which is often followed by cellular activation. Some mitogens preferentially activate B-cells or T-cells and some activate both cell types. Three common lectins are Concanavalin A (Con A), Pokeweed mitogen (PWM) and Phytohaemagglutinin (PHA). Each of these proteins bind to different carbohydrate residues in glycoproteins and are able to cross-link these molecules on the surface of cells. Con A and PHA are T-cell mitogens and PWM activates both T- and B-cells. The mitogenic response to PHA and Con A is dependent on the presence of macrophages (Rosenberg *et al,* 1976; Thiele *et al,* 1983).

Not all mitogens are lectins. The lipopolysaccharide (LPS) component of the gram-negative bacterial cell wall functions as a B-cell mitogen. The mitogenic activity of LPS is due to its lipid moiety, which is thought to interact with the plasma membrane, resulting in cellular activation (Wright *et al*, 1990). Research into the actions of LPS has been greatly advanced by the use of LPS non-responder mice called C3H/HeJ, the

B-lymphocyte and macrophage of this strain fail to be activated by LPS (Sultzer *et al,* 1993). Currently it is thought that their lack of responsiveness is due to the lack of, or defective signal receptor, as the LPS intracellular signalling system appears to be intact in non-responding cells. There is already an identified receptor for LPS on B-cells; the CD14 molecule (Maliszewski, 1991; Ziegler-Heitbrock & Ulevitch, 1993), the ligation of which with soluble LPS or LPS binding protein results in TNF-α (Wright *et al,* 1990) and PAF (Camussi *et al,* 1995) synthesis. Other B-cell mitogens include dextran sulphate and Poly[I][C], which act as mitogens by cross-linking receptors on the B-cells due to repeating epitopes.

The name mitogen was originally derived because they induce cells to enter mitosis. As mitosis involves the synthesis of new DNA, the ability of an agent to act as a mitogen can easily be determined by adding Tritiated Thymidine to cultures of cells and quantifying the amount of the isotope incorporated into the newly synthesised DNA. Naive, or resting B-cells are non-cycling cells in the G_0 stage of the cell cycle. Activation drives the resting cell into the cell cycle, progressing from G_1 into the S phase, in which DNA is replicated therefore Tritiated Thymidine can be incorporated into the cell at this point. The G_1 to S transition represents a critical restriction point in the cell cycle. Once a cell has reached S, it completes the cell cycle moving through G_2 and into mitosis (M). After analysing the events in progression of lymphocytes from G_0 to S phase, Cooper, 1963 noted a number of similarities with events that had been identified in fibroblasts. He divided these signals into competence and progression signals. Competence drives the B-cell from G_0 into early G_1, progression signals then drive the cell from G_1 to S and ultimately to cell division and differentiation. Transition or restriction points appear to be under the control of cyclins and cyclin-dependent kinases, competence and progression factors may therefore activate these.

1.2.2 Regulation of B-lymphocytes by Soluble Mediators and Receptor Binding

The regulation of B-cell differentiation by antigen or cytokines is complicated by the range of biological responses that a B-cell can make to the same stimulus depending on

the differentiation state of the lymphocyte. Thus, like antigen certain cytokines mediate quite different effects on B-cells. The result of an encounter with a soluble stimulus can lead to the generation of other soluble products by the B-cell, and these other products can influence B-cell differentiation by paracrine or autocrine mechanisms. Examples include cytokines, cleaved forms of plasma membrane differentiation antigens and Ig. The main features of the biological responses induced in B-lymphocytes by soluble mediators are reviewed in the following section.

1.2.2.1 Soluble Antigen

The effect of an antigen on the biochemistry of B-cell activation, growth and differentiation depends to a very large extent on the data from studies using soluble anti-Ig reagents to mimic the effect of antigen. This information must be used with caution as even monoclonal antibodies can cross-link adjacent membrane Ig (mIg) molecules and polyclonal anti-Ig can bind to Fc receptors on the B-cell surface, which may transmit a negative signal to the B-cell; known as abortive activation. This could be avoided by only using the Fab fragment of the antibody.

Anti-Ig studies have demonstrated that in immature B-cells (mIgM+ and mIgD-) ligation of the B-cell antigen receptor results in clonal deletion via apoptosis. The reason for this is that at different states of differentiation gene expression varies, hence stimulation of the same receptor with the generation of similar signals can result in many different biological responses. For example apoptosis, or escape from apoptosis in immature and mature virgin B-cells respectively or activation and proliferation in mature B-cells leading to antibody production and memory B-cell formation.

1.2.2.2 Cytokines

Cytokines critically influence B-cell development. They are derived from a range of sources, including monocytes, mast cells, lymphocytes and stromal cells (Tucci *et al,* 1992; Pistoia and Corcione 1995). An individual cytokine can display a range of

activities towards B-cells, and the particular biological response elicited depends on the state of differentiation of the B-cell and other stimuli involved. Cytokines are protein or glycoprotein in nature, have short half-lives and demonstrate pleiotropy. Discussed below are the actions of cytokines produced by macrophages and lymphocytes which may be important in responses to mitogen.

Interleukin-1 is produced by many cell types, particularly monocytes and macrophages (Durum *et al*, 1986). It is therefore of particular interest in this work as macrophage products may influence lymphocyte responses to mitogen. B-cells also produce IL-1 and this can have implications for autocrine B-cell stimulation (Takeuchi and Katayama 1994), or it may mediate local activation of T-cells which interact physically with the B-cell. IL-1 does influence B-cell growth and differentiation, however it fails to activate without another mitogenic stimulus e.g. anti-Ig or Dextran sulphate (Booth and Watson, 1984). Both IL-1α and IL-1β have distinct but potentially synergistic roles in sustaining the growth of activated B-cells (Takeuchi and Katayama 1994).

Activated T-cells of the Th1 subset are the main source of IL-2 (Moreau *et al,* 1995) and it is principally a T-cell growth factor, but does exert a range of effects on activated B-cells with little or no effect on high density quiescent B-cells. In the mouse IL-2 supports B-cell proliferation only when two other activation signals are present; anti-Ig and LPS (Zubler *et al*, 1984). Other data from single cell systems using TI antigens as the stimulant showed that IL-2 could act directly on B-cells (Endres *et al,* 1983; Mond *et al,* 1987). Finally, IL-2 was shown to promote Ig secretion from activated B-cells and to replace T-cells in direct plaque forming cell (PFC) assays (Moreau *et al,* 1995).Th2 lymphocytes produce IL-4 and it exerts a wide range of effects on B-cells at various stages in their development (Stack *et al,* 1994). The combination of IL-4 and anti-Ig cultured with B-cells results in co-stimulation of quiescent (G_0 and G_1) B-cells. In fact it appears that IL-4 primed the B-cells to make a more vigorous response upon stimulation by anti-Ig or LPS (Stack *et al,* 1994). IL-4 can also work at the level of the pre-B-cells, by up-regulating CD23 (IgE receptor) expression and MHC Class II antigens (this does not happen in mature B-cells) (Kawabe *et al*, 1988). Not all of the

effects of IL-4 on surface expression are positive, as it appears to down-regulate CD5 antigen expression on activated B-cells (O'Garra *et al*, 1986). Studies of LPS-activated murine B-cells indicated that these cells responded to IL-4 in a manner distinct from that observed in resting populations. Increased levels of IgG_1 and IgE were found in the supernatants compared to IgM and IgG3 found in the supernatants of control cultures exposed to LPS only (Punnonen *et al,* 1994). Interleukin-4 can also influence the expression of other cytokines and cytokine receptors by B-cells (Moreau *et al*, 1995).In murine, but not human, B-lymphocytes IL-5 can stimulate proliferation but only after the cells have been primed with a mitogen. It may facilitate passage from G_2 into mitosis (O'Garra *et al*, 1986). The response is restricted to large low density B-cells although it may promote expression of IL-2 receptors on refractory high density B-cells. IL-5 also has a role in Ig secretion and isotype switching in activated murine B-cells, leading to an increase in IgM and IgA secretion (Coffman *et al*, 1988).

Like IL-1, IL-6 plays a pivotal role in the inflammatory response and acts on a range of cell lineages functioning as a fibroblast mitogen, and in the induction of acute phase protein biosynthesis in hepatocytes (Van Snick, 1990). The main function of IL-6 on human B-lymphocytes appears to be to sustain the growth of already synthesising cells and in certain instances to accelerate Ig secretion (Van Snick, 1990). IL-6 therefore acts at the terminal stages of B-lymphocyte differentiation, and cells require activation before they can respond to IL-6. In murine B-cells however, IL-6 shows poor growth promoting activity, but can synergise with anti-Ig, particularly if IL-1 is present (Kishimoto *et al*, 1992).

IL-10 is produced by Th2 cells, monocytes and B-cells (O'Garra, *et al,* 1990; Fine *et al,* 1994). The capacity of IL-10 to direct immune responses towards the Th2 axis depends upon its ability to inhibit cytokine biosynthesis and secretion (Cassatella *et al,* 1993). For example it inhibits the secretion of IFN-γ by Th1 cells and exerts inhibitory effects on macrophage activation processes, such as the expression of MHC class II antigens and the production of the inflammatory mediators IL-6 and IL-8. IL-10 displays stimulatory effects towards B-cells (Fluckiger *et al,* 1994; Burdin *et al,* 1995). These findings not only demonstrate that IL-10 may be one of the many macrophage products

which exert an effect on lymphocytes but also that the lymphocyte and macrophage can influence each other's actions by the release of cytokines. Quiescent B-cells also respond to IL-10 by up-regulation of class II MHC antigens and it promotes the proliferation and differentiation of B-cells preactivated with either anti-Ig or combined anti-CD40 and IL-4 (Jumper *et al,* 1995).

IFN-α is produced by a wide range of cell types including macrophages and lymphocytes, whereas IFN-γ or immune interferon is produced predominantly by Th1 cells (Mosmann and Coffman, 1989). The anti-proliferative and anti-viral effects of all forms of IFN are well documented and each also has a range of effects on B-cells (Ijzerman *et al*, 1990). IFN-γ acts as a growth enhancing factor for activated B-cells and can synergise with IL-2 and IL-4 (Kishimoto, 1984). Both IFN-α and IFN-γ can potentiate the stimulation of B-cells caused by anti-Ig (Morikawa *et al*, 1987). Data suggests that IFN-γ acts early in the response and high density, quiescent B-cells are the target of IFN-γ action. However if it is added 36 hours after the initiation of the culture it fails to accelerate growth (Trubiani *et al,* 1994). This evidence suggests that pre-activated cells lose the capacity to respond to the growth promoting effects of IFN-γ. High doses also alter the distribution of B-cells and macrophages in the spleen (Steiniger *et al,* 1993), thus it may influence B-cell-macrophage interactions.

Both TNF-α and TNF-β (lymphotoxin), despite showing little structural homology, act via the same receptor when mediating the killing of cancer cells (Lopez-Cepero, 1994; Matsui and Patek, 1994). In B-cell assays both forms show positive effects on lymphocyte proliferation and synergise with IL-2, IL-4 and IFN-γ in supporting B-cell proliferation (Kehrl *et al,* 1987; Bundschuh *et al,* 1997). TNF-α and TNF-β also enhance growth in B-CLL cells (Mapara *et al,* 1994) and B lymphoblastoid cell lines synthesise and secrete TNF-β, which could potentially act as an autocrine growth factor (Boussiotis *et al,* 1994).

Transforming growth factor (TGF)-β has a range of positive and negative effects on cells of the immune system. This cytokine, produced by T-cells, B-cells and

macrophages exists in two forms, TGF1 and TGF2, with 70 % homology but acting via different receptors (Kehrl *et al*, 1989).

1.2.3 Regulation of B-Lymphocytes By Their Products.

The regulation of B-cell proliferation and activation by soluble factors is largely mediated via classical paracrine mechanisms, that is via cytokines that are not B-cell derived. However, B-cells are also under some autocrine control and this section aims to describe some of these.

1.2.3.1 Immunoglobulin and CD23

The protein end product of B-cell differentiation is secreted Ig. In some instances antibody and antigen combine to form immune complexes which are then removed by the liver. Cells that express specific Fc receptors for Ig, bind to either free antibody or immune complexes to transmit regulatory signals to the cell. B-cells possess these receptors and the subsequent effect may be either positive or negative.

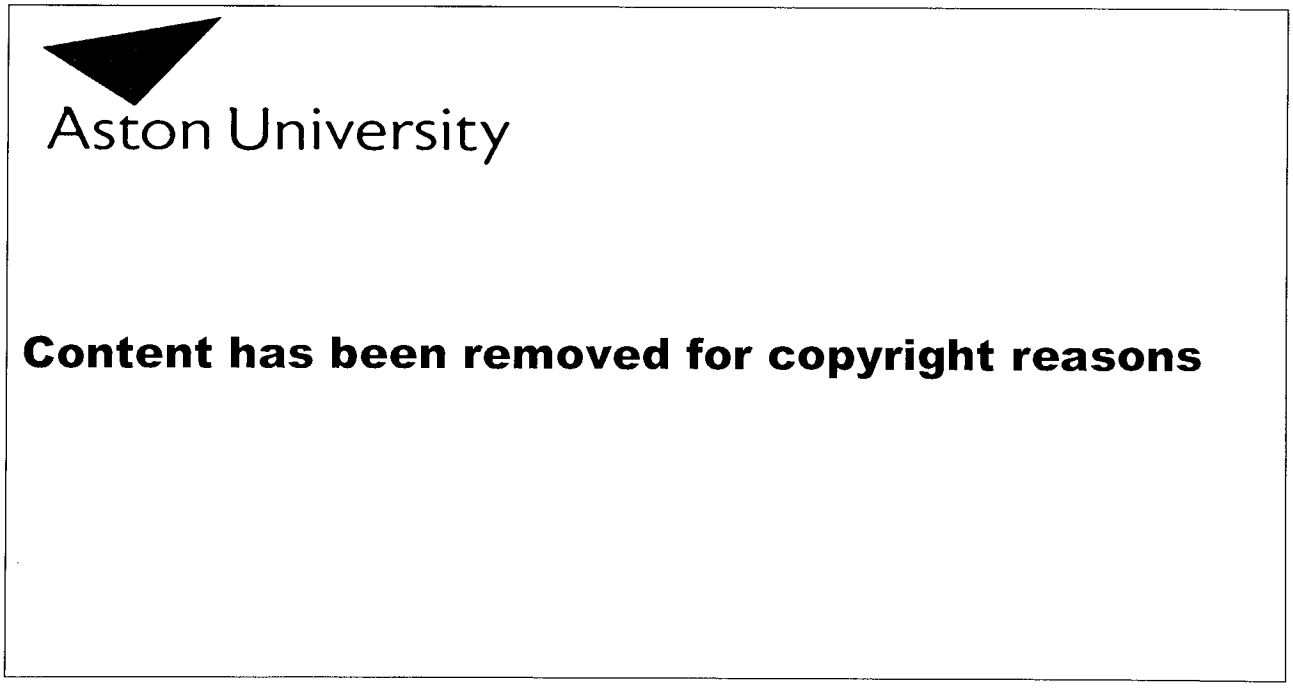
Occupation of the low-affinity receptor for IgG on B-cells sends a profoundly negative regulatory signal to the B-cell, which can only be overcome by IL-4 (Stack *et al*, 1994). IL-4 also causes an increase in the expression of the low-affinity receptor for IgE (CD23) (Kawabe *et al*, 1988). The secreted form of this molecule possess novel mitogenic properties and therefore elicits a positive signal (Mossalayi *et al*, 1990).

1.3 The Macrophage

Having discussed the lymphocyte and how it responds to cytokines, mitogens, antigens and autoregulatory products, this following section aims to describe the macrophage and the possible regulatory role it may have on these various lymphocyte responses.

The mononuclear phagocytic system consists of circulating monocytes in the blood and macrophages in the tissues. During haematopoiesis in the bone-marrow granulocyte-monocyte progenitor cells differentiate into promonocytes, which enter the blood, where they differentiate into mature monocytes. Monocytes circulate in the blood for 8 hours, during which time they enlarge (5-10 fold). They then migrate into the tissues and differentiate into specific macrophages with increased organelles, hydrolytic enzymes and secretory products. The tissue or resident macrophages, in particular new arrivals, can develop along a variety of disparate routes (Cohen *et al,* 1983; Adams *et al,* 1990a). The activation of macrophages has been traditionally described as the development of competence for microbicidal and/or tumoricidal function, but an alternative definition is the ability to perform any complex function, such as the ability to present antigen to T-cells. In murine macrophages, lytic functions can be induced *in vivo* and *in vitro* by a variety of signals that operate both synergistically and in a defined sequence. The first signal is from a class of lymphokines operationally termed macrophage activation factors (MAF) these lower the dose requirement for a secondary signal which is frequently supplied, for example by LPS.

Figure 1.1 A Model Of Macrophage Activation (Adams *et al,* 1990b).

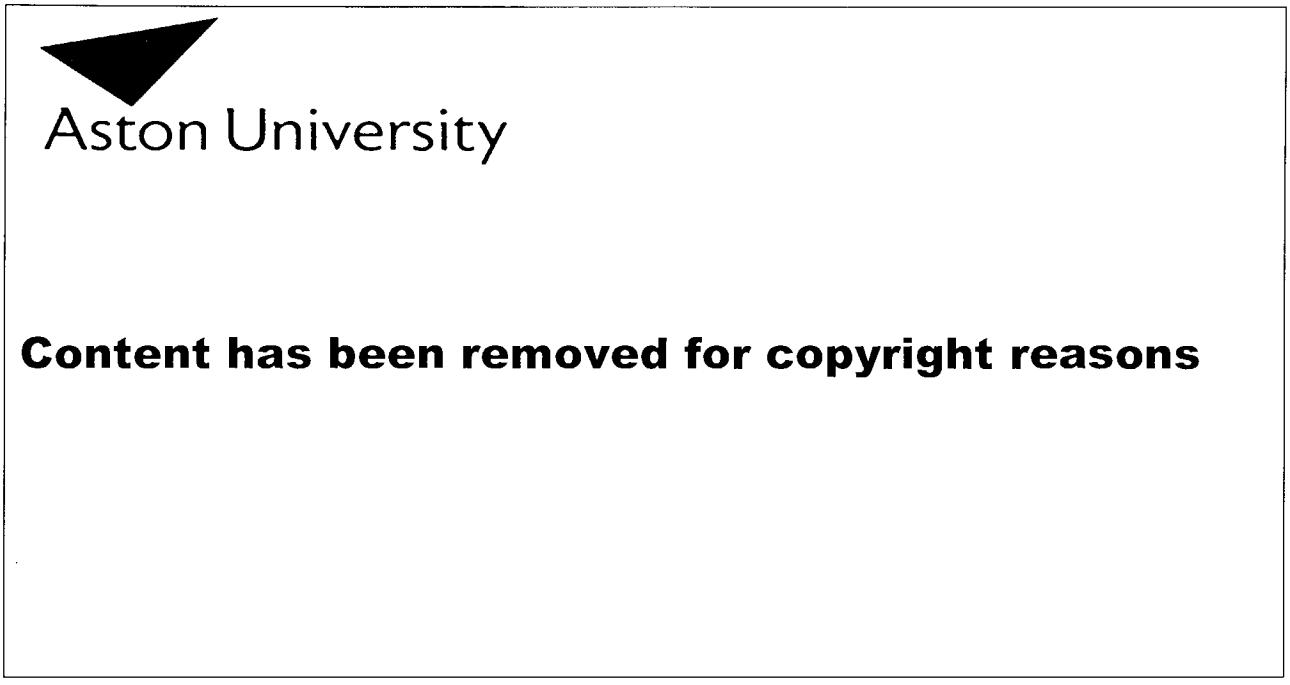


IFN-γ is produced by T-helper cells (Paul *et al,* 1994) and is a potent MAF both *in vivo* and *in vitro* (Nathan *et al,* 1984), as is PMA *in vitro*. A specific receptor for IFN-γ has been identified on murine macrophages and human monocytes. A variety of signals then push primed macrophages into the fully activated stage. These second signals include LPS (Uhing *et al,* 1989), high concentrations of crude lymphokine such as IL-1, IL-2 or IL-4, tumour cell supernatants, heat-killed Gram-positive bacteria and liposome encapsulated muramyl dipeptide (Adams, 1991). Activated mononuclear phagocytes express more than thirty distinct receptors that enable them to recognise and interact with various molecules in their external environment. They also possess the capacity to synthesise and secrete more than one hundred defined substances that act both intracellularly and extracellularly (Nathan *et al*, 1987). For example the role of their Fc receptor is to facilitate phagocytosis and antibody-dependent cell mediated cytotoxicity. The C3 receptors on phagocytes (CR1 and CR3) recognise C3b and C3bi respectively to mediate phagocytosis and prostaglandin release (Hourcade *et al*, 1989). When Fc and C3 receptors are both engaged there is a synergistic enhancement of phagocytosis. The molecular transductional events which occur in macrophage activation after association with a number of agents are very complex. The engagement of the IFN receptor results in direct PKC activation whereas LPS or TNF activate the phospholipase C (PLC) second messenger cascade (Adams *et al*, 1990a).

1.3.1 Macrophage Function

The macrophage carries out five main functions within the immune system, these are phagocytosis, chemotaxis, the processing and presentation of antigen, the secretion of products and tumour cell control and these functions will now be discussed. Macrophage secretion has been described in the greatest detail as the possible influences the macrophage has on lymphocytes may be mediated through soluble products.

Figure 1.2 The Process Of Phagocytosis And Presentation Of Exogenous Antigen. From Kuby, J. Immunology. 1994. W.H. Freeman and Company, New York.



Antimicrobial and cytotoxic substances are produced by activated macrophages. The toxic effects of these substances involve both oxygen-dependent and independent mechanisms. Research has shown that that the oxidative burst and anti-microbial defence can be dissociable (Chateau *et al,* 1993), as other substances such as defensins, hydrolytic enzymes and lysozyme can also kill bacteria. During phagocytosis reactive oxygen intermediates (ROI) are produced. This is known as the oxidative or respiratory burst and results in activation of membrane bound oxidases that catalyse the reduction of oxygen to the superoxide anion $O_2^{\cdot-}$ which is extremely toxic (Johnston *et al,* 1985).

$$2O_2 + NADPH \xrightarrow{\text{NADPH OXIDASE}} 2O_2^{\cdot-} + NADP + H^+$$

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SUPEROXIDE DISMUTASE}} H_2O_2 + O_2$$

Activated macrophages can differ in their production of the superoxide anion and therefore their ability to carry out intracellular and anti-microbial killing (Klebarry *et al,* 1975; Murray *et al,* 1980). The oxidative burst also generates other powerful oxidising agents including hydroxyl radicals ($OH^{\cdot}$) formed in the Haber Weiss reaction

in which superoxide and hydrogen peroxide react in the presence of iron to produce the hydroxyl radical, singlet oxygen (O_2) and hydrogen peroxide (H_2O_2), these are known as myeloperoxidase-independent mechanisms. During phagocytosis the lysosome fuses with the phagosome and the contained myeloperoxidase together with a halide ion act on the H_2O_2 to produce long-lived oxidants, for example, hypochlorite (HOCl) and monochloroamine (NH_2Cl) which are both cytotoxic. (These two reactions are catalysed by myeloperoxidase (MPO)). They only occur in monocytes and polymorphonuclear cells (PMNs), as macrophages possess very low levels of MPO and thus rely on myeloperoxidase-independent defense mechanisms. Reactive nitrogen intermediates are also formed (Adams *et al*, 1990c). Some examples of these are nitric oxide (NO), nitrogen dioxide and nitrous acid. These combine with the superoxide anions to produce extremely potent antimicrobial substances directed against fungal, helminthic, bacterial and protozoal pathogens. Nitric oxide has also been shown to inhibit proliferation of T- and B-cells (Eisenstein *et al,* 1994; Liew 1995).

Another important function of the macrophage is chemotaxis, which refers to the directed movement of cells along a concentration gradient of a 'chemotactic factor', such factors include Leukotriene B_4 (LTB_4), C5a, IFN-γ and N-formylated peptides (e.g. f-MLP (f-Methionine-Leucine-Phenylalanine)) (Brown *et al*, 1988). Chemotactic factors initiate a leucocyte response after binding to specific receptors which are present and this activates the cell's PKC pathway (Nishizuka *et al*, 1984). These receptors can be up or down-regulated in response to other cues and when occupied result in such abilities as attachment to the endothelium, proper orientation of the cell, shape change due to cytoskeletal rearrangement and movement across the endothelial layer (diapedesis) (Brown *et al*, 1988). Chemotaxis is an important part of the immune response and disorders can lead to dramatic and often life-threatening host defence problems (Brown *et al*, 1988).

One of the major functions of the macrophage is to assist in initiating and facilitating cell-mediated immune responses against pathogens. The macrophage can phagocytose antigen without help from Fc receptors. After uptake the antigen is processed inside the cell and in the case of protein antigens, presented as part of Class I or II major histo-

compatibility complex, depending on the type of intracellular processing which occurs. Antigens presented in the context of Class I molecules have been processed in the cytoplasm before being released from the ER (Moore *et al*, 1988), whereas Class II antigens have been processed in endosomes resulting in the activation of T cytotoxic and T helper cells respectively (Qin *et al*, 1989).

Macrophages infiltrate tumours and the lysis of cells is thought to be one mechanism of host defence against tumours. This is cell-contact-dependent and occurs after macrophages recognise and bind to neoplastic cells or antibody-coated cells. The release of cytotoxic substances then results in the lysis of bound tumour cells. They can also inhibit cell growth by secreting IFN-α (Einhorn *et al*, 1982), or stimulate cell lysis by secreting TNF-α (Fidler & Ichinose, 1989).

1.3.2 Macrophage Products

The macrophage not only possesses phagocytic and immunomodulatory properties, but has an extensive secretory capability. Over one hundred substances have been reported to be secreted by the macrophage, some of which are displayed in Table 1.3.

Table 1.3 Macrophage Products. Adapted from: Lewis, C.E., McGee, J.O'D (eds). 1992. The Natural Immune system: The Macrophage, pp 39-42. Oxford University Press.

Enzymes	Inhibitors	Eicosanoids	Growth Factors	Proteins	Coagulation factors	Other
glycosidases	α2macro-globulin	prostaglandins	epidermal GF	complement proteins	tissue factor	apolipo-protein E
lysozyme	plasminogen activator inh.	leukotrienes	fibroblast GF	IL-1	prothrombin activator	PAF
proteases	collagenase inhibitor	thromboxanes	G-CSF	IL-6	coagulation factors	thymidine
lipases	anti-trypsin inhibitor	prostacyclin	M-CSF	IL-8	plasminogen activator	oxygen metabolites
phosphatases	phospholipase inhibitor		GM-CSF	IL-10		Thrombo-spondin
colleganases	IL-1 inhibitors		PDGF	TNF-α		fibronectin
myelinases			TGF-β	IFN-α and -β		transferrin
sulphatases				fibronectin		uric acid
elastases						neopterin
arginases						lipocortin
angiotensin convertase						glutathione

1.3.3 Macrophage Heterogeneity

The seeding of monocytes to different tissues where they remain as macrophages is apparently random as there is no evidence that the tissue destination is pre-programmed. Resident macrophages are widely distributed in the absence of any inflammatory signal and display regional heterogeneity. Functional, morphological and phenotypic heterogeneity may reflect the local environment of the macrophage and the involvement in various physiological or pathological processes.

As previously discussed the spleen contains a heterogenous population of macrophages and related cells. Differences reported in phenotype and appearance probably reflect the different functions they undertake. These include; trapping and processing foreign

antigens (Claassen *et al*, 1986), particularly thymus-independent antigens in the marginal zone, specialised interactions with T- and B-cells in the lymphoid areas (Van den Eertwegh *et al*, 1994), and phagocytosis/degradation of red blood cells in the red pulp (Buckley *et al*, 1987).

The gut lamina propria in both the large and small intestine contains large populations of macrophages. They are also present in the specialised gut-associated lymphoid tissue with a well-defined structure, such as the tonsils and Peyer's patches. They are mainly involved with the phagocytosis of foreign or dead material (Hume *et al*, 1987).

Macrophages are a major cellular component of the lung and several functionally and biochemically distinct sub-populations of alveolar macrophages have been discovered (Sandron *et al*, 1986). Their most characteristic ultra-structural feature is the abundance of membrane bound cytoplasmic inclusions containing proteolytic enzymes (Nakstad *et al*, 1989). Analysis of the tissue distribution of a novel monocyte sub-population, forming about 13 per cent of circulating monocytes, revealed large numbers of these CD14+, CD16+ cells in the alveolar space (Passlick *et al*, 1989). Alveolar macrophages may represent a self-replicating population as they proliferate readily in response to colony-stimulating factors *in vitro* and maintain their numbers after bone marrow ablation (Tarling *et al*, 1987). Macrophages in the lung are involved in local defence against a variety of pathogenic and particulate entrants via the airway and play an early role in inflammation and the control of infection.

The resident macrophages of the liver are involved in the clearance of particulate and soluble substances and express Fc and other receptors, such as Mannosyl-Fucosyl receptor (MFR), CD14 and CD33. It is believed that the response of Kupffer cells to LPS and other gut-derived stimuli may be important in their interactions with hepatocytes (Fuller *et al*, 1987). Indeed, these cells may at least be partially responsible for regulating the acute phase protein response in injury and malignancy by producing IL-6 which appears to deliver the final signal to hepatocytes to trigger the altered metabolism associated with this response (Van Snick, 1990). Large numbers of

monocytes are recruited to the liver following the uptake of micro-organisms by Kupffer cells, these therefore contribute to the immune response against invaders (Buckley *et al*, 1987).

The specialised actions of macrophages in relation to where they reside, is a very important issue in this study. In fact the whole ability of the macrophage to interact with lymphocytes and enable them to respond to certain antigens may be due to the characteristics and functions of macrophages found within this location, in this instance the spleen. Splenic macrophages have therefore developed to efficiently respond to blood borne infections and this is achieved by phagocytosis, close associations with lymphocytes and the release of soluble mediators.

1.4 Lymphocyte-Macrophage Interactions

1.4.1 Evidence for Associations

One of the first experiments suggesting the requirement for accessory cells in the responses of lymphocytes to antigens demonstrated that if the spleen cells of unimmunised mice were depleted of adherent cells, they would not secrete antibody when stimulated by a TD antigen, sheep red blood cells. Responsiveness was restored by the addition of non-lymphoid cells such as macrophages (Hoffmann *et al*, 1979). Further experiments have shown the obligate need for macrophages in T- and B-cell responses to mitogen and antigen.

Macrophages and other accessory cells can perform various functions in the induction of an immune response (Rosenberg and Lipsky, 1979; de Vries *et al,* 1979). Firstly, they might be necessary in presenting antigen to T- and B-cells, thereby inducing both cytokine secretion and clonal proliferation (Claassen *et al,* 1986; Chao and MacPherson, 1990; Aversa *et al,* 1994). Secondly, they secrete cytokines which induce proliferation in both T- and B-cells (Hoffmann *et al,* 1979; Ryffel *et al,* 1997). Lastly, the very young and splenectomised patients are unable to respond effectively to certain

highly virulent strains of bacteria such as *Neisseria meningitidis* and *Streptococcus pneumoniae* (Timens *et al*, 1989). This lack of response corresponds to the immaturity of the marginal zone in the spleen. In this zone specialised interactions occur between B-cells and macrophages. Perhaps the ability to respond to such infections is related to these interactions. A more detailed description of these findings now follows.

1.4.2 TI Responses

The role of T-cells, accessory cells and cytokines in TI immune responses is still under discussion. The original definition of TI antigens reflects their ability to stimulate humoral immunity in nude mice *in vivo*, and in T-cell depleted cultures *in vitro*. These criteria are relatively non-stringent, in that nude mice and T-cell depleted cultures have been shown to possess small numbers of T-cells. Rigorous elimination of T-cells reduces *in vitro* immune responses to TI-2 antigens, indicating that these antigens are T-cell dependent to some degree (Baker, 1992). However TI-1 antigens appear to be totally T-cell independent, as splenic B-cells, prepared by depletion of T-cells (by multiple treatments with anti-T-cell antibodies) do not respond to the strong TI-1 antigen LPS (Corbel and Melchers, 1983). Supplementation with adherent accessory cells or factors derived from them reconstitutes the LPS response (Corbel and Melchers, 1983) which indicates that accessory cells are even more important in B-cell responses to TI antigens. B-cell responsiveness to TI antigens could also be enhanced by the addition of the T-cell-derived cytokine IL-2 (Endres *et al,* 1983; Mond *et al,* 1987) or recombinant IL-1 (Garg *et al,* 1996) which could be derived from the macrophage. The mechanism of lymphocyte activation was also investigated by Dekruyff *et al,* 1985, who demonstrated that when T-cells were activated by the TI-2 antigen, TNP-Ficoll, they were able to directly stimulate B-cells to proliferate and secrete cytokines. This has since been confirmed by Van den Eertwegh, 1992. They suggested that these cell factors activated accessory cells to supply additional factors required for TI immune responses. Accessory cells were found to be essential for an optimal TI immune response. In addition there are a number of reasons why it is unlikely that accessory cells present antigen to B-cells. For example their function can

be replaced by soluble factors (Kraal, 1989; Shieh and Tseng, 1994) and Ia positive and Ia negative cells are equally efficient in reconstituting the TI-1 response of purified B-cells (Fultz *et al,* 1989). However, Claassen *et al*, 1986 did demonstrate that marginal zone macrophages were directly involved in the processing and presentation of TI-2 antigens to B-lymphocytes. This was achieved by injecting mice with dichloromethylene diphosphate encapsulated in liposomes to eliminate MZ macrophages. Subsequent immunization with TNP-Ficoll resulted in a strong decrease in the antibody response in macrophage-depleted animals, in fact the number of antibody forming cells in the spleen dropped 30 to 60-fold. They concluded that MZ macrophages are indispensable for the formation of splenic anti-TNP AFC after immunization with a TI-2 antigen and that the stimulated B-cells migrate out of the MZ very shortly after being 'instructed' by the MZ macrophage. The actual production of antibody takes place in the periphery of the PALS. Chao *et al*, 1990 also provide evidence that MZ macrophages can selectively take up TI-2 antigens by a receptor mediated uptake system. The ligand specificity showed cross-reactivity with the mannosyl-fucosyl receptor with high affinity for mannan and zymosan. They therefore suggest that the TI-2 antigen uptake system may also mediate phagocytosis. Thus, macrophage-B-cell associations or the presentation of antigen by macrophages to B-cells may be required to enable macrophages to provide the necessary signals for B-cell activation in response to certain TI-2 antigens, but not TI-1 antigens. T-cells may also enhance some B-cell responses to TI-2 antigens, but again they have no effect on TI-1 antigen responses. The secretion of a macrophage factor(s) is also important in responses to both TI-1 and TI-2 antigens. There is some evidence to show that T-cells require macrophages to respond to the mitogens Con A and PHA (Rosenberg *et al,* 1976; Thiele *et al,* 1983) too, but the mechanism is unknown. These findings suggest that the macrophage is required by the B-cell in responses to TI-1 and TI-2 antigens.

1.4.3 Cytokines

It is generally assumed that for a thymus-dependent response, the presence of accessory cells, B-lymphocytes and T-lymphocytes are required, (DeFranco *et al,* 1988). Cell-

cell contact and cytokine production by these cells are essential events in the differentiation process of B-cells, as demonstrated in *in vitro* studies (Noelle *et al,* 1990; Parker *et al,* 1990). They showed that direct cell-cell contact between antigen-presenting B-cells and T helper cells is required for the activation of T-cells. Activation induces the expression of novel surface antigens on these T-cells and the secretion of cytokines, both of which are needed to provide the major growth stimulus to B-cells. These cytokines act at different steps in the B-cell activation pathway. Cytokines, like IL-1, IL-2 and IL-4 support the growth of activated B-cells, while others such as IL-6 and IFN-γ participate as differentiation factors.

Although the role of the cytokine in TD immune responses is very well understood, currently it is unclear how soluble factors derived from accessory cells affect B-cell responses to TI antigens. First, cell cultures in which single B-cells are placed respond to TI stimuli directly in the presence of cytokines (Mond *et al*, 1987; Garg *et al*, 1995). The accessory cells, or factors, may also induce residual T-cells to secrete factors necessary for differentiation (Unanue and Allen, 1987). To support this, IL-5, a T-cell derived factor, substitutes for accessory cells in certain TI-2 antigen responses (O'Garra *et al*, 1986). Further research is therefore required to create a fuller understanding of how important the accessory cell is in lymphocyte responses and in what way the B-cell is dependent on the accessory cell.

1.4.4 CD21

The immune response to polysaccharide antigen found in the capsule of certain virulent bacteria has been shown to be related to a functionally active spleen. This immune response is almost completely defective in infancy (Timens *et al*, 1989). Whereas all other cellular compartments had completed their maturation in the young, the MZ had not. The marginal zone B-cells were essentially different compared with the adult situation. The main characteristics of the immature marginal zone B-cell was the absence of CD21/CR2 (C3d receptor) expression and the high percentage of cells co-expressing IgM and IgD (Timens *et al,* 1989). The marginal zone is supposed to be the

site of initiation of the immune response to polysaccharide antigen and there is remarkable coincidence between the first appearance of the MZ B-cell with adult features, and the time of acquisition of the ability to mount an immune response to polysaccharides. It is thus tempting to suggest that CD21 expression plays a role in the immune response to polysaccharide antigen. Because CD21 can function as the receptor for the complement component C3d there may be a possible interaction with marginal zone macrophages which express the ligand to CD21, CD23 (Aubry *et al,* 1992). Another explanation is that the synergistic signalling of the complement receptor and mIg could provide an additional activation signal for B-cells (Carter *et al,* 1988 & 1989), and TI-2 antigens are able to activate complement via the alternative pathway (Griffeon *et al,* 1991). Moreover, they showed that polysaccharides complexed with C3d were able to bind to the complement receptor on B-cells. These data suggest that TI-2 antigens complexed with C3d are able to activate B-cells directly. However cytokines produced by T-cells and macrophages are essential for a full immune response. Marginal zone macrophages do act as an antigen presenting cell in the immune response to polysaccharide, as only marginal zone macrophages have been shown to specifically process and present neutral polysaccharide antigen (Amlot *et al,* 1985; Humphrey *et al,* 1981; Humphrey *et al,* 1985). The response to this antigen is abrogated after selective elimination of marginal zone macrophages (Claassen *et al,* 1986). This gives some evidence that associations between B-cells and macrophages are important in responses to thymus independent antigens and also highlights some of the views on how the macrophage interacts with the B-cell to produce an immune response to these antigens.

1.5 Aims of This Study

The immune response to thymus-independent antigens is a fundamental defence mechanism for the protection against virulent bacteria with polysaccharide capsules such as *Streptococcus pneumoniae, Neisseria meningitidis* or *Haemophilus influenzae.* Marginal zone B-cells in infants are unable to respond to such bacteria. This may be due to the lack of CD21 expression on immature B-cells which could associate with its

co-receptor (C3d) or CD23 on adjacent macrophages. In fact, purified B-cells fail to proliferate in response to the strong TI-1 antigen Lipopolysaccharide (LPS) in the absence of macrophages (Corbel and Melchers, 1983) and Claassen *et al*, 1986 have demonstrated that marginal zone macrophages are directly involved in the processing and presentation of TI-2 antigens. The mitogenic response to PHA and Con A is also dependent on the presence of macrophages (Rosenberg *et al,* 1976; Thiele *et al,* 1983). B-cell responsiveness to TI antigens could be enhanced by the addition of the T-cell-derived cytokine IL-2 (Endres *et al,* 1983; Mond *et al,* 1987) or macrophage-derived IL-1 (Garg *et al,* 1996). This thesis was based upon the hypothesis that the macrophage is important in T- and B-cell responses to TI antigens and mitogens. The focus of this thesis was to characterise this requirement. The aims were as follows:

(1). To gain a better understanding of the macrophage, including: differences between monocytes and macrophages/ primed and un-primed macrophages; macrophage heterogeneity; macrophage differentiation; macrophage activation.

(2). To establish whether the macrophage was required by the lymphocyte in response to a range of T- and B-cell mitogens and TI-1 and TI-2 antigens, including Con A, LPS, PWM, Dextran sulphate, PHA-P and Poly[I][C] and to determine the macrophage: lymphocyte ratio able to respond to mitogen.

(3). To determine whether the requirement for the macrophage is via cell surface contact or soluble mediators, or both. To examine the relationship between T-cells, B-cells and macrophages in response to the six mitogens previously named.

(4). To determine the splenic macrophage product profile and isolate possible co-mitogenic factors.

2. MATERIALS AND METHODS

The work described in this thesis was designed to investigate the contribution which cells of the monocyte lineage might play in lymphoid cell proliferation in general and B-lymphocyte mitosis in particular. Thus, various activation protocols were employed to stimulate macrophages or macrophage-like cells of mouse and human origin and the level of stimulation was determined. These macrophages and, or their secreted products were then co-cultured with lymphocytes under different regimes to assess their role.

2.1 Cell Culture

2.1.1 Primary Mouse Splenocytes, Peritoneal Macrophages, Kupffer Cells and Alveolar Macrophages

To prepare the various cell suspensions adult male, MF1 outbred mice and adult male Balb/c inbred mice were killed by cervical dislocation under diethyl ether anaesthesia. In order to remove the spleen the mouse was placed on to its right side. The animal was swabbed with 70 % alcohol and sterile forceps and scissors were used to take out the spleen. Initially the fur was removed to reveal the abdominal wall, which was then swabbed with ethanol before extracting the spleen. The spleen was then put into a 10 cm sterile petri dish (Falcon) containing approximately 10 mls of unsupplemented RPMI 1640 tissue culture medium (Sigma). Splenocytes were mechanically dispersed aseptically using forceps to yield a cell solution containing mainly T- and B-cells, follicular dendritic cells and resident macrophages. The cell mixture was then transferred to a 15 ml sterile conical tube (Sarstedt) and the debris allowed to settle to the bottom of the tube. The single cell suspension was aspirated from the top of the tube and put into a fresh tube. The cells were then washed three times in unsupplemented RPMI 1640 medium by gently centrifuging them for 10 minutes at 1200 rpm and decanting the supernatant, replacing with fresh medium and resuspending

the cell pellet. All spins were performed in an IEC Centra-3C Centrifuge. The cells were then ready for use in an appropriate experiment.

The murine peritoneal macrophages were extracted by injecting 5 mls of warmed, sterile RPMI 1640 medium supplemented with 10 % foetal calf serum (Gibco) and 100 μg/ml Penicillin and 100 IU/ml Streptomycin (Sigma) into the peritoneum and gently massaging the area. The medium was then removed using a syringe (Plastipak) and placed in a 10 cm petri dish and incubated in 5 % CO_2 and 95 % air at 37°C for 6 to 24 hours to allow the macrophages to adhere. Three washes with cold (4°C) phosphate buffered saline (Gibco) were carried out to remove non-adherent cells. The macrophages were then removed by mechanical scraping using a cell scraper (Costar), placed in a 15 ml sterile conical tube and washed three times by centrifugation to yield approximately 3 x 10^6 peritoneal macrophages per mouse.

Kupffer cells and alveolar macrophages were obtained by the aseptic removal of the liver and the lungs. The organs were transferred to sterile petri dishes containing 10 mls of warmed supplemented RPMI 1640 medium. The tissues were then mechanically dispersed by the use of sterile forceps and transferred into a 15 ml sterile conical tube. After a brief period of time to allow the clumps to settle the single cell suspension was aspirated into a fresh tube. The cells were then washed three times by centrifuging them for 10 minutes at 1200 rpm and decanting the supernatant, replacing with fresh medium and resuspending the cell pellet. After preparation of the cells they were placed into a petri dish and left for 6-24 hours to allow the macrophages to adhere to the bottom. The same technique was used to obtain adherent splenic macrophages (see Photo 2.1). The cells were removed from the petri dish in the same manner as for peritoneal macrophages. Cultured cells were routinely inspected using an inverted microscope at low power (X100) with a dark phase lens.

2.1.2 Cell Counts

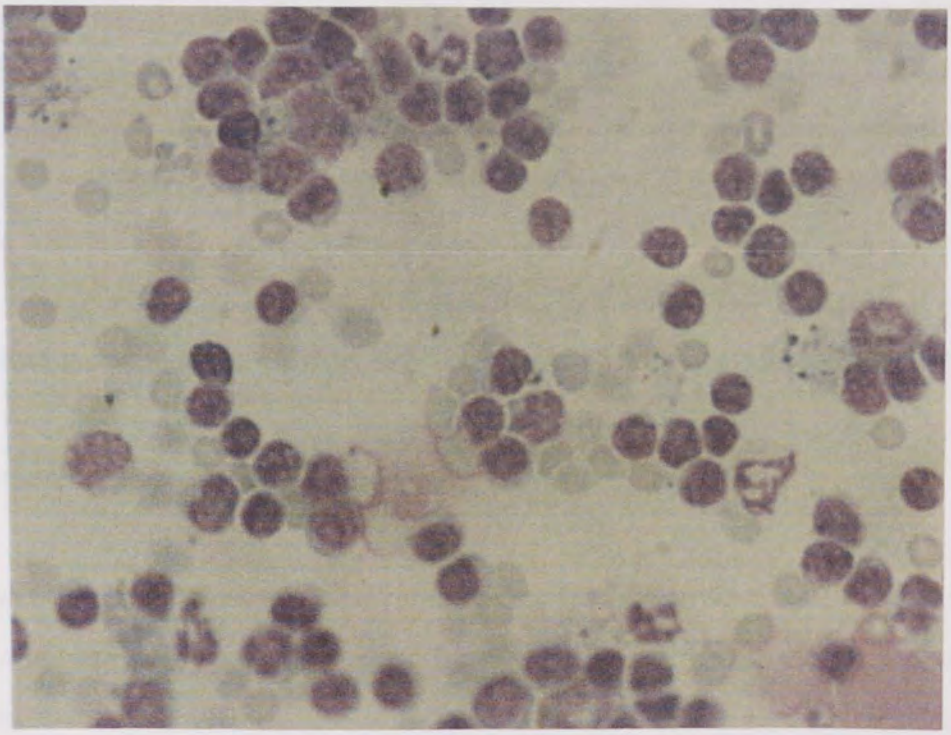
Cells were routinely counted twice on a haemocytometer. The accuracy of cell numbers obtained using a haemocytometer was determined by taking four samples of cells from the same culture and counting each four times. The overall mean (n=16) and SEM were 43.375 +/- 0.970, giving an error of less than 3 %.

2.1.3 Adherent Cells

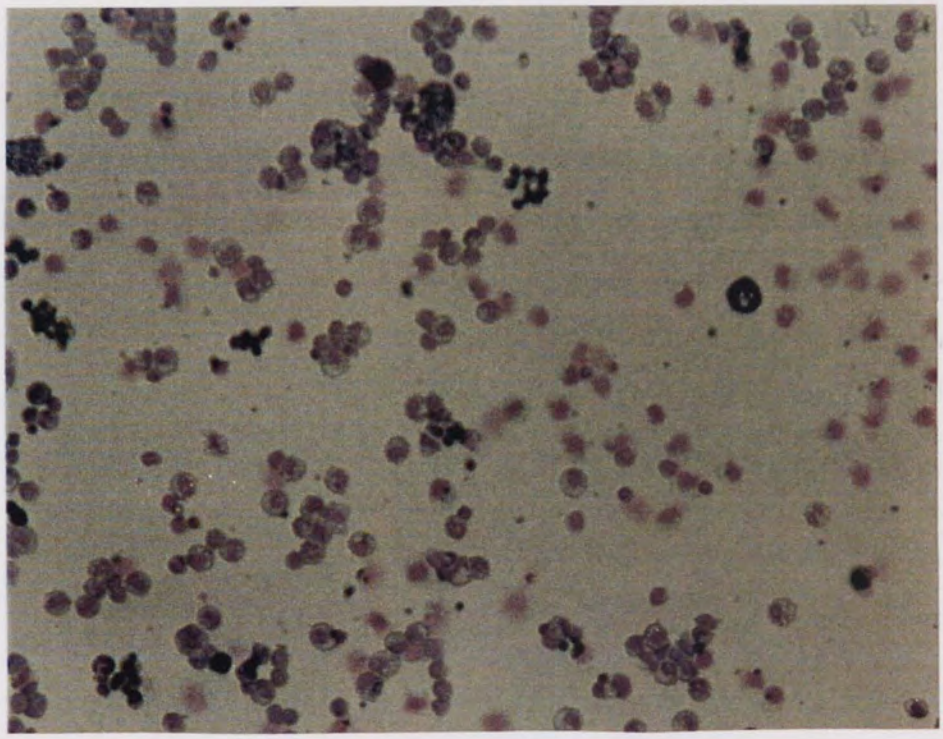
The murine macrophage-like cell line RAW 264.7. (Ralph and Nakoinz, 1977 & Aksamit *et al*, 1986) was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented in the same manner as RPMI 1640 in section 2.1.1. Cultures were passaged once a week using pre-warmed non-enzymatic cell dissociation solution, containing EDTA, glycerol and sodium citrate (Sigma) and a cell scraper. This was achieved by first removing all medium and then rinsing with sterile phosphate buffered saline (PBS) without calcium and magnesium (PBS 'A'). The vessel was gently rocked for approximately one minute and then the buffer was removed, 5 mls of dissociation solution were added to the 75 cm^2 flask (Corning) and the vessel was again rocked to cover the cell monolayer with solution and incubated for 10 minutes at 37°C. The cells were dislodged by sharply tapping the side of the flask with the palm of the hand. Strongly adherent cells were removed using the cell scraper. The cell suspension was then washed and counted and 3 x 10^6 cells were placed into a fresh sterile 75 cm^2 flask with 30 mls of supplemented DMEM. Cell Clumps were dissociated by repeatedly pipetting the suspension. RAW 264.7 cells were fed once a week by decanting off the medium and replacing with fresh supplemented DMEM. Cells were also grown in suspension on Cytodex 1 beads (Pharmacia Fine Chemicals). The dry micro-carrier beads were prepared by hydrating 5 g of beads in 300 mls of PBS 'A' for 3 hours at room temperature. The supernatant was decanted and the micro-carriers were left for one minute in 200 mls of PBS 'A'. The cytodex beads were resuspended in 200 ml of PBS 'A' and sterilised by autoclaving. They can be autoclaved five times without affecting performance.

Photo 2.1 Mixed Murine Splenocytes

a)



b)



Cytospin preparations of splenocytes stained with Diff-Quick are shown. Mixed splenocytes a) were incubated in 5 % CO_2 and 95 % air at 37°C in a petri-dish for 6 hours, X250 and the adherent cells, b) were harvested, X125.

Cytodex 1 beads are based on a cross-linked Dextran matrix which is substituted with positively charged N,N-diethylaminoethyl groups. Charged groups are distributed throughout the micro-carrier, to produce a biologically inert strong but non-rigid structure for growth of anchorage-dependent cells. The micro-carriers are transparent making microscopic examination easy which enables quick monitoring of cell growth. The most effective cell growth on the beads was achieved when 3 mls of the bead preparation was incubated in 30 ml of RAW 264.7 cells at a density of 1 x 10^5 cells per ml. All cultures were maintained at 100 % humidity in 5 % CO_2 and 95 % air at 37°C.

2.1.4 Quantification of Cell Number using CytoPro Kit

Adherent cells grown in suspension on Cytodex beads (see section 2.1.2.) cannot be counted at all using a haemocytometer, so the CytoPro 480 kit (BioOrbit) was used to assess cell number. This is a bioluminescence assay which measures the amount of Adenosine Triphosphate (ATP) released from lysed cells (Leach, 1981). A sample of cells from the population in question can be taken and the cell density quickly measured using this technique. The reagents in the kit were formulated to provide a linear, time-independent light output over a concentration range of 10^{-11} to 10^{-6} M ATP. Known cell numbers in a volume of 100 µl of medium were first incubated at room temperature with 700 µl of Somalyze for 10 minutes in a cuvette to release ATP by lysing the cells. Two-hundred microlitres of ATP monitoring agent (containing Luciferase from the firefly) were then added to the cuvette and it was gently shaken. This generated light via the Luciferase reaction, which is shown below:

$$\text{ATP} + \text{Luciferin} + O_2^{\cdot} \xrightarrow{\text{LUCIFERASE}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{Light}$$

Luminescence was measured on a BioOrbit 1253 luminometer and a standard curve produced, which was calibrated using the Bio-Orbit ATP standard where the light generated from known concentrations of ATP was recorded (see Figure 2.1 and 2.2 respectively).

2.1.5 Non-Adherent Cells

The human myelomonocytic cell line U937 (Sundstrom and Nilsson, 1977, Nilsson *et al*, 1980, Sundstrom *et al*, 1982) was maintained in RPMI 1640 supplemented with 10 % heat-inactivated foetal calf serum (FCS), and 100 μg/ml Penicillin, 100 IU/ml Streptomycin and 2mM L-Glutamine. To prime cells, one millilitre of human recombinant IFN-γ (10,000 International Units (IU)/ml) (R& D Systems) was added to a 75 cm^2 tissue culture flask containing 30 mls of U937 cells seeded at 1 x 10^5 cells per ml, giving a concentration of approximately 330 IU/ml human recombinant IFN-γ.

They were passaged once a week by decanting the cells into a sterile 50 ml tube and centrifuging for 10 minutes at 1200 rpm. The cell pellet was resuspended in 5 mls of medium and the cells were then counted using a haemocytometer. Cell number was adjusted to 3 x 10^6 cells per ml and 1 ml of cells was added to 30 mls of medium in a 75 cm^2. The U937 cells were therefore seeded at 1 x 10^5 cells per ml being incubated at 100 % humidity in 5 % CO_2 and 95 % air at 37°C.

2.1.6 Freezing Down Cells

Long term storage of cell lines was achieved by freezing ~ 1 x 10^8 cells in 1 ml of freezing medium (Sigma), containing Dimethyl Sulphoxide (DMSO) in a cryovial, which was then placed into an appropriate cell box and stored for 24 hours at -70°C. They were then transferred to the Cell Bank at -196°C. When resurrecting cells they were thawed as quickly as possible, washed twice and suspended in 10 mls of medium containing 20 % FCS.

Figure 2.1 Standard Curve Using CytoPro-480 Kit (n=6)

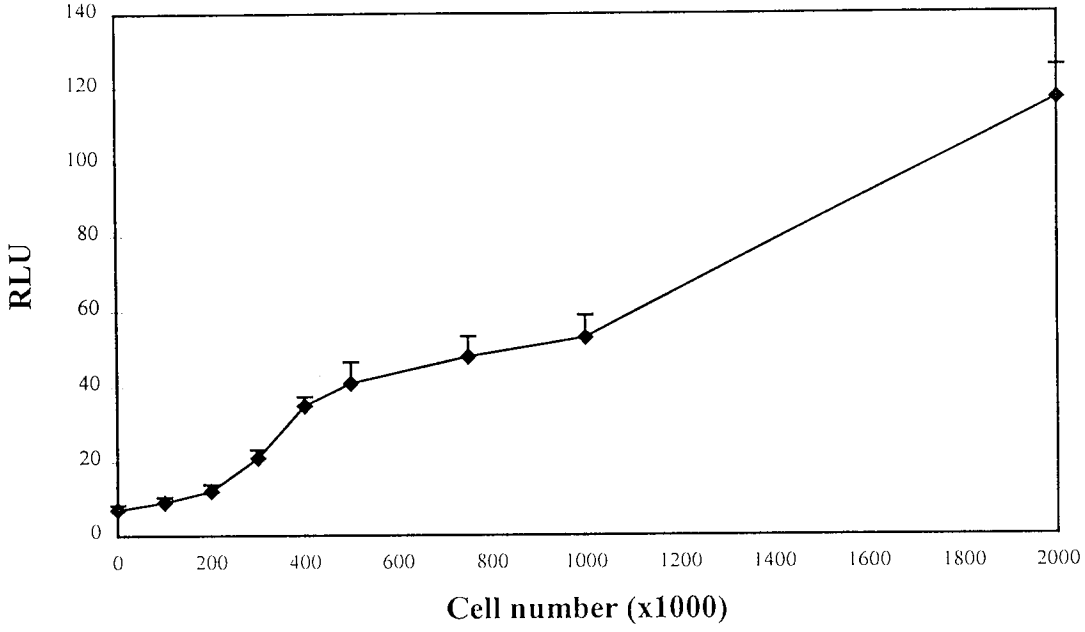
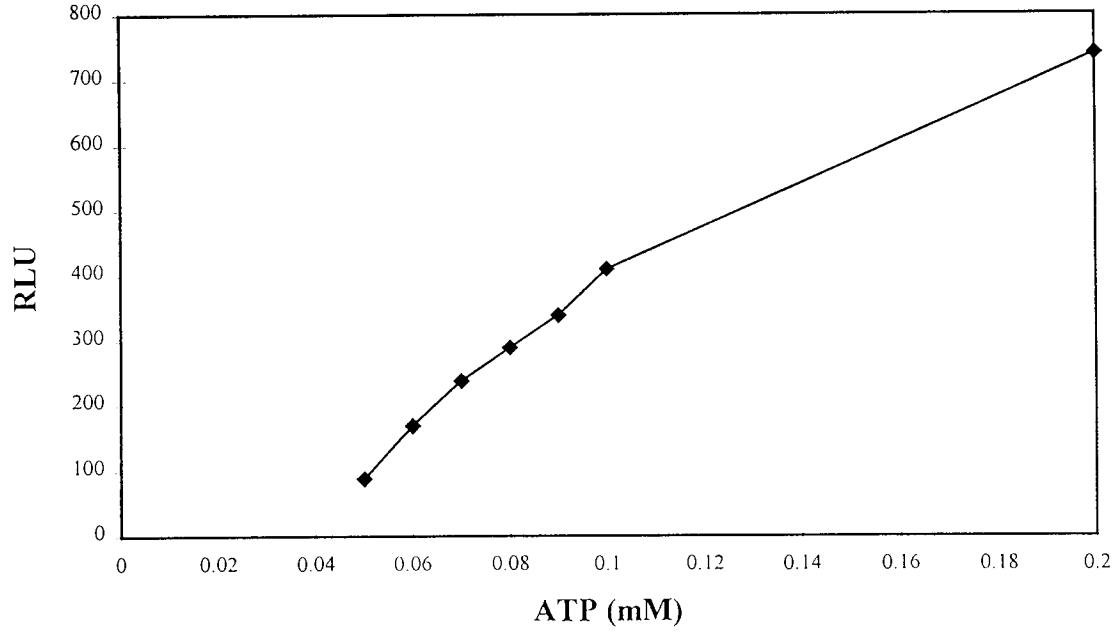


Figure 2.2 ATP Standard Curve (n=6)

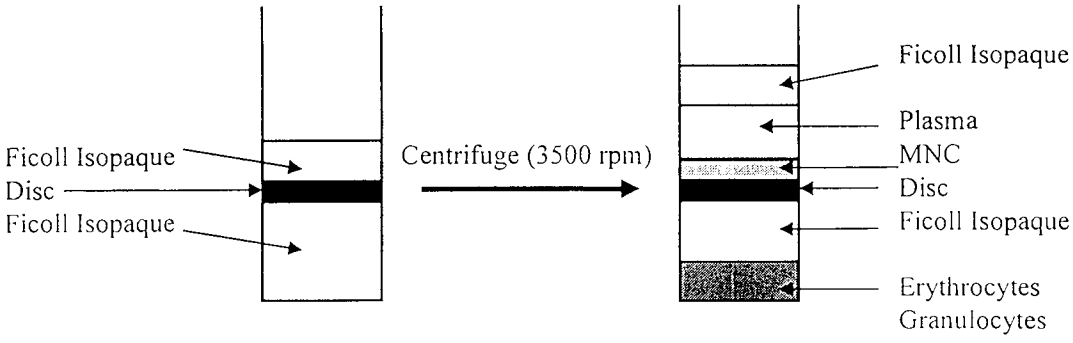


Cell numbers could accurately be determined between the range of 1 x 10^5 to 1 x 10^6 cells. The experiment was performed six times and SEMs are shown.

2.1.7 Primary Human Mononuclear Cells (MNC)

The required amount of blood was obtained by venepuncture being performed on healthy volunteers. The blood was collected in the appropriate sized syringe and wide bore needle and transferred to a heparin coated 10 ml tube (L.I.P. Equipment Services Ltd.). In the first step, Ficoll-Isopaque-solution (Sigma) was warmed to room temperature and 15 mls was pipetted into a Leucosep tube (Greiner Labortechnik). This was centrifuged at 3500 rpm for 30 seconds so that the isopaque settled to the bottom of the tube above and below the disc which was in the leucosep tube already (see Figure 2.3). Next, the fresh anti-coagulated blood was diluted 1:2 with warmed Hanks balanced salt solution (Sigma) and poured quickly into the Leucosep tube (30 mls per tube) and then spun at 3500 rpm for 10 minutes. This process separated out the mononuclear cells and after centrifugation the sequence of the tube was as follows (top to bottom): plasma - MNC - Ficoll Isopaque - disc - Ficoll-Isopaque - granulocytes and erythrocytes. Again this is clarified in Figure 2.3. The plasma layer was aspirated and disposed of appropriately. The mononuclear cells were then collected and placed in a 15 ml tube. The cells were washed twice by adding 10 mls of PBS and centrifuging at 1200 for 10 minutes. Due to the shorter centrifugation times the cells are less stressed compared to other methods. The cell pellet was resuspended in 5 mls of supplemented RPMI 1640 medium. Cell number was adjusted as appropriate.

Figure 2.3 MNC Separation from Whole Blood Using Leucosep Tube



2.1.8 Fixing cells

Macrophages were fixed after various activation protocols to determine the effect of cell surface ligands on lymphocyte proliferation. The appropriate number of cells were washed twice and transferred to a suitable tube. Two mililitres of medium and 2 mls of 4 % paraformaldehyde (Sigma) were added to fix them. The 4 % paraformaldehyde was prepared by dissolving 4 g per 100 ml of unsupplemented medium and warming to 60°C for 1 hour in a water bath. The cells and paraformaldehyde were incubated for 10 minutes at 37°C, washed once in PBS and stored at +4°C for a maximum of 24 hours. The fixing procedure was also employed when using cells for FACscan analysis. In this instance paraformaldehyde was an ideal fixative as it did not interfere with the fluorescence of the cells.

2.2 Microscopy

2.2.1 Detection of Phagosomes

The uptake of particulate matter was assessed in a number of cell types to investigate cellular activation. Slides were prepared by taking a sterile 15 ml tube containing 1 x 10^6 cells in 300 µls of unsupplemented medium and 100 µls of opsonised Zymosan A (5 mg/ml, Sigma) or antibody-coated sheep red blood cells (3 mg/ml, ICN) or latex beads (3 mg/ml, Sigma) and centrifuging at 1200 rpm for 10 minutes (for preparation of opsonised Zymosan A see section 2.7.1). The cell-particle mixture was incubated for 30 minutes and then washed three times by centrifuging and resuspending in fresh unsupplemented medium. The slide (BDH) was prepared using a Cytospin 3 (Shandon). Cytospins were generated as opposed to cell smears to give a dense cell preparation, making slide examinations faster. The cells in question were re-suspended in complete media at a density of 5 x 10^5 cells per ml and 100 µls was spun down onto a slide in a cyto-centrifuge. After a 3 minute spin at 300 rpm the slides were removed and air dried for 10 minutes. The slides were then fixed and stained using Diff-Quick

(Baxter-Dade). The number of cells containing phagosomes were counted by two independent observers with an oil immersion objective at 1250X magnification.

2.3 Luminometry

2.3.1 The Oxidative Burst in Non-Adherent Cells

The oxidative burst was assessed by Lucigenin enhanced chemiluminescence. The basis of the experiment is the measurement of light produced as a result of adding Lucigenin to the phagocytic system used. Lucigenin interacts with the oxidising species released by the phagocyte to produce a measurable amount of light at a peak wavelength of approximately 425 nm. A generalised equation for this is:

$$\underset{\text{reactant}}{\text{reduced luminescent}} + H_2O_2 \text{ -------> } \underset{\text{reactant}}{\text{oxidised luminescent}} + \text{light (425 nm)}$$

Macrophage populations were harvested and re-suspended at 1 x 10^6 per 300 µls. The suspension was placed in a 75 x 11 mm plastic tube (Sarstedt) only after Lucigenin (100 µl at 5 x 10^{-4}M) (Sigma) and 100 µl of the test compound had been added (the details of compounds utilised is described in section 2.8). The cells were added last so that readings could be taken at zero time. The tubes were shaken gently and placed in the luminometer (BioOrbit 1253) and read immediately. Tubes were incubated at 37°C in the dark. Chemiluminescence readings were taken at 5 - 10 minute intervals over a 90 minute period, or as appropriate. The results were expressed as relative light units (RLU) x 1000 per 1 x 10^6 cells. A photomultiplier detects changes in the light being produced which is then converted into mVolts and displayed as a digital readout in Relative Light Units (RLU).

2.3.2 The Oxidative Burst in RAW 264.7 Cells

The cell number of the adherent cells grown on cytodex beads was elucidated using the ATP monitoring kit and standard curve (see 2.1.4) and adjusted to 1 x 10^6 per 300 µls. The method was then carried out as for non-adherent cells (2.3.1).

2.3.3 Sheep Red Blood Cells (SRBC) Coated with SRBC Antibodies

Sheep red blood cells (ICN) were coated with SRBC antibodies in an attempt to engage cross-linked Fc receptors on the macrophage to produce an oxidative burst. To achieve this adult, male MF1 mice were injected intra-peritoneally with 4 mls of sheep red blood cells. The mice were left for a week and then re-injected. They were again left for one week. The antibodies to sheep red blood cells were harvested by extracting blood directly from the heart (~ 1.5 mls per mouse). The serum was collected. Sheep red blood cells were coated with this serum at the appropriate dilution (as elucidated by a haemagglutination test). Briefly, serial dilution's of the serum were made by diluting the serum with PBS and 100 µls of these solutions were pipetted in triplicate into a 96 well V bottomed plate. To this 100 µls of fresh SRBCs were added, the plates were left at room temperature to allow agglutination to take place. A haemagglutination test was used to assess the anti-SRBC antibody titre and sheep red blood cells were subsequently coated with this serum. The same protocol for coating Zymosan A with guinea pig complement (2.7.1) was employed. The cells opsonised with antibody were kept at +4°C until required.

2.4 Proliferation Assays

2.4.1 Cell Separation

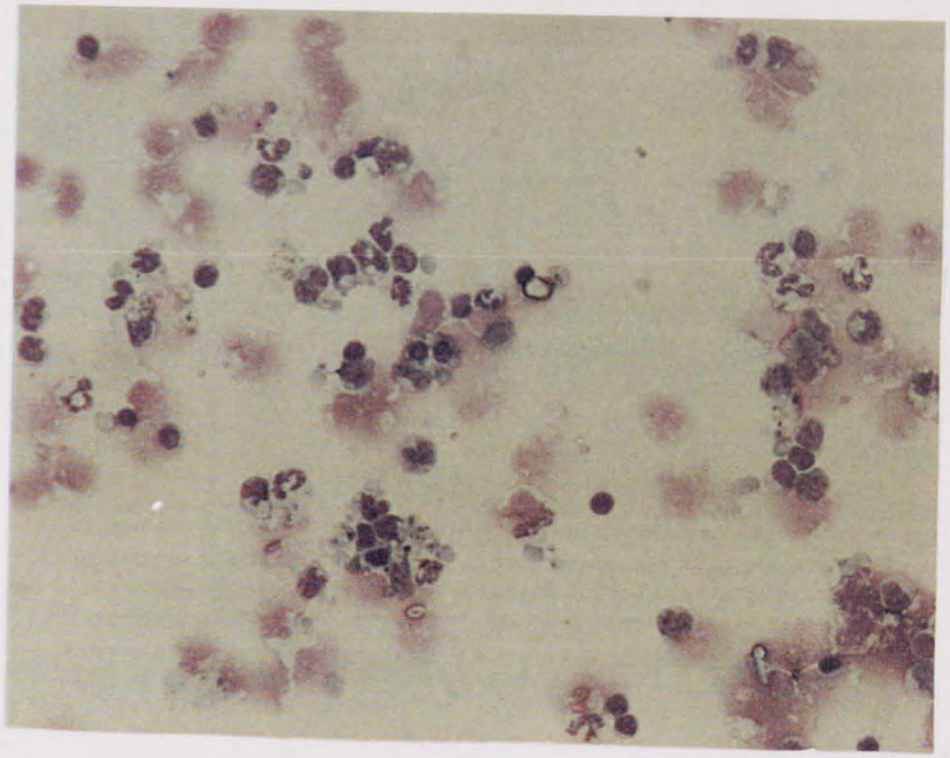
After isolation of murine splenocytes (2.1.1) cells were washed three times and re-suspended in un-supplemented medium to give 1 x 10^7 per ml. To separate the cells a magnetic activated cell sorting (MACS) system was employed. Thus cells in

suspension can be separated to very high purity by means of cell specific surface antigens. Cells were labelled with MACS microbeads (Miltenyi Biotech Ltd.) which are coated with the antibody to a particular surface marker (expressed on the cells that are to be separated). The beads are only 50 nm in diameter and are therefore unlikely to affect cell surface expression or receptor binding. The cells were then separated by placing a magnetic separation column in a strong magnetic field. The magnetically labelled cells were retained in the column while the un-labelled cells (the 'negative fraction') passed through and were collected (see Photo 2.2). When the column is removed from the magnetic field, the magnetically retained cells (the 'positive fraction') were eluted using the plunger provided. Both labelled and non-labelled cells can be completely recovered. The protocol was as follows: sterile tubes containing 1 ml of cells were centrifuged at 1200 rpm for 10 minutes. The cell pellet was then re-suspended in 10 µl of miniMACS microbeads and 90 µl of un-supplemented medium (in this protocol it will be referred to as 'buffer'). To isolate murine B-cells the B220 bead was required (it carries the antibody to CD45R). For human B-cells the CD19 bead was used and for both murine and human monocytes/macrophages the CD11b microbead can be employed. The cells were then incubated for 15 minutes at 6°C to 12°C to allow the magnetic labelling to take place. After the addition of 150 µl buffer the cells were centrifuged at 1650 rpm for 10 minutes and resuspended in 40 µl. The miniMACS separation column was placed in the miniMACS separation unit (Miltenyi Biotech Ltd.) and washed with 2 mls of buffer to remove the preservative. The labelled cells were then passed down the column and washed with 4x500 µl of buffer, the negative fraction being collected at the bottom. To remove the positive fraction, the separator was placed on a 15 ml sterile tube. One ml of buffer was pipetted on top of the column and the magnetically labelled cells were flushed through using the plunger.

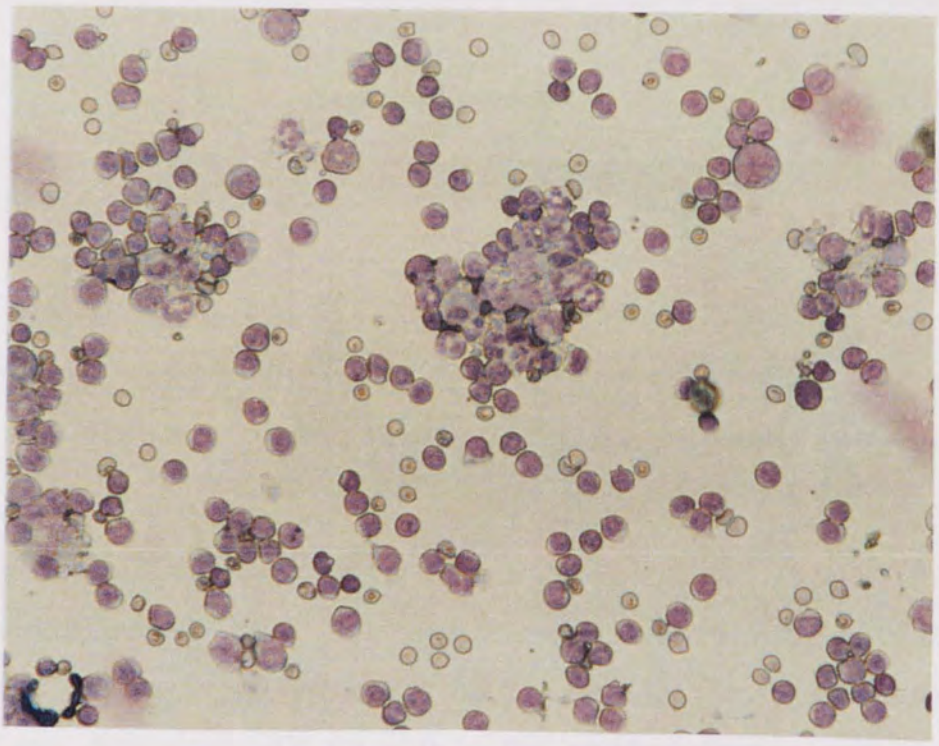
The cells were counted on a haemocytometer washed and adjusted to the appropriate concentration. The purity of the cells was assessed by labelling with the appropriate antibody conjugated to FITC (e.g. the antibody to CD19, CD11b or CD45R, obtained from R&D Systems). The purity was then examined under a fluorescent microscope (Jenamed). A FACScan was also used to assess purity (see section 2.6 and Figure 2.4).

Photo 2.2 Macrophage Separation using MiniMACS

a)

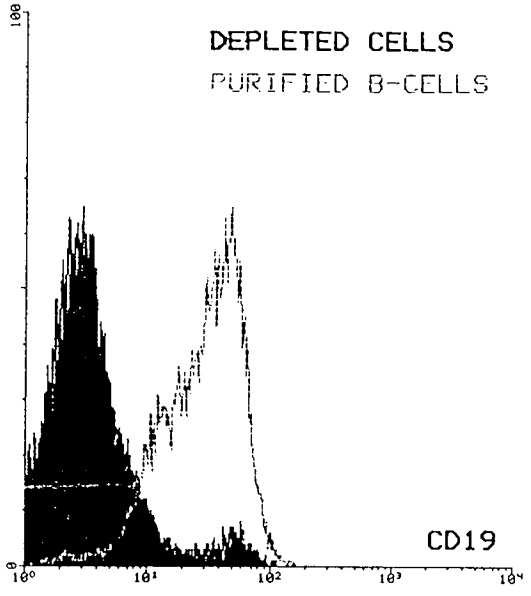


b)

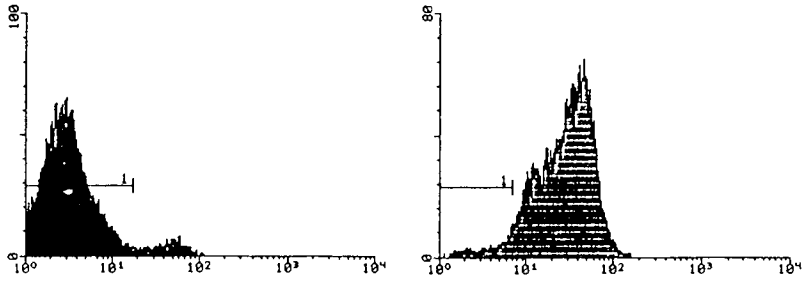


The 'positive fraction', a) consists of cells expressing CD11b (predominantly macrophages) stained with Diff-Quick, X125. The 'negative fraction' b) was also stained with Diff-Quick and contains cells that do not express CD11b (mainly lymphocytes), X125.

Figure 2.4 Cell Purity Using miniMACs Separation



Negative Fraction **Positive Fraction**



1×10^5 miniMACS purified human B-Cells or the depleted cell 'negative' fraction in 1 ml of un-supplemented RPMI 1640 medium were incubated with 5 µl of stock FITC conjugated anti-human CD19 antibody for 15 minutes. The cells were washed and fluorescence determined. Of the cells in the 'positive' fraction, +98 % expressed CD19, thus were B-cells.

2.4.2 Preparation of 96 Well Microtitre Plate

Half a million viable cells were cultured in 50 µl of supplemented RPMI 1640 in a flat bottomed 96 well microtitre plate. Additions of mitogens, supernatants or other cells to the culture were made in 10 µl of the same media and the final volume was made up to 100 µl. Cultures were incubated for 48 to 72 hours in 5 % CO_2 and 95 % air at 37°C.

2.4.3 Incorporation of Tritiated Thymidine

Cultures were pulsed after the appropriate time course with 18.5K Bq per well (0.5 µCi/well) [^{3}H]TdR (Amersham) with a specific activity of 185 GBq (5.0 Ci) per mmol. This was achieved by diluting the Tritiated Thymidine 1:20 with un-supplemented RPMI 1640 medium and adding 20 µl of this per well. They were incubated for a further 4 hours at 37°C in 5 % CO_2 and 95 % air at 37°C.

2.4.4 Cell Harvesting

After incubation cells were harvested using a Titertek Cell Harvester on to glass fibre filter mats (Titertek). Specific incorporation of Tdr was analysed using liquid scintillation counting, by immersing each dried mat in 5 ml of Optiphase scintillant (LKB) and counting on a β-counter (1600 TR Packard Liquid scintillation analyser). Proliferation was quantified by use of the Stimulation Index (SI). This was calculated:

SI = Average counts per minute (cpm) test wells / Average cpm control wells

2.4.5 The 5-bromo-2'-deoxyuridine (BrdU) Labelling Kit

The use of BrdU instead of ^{3}H-thymidine offers a novel alternative to the radioactive proliferation assay. Like Thymidine, the Pyrimidine analogue BrdU is incorporated into the DNA when cells enter the S-phase. Cells which have incorporated BrdU into their DNA can be quickly detected using a monoclonal antibody against BrdU, an

enzyme-conjugated second antibody and Chromogen, enabling detection of dividing cells/coloured cells under a light microscope. This technique was used to determine the percentage of cells synthesising DNA after mitogenic stimulation. The slides were prepared by labelling 1 x 10^6 of the cells in question (i.e. after a 48 h or 72 h incubation with the appropriate mitogen) with 500 µls of BrdU labelling medium (all reagents were provided in the kit by Boehringer Ingelheim) in a sterile 15 ml tube for 60 minutes at 37°C, 5 % CO_2, 95 % air. The cells were then washed three times by centrifugation using the washing buffer provided and 3 x 10^5 cells were re-suspended in 1 ml of PBS with 5 % albumin. One hundred microlitres of this sample was then cyto-centrifuged on a poly-L-lysine coated slide from BDH (see section 2.2.1 for centrifugation technique). The cells were fixed in a 70 % ethanol glycine buffer at pH 2.0 for 20 minutes at -20°C and then washed in buffer three times. The peripheral area of the slide was dried. The cells were then coated in a sufficient amount of anti-BrdU solution and incubated for 30 minutes in a humid atmosphere at 37°C. The slides were again washed three times in washing buffer. Bound anti-BrdU antibody was detected by incubating the cells with the anti-mouse-Ig-acid phosphatase (AP) conjugate for 30 minutes under the same conditions. To obtain the stained cells the colour-substrate was now added after three washes with buffer to remove unbound AP conjugate and left on for 30 minutes. The slides were washed for a last time in buffer and left to dry. Once dried they were mounted using Delafield's Mountant (BDH) and evaluated under a light microscope.

2.5 Analysis of Supernatants

To assess whether a range of products were being released by macrophages, after various activation regimens supernatants were collected and their contents analysed using ELISAs.

2.5.1 Collecting Supernatants

Supernatants were collected after the cells had been incubated for a given time period with the appropriate treatment. To achieve this the cells were centrifuged at 1200 rpm for 10 minutes and the supernatant was decanted into a fresh tube. The supernatants were then either used immediately or aliquoted into Eppendorf tubes (Sarstedt). The tubes were labelled and stored at -70°C, for no longer than 2 weeks.

2.5.2 Macrophage Product Profiles Using ELISAs

2.5.2.1 Cytokines

The cytokines IL-1β, IL-10, IFN-α and TNF-α were measured in the cell supernatants using murine Quantikine ELISA kits (R & D Systems). The kits were all sandwich enzyme immunoassays able to detect cytokine concentrations as low as 5.0 pg/ml. A brief description of the protocol follows. An affinity purified polyclonal antibody specific for the mouse cytokine was pre-coated on to the 96 well micrtotitre plate provided. Standards, controls and samples were pipetted into the well and any cytokine present was bound by the immobilised antibody. After washing away any of the unbound substances, an enzyme-linked polyclonal antibody was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue colour, that turned yellow when the stop solution was added to prevent any further reaction taking place. The intensity of the colour was therefore directly proportional to the amount of cytokine bound in the initial step. The optical density was read within 30 minutes at 450 nm with a correction wavelength of 540 nm on a Titertek Multiscan plate reader. The sample values were then read off their respective standard curves (see Figures 2.5-2.8).

2.5.2.2 Eicosanoids

The assays used to measure the eicosanoids PGE_2, LTB_4 and TXB_2 were based on the competition between free eicosanoid and an eicosanoid tracer linked to an acetylcholinesterase molecule for a limited number of eicosanoid-specific rabbit antiserum binding sites (Cayman Chemical Company). The concentration of eicosanoid tracer stayed constant whereas the concentration of free eicosanoid (i.e. the sample or standard) varied. Thus, the amount of eicosanoid tracer that was able to bind to the rabbit antiserum was inversely proportional to the amount of free eicosanoid in the well. This rabbit antiserum-eicosanoid (either free or tracer) complex bound to the monoclonal rabbit antibody that had been previously attached to the well in a 96 well microtitre plate by the manufacturer. The plate was washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate for acetylcholinesterase) was added to the well. The product of the enzymatic reaction had a distinct yellow colour that absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of eicosanoid tracer bound to the well, which is inversely proportional to the amount of free eicosanoid present in the well during the incubation (see Figures 2.9-2.11); or

$$\text{Absorbance} \propto [\text{Bound eicosanoid tracer}] \propto 1/[\text{ free eicosanoid}]$$

2.5.2.3 Nitric Oxide

The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-) which can be formed by the following reactions:

$$NO + O_2^- \text{---------->} ONO_2^- + H^+ \text{---------->} NO_3^- + H^+$$
$$2NO + O_2 \text{----------} N_2O_4 + H_2O \text{----------> } NO_2^- + NO_3^-$$
$$NO + NO_2 \text{ ----------> } N_2O_3 + H_2O \text{ ----------> } 2NO_2^-$$

The relative proportion of NO_2^- and NO_3^- is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both nitrate and nitrite. The assay kit (Cayman Chemical Company) used to measure total nitrate and nitrite production was an accurate and convenient two-step process (see below). The first step was the conversion of Nitrate to Nitrite utilising Nitrate Reductase. The second step was the addition of the Griess Reagents which convert nitrite into a deep purple azo compound. Photometric measurement of the Absorbance due to this Azo Chromophore accurately determined NO_2^- concentration at 540 nm, see Figure 2.12.

NITRATE REDUCTASE

$$NO_3^- \text{ ----------> } NO_2^-$$

$$NO_2^- + \text{Sulphanilamide [Griess Reagent 1]} \xrightarrow[H+]{\text{----------}} \text{Product A}$$

$$\text{Product A + N-(1-Naphthyl) ethylenediamine [Griess Reagent 2]} \text{ ----------> Azo Product (540 nm)}$$

Figure 2.5 IL-1-β Standard Curve

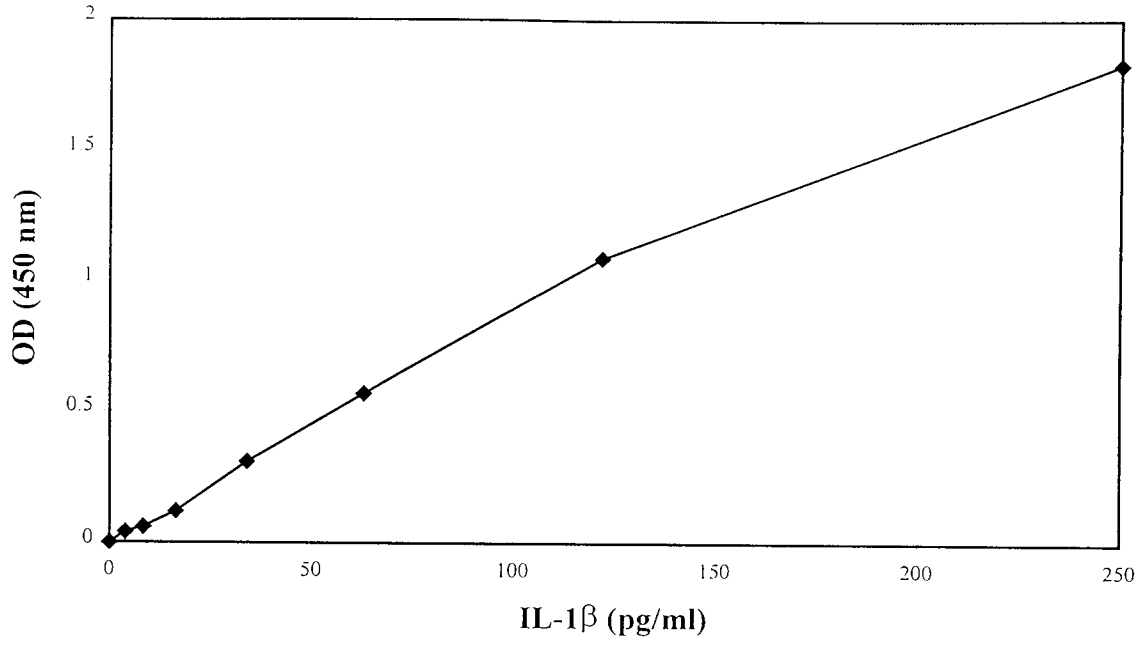
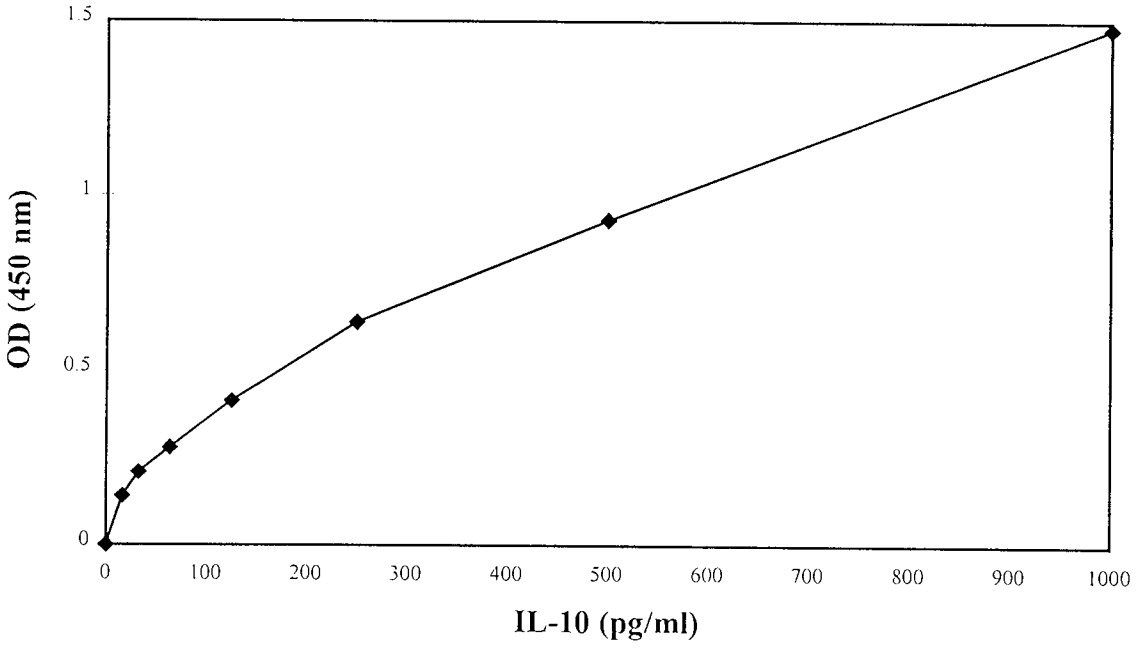


Figure 2.6 IL-10 Standard Curve



Standards were carried out in duplicate. Serial dilutions were performed between 250 pg/ml and 3.9 pg/ml for IL-1 and between 1000 pg/ml and 15.6 pg/ml for IL-10. The detection levels were 0.3 pg/ml and 4.0 pg/ml for IL-1 and IL-10 respectively.

Figure 2.7 IFN-γ Standard Curve

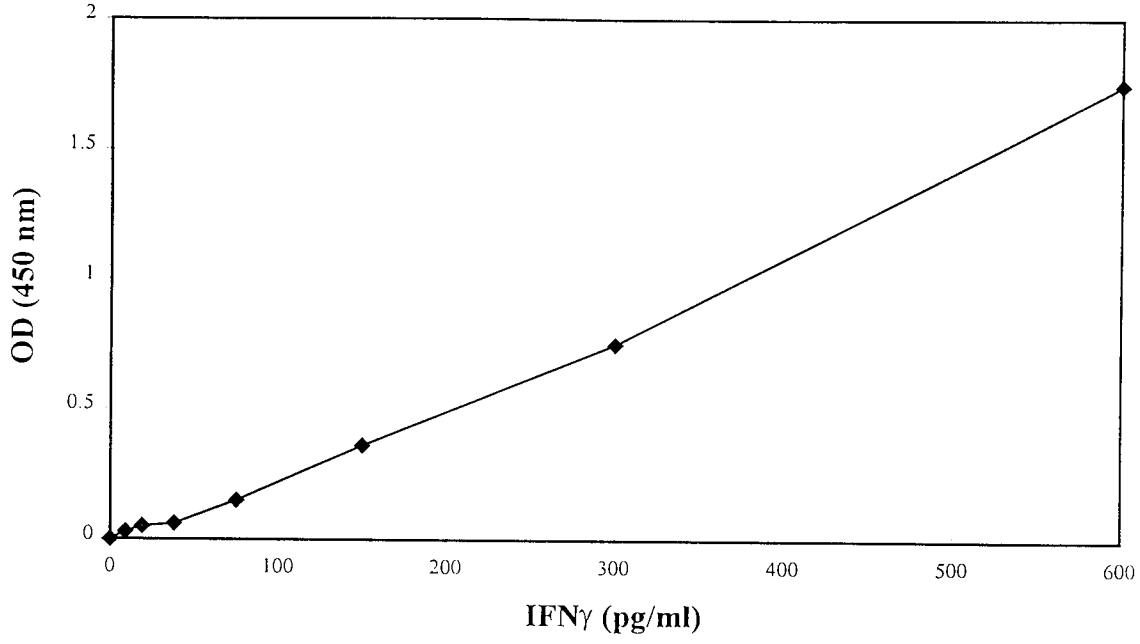
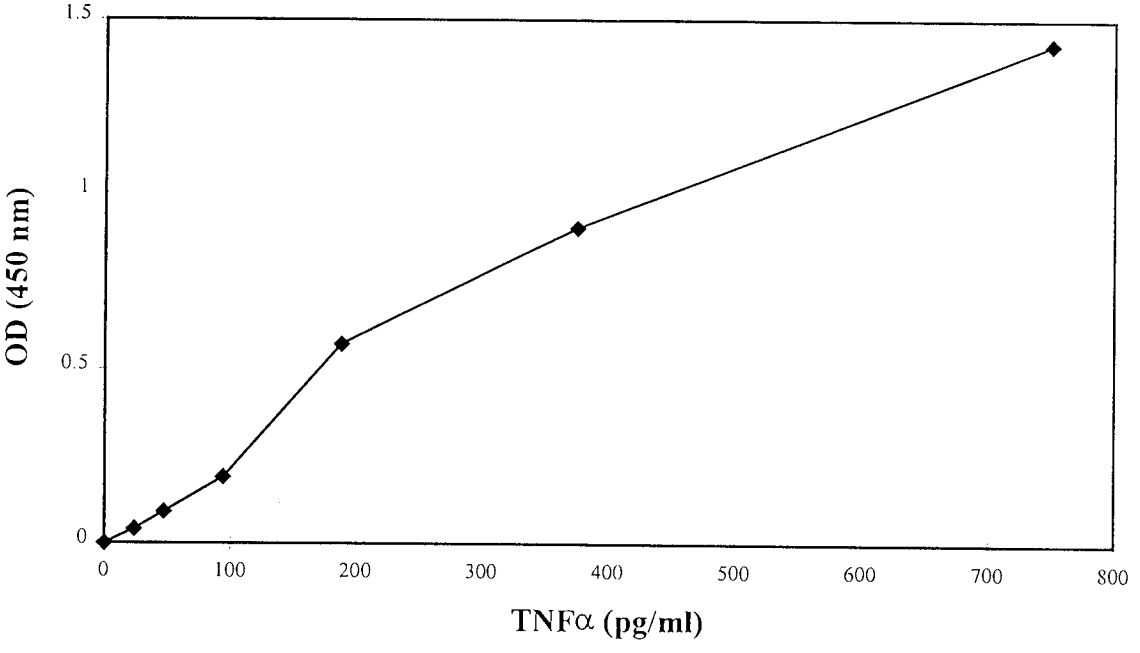


Figure 2.8 TNF-α Standard Curve



Standards were carried out in duplicate. Serial dilutions were performed between 600 pg/ml and 9.6 pg/ml with IFN-γ and between 750 pg/ml and 23.4 pg/ml with TNF-α. The detection levels were 2 pg/ml and 5.1 pg/ml for IFN-γ and TNF-α respectively.

Figure 2.9 LTB_4 Standard Curve

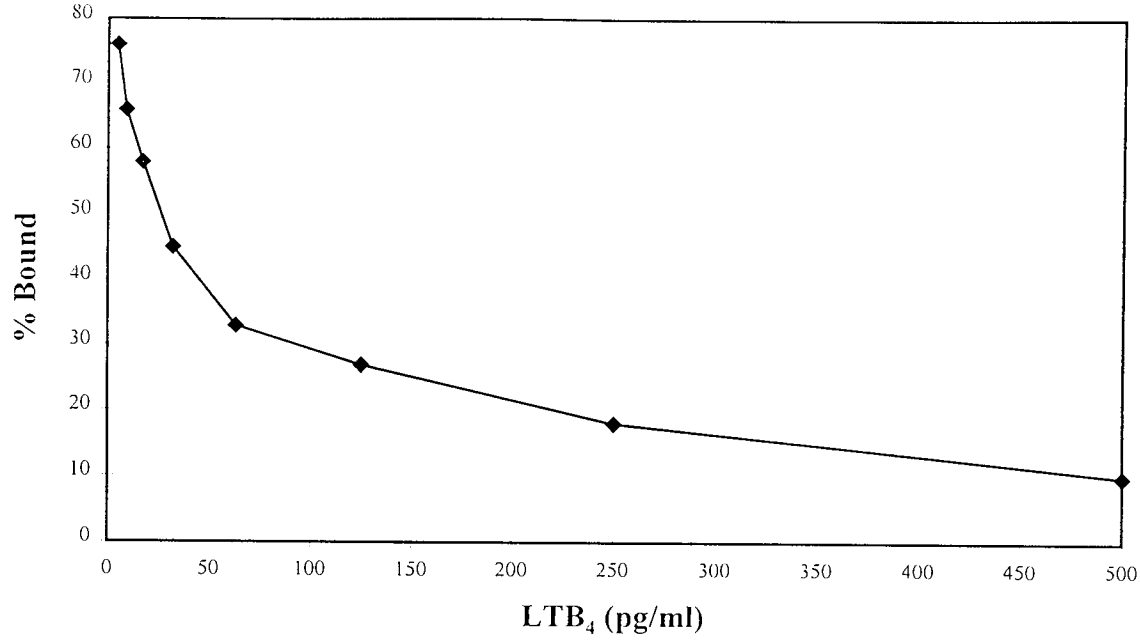
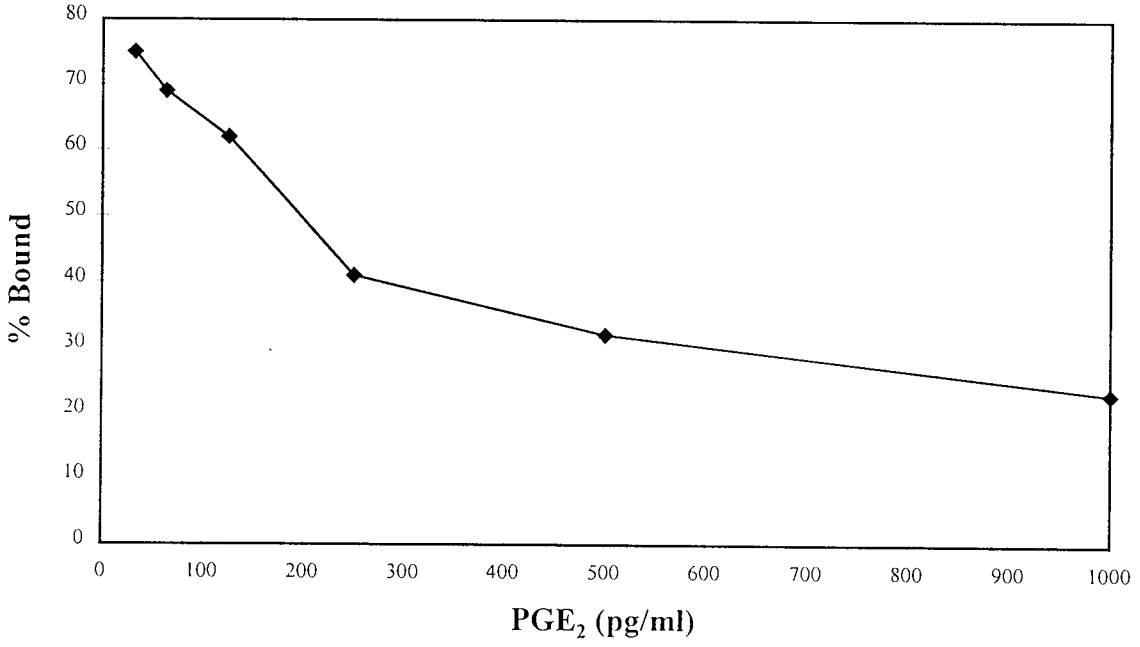


Figure 2.10 PGE_2 Standard Curve



The standards were performed in duplicate over the range of 500 pg/ml to 3.9 pg/ml and 1000 pg/ml to 7.8 pg/ml for LTB_4 (sensitivity 7 pg/ml) and PGE_2 (sensitivity 29 pg/ml) respectively.

Figure 2.11 TXB_2 Standard Curve

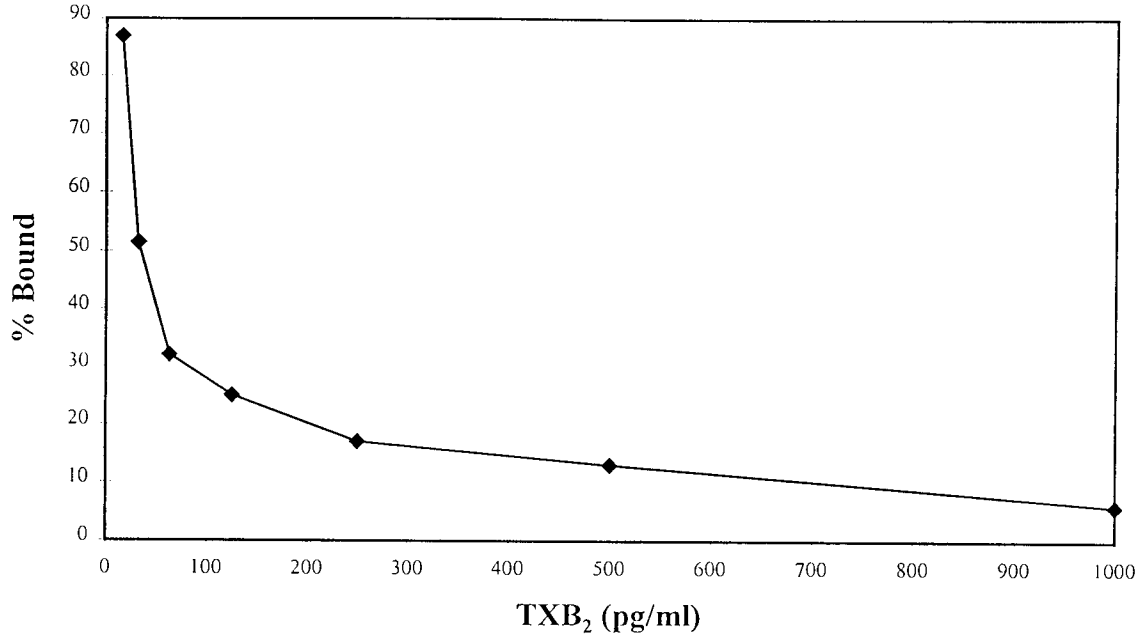
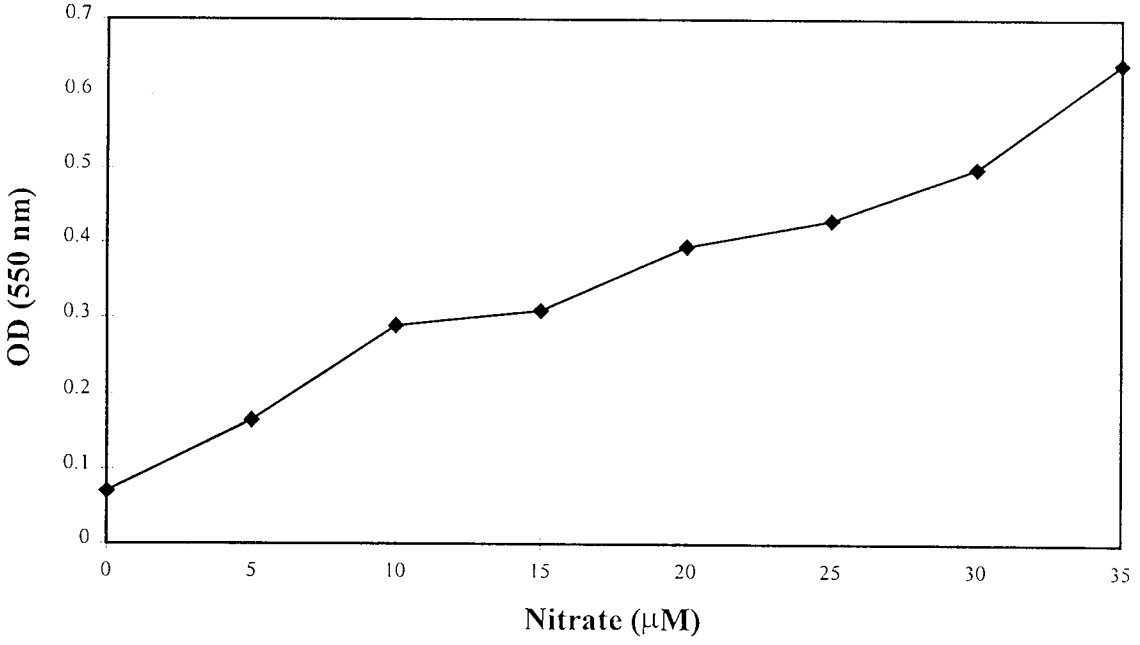


Figure 2.12 Nitric Oxide Standard Curve



Standards were performed in duplicate over the range 1000 to 7.8 pg/ml with TXB_2 and 0 to 35 µM with Nitrate. The level of sensitivity was 13.3 pg/ml and 2.5 µM for TXB_2 and Nitrate respectively.

2.6 Receptor Expression

2.6.1 FITC Conjugation to Antibody

One milligram of the purified protein (antibody) was dissolved in 300 µl of sodium bicarbonate buffer at pH 9.0. To this 30 µl of pure Fluorescein iso-thiocyanate (FITC) attached to a celite freeze dried polymer (BDH) was added to label the antibody. This took place for 18 h on a gentle rotator, in the dark at 4°C. In alkaline conditions the FITC leaves the celite in favour of the antibody in a neutrophillic reaction. To gain a concentrated protein sample it was then passed through a Sephadex G50 tube in saline. Due to the different sizes of the compounds, all of the FITC bound to antibody can be collected as the first yellow fraction to pass through the column. The fluorochrome to protein ratio was measured on a spectrophotometer at an optical density of 280 nm and 495 nm, measuring FITC and protein respectively. The formula to work out the ratio was: (2.87 x OD 495 nm) divided by [OD 280 nm - (0.35 x OD 495 nm)]. The average number of FITC molecules per protein was 4, which is an ideal amount to allow the binding of antibody and subsequent fluorescence.

2.6.2 Preparing Labelled Cells

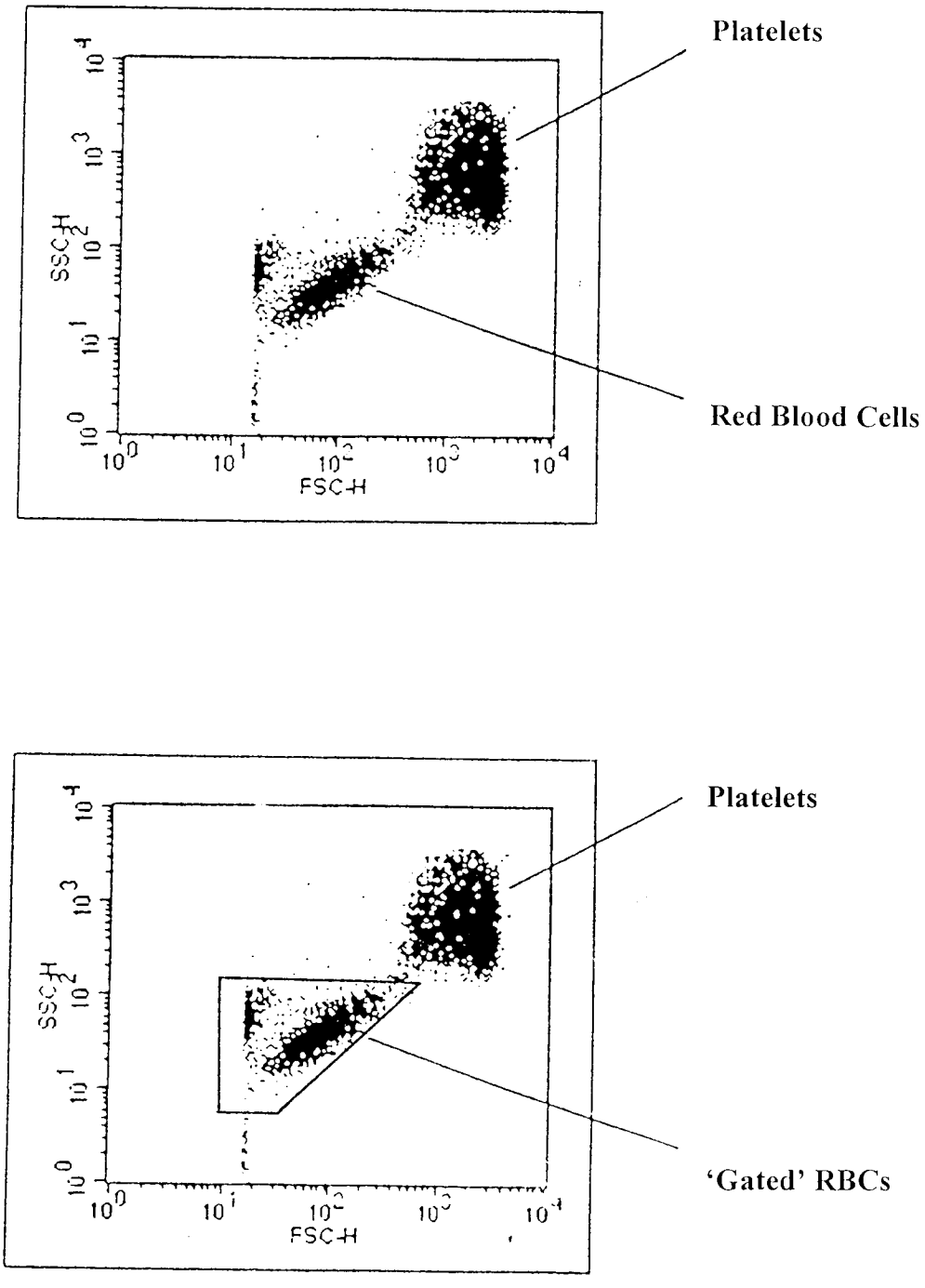
Expression of the early activation marker CD69 and the cell surface marker CD11b, expressed on monocytes were measured using a Fluorescent Activated Cell Scanner (FACScan, Becton Dickinson). This was carried out to assess both cell activation and purity. The cells were treated in the appropriate regime to investigate activation and fixed in 4 % paraformaldehyde (see section 2.1.8). They were labelled with antibodies specific for the surface receptors to be measured. These antibodies had been conjugated to Fluorescein Isothiocyanate (FITC) or R-Phycoerythrin (R-PE). To assess monocyte activation one million cells in 100 µls of PBS 'A' were incubated with 2 µls of undiluted (0.5 mg/ml) FITC anti-mouse CD11b (Vieth *et al,* 1994) and 10 µls of undiluted (0.2 mg/ml) R-Pe anti-mouse CD69 (both Pharmingen). The cells were incubated for 20 minutes at room temperature. This was sufficient time to ensure

complete labelling of the markers. The antibody in each instance was in excess. The cells were washed by centrifuging at 1200 rpm for 10 minutes and resuspending in PBS 'A'. They were then ready for FACS analysis.

2.6.3 FACScan

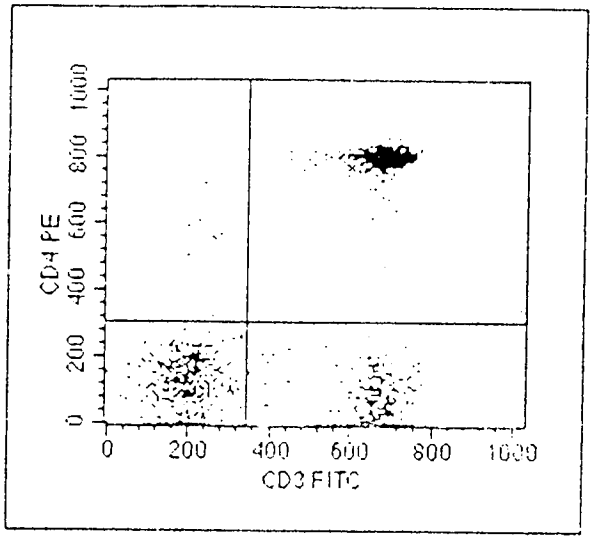
The FACScan analyses cells as they travel in a moving fluid stream past a fixed laser beam. As a cell passes in front of the laser, several measurements are made based on the physical characteristics of the cell. These characteristics, which pertain to how the cell scatters the laser light and emits fluorescence, provide us with information about the cell's size, internal complexity and relative fluorescence intensity. This information is collected and transmitted to the computer. FACScan can measure up to five parameters; forward light scatter (FSC), side light scatter (SSC) and three fluorescent parameters. In this system two fluorescent parameters were measured (FITC and R-PE), in addition to FSC and SSC. A sample of the cells was pipetted into the appropriate sample tube (Falcon) and placed in the sample injection port. The cell population to be analysed by the FACScan for fluorescence was then 'gated' to optimize the assessment (i.e. the appropriate cells were chosen according to their size and fluorescence and only these were analysed, see Figures 2.12 and 2.13). Two-way analysis was performed to determine the number of activated moncytes/macrophages, by measuring CD11b (Mac-1) against CD69.

Figure 2.13 Optimising the FACScan to Analyse the Appropriate Cell Population



FSC and SSC are measured by the FACScan as shown above. The appropriate cells can be selected or 'gated'. Thus future work will only analyse the cells which appear within the FSC and SSC range which has been chosen. This is therefore known as optimising the FACScan.

Figure 2.14 Two-way Analysis of CD3 and CD4 Expression on T-Cells



This figure clearly demonstrates three distinct T-cell populations; those which express high-levels of both CD4 and CD3, those which express low-levels of both of the surface markers and the remaining group express CD3 but very little CD4. This distinction can be easily made by labelling the cells with two different fluorescent markers, i.e. FITC and R-PE.

2.7 Preparation of Chemicals

2.7.1 Opsonised Zymosan

Zymosan A (Sigma) was opsonised by suspending 5 mg in 1 ml of Guinea pig serum (Gibco BRL) or human serum (freshly prepared) in an epindorph and incubating at 37°C for 20 minutes. This was an adequate time period for the zymosan A to be coated in complement components e.g. C3 (Guinea pig serum is often used for this due to the high levels of complement in it). Complement-coated zymosan A preparations were

then washed three times in PBS and resusupended in supplemented RPMI medium and stored at 4°C.

2.7.2 Mitogens

Stock solutions of 1 mg/ml were prepared of the six mitogens; the T-cell mitogens Concanavalin A (Gunther *et al,* 1973) and Phytohemagglutinin (PHA-P), the B-cell mitogens Lipopolysaccharide (LPS), Dextran sulphate (DS), Polyinosinic-Polycytidylic acid phosphate (Poly[I][C]) and the both T- and B-cell mitogen, Pokeweed mitogen (PWM). All were purchased from Sigma. The mitogens were obtained as lyophilised powders that were readily soluble in supplemented RPMI 1640 medium. The solutions were then aliquoted into Eppendorf tubes (200 µl per tube), labelled and stored at -20°C until required. The aliquot was only ever thawed once when a working solution was prepared. The mitogens were diluted in supplemented RPMI 1640.

2.7.3 Polymyxin B and Methyl D Mannopyranoside

Polymyxin B (PB) is a cationic peptide which at low doses inhibits LPS-induced DNA synthesis. PB (Sigma) binds with LPS and the resulting complex has a much reduced mitogenic capacity. It may still bind with B-cells but is unable to trigger them (Jacobs and Morrison, 1977). It was prepared in the same manner as the mitogens, i.e. a 1 mg/ml stock was aliquoted and stored at -20°C until required. At which time it was thawed and diluted appropriately, the remaining PB being discarded to avoid repeat freeze-thawing. The method of inhibition of Con A-stimulated DNA synthesis by methyl D Mannopyranoside (Sigma) was similar to that of PB (Ballou and Raschke, 1974). It prevents the binding of Con A to T-cell surface glycoprotein residues by binding to the Con A. It was prepared in the same manner as PB.

2.7.4 PMA and Ionomycin

The Phorbol ester, Phorbol 12-Myristate 13-Acetate (PMA) was employed to activate cells directly via PKC. Stock solutions of PMA and its inactive analogue 4α-phorbol 12,13-didecanoate (as a control) were prepared in Dimethylsulfoxide (DMSO) at a concentration of 10^{-3}M, as was the calcium ionophore Ionomycin (all Sigma). The solutions were aliquoted in to Eppendorf tubes (100 µl per Eppendorf) and stored at -20°C. Further dilutions were carried out using supplemented RPMI 1640 medium and the chemicals were only thawed once.

2.7.5 f-MLP

Macrophage activation was assessed in response to the chemotactic peptide f-MLP (Chapter 3). Stock solutions of f-MLP were prepared in DMSO at a concentration of 10^{-4} M, 100 µl samples were aliquoted into Eppendorf tubes and stored at -20°C. Further dilutions were carried out using supplemented RPMI 1640 medium when preparing working solutions.

2.7.6 Cytokines and Eicosanoids

The murine cytokines IL-1-β, IFN-γ and TNF-α were a kind gift from NIBSSC. They were obtained as a lyophilised powder which was then suspended in PBS at a concentration of 0.1 mg/ml (1 x 10^7 IU/ml). The cytokines were then aliquoted (100 µls per epindorph). Human IFN-γ (R & D systems) was reconstituted in 10 mls of Tris-buffered saline pH 7.3 containing 0.1 % bovine serum albumin (BSA) and 100 µl samples were aliquoted into Eppendorf tubes which gave a cytokine concentration of 10,000 IU per tube. The eicosanoid Thromboxane B_2 (Sigma) was reconstituted in 1 ml of PBS to yield a stock solution of 1 mg/ml. This was aliquoted into 50 µl samples. In all cases the aliquots were stored at -70°C until required and repeated freeze-thawing was avoided.

2.7.7 Mouse Anti-Thromboxane B_2 Antibody

The neutralising antibody to TXB_2 was raised in the rabbit (Sigma) and obtained as a vial of lyophilised powder. To this 5.0 ml of 0.01 M sodium phosphate buffered saline, pH 7.4, containing 0.1 % BSA and 0.1 % sodium azide was added and the antibody left to dissolve. This was separated into 50 µl aliquots and stored at -20°C. To achieve a working solution, (100 pg/ml) the aliquots were thawed and diluted 10-fold with supplemented RPMI 1640.

2.7.8 Matrix Metalloproteinase Inhibitors (MMPI)

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that degrade all of the major components of the extracellular matrix. Upon activation they are subject to local control by inhibitors called tissue inhibitors of metalloproteinases (TIMPS), which are of great therapeutic importance and much research into these inhibitors is currently underway. The MMPI used in this research was gratefully received from British Biotech Ltd. The MMPI named BB3103, was reconstituted in DMSO to give a stock solution of 10 mM, which was kept in the dark at room temperature. To obtain a working solution of 10µM, the MMPI was diluted 1000-fold with supplemented RPMI 1640 medium.

2.7.9 Cycloheximide, Dexamethasone and Hexanolamine PAF

Cycloheximide is a Glutarimide antibiotic that inhibits protein biosynthesis (Obrig *et al,* 1971). The inhibition has been shown to involve one or more steps in the reaction sequence by which amino acids are transferred from Aminoacyl transfer RNA into nascent peptides on ribosomes. A 10 mg/ml stock solution of Cycloheximide (Sigma) was prepared by dissolving 100 mg of Cycloheximide in 10 mls of DMSO. They were aliquoted into 500 µl samples and stored at -20°C until required. At which time they were diluted with supplemented RPMI 1640 to give a working solution of 10 µg/ml.

The glucocorticoid Dexamethasone is a potent inhibitor of Arachidonate metabolism (Manz *et al,* 1983; Shappiro *et al,* 1991). Water-soluble Dexamethasone was obtained from Sigma. It was dissolved in un-supplemented medium to yield a 10 mM solution, aliquoted (500 µl per epindorph) and stored at -20°C until required. A working solution of 5 µM was obtained by further diluting with supplemented RPMI 1640.

Hexanolamine PAF C16 is a platelet activating factor (PAF) inhibitor (Grigoriadis and Stewart *et al,* 1991). It is an analogue of PAF that inhibits phospholipid turnover and secretion. It was received from Calbiochem NovaBiochem as a lyophilised solid and dissolved in ethanol at a concentration of 1 mg/ml. This was separated into 100 µl aliquots and stored at -20°C. When required it was diluted in supplemented RPMI 1640 to 1 µM.

2.8 Statistical Analysis

Data was analysed using the Students Paired T-test or ANOVA with groups larger than two. All data was expressed as mean +/- standard error of the mean (SEM). Statistical significance was considered if p values were less than 0.05. Statistical evaluation was only performed if sample groups exceeded n = 3.

3. MACROPHAGE ACTIVATION AND HETEROGENEITY

3.1 Introduction

The macrophage carries out five main functions within the immune system, these are phagocytosis, chemotaxis, the processing and presentation of antigen, secretion of products and tumour cell control. Demonstration of one of these features is thus an indication of a state of activation. In this chapter phagocytic activity was measured as an index of activation.

Phagocytosis may result in an oxidative burst during which reactive oxygen intermediates are produced to enable the killing of ingested pathogens. Macrophages produce superoxide anion (O_2-) in response to both particulate and soluble antigen (Drath and Karnovsky, 1975). Superoxide then enters into reactions culminating in the generation of bactericidal products. Reactive oxygen intermediates can be generated by both myeloperoxidase-dependent and -independent mechanisms (Curnette and Baboir, 1987). Macrophages as opposed to PMNs and monocytes, have very low levels of myeloperoxidase (MPO) and therefore rely upon myeloperoxidase-independent mechanisms. In this system, highly reactive oxygen intermediates appear to be derived from the interaction of superoxide and hydrogen peroxide (H_2O_2). The principal toxic reactants formed are hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen (O_2-) (Shepherd, 1986). $OH^{\cdot}$ is formed with the aid of an iron catalyst as follows:

$$O_2\text{-} + O_2\text{-} + 2H+ \rightarrow H_2O_2 + O_2$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH\text{-}$$
$$Fe^{3+} + O_2\text{-} \rightarrow Fe^{2+} + O_2$$
$$O_2\text{-} + H_2O_2 \rightarrow OH^{\cdot} + OH\text{-} + O_2$$

Myeloperoxidase-dependent mechanisms are employed by both monocytes and PMNs. In this system, superoxide anion dismutates to form hydrogen peroxide. Myeloperoxidase

then catalyses the oxidation of chloride to hypochlorite anion (OCl-), from which singlet oxygen appears to be derived.

There are numerous methods described to measure phagocytic activity ranging from; quantitative analysis of heat killed *Candida albicans* or polystyrene beads using a spectrophotometer (Roberts *et al,* 1963), analysis of Zymosan A ingestion using oil immersion microscopy, or quantification of phagocytic and bactericidal activity of polymorphonuclear cells (PMN) using a differential centrifugation technique (Mandell *et al,* 1969). The respiratory burst, central to the phagocytic activity of the macrophage can also be measured. For example, H_2O_2 detection using phenol red (Lee *et al,* 1980), or monitoring superoxide production with Ferricytochrome c (Pick and Mizel, 1981; Secombes *et al,* 1988). Hydrogen peroxide production can be detected by Lucigenin-enhanced chemiluminescence (Clement *et al,* 1983). This method was found to be the most appropriate for the measurement of monocyte/macrophage activation as it is very sensitive and allows the kinetics of monocyte/macrophage activation to be monitored over a continuous time course, both before and after the introduction of pharmacological or mitogenic reagents. Hydrogen peroxide is produced in both MPO-dependent and -independent mechanisms, hence the activation of macrophages and monocytes can be measured chemiluminometrically.

An important point to note when deciding which macrophage populations were to be studied, was that resident macrophages are widely distributed in the absence of any inflammatory signal and display regional heterogeneity. Functional, morphological and phenotypic heterogeneity may reflect the local environment of the macrophage and the involvement in various physiological or pathological processes. For this reason cell lines were chosen as they are a homogenous population of cells. The human myelomonocytic cell line U937 and the mouse macrophage line RAW 264.7 were used to investigate macrophage activation. However, due to the transformed nature of these cells it was important to make sure they behaved in the same manner as primary cell cultures, thus

peritoneal macrophage were also evaluated. The resident macrophages of the gut were selected as they are highly responsive and, therefore, possible methods of macrophage activation can be easily investigated and this information then used on other macrophage populations.

The primary objective of this chapter is to determine the best regimen for activating macrophages, using the cell lines U937 and RAW 264.7 and murine peritoneal macrophages. These findings can then be applied to investigate the importance of (activated) macrophages in lymphocyte proliferation. The secondary objective is to compare the response of different macrophage populations to this regimen.

3.2 Experimental Design

U937 cells and RAW 264.7 macrophages were cultured as described previously (Chapter 2). Growth curves were produced and showed a doubling time of approximately 24 hours and 22 hours respectively. Due to the immature nature of U937 cells, they required priming to become fully differentiated macrophages. IFN-γ was chosen as the priming agent as it is a potent macrophage activation factor (Nathan *et al,* 1984). The concentration of IFN-γ required to prime U937 cells was analysed. Between 100-1000 IU/ml IFN-γ appeared to prime the promonocytes equally well, this agrees with studies of Finbloom in 1991. The amount chosen routinely was 330 IU/ml as this was well within this limit and cost-effective. Although IFN-γ is known to have an anti-proliferative effect on cells (Herrmann *et al,* 1985), at this concentration the growth kinetics of U937 cells were not significantly affected and the cells were primed most effectively after 72 hours. It must therefore take 72 hours to up-regulate cellular machinery involved in the oxidative burst and *de novo* synthesis of proteins is required in U937 cells. Priming was not essential for the achievement of an oxidative burst in RAW 264.7 macrophages as they are partially differentiated. Peritoneal macrophages are fully differentiated resident macrophages they therefore do not require priming.

The effect of cell number on the oxidative burst was investigated using the chemiluminometric assay described in chapter 2. The cells were activated with opsonized Zymosan A (OpZ) and the oxidative burst was found to be directly proportional to the number of cells in the assay over the range of 0.5×10^6 to 1×10^7 cells per. Therefore, cell number was accurately determined and the same concentration of cells (1×10^6 per cuvette) was used in all experiments.

Dose response curves were utilised to establish the optimal dose of agents to be used in subsequent studies on macrophage activation. In every case, the concentrations found to elicit the optimum oxidative burst were the same for all three cell types. The activating

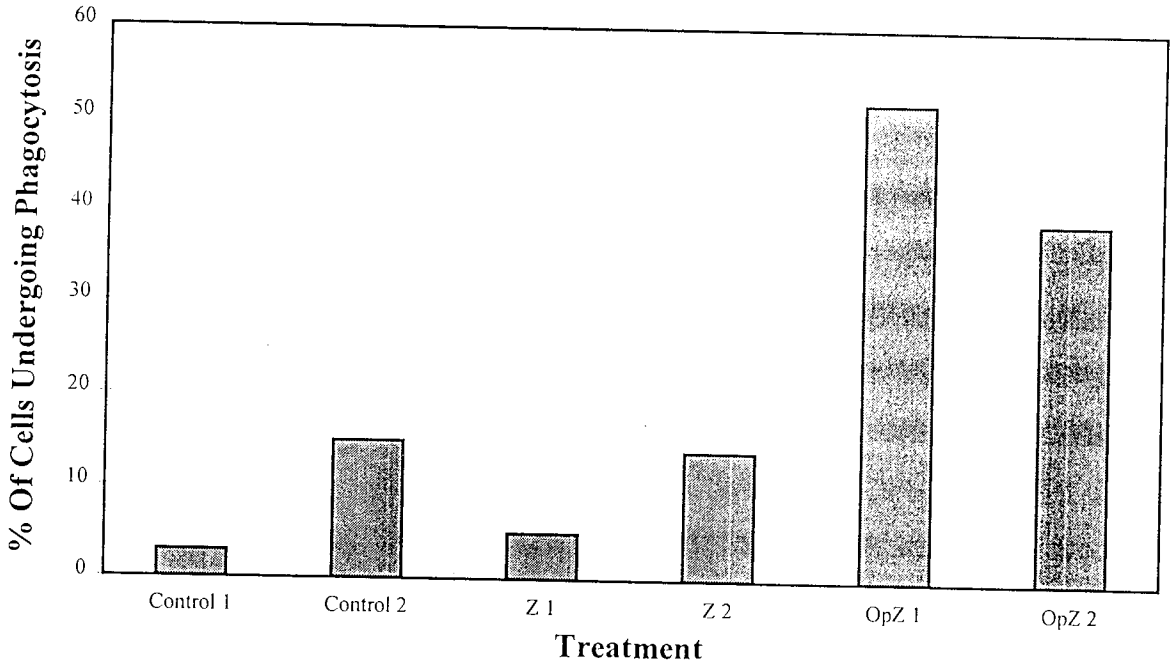
agents examined were OpZ, f-MLP, Concanavalin A (Con A), Lipopolysaccharide (LPS), Pokeweed Mitogen (PWM), PMA and Ionomycin, preparation of which was also described in chapter 2. The doses employed are specified in the figure legends.

3.3 Results

3.3.1 Microscopic Detection of Phagosomes

Control U937 cells primed with IFN-γ exhibited low spontaneous phagocytic activity of approximately 10% (Figure 3.1), but it should be noted that different individuals counting phagosomes produced counts differing by up to 4% in control slides. The presence of Zymosan A did not elicit additional phagocytic activity. However OpZ increased the percentage of cells bearing phagosomes to ~ 45 % but individual counts varied by as much as 15 % (these results were only taken from two experiments). Clearly only gross changes can be detected by this method and more subtle changes of 10-15% would be impossible to assess with any certainty.

Figure 3.1 Effect of Zymosan A on Phagocytosis in U937 Cells



U937 Cells were primed for 72 h with 330 IU/ml IFN-γ. Slides were prepared from these various treatments (control, Zymosan A (Z) and OpZ (1 mg/ml)). Phagosomes were counted under a light microscope using oil immersion. Two independent observers counted at least 600 cells on one preparation the percentage of cells with phagosomes is shown.

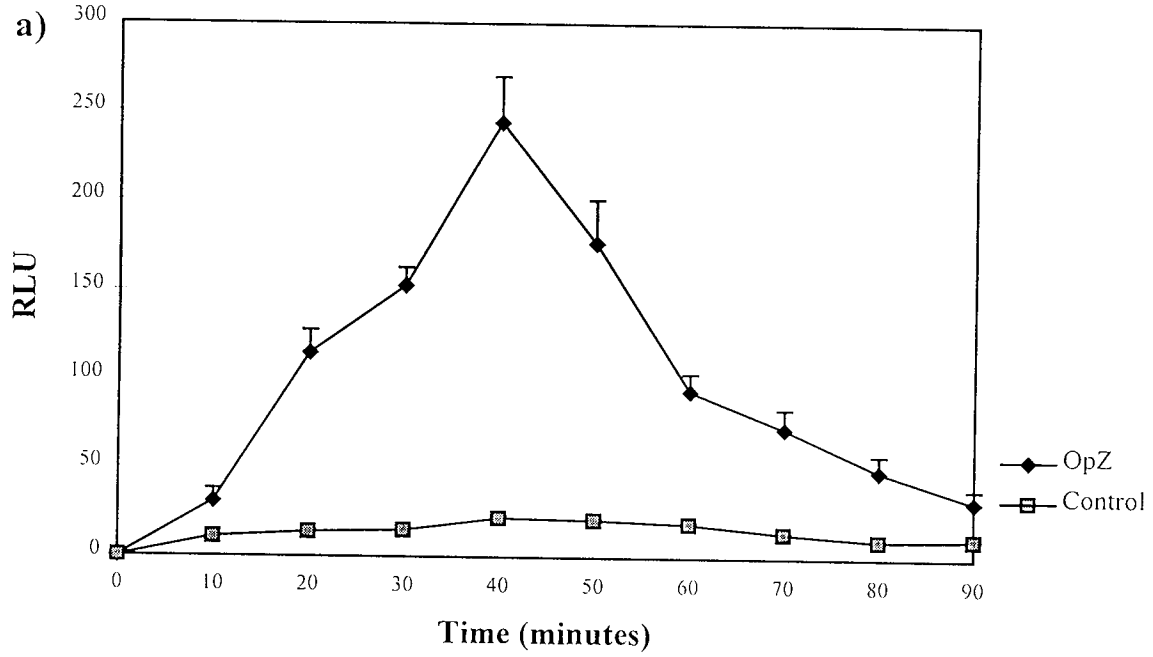
3.3.2 Measurement of Oxidative Burst in U937 Cells, RAW 264.7 and Peritoneal Macrophages

When IFN-γ primed U937 cells were incubated with lucigenin and OpZ at 1 mg/ml an oxidative burst was produced (Figure 3.2a). The burst peaks after approximately 40 minutes and returns to control levels after 100 minutes. RAW 264.7 macrophages displayed a similar response to OpZ over 100 minutes, however the maximal burst was significantly smaller than that elicited by U937 cells (Figure 3.2b). The response of peritoneal macrophages to OpZ was at least twenty times greater than the burst elicited by U937 cells and RAW cells (Figure 3.2c) and the peak response was achieved more rapidly.

Zymosan A opsonized with human serum elicited a burst in U937 cells ten fold larger than that produced with Guinea pig serum and the burst was slightly larger when fresh serum was used. This shows that there is some species-specificity of the C3b receptor and in our hands it was the most powerful activator of the respiratory burst. However attempts at opsonisation with antibody (engaging Fc receptors) were not successful but theoretically this response maybe larger. This was attempted with RAW cells and peritoneal macrophages using sheep red blood cells (SRBC) coated with mouse anti-serum to SRBCs. The SRBCs were therefore coated with antibodies and should display Fc regions. The Fc receptors should have cross-linked to induce an oxidative burst, but a burst did not occur (data not shown). Other methods of macrophage activation were therefore investigated.

The chemotactic peptide f-MLP activated U937 cells (Figure 3.3), RAW 264.7 and peritoneal macrophages (data not shown) for a very brief period of between 25 and 100 seconds. However, in all cases this response was less than one fifth of that produced with OpZ. The same pattern of responsiveness was found for all three cell populations.

Figure 3.2 Effect of Opsonised Zymosan A on Oxidative Burst (n=12)



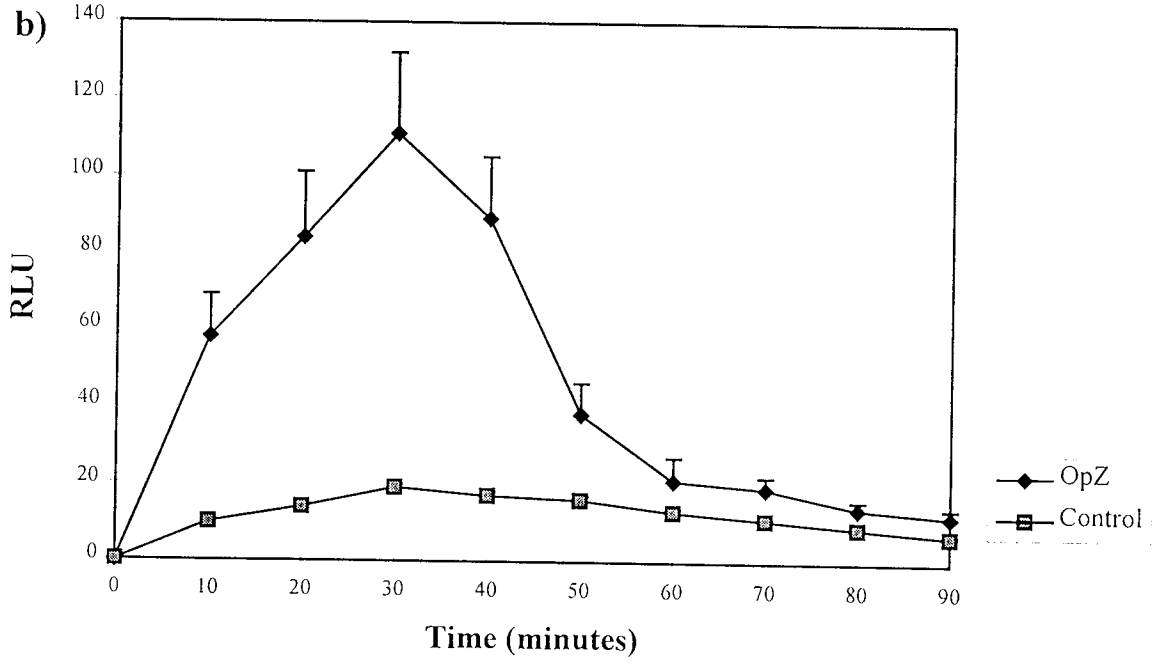
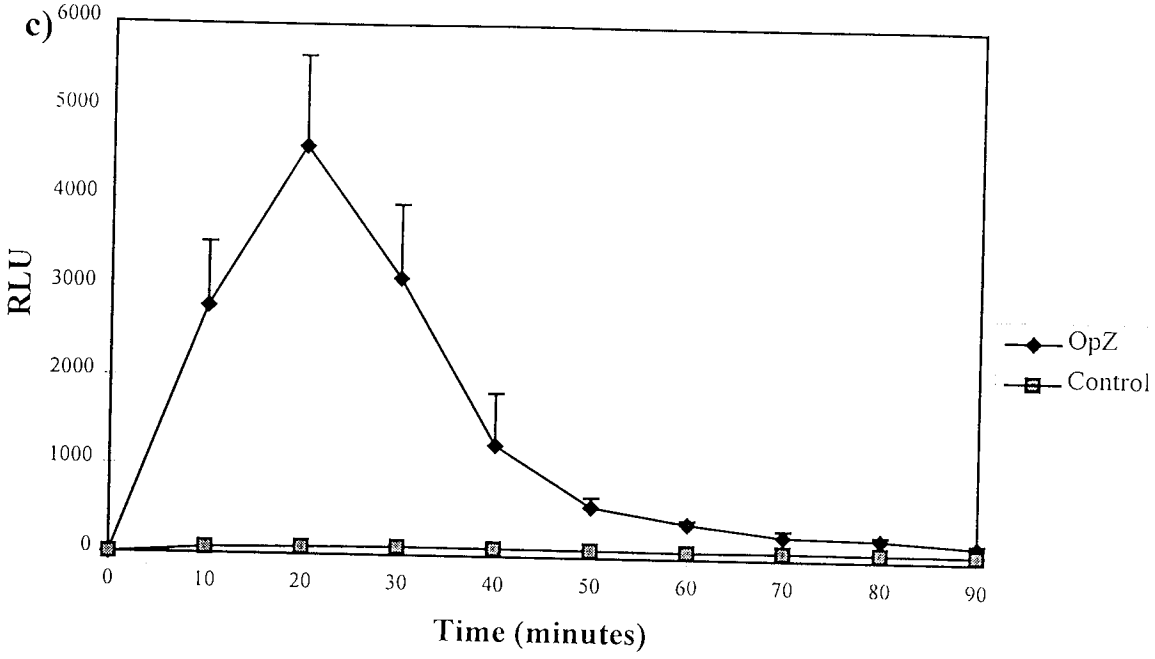
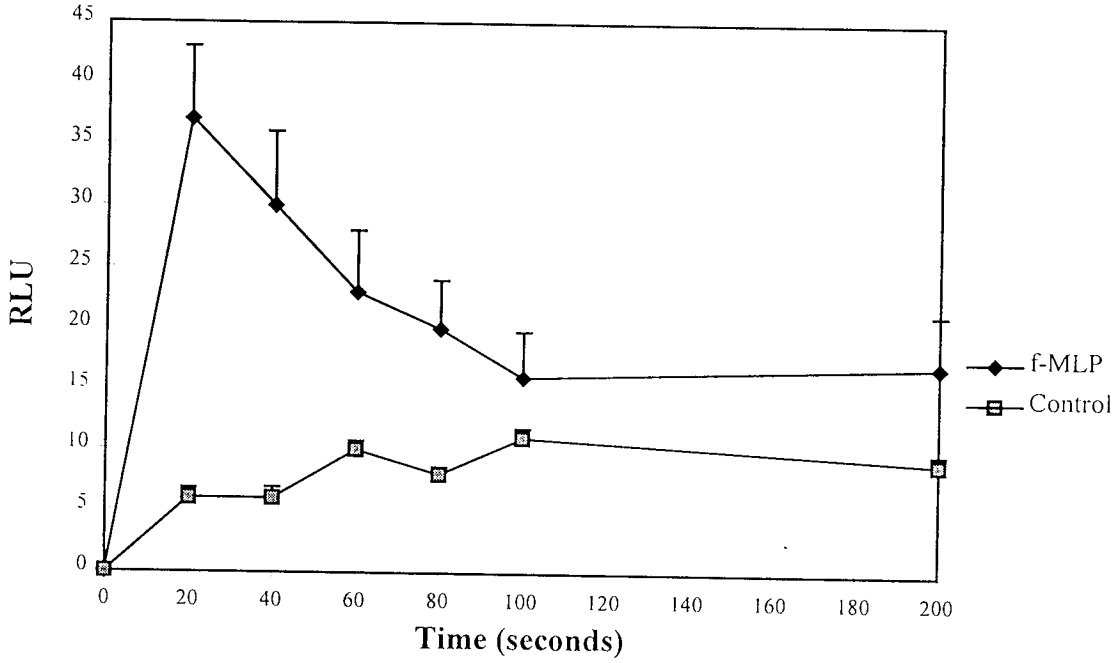


Figure 3.2 Effect of Opsonised Zymosan A on Oxidative Burst (n=12)



1 x 10^6 primed U937 cells (a), RAW 264.7 cells (b) and peritoneal macrophages (c) were incubated with 1 mg/ml of opsonised Zymosan A (OpZ) for 90 minutes. The oxidative burst produced was measured on a luminometer every 10 minutes during this period.

Figure 3.3 Effect of f-MLP on the Oxidative Burst in U937 Cells (n=6)



1 x 10^6 primed U937 cells were incubated with the chemotactic peptide f-MLP (10^{-5}M) for 200 seconds. A small amount of short-lived burst activity was measured luminometrically.

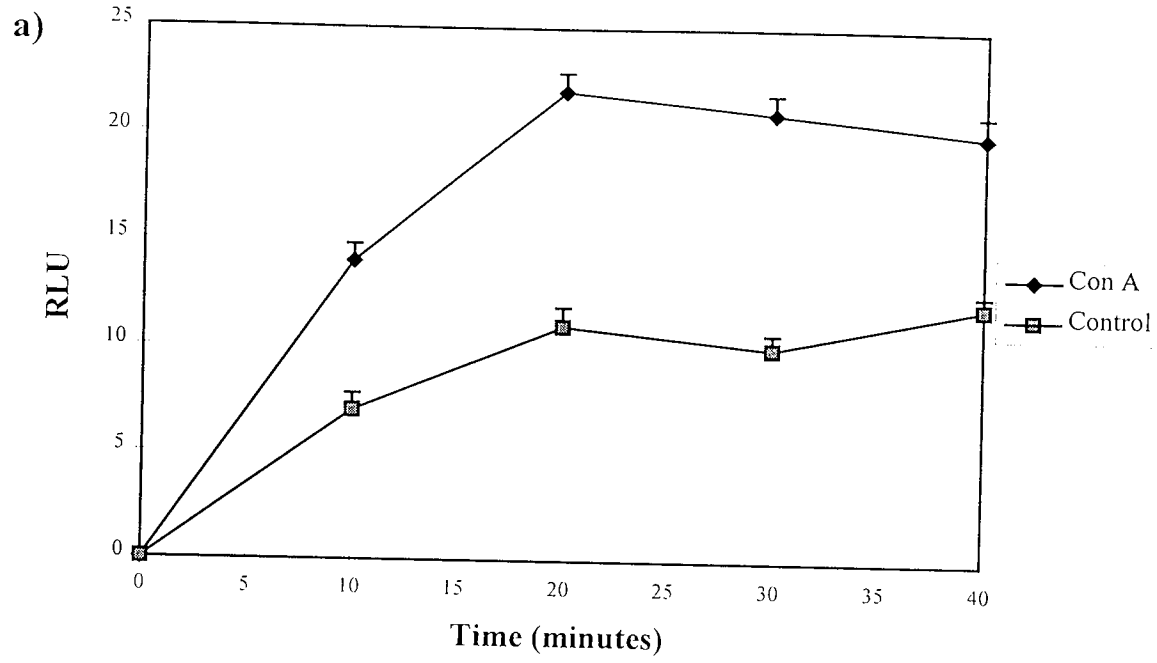
3.3.2 Measurement of Oxidative Burst in U937 Cells, RAW 264.7 and Peritoneal Macrophages

The mitogens Con A, LPS and PWM did not produce a burst of any significance compared to OpZ in U937 cells, RAW 264.7 cells and peritoneal macrophages (Figure 3.4-3.6). The cells were also incubated with the mitogens and latex beads (data not shown), to identify whether they were activated but required phagocytic material to facilitate a substantial burst. However, this did not affect the burst produced and was therefore not essential. Although these mitogens were unable to stimulate a respiratory burst they may have been having other effects on the cells.

PMA the PKC activator did stimulate U937 cells and RAW 264.7 cells to elicit a burst which was approximately half the size of that with OpZ (Figures 3.7a and 3.7b respectively). DMSO, the PMA vehicle had no effect. When both cell types were incubated with the calcium ionophore Ionomycin and PMA they produced a burst of similar magnitude to that of PMA alone (Figures 3.8a and 3.8b), the influx of calcium does not appear to increase burst activity further. PMA partially inhibited the spontaneous burst activity of peritoneal macrophages seen in control cultures (Figure 3.7c). This spontaneous burst activity was in fact larger than the maximum response of both U937 cells and RAW 264.7 cells to OpZ. Clearly there is a great difference between primary cultures of cells and transformed cells. The high levels of burst activity in control cultures of peritoneal macrophages may be due to them being partially activated. This could be attributed to the cell extraction process or the presence of flora or pathogens within the gut. Whilst PMA alone inhibited the oxidative burst the addition of Ionomycin led to a large increase in burst activity, Figure 3.8c. Clearly the combined effect of PKC activation plus an influx of calcium gives the greatest activation stimulus for a respiratory burst in peritoneal macrophages. The morphological features of U937 cells treated with OpZ and PMA are displayed in Photo 3.1. Opsonised Zymosan A is readily phagocytosed by these cells whereas PMA has stimulated vacuolarisation within the cytoplasm of the U937 monocytes.

To establish whether the activators OpZ, LPS and PMA utilised different second messenger systems within the macrophage they were incubated in combination (Figure 3.9a-c). All combinations except LPS with PMA did not enhance the oxidative burst produced by RAW 264.7 cells and peritoneal macrophages any more than the chemicals given individually and, PMA inhibited by 9 % the oxidative burst produced by peritoneal macrophages after treatment with OpZ. In addition the treatment of combinations of OpZ, LPS and PMA in U937 cells resulted in increased burst activity. This could either be due to the involvement of different signals or enhancement of the same signal.

Figure 3.4 Effect of Con A on the Respiratory Burst (n=6)



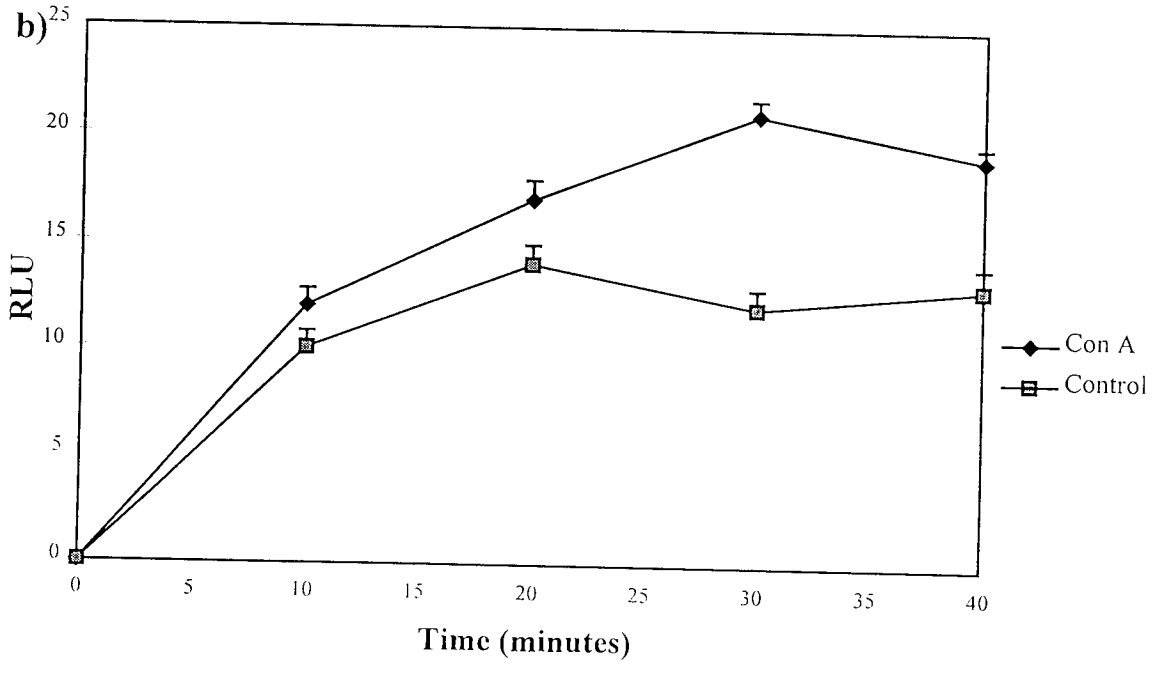
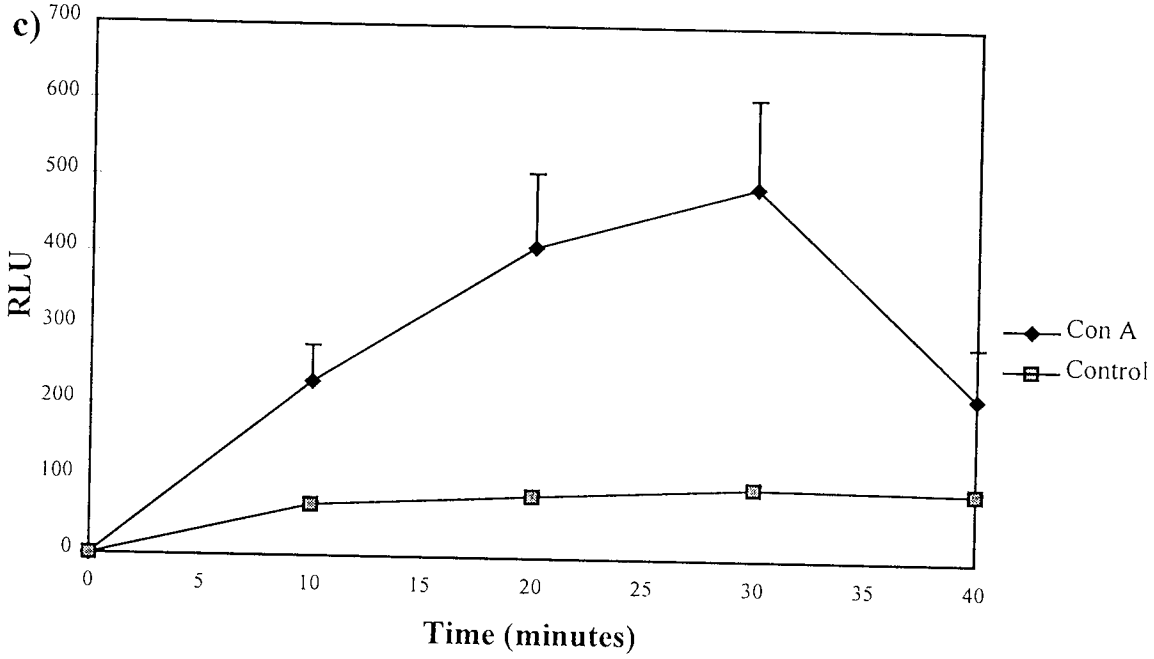


Figure 3.4 Effect of Con A on the Respiratory Burst (n=6)



1 x 10^6 primed U937 cells (a), RAW 264.7 cells (b) and peritoneal macrophages (c) were incubated with 1 μg/ml Concanavalin A (Con A) for 40 minutes. The oxidative burst produced was measured on a luminometer every 10 minutes during this period.

Figure 3.5 Effect of LPS on the Production of ROIs (n=6)

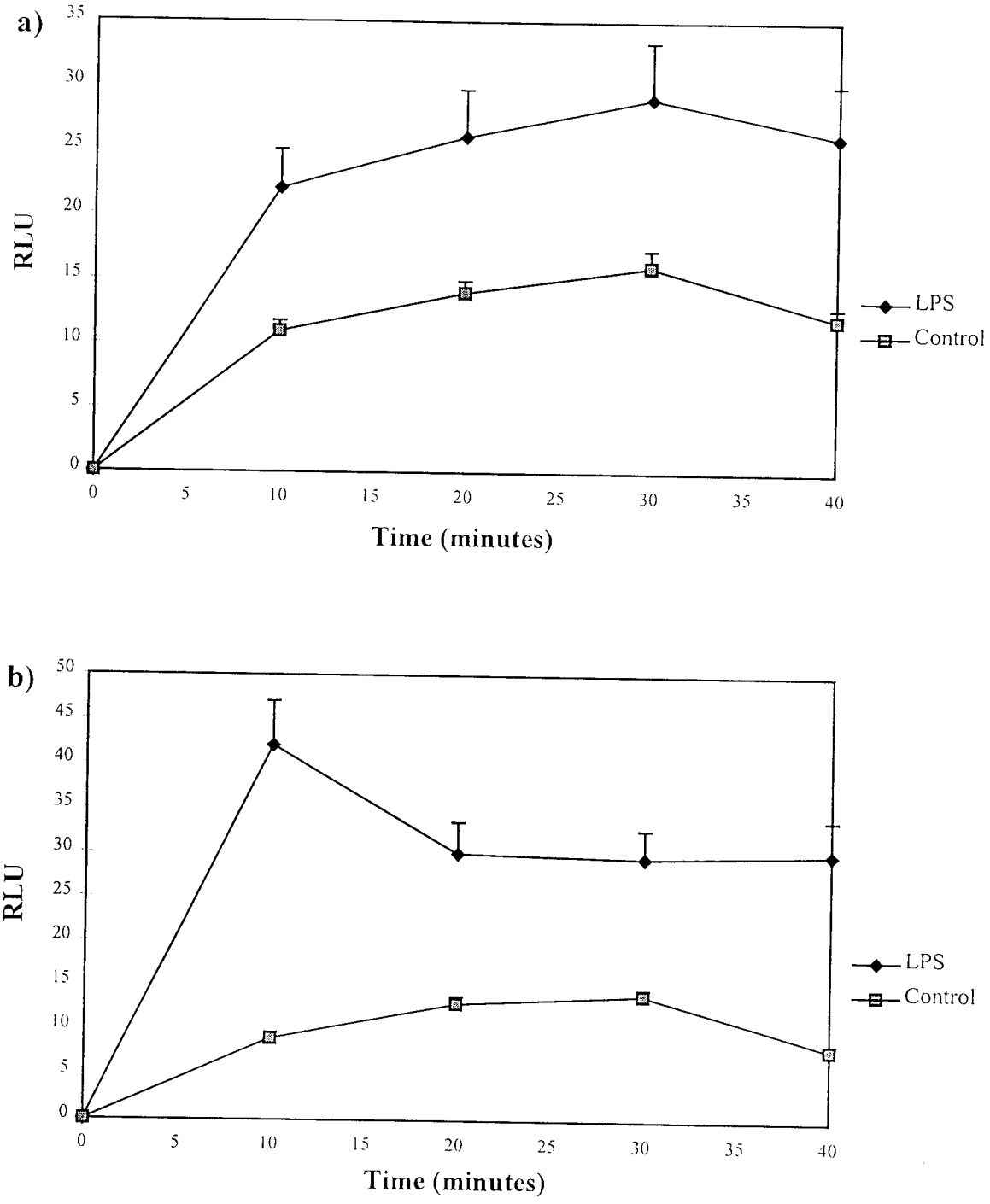
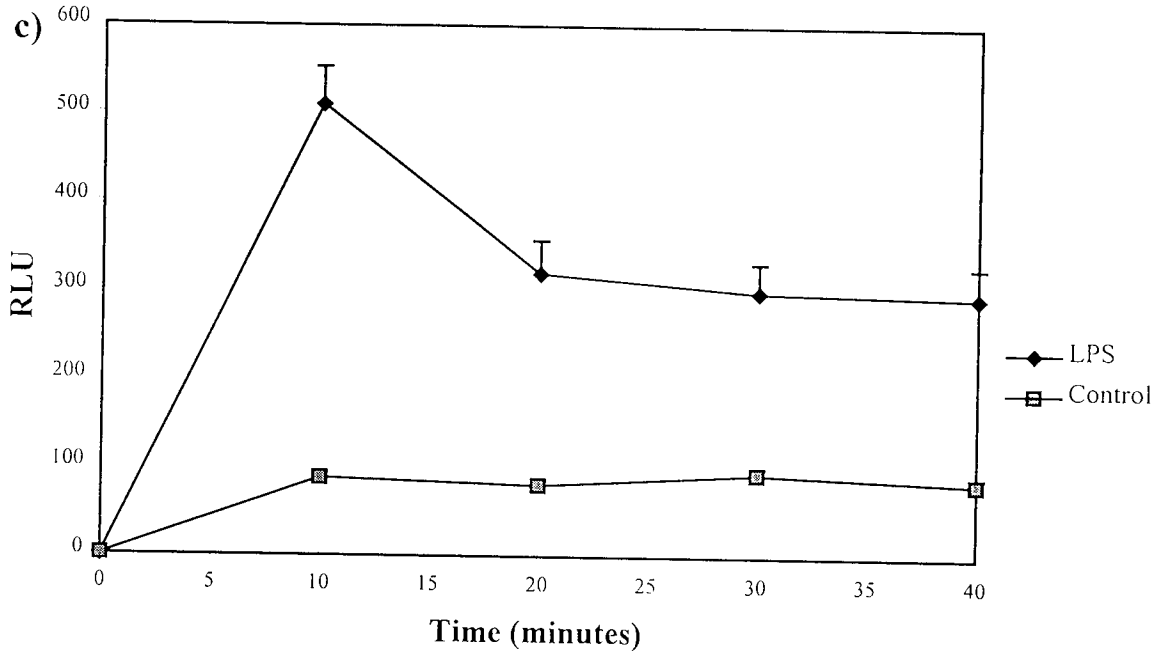


Figure 3.5 Effect of LPS on the Production of ROIs (n=6)



1 x 10^6 primed U937 cells (a), RAW 264.7 cells (b) and peritoneal macrophages (c) were incubated with 1 μg/ml of Lipopolysaccharide from E. coli serotype 055:B55 (LPS) for 40 minutes. The oxidative burst produced was measured on a luminometer every 10 minutes during this period.

Figure 3.6 Effect of PWM on the Oxidative Burst (n=6)

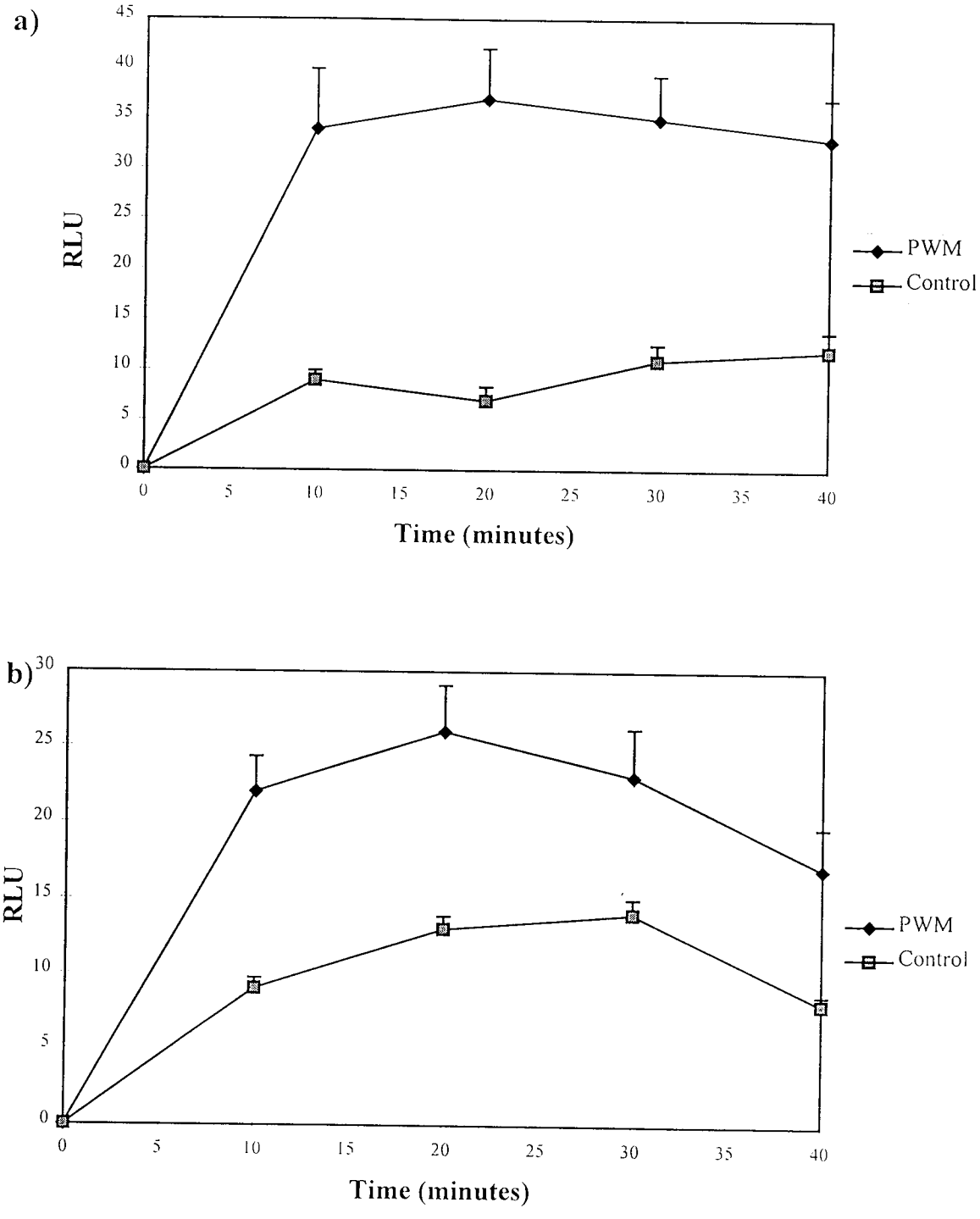
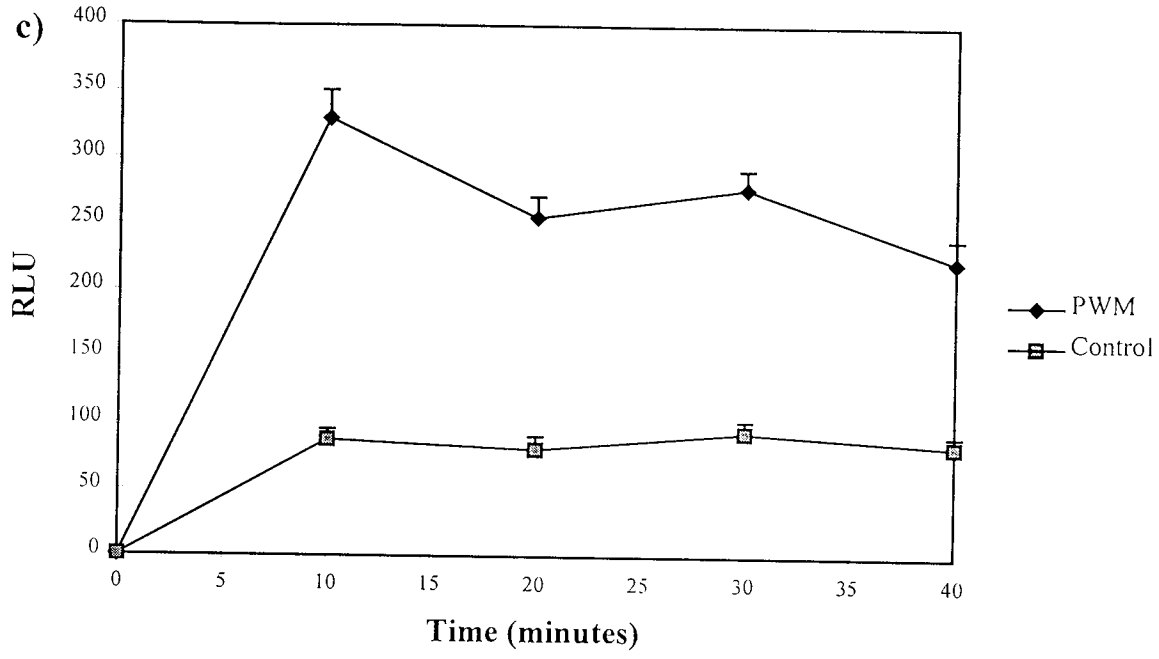


Figure 3.6 Effect of PWM on the Oxidative Burst (n=6)



1 x 10^6 primed U937 cells (a), RAW 264.7 cells (b) and peritoneal macrophages (c) were incubated with 1 μg/ml of Pokeweed Mitogen (PWM) for 40 minutes. The oxidative burst produced was measured on a luminometer every 10 minutes during this period.

Figure 3.7 Effect of PMA on the Respiratory Burst (n=6)

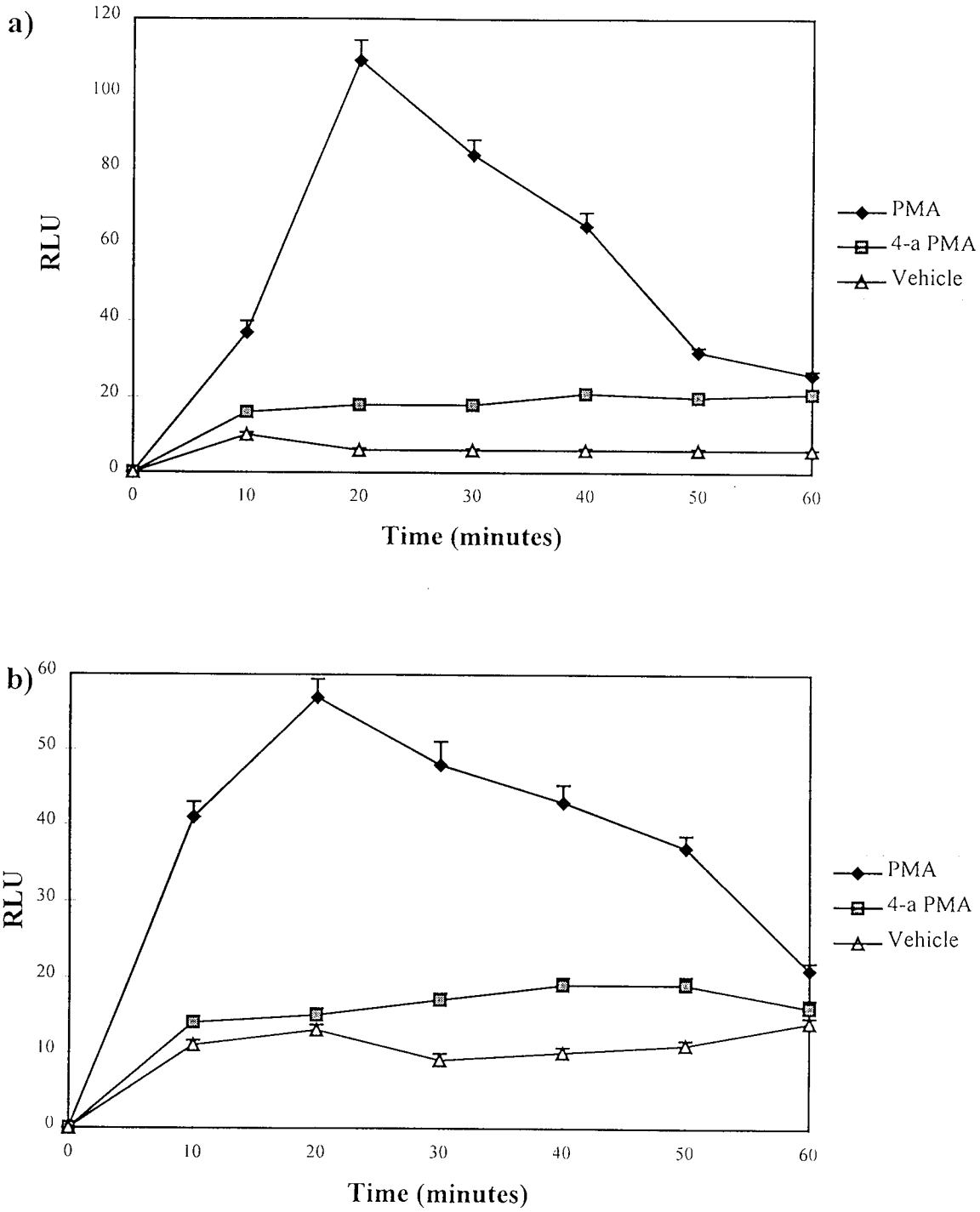
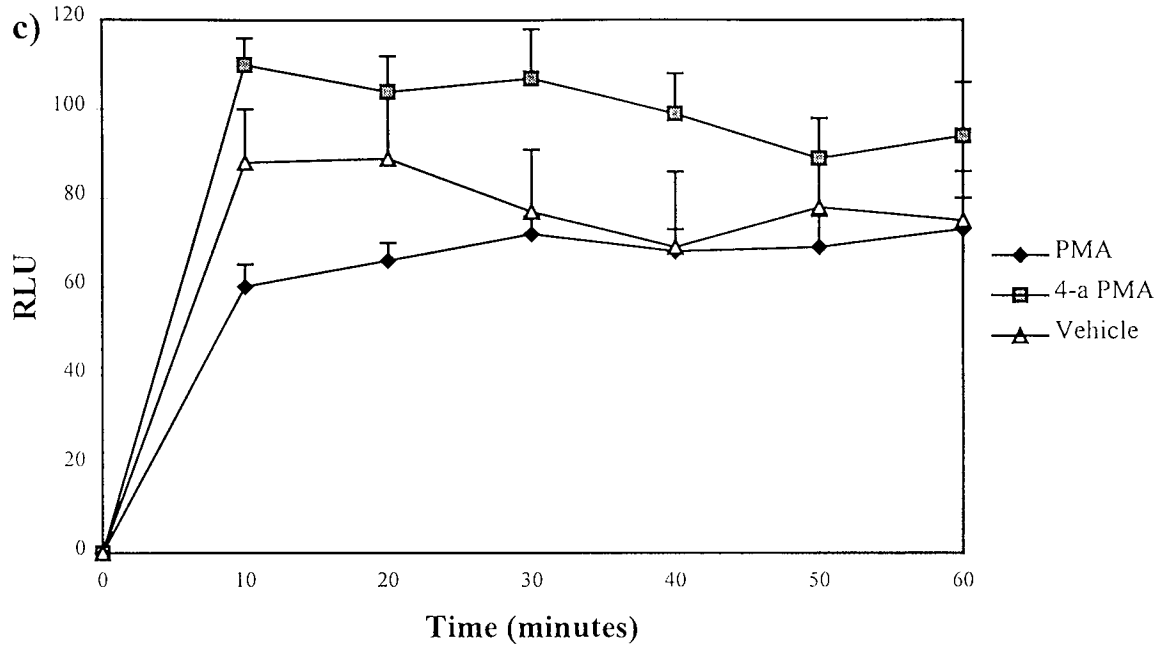


Figure 3.7 Effect of PMA on the Respiratory Burst (n=6)



1 x 10^6 primed U937 cells (a), RAW 264.7 cells (b) and peritoneal macrophages (c) were incubated with the PKC activator Phorbol 12-myristate 13-acetate (PMA), 4-α-PMA (the in-active form of PMA), both at 10^{-8}M, or the PMA vehicle Dimethylsulfoxide (DMSO) for 60 minutes. The oxidative burst produced was measured on a luminometer every 10 minutes during this period.

Figure 3.8 The Effect of PMA and Ionomycin on the Production of ROIs (n=4)

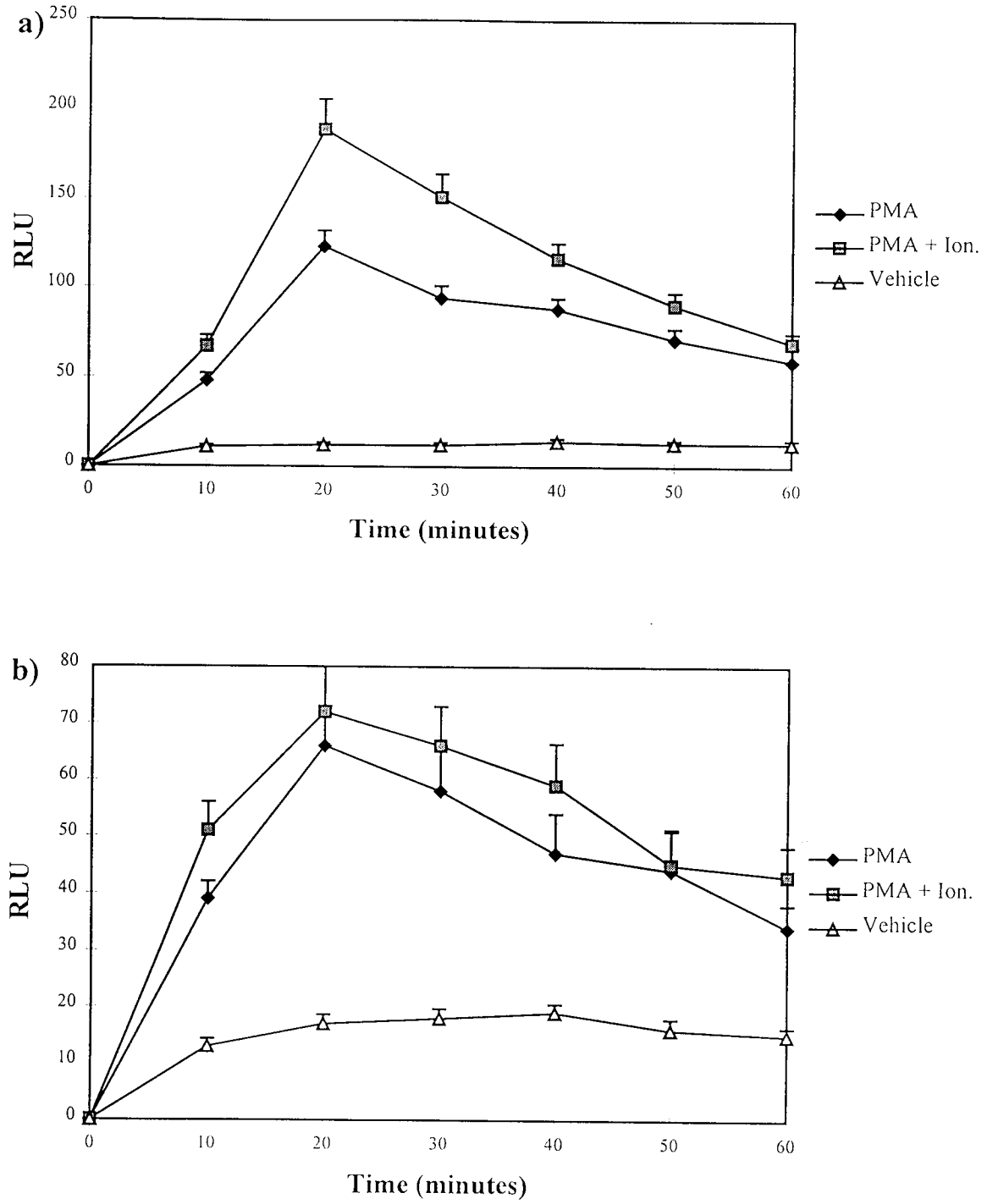
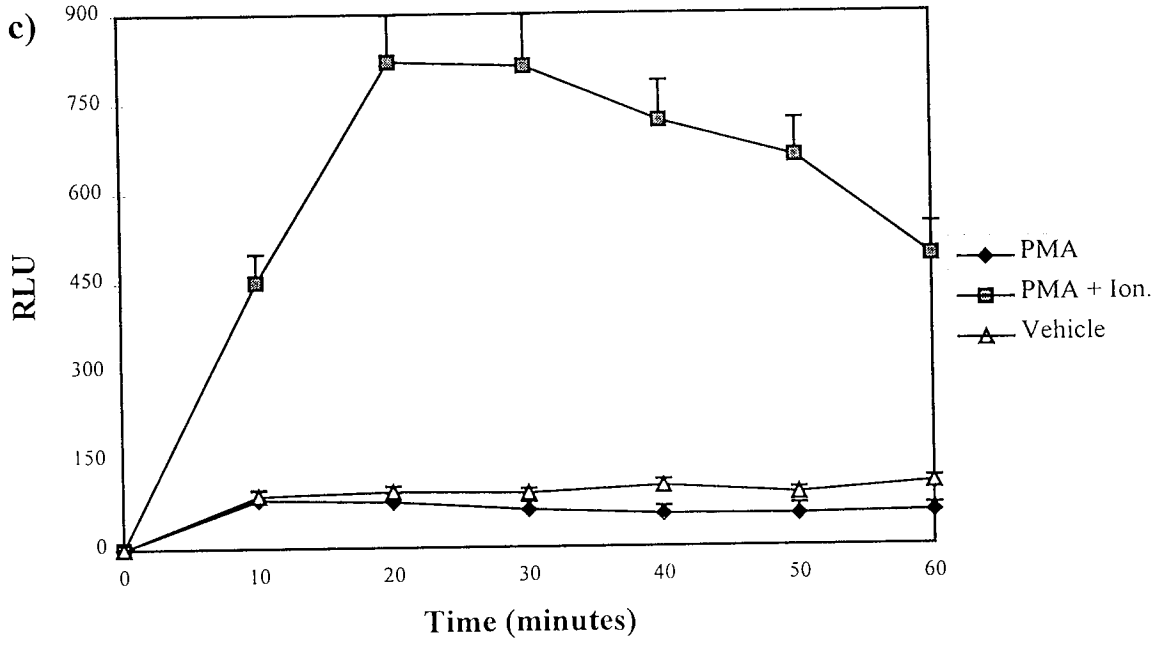
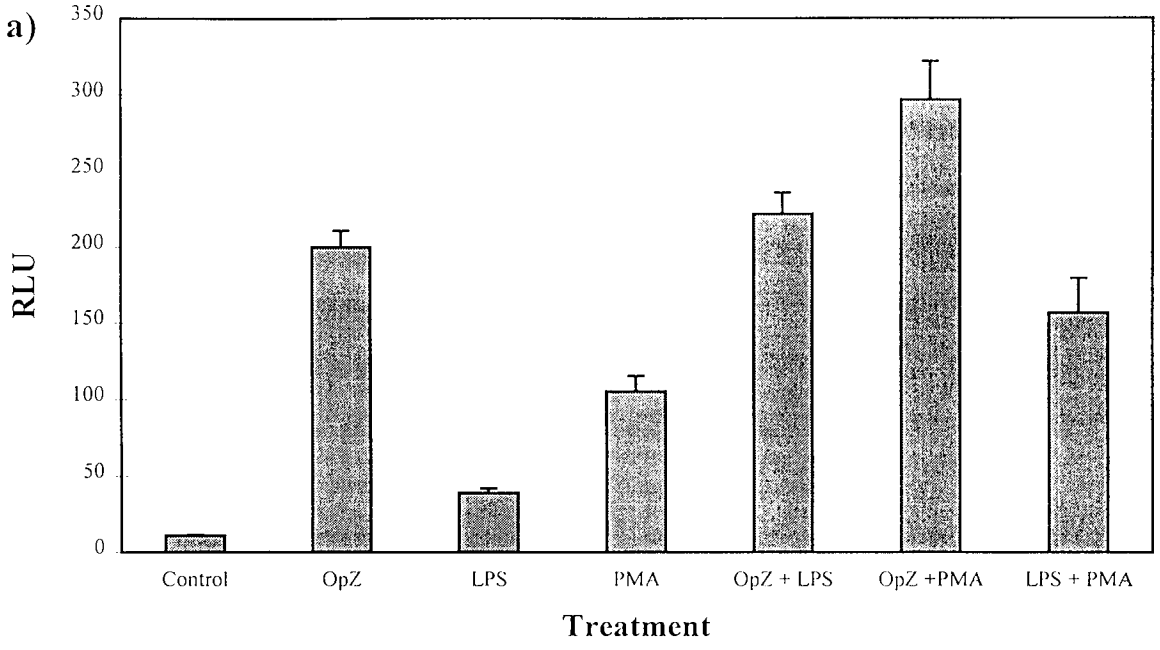


Figure 3.8 The Effect of PMA and Ionomycin on the Production of ROIs (n=4)



1 x 10^6 primed U937 cells (a), RAW 264.7 cells (b) and peritoneal macrophages (c) were treated with PMA (10^{-8}M) and/or the calcium ionophore Ionomycin (10^{-8}M) for 60 minutes. The oxidative burst produced was measured on a luminometer every 10 minutes.

Figure 3.9 The Effect of Combinations of Activators on the Oxidative Burst (n=4)



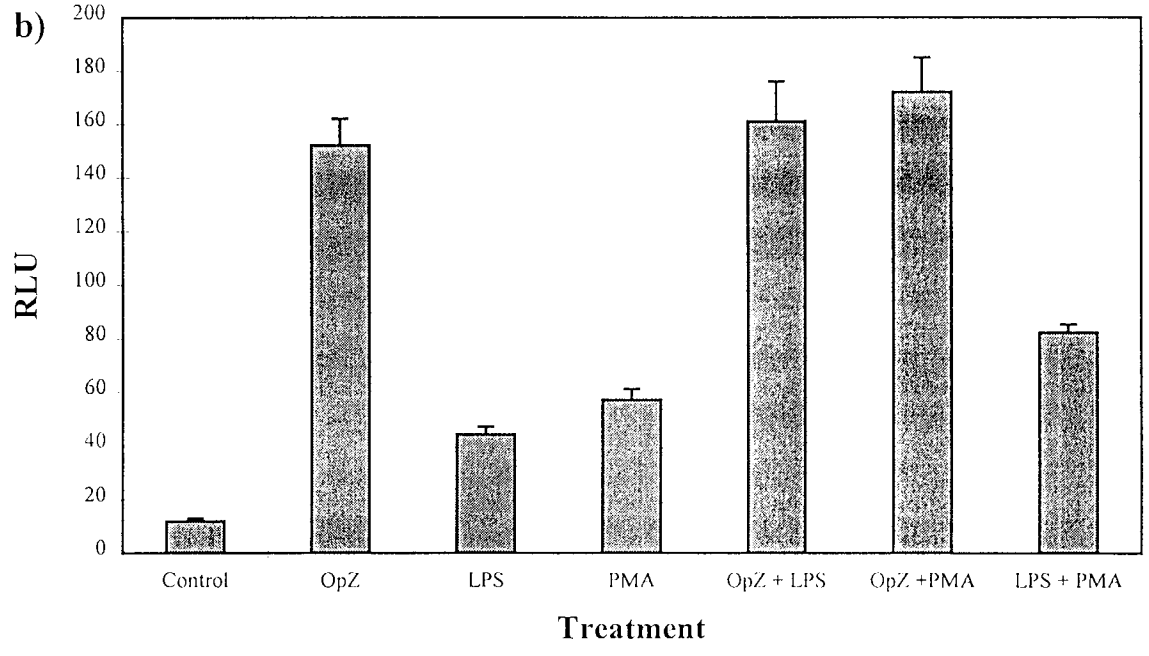
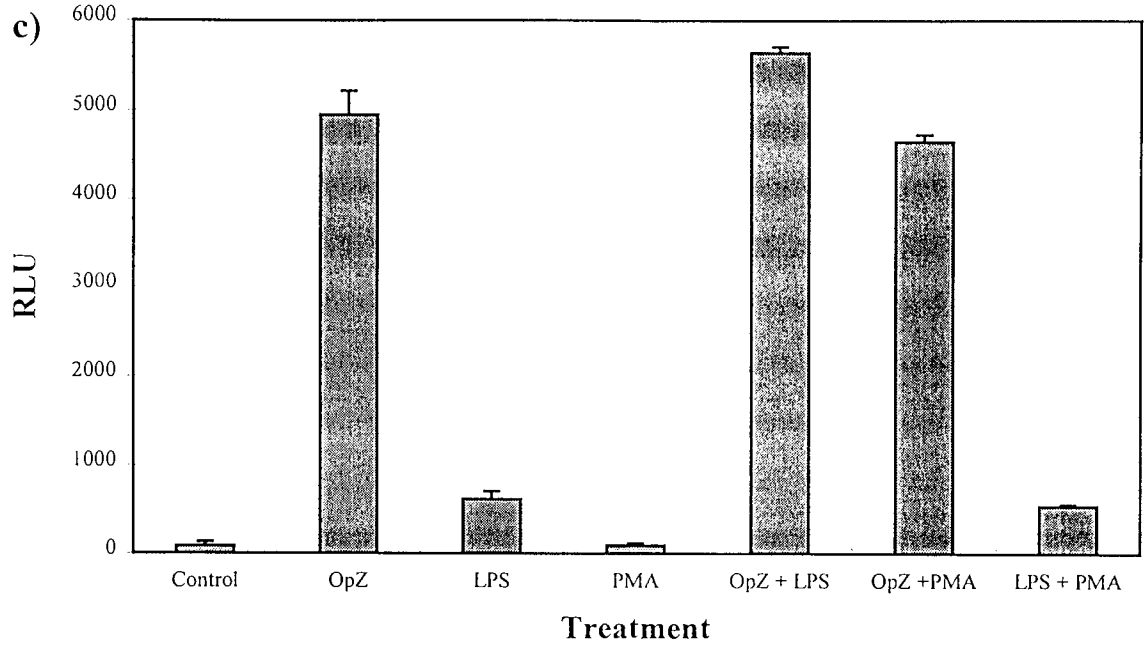


Figure 3.9 The Effect of Combinations of Activators on the Oxidative Burst (n=4)

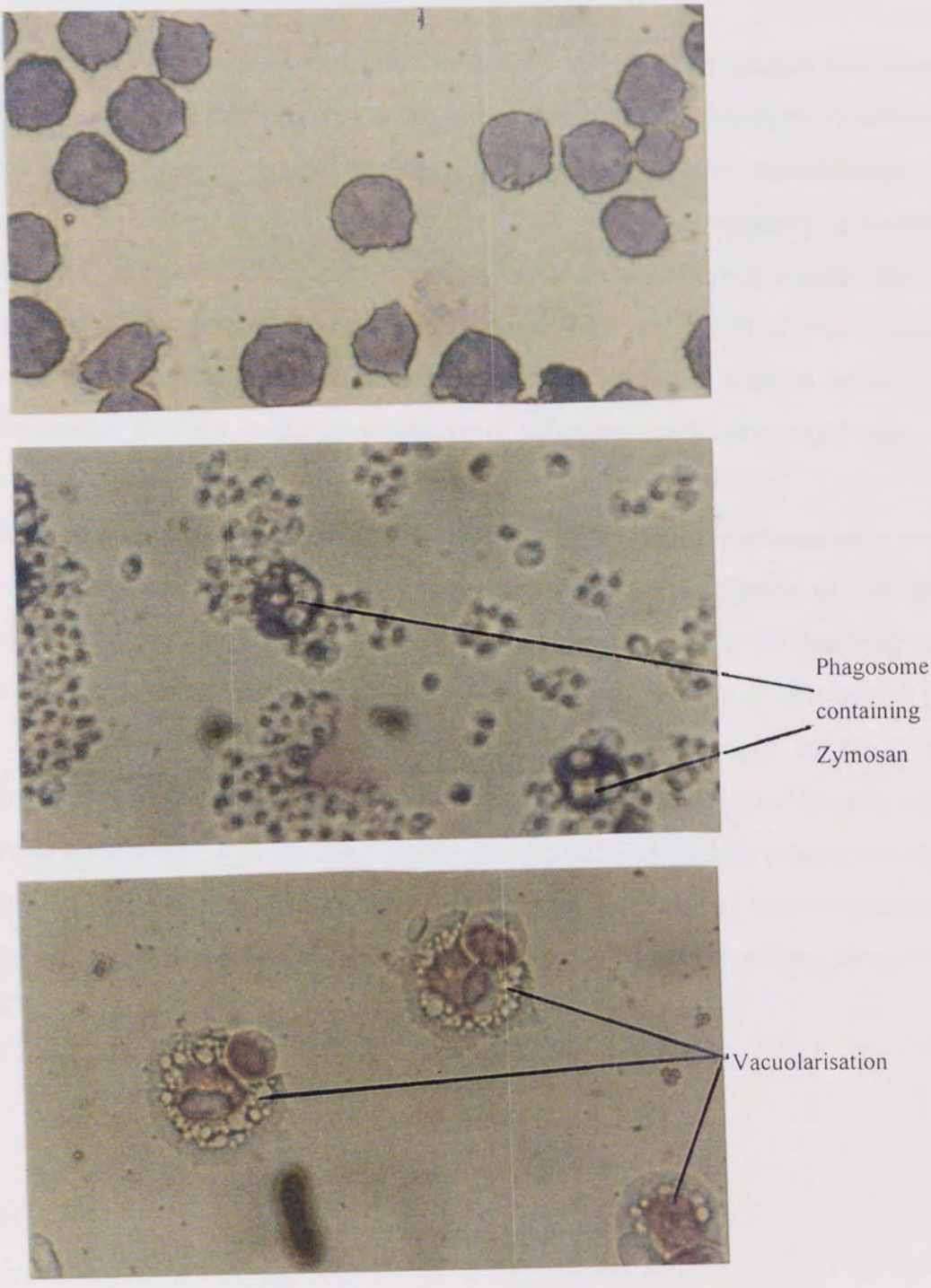


The oxidative burst of primed U937 cells (a), RAW 264.7 (b) and peritoneal macrophages (c) was measured after treatment with OpZ (1 mg/ml), LPS (1 μg/ml) and PMA (10^{-8}M), and in combinations to establish whether any synergy existed between these compounds.

Photo 3.1 U937 Cells Cultured with OpZ and PMA

a)

b)



c)

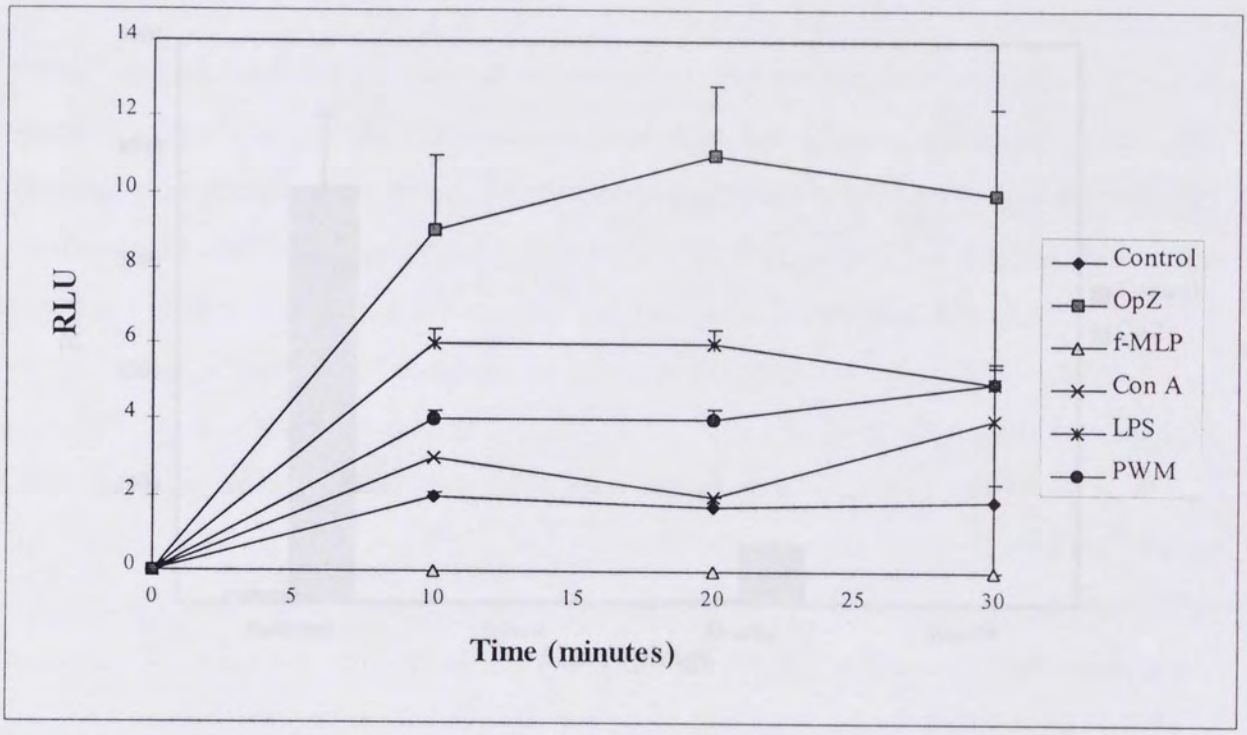
Cytospin preparations were produced from 1 x 10^6 primed U937 cells incubated alone (a), with OpZ (b) and with PMA (c) for 60 minutes. The cells were stained with Diff-Quick and viewed under a light microscope (X500).

3.3.3 Macrophage Heterogeneity

The production of reactive oxygen intermediates by splenic macrophages was determined to investigate whether they behaved in the same manner as the other primary cells studied, peritoneal macrophages. Unlike peritoneal macrophages, splenic macrophages did not display substantial burst activity, demonstrating macrophage heterogeneity in the functions they perform. However a very modest respiratory burst significantly greater than control values ($p<0.05$) was produced in response to OpZ, LPS and PWM (Figure 3.10). This assay was limited in that it does not determine whether these cells were in fact activated to perform another function, such as presentation of antigen or secretion of cytokines.

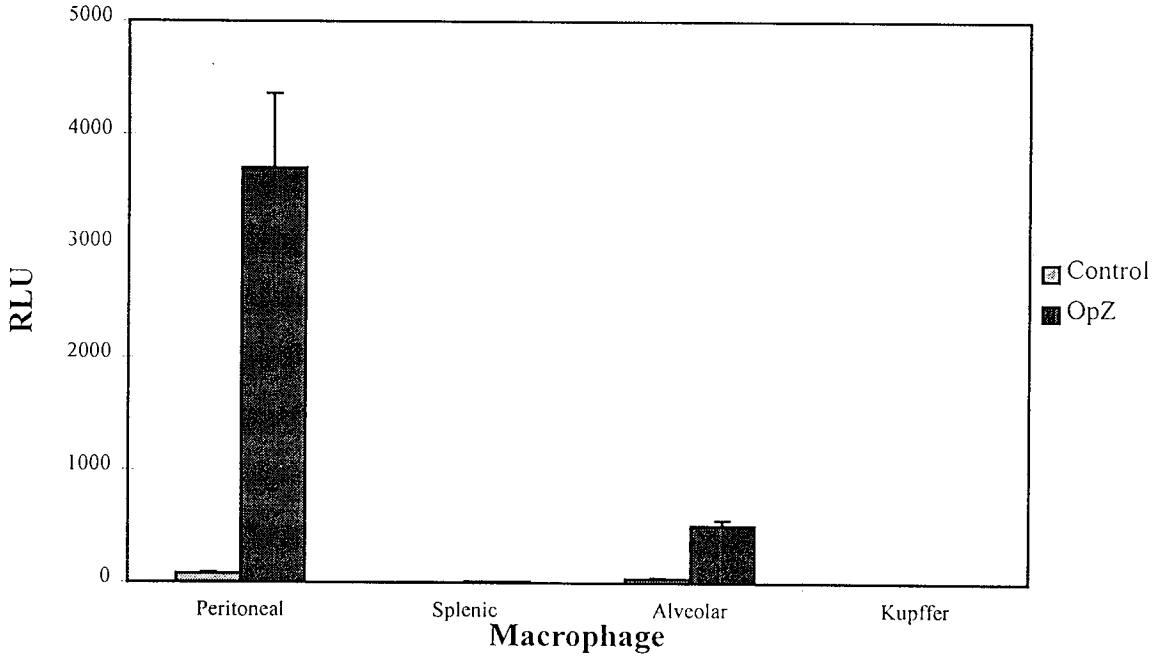
To further demonstrate this heterogeneity, resident macrophages were taken from a number of sites in the mouse (peritoneum, lung, liver and spleen), a description of how this was carried out can be found in chapter 2. The response of these various macrophages produced after a twenty-five minute incubation with OpZ was then determined (Figure 3.11). Peritoneal and alveolar macrophages responded strongly to OpZ, splenic macrophages were able to produce a small oxidative burst, whereas Kupffer cells were not. This demonstrates how different macrophage populations vary in their phagocytic ability in response to OpZ. Other compounds may alter the responsiveness of these various types of macrophage, for example Kupffer cells respond strongly to LPS to secrete IL-6 (Fuller *et al*, 1987).

Figure 3.10 Effect of Various Activators on the Respiratory Burst in Splenic Macrophages (n=4)



1 x 10^6 splenic macrophages isolated by adherence (see section 2.1.1) were incubated for 30 minutes with OpZ (1 mg/ml), f-MLP (10^{-5}M), Con A, LPS and PWM (all 1 μg/ml). The respiratory burst elicited was measured on a luminometer at ten minute intervals.

Figure 3.11 Oxidative Burst of Macrophages From Different Anatomical Sites



A comparison was made between the oxidative burst produced by 1 x 10^6 murine peritoneal, splenic or alveolar macrophages and Kupffer cells in response to 1 mg/ml of OpZ after a twenty-five minute incubation..

3.4 Discussion

The U937 and RAW 264.7 cell lines represent highly atypical cells with profoundly altered gene expression rather than cells arrested at a defined stage of myeloid lineage (Palumbo *et al,* 1984). This must be taken into account when using transformed cells to investigate biological functions, as they may not behave in the same way as cells *in vivo*. Presumably primary cultures of mouse peritoneal macrophages are a better reflection of the true physiological response. However, removal of non-adherent cells in peritoneal macrophage exudate may also result in altered properties of macrophages. This may be due to the treatment used in the cell separation, or to the removal of interacting cells (Aksamit, 1981). Therefore, in all instances, these cells do not give definitive information on cell behaviour *in vivo* as the experiments were carried out *in vitro*. It is important to take this into account when analysing data obtained from cell culture experiments.

Preliminary experiments on phagocytosis using U937 cells revealed wide variations between two independent observers' subjective cell counting procedures and although the crude agreement was reached that OpZ promoted phagocytosis good reproducibility was not obtained (Figure 3.1). This method was therefore abandoned as a realistic quantitative technique. In contrast luminometric studies gave more reliable objective results. Inter-assay variation was minimised by maintaining U937 cells in log phase growth before priming (Hadley *et al,* 1992). Initial chemiluminometric studies revealed that un-opsonized Zymosan A (1 mg/ml) did not elicit an oxidative burst of significant magnitude (Figure 3.2). The ability of Opsonised Zymosan A (OpZ) at this same concentration to elicit a large oxidative burst must therefore be due to the presence of C3b on the surface of the Zymosan A. All phagocytes express complement receptors, such as CR1 which binds C3b, and binding to these receptors facilitates macrophage activation leading to phagocytosis and enhanced oxidative metabolism. Clement and Lehmeyer, 1983 also demonstrated enhanced burst activity in human monocytes when Zymosan A was opsonised with complement components. The optimum concentration of OpZ able to elicit

a respiratory burst was 1 mg/ml and with non-opsonized Zymosan A it was 20 mg/ml. Therefore, at 1 mg/ml there must be the optimum amount of C3b to bind to CR1 receptors on U937 cells; increasing Zymosan A concentrations further resulted in too much C3b binding leading to desensitisation (at less than 1 mg/ml some CR1 receptors are no doubt free). In the absence of C3b on Zymosan A particles increased concentrations of Zymosan A have a greater chance of being inherently phagocytosed by U937 cells.

In contrast to the OpZ response, the chemotactic peptide f-MLP only generated a very small, short-lived response in U937 cells (Figure 3.3). Coffey *et al,* 1991 found that f-MLP increased PLC activity, IP_3 turnover and raised intracellular calcium in macrophages which may have resulted in this short-lived burst.

Mitogens have a variety of effects on macrophages. For example Con A and LPS increase the number of villi outside and lysosomes inside macrophages and enhance Antibody-dependent cell cytotoxicity (Larrick *et al,* 1980). TNF-α and IL-6 production in macrophages is stimulated by PWM and TNF stimulates IL-1 production (Waldman *et al,* 1982). PWM also weakly stimulates C3 production in human macrophages. These cytokine and surface molecule changes may affect lymphocyte-macrophage interactions. In these experiments, the mitogens Con A, LPS and PWM, failed to stimulate ROI production in U937 cells to the same magnitude as OpZ. However it has been proven that macrophage activation and the oxidative burst are dissociable (Chateau *et al,* 1993) which suggests that although they did not elicit a sizeable oxidative burst, they may have activated the cells in other ways. Phagocytosis could not be enhanced by incubation with latex beads in addition to the mitogenic agents, showing that these chemicals have no significant effect on phagocytosis. The only other agent found to produce a significant oxidative burst in U937 cells was the phorbol ester PMA (Figure 3.6). The burst was only half the size of that produced by incubation with OpZ. Not only did PMA enhance H_2O_2 production, but it also produced a number of morphological changes (Photo 3.1). For example, increased cellular adherence and pseudopodial extensions. U937 cells must be

primed for PMA to elicit a respiratory burst. It produced a maximum response at 10^{-6} Molar. Interestingly, when the calcium ionophore Ionomycin was incubated with U937 cells as well as PMA, the burst although larger, still did not reach the magnitude of that produced after treatment with OpZ. This shows that PKC activation and increased cytosolic Ca^{2+} may not be the only mechanisms involved in the oxidative burst. Ionomycin does cause a sharp increase in intracellular calcium in U937 cells (Maudsley and Morris, 1987), this and PMA are sufficient to activate PKC and mobilise intracellular calcium. Possibly other intracellular activation pathways are involved e.g. the activation of Protein Tyrosine Kinases (PTK) or increased synthesis of calmodulin-related proteins. U937 cells produced an oxidative burst ten-times larger when Zymosan A was opsonized with human and not guinea-pig serum (data not shown) demonstrating CR1 species-specificity. However there was some homology between human and guinea pig complement proteins, which was enough to facilitate ligation of CR1 receptors. This re-enforces the assumption that other secondary messenger activation pathways are required in the oxidative burst as PKC activation alone only produced one twentieth of this response. Also, the response of U937 cells to OpZ was not maximally exhaustible as LPS and PMA both further enhanced the oxidative burst produced, but without synergy. This could be investigated by pre-incubating with PMA to exhaust intracellular PKC to investigate the effects on the respiratory burst produced by OpZ. The extent to which the response is reduced will thus relate to how much PKC contributes normally to this response.

RAW 264.7 cells do produce an oxidative burst without prior incubation with a priming agent such as IFN-γ (Figure 3.2b). This is due to their partial differentiation. However, priming does increase further their ability to produce an oxidative burst in response to OpZ and this increases with priming time. This is probably due to enhanced up-regulation of oxidative enzyme precursors and/or increased expression of surface receptors for C3b. In these studies, the cells were unprimed as they still elicited an adequate oxidative burst and this saved time and money. Again, the maximum response to OpZ was at 1 mg/ml when concentrations of 0.2 mg/ml to 20 mg/ml were analysed. This may also be due to there

being optimum C3b receptor occupancy and thus second messenger generation at this concentration. The agents used to activate RAW 264.7 were, the PKC activator, PMA (Figure 3.7b) and Ionomycin (Figure 3.8b) and the mitogens Con A, LPS and PWM (Figure 3.4b, 3.5b and 3.6b respectively). PMA elicited a significant oxidative burst at 10^{-6} M, however this was again only half the size of that elicited by RAW cells in response to OpZ. RAW 264.7 macrophages were more responsive to LPS than the human cell line U937.

In comparison a huge respiratory burst was achieved when peritoneal macrophages were incubated with OpZ. It was approximately twenty times larger than that produced by the same number of U937 and RAW 264.7 cells to this same stimulus (Figure 3.2c). This burst would possibly be even larger if the Zymosan A had been coated in mouse serum. However it was still approximately twice as large as the response produced by the human cell line, U937 to Zymosan A coated with human serum. These results demonstrate the difference between transformed cells and primary cells. It is therefore essential that experiments are carried out on primary cells as well as transformed cells to gain a more reliable idea of how these cells function *in vivo*. Peritoneal macrophages also exhibit differences in basal activity. Spontaneous burst activity was greater than the responses of both U937 cells and RAW 264.7 to OpZ. The phorbol ester PMA inhibits this spontaneous burst activity at all concentrations over the range 10^{-5} M to 10^{-10} M (Figure 3.7c). Maybe the activation of peritoneal macrophages with PKC alone causes them to differentiate towards another function (e.g. secretory). PKC activation plus raised intracellular calcium does activate the respiratory burst. However, Kitagawa *et al,* 1986 did find that respiratory burst activity was de-activated by changes in signal transduction to respiratory enzymes produced by PMA which may explain this phenomenon. PMA cannot fully inhibit the oxidative burst as incubation with OpZ and PMA only slightly reduced the burst produced (Figure 3.9c). It can therefore be overridden by other second messenger pathways such as PLC, PTK, calmodulin related proteins etc. As in U937 cells and RAW 264.7 cells the maximum response of peritoneal macrophages to OpZ was at 1 mg/ml, and LPS was 1

μg/ml respectively, but the response to LPS was only one-tenth that of the response to OpZ (Figure 3.8c). The Ca^{2+} ionophore Ionomycin did elicit a small respiratory burst in peritoneal macrophages which was approximately five-fold smaller than that produced by OpZ. PMA was unable to inhibit spontaneous burst activity in the presence of Ionomycin. In fact, there was an increase in burst activity. The combination of both PMA and Ionomycin did not result in full activation i.e. a response of the magnitude produced by OpZ. Perhaps there are other activation pathways involved in the production of the respiratory burst leading to H_2O_2 production. The greater ability of peritoneal macrophages to respond to OpZ than U937 cells and RAW 264.7 may be due to their differentiation state. When peritoneal macrophages were incubated with combinations of OpZ, PMA and LPS there was no synergy (Figure 3.9c). PMA partially inhibited the original burst produced by OpZ and LPS alone, indicating that more than one activation mechanism is required for the production of an oxidative burst. This no doubt depends on which receptors are ligated. The importance of PKC in macrophage responses to OpZ could be investigated by incubating the cells with OpZ and a PKC inhibitor, such as Hexadecyclophosphocholine or Staurosporine.

In summary U937 cells, RAW 264.7 and peritoneal macrophages are the most responsive to OpZ at 1 mg/ml in producing an oxidative burst. They only marginally respond to Zymosan A that is not opsonized. When the U937 human cell line was coated with human serum this response was increased by ten-fold. This could be investigated with the murine equivalent. Although PMA could activate U937 and RAW cells the response was only about half the size of the respiratory burst produced with OpZ in these cells. In peritoneal macrophages PMA slightly reduced both spontaneous burst activity and the OpZ-mediated burst. Incubation with Ionomycin as well as PMA potentiated the oxidative burst produced by both U937 cells and peritoneal macrophages. Maximal activation was not achieved by this means therefore additional factors may also be involved. The second messenger systems involved in macrophage activation are clearly highly complex and deserve further investigation. The endotoxin LPS only had a weak effect on all three cell types but in each

case the optimum concentration was 1 μg/ml. The lectins Con A and PWM, and the chemotactic peptide f-MLP did not elicit a significantly substantial oxidative burst in all three cell types. However, they may have activated the monocytes in other ways e.g. by altering their cytokine profiles, or up/down regulation of surface molecules. The most responsive cell type in producing an oxidative burst was the peritoneal macrophage. Heterogeneity between different resident macrophage populations was demonstrated. Their function was related to their location in the body, therefore this must be taken into account when drawing conclusions from experiments involving macrophages.

The optimal conditions have been established for the activation of a respiratory burst in cells of the monocyte/macrophage lineage. Although such an activation may be dissociated from or not associated with other forms of macrophage activation e.g. cytokine release or up-regulation of cell surface receptors it will be used in the first instance as a convenient and reproducible means of monitoring activation. The effects of such provocative stimuli on B- and T-cell activation can now be assessed. In cultures of purified murine splenic B- and T-lymphocytes the effects of the macrophage, supernatants from activated macrophage cultures or the effects of surface membranes of such cells could be measured in terms of the DNA-synthesis. This will be investigated in the following chapters.

4. REQUIREMENT FOR THE MACROPHAGE IN THE LYMPHOCYTE RESPONSE TO MITOGEN

4.1 Introduction

Early studies by Corbel and Melchers (1983) demonstrated that murine B-cells lose their ability to respond to LPS or anti-Ig antibodies after extensive depletion of accessory cells. In the model proposed by Melchers and Anderson (1984), three restriction points control the cell cycle of activated murine B-cells. The first restriction point occurs just after mitosis and progression to the second point of arrest in G_1, is controlled by the antigen (or anti-Ig antibodies). Progression from the second point, in S, to the third point and through G_2, depends upon macrophages (or α factors). T-cells (or β factors) allow completion of the cell cycle by inducing mitosis. B-cell responses to TI-1 antigens may not need β factors to stimulate cell division as these antigens are totally T-cell independent.

Macrophages and other accessory cells can perform various functions in the induction of an immune response (Rosenberg and Lipsky, 1979; de Vries *et al,* 1979). However in many instances the mechanisms underlying their contributions have not been elucidated. They might be necessary in presenting antigen to T- and B-cells, thereby inducing both cytokine secretion and clonal proliferation (Claassen *et al,* 1986; Chao and MacPherson, 1990; Aversa *et al,* 1994). This is probably most important for primary antibody responses. Macrophages secrete cytokines which may induce proliferation in both T- and B-cells (Hoffmann *et al,* 1979; Ryffel *et al,* 1997). These cytokines include IL-1, IL-6, IL-10, TNF, PAF and IFN-α. Thus, this study aims to identify which macrophage products (if any) are required by lymphocytes in responses to mitogen.

The lack of expression of CR2 (CD21), the receptor for C3, on marginal zone B-lymphocytes in the spleen of the very young, may result in the inability of B-cells to respond effectively to certain highly virulent strains of bacteria such as *Neisseria*

meningitidis and *Streptococcus pneumoniae* (Timens *et al*, 1989). This may be due to the fact that macrophages express a ligand for CD21 (CD23) and so without the close association between these cells an effective immune response cannot take place (Aubry *et al*, 1992). Splenectomised patients also suffer from the same vulnerability to such infections.

This chapter aims to identify the importance of the macrophage in lymphocyte responses to a range of mitogens and establish by which means they are required. For example does the macrophage secrete an essential product(s), or does it mediate lymphocyte activation via receptor binding.

4.2 Experimental Design

Splenocytes were the chosen cell type to investigate lymphocyte-macrophage interactions as evidence suggests that close physical associations between these cell types occurs (Claassen *et al*, 1986; Van den Eertwegh *et al*, 1992). The previous chapter demonstrated heterogeneity between macrophage populations. It was therefore essential that splenic macrophages were used when investigating interactions with splenic lymphocytes so that valid assumptions could be drawn. This chapter initially examines splenocyte proliferation as assessed by Tritiated Thymidine incorporation in response to a range of mitogens. The effect of macrophage removal from these cultures is then investigated. And finally the macrophages are returned either in a viable state or fixed, to establish how important cell-cell contact is between the lymphocyte and macrophage in response to mitogen.

Splenocytes prepared as described previously in section 2.1.1 were cultured in supplemented medium at a density of 5 x 10^5 per well in 96 well plates. To this mitogens were added in triplicate wells to initiate proliferation within these cultures. Dose response curves were utilised to ascertain the optimum concentration of mitogen able to induce cell proliferation and a reliable method was established. The preferred concentrations of Con A and PWM were 1 μg/ml, Dextran sulphate (DS) was 10 μg/ml, LPS was 20 μg/ml and both Poly[I][C] and PHA-P were 50 μg/ml, in this system. Cultures were incubated for 48 or 72 hours, all mitogens except DS induced a peak in proliferation after 48 hours. Therefore all future experiments were carried out using the optimum concentrations of mitogen incubated for 48 hours.

Macrophages were initially removed from splenocyte cultures by adherence, as described in section 2.1.1. (and in some instances re-added). The miniMACS cell separation technique was also employed which was described in section 2.4.1. The effect of miniMACS-separated macrophages at various densities (0-10,000 per well) on lymphocyte proliferation in response to mitogen was assessed, as was the effect of macrophages pre-treated for 1 hour with a range of activators and then fixed with

paraformaldehye (see section 2.1.8).

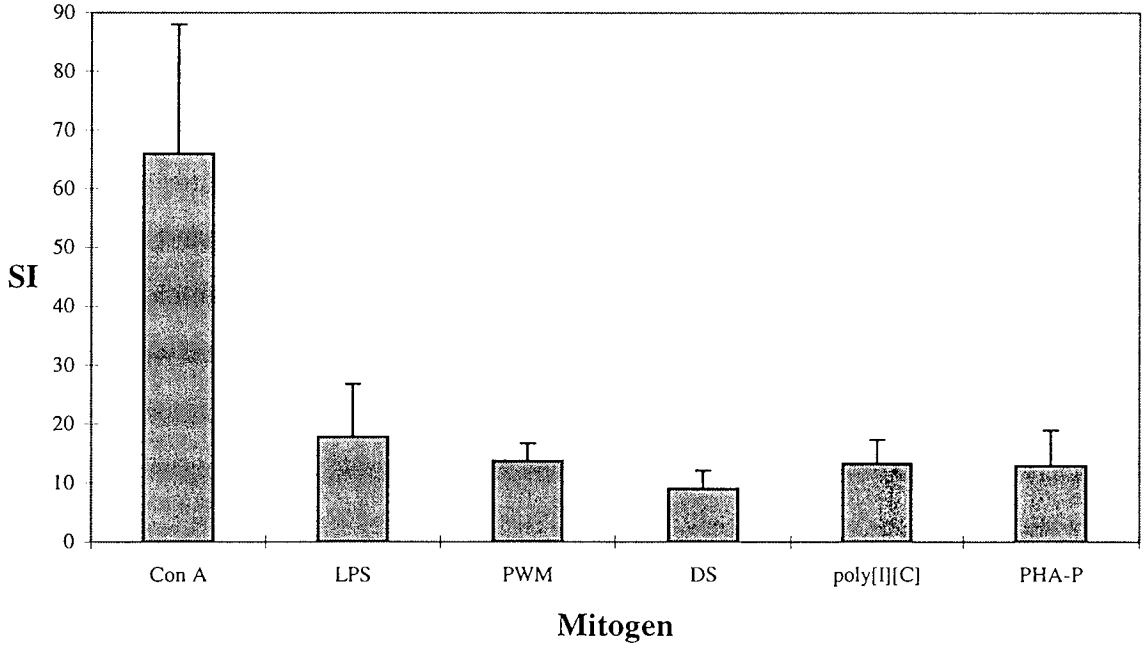
To quantify the number of lymphocytes undergoing DNA synthesis the BrdU kit was employed. The kit stains all cells synthesising DNA, a full explanation of the technique was described in section 2.4.5. This was used to determine the extent of cell recruitment into DNA synthesis by a specific mitogen.

4.3 Results

4.3.1 Proliferation of T- and B-lymphocytes

There was a sixty-six-times greater synthesis of DNA in Con A-treated splenocytes compared to control cultures and this was at least three-fold higher than the other five mitogens used (Figure 4.1). LPS was the second most potent stimulator of proliferation in MF1 splenocytes, causing an approximately 18-fold increase from resting control splenocytes. The mitogens PWM, Poly[I][C] and PHA-P all induced an approximately thirteen-fold increase in proliferation, whereas DS only induced a nine-fold increase. A similar pattern of findings was produced when Balb/c splenocytes were examined.

Figure 4.1 Effect of Six Mitogens on the Proliferation of Murine Splenocytes (n=18)



5 x 10^5 splenocytes were incubated with the mitogens Con A (1 μg/ml), LPS (20 μg/ml), PWM (1 μg/ml), Dextran sulphate (10 μg/ml), Poly[I][C] (50 μg/ml) and PHA-P (50 μg/ml). Proliferation was assessed after 48 hours by Tritiated Thymidine incorporation. The Stimulation Index was calculated as indicated in section 2.4.4.

4.3.2 Effect of Proliferation after Removal of Macrophages by Adherence

The lack of macrophages in adherence-depleted splenocyte cultures resulted in significantly reduced proliferation in response to all these same six mitogens (Con A, LPS, PWM, DS, PHA-P and Poly[I][C]) ($p<0.05$). Replenishment of viable macrophages (1000 per well) isolated by adherence to plastic for at least six hours did not however restore lymphocyte responsiveness to the mitogens ($p>0.05$), see Figure 4.2.

4.3.3 Effect of Proliferation after Removal of Macrophages by miniMACS

When purified splenic B-lymphocytes from MF1 mice were cultured with LPS and purified splenic macrophages there was a nine-fold increase in proliferation compared with cells cultured without LPS. The effect of PWM, DS and Poly[I][C] resulted in an approximate six-fold increase in DNA synthesis. However, when purified B-lymphocytes were cultured without macrophages the response to mitogen was abrogated ($p<0.05$), Figure 4.3a.

Splenocyte cultures depleted of B-lymphocytes by miniMACS, resulting in a culture consisting of mainly T-cells and macrophages responded to Con A with a twenty-fold increase in proliferation, and PWM and PHA-P by approximately twelve-fold. Removal of macrophages from this culture, leaving a predominantly T-cell population again abrogated the response to these mitogens (Figure 4.3b) ($p<0.05$).

These experiments were also carried out on the inbred strain of mice, Balb/c and a very similar pattern of results was obtained (data not shown). It is interesting to note that purified populations of B-cells or T-cells with macrophages did not respond to their appropriate mitogens as well as when the other type of lymphocyte was also present.

Figure 4.2 Proliferation of Splenocytes +/- Adherence-Separated-Macrophages (n=6)

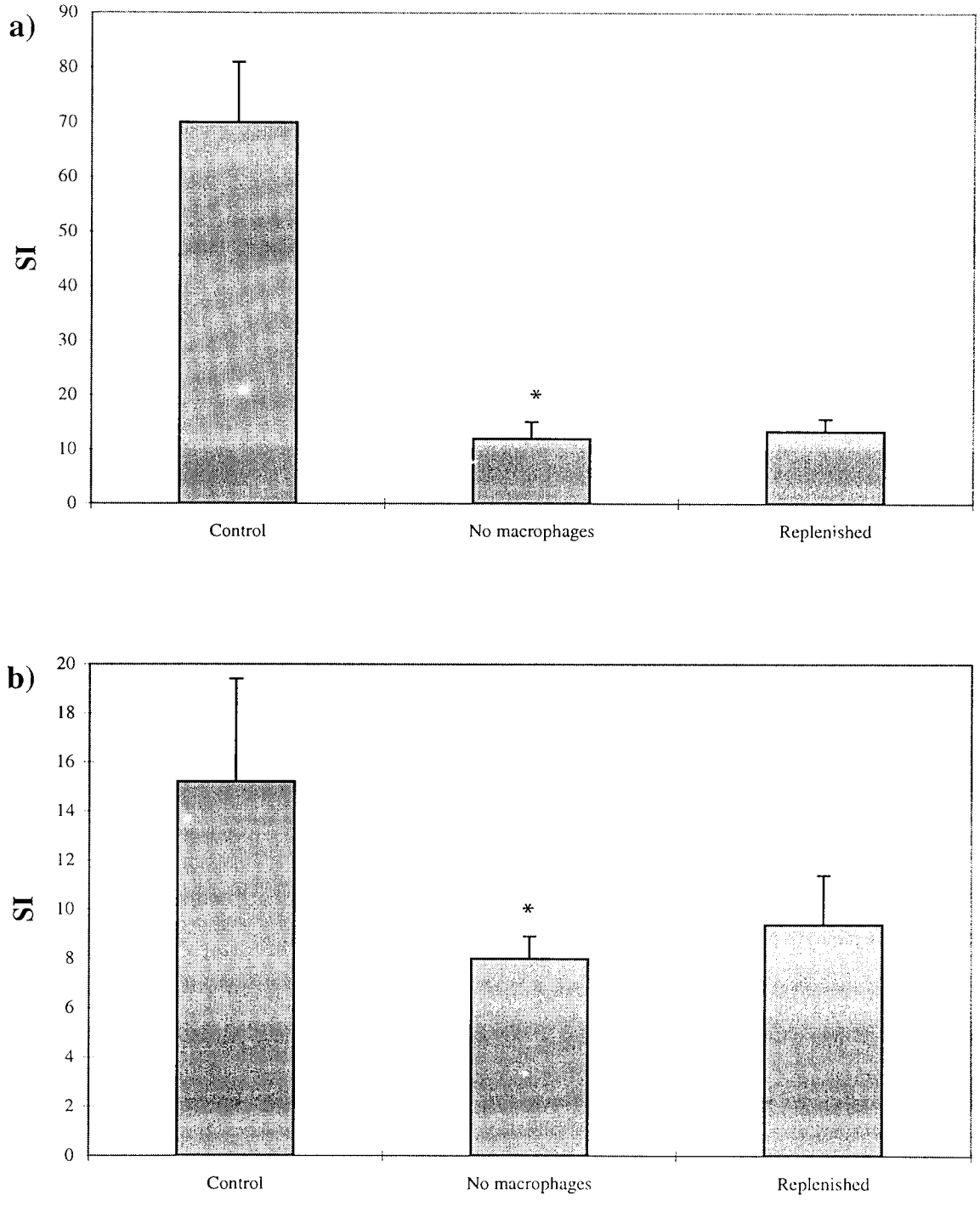


Figure 4.2 Proliferation of Splenocytes +/- Adherence-Separated-Macrophages (n=6)

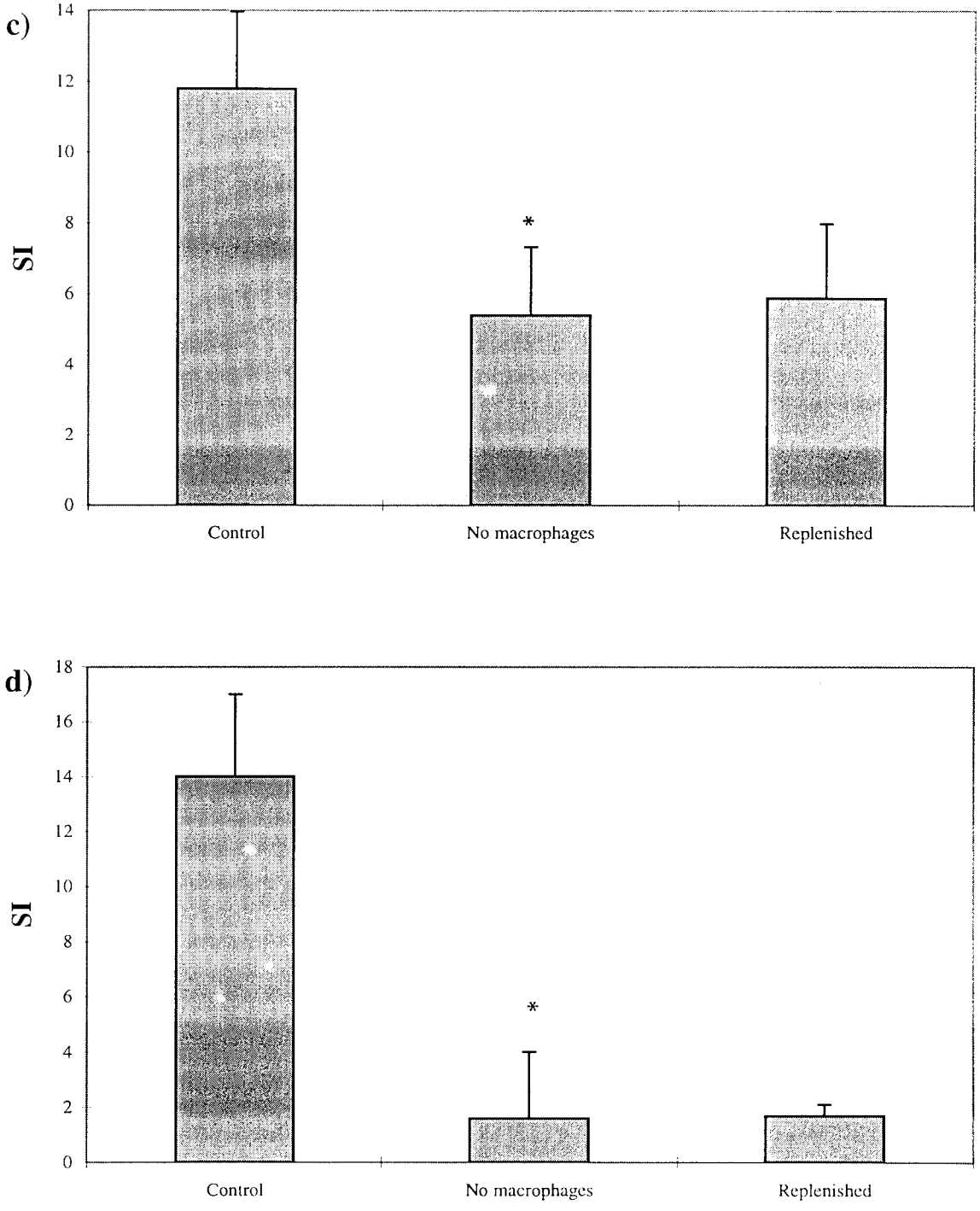
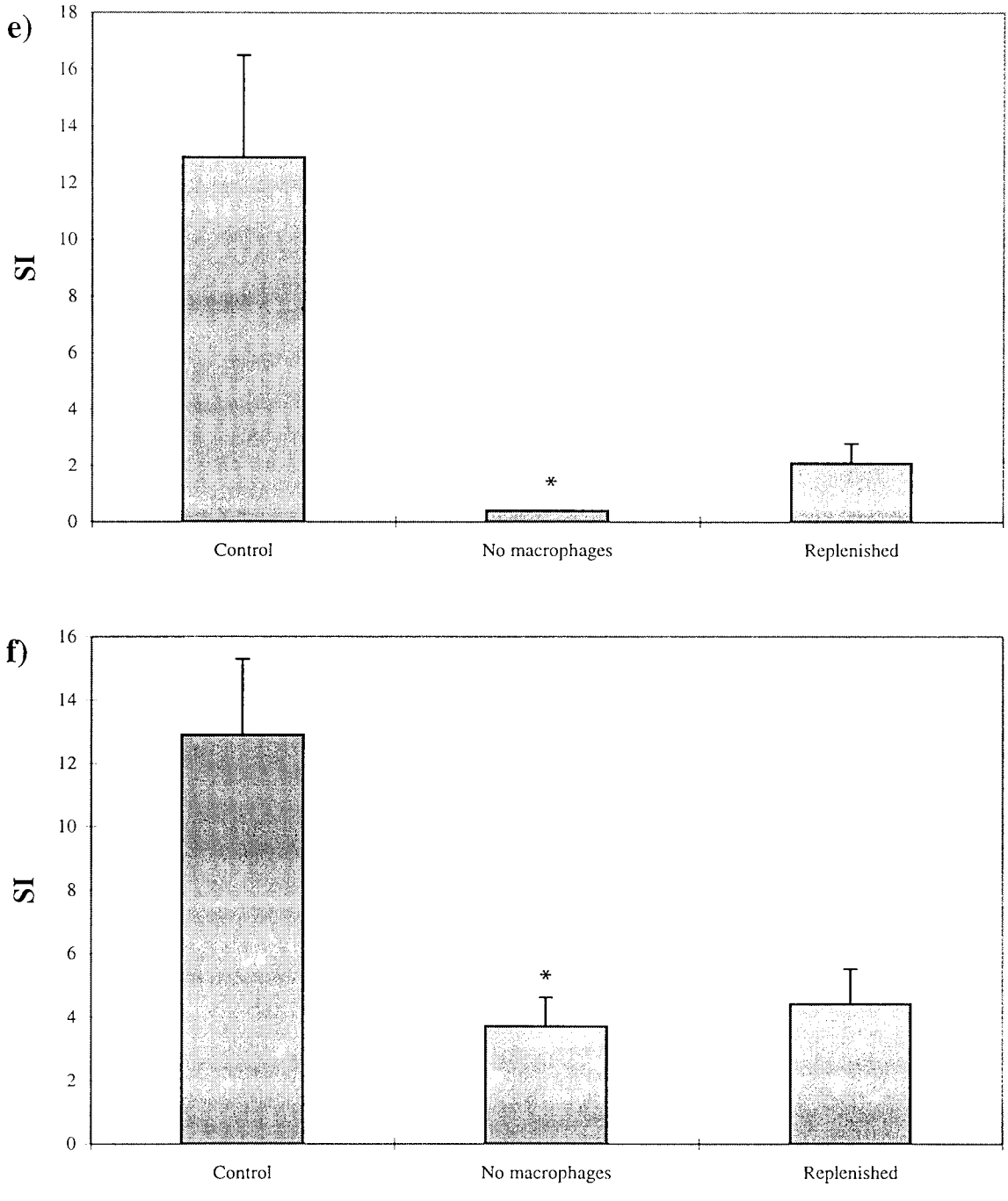
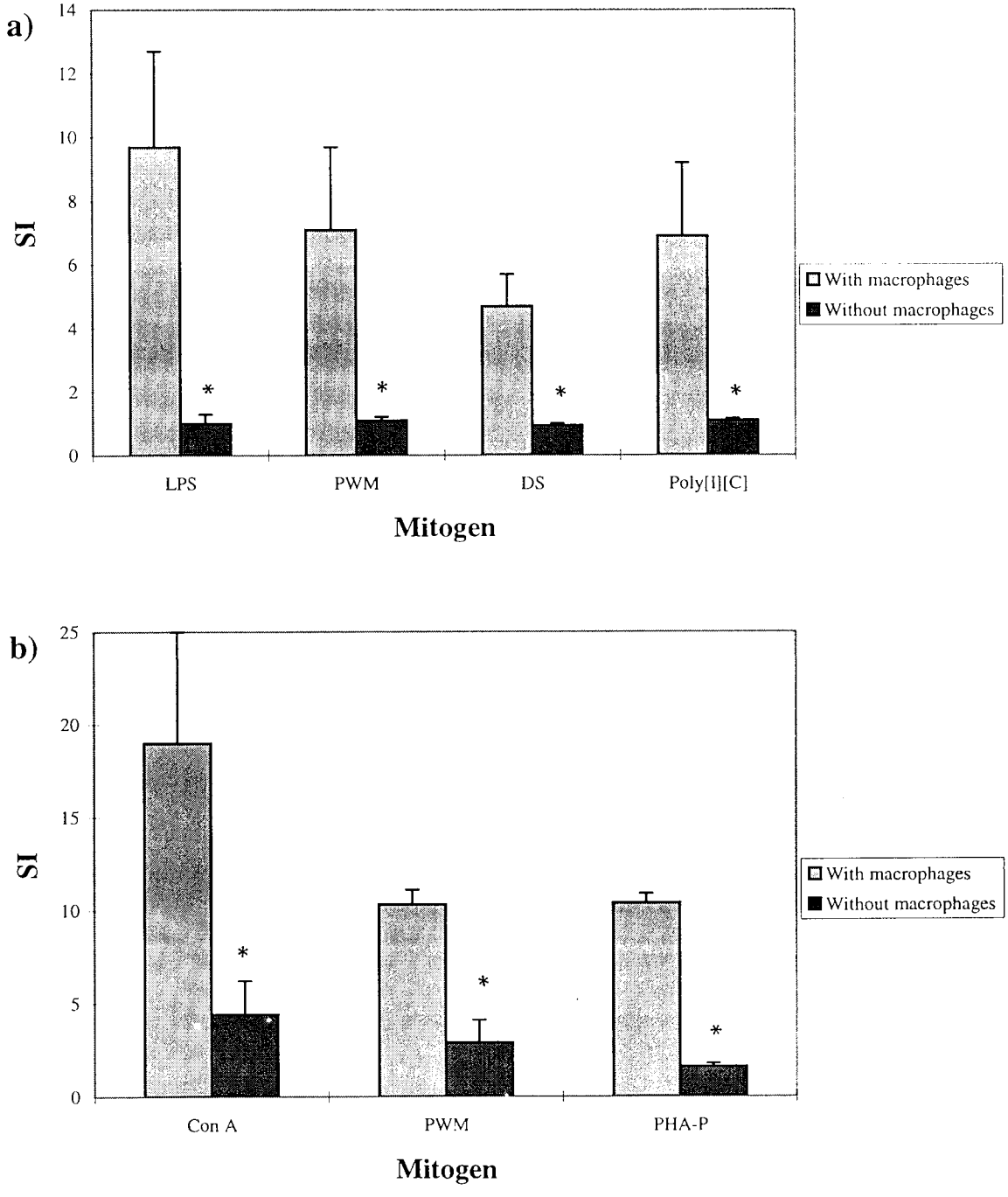


Figure 4.2 Proliferation of Splenocytes +/- Adherence-Separated-Macrophages (n=6)



Mixed splenocyte cultures (control), cultures with macrophages removed by adherence (No macrophages) and with 1000 viable macrophages per well returned (Replenished), were incubated at a density of 5 x 10^5 cells per well with Con A (a), LPS (b), PWM (c), DS (d), Poly[I][C] (e) and PHA-P (f). In all instances removal and replenishment of these macrophages significantly reduced proliferation in response to mitogen ($p<0.05$) *.

Figure 4.3 Stimulation of Purified T- or B-cells +/- Macrophages (n=4)



5×10^5 purified splenic B-cells (a) and T-cells (b) were cultured with or without 1000 purified macrophages per well and a range of mitogens. The presence of macrophages in cultures significantly enhanced the mitogenic response ($p<0.05$)*.

4.3.4 The Number of Macrophages Required in Lymphocyte Responses to Mitogen

The number of macrophages required to restore the response of macrophage-depleted lymphocyte cultures to the six mitogens Con A, LPS, PWM, DS, PHA-P and Poly[I][C] was investigated in cells from MF1 mice (Figure 4.4). The macrophages were removed by miniMACS separation and returned to the cultures (50-10000 per well). It was shown that as few as 500 macrophages per well produced a partial, if not total restoration of the response to all mitogens, representing a macrophage:lymphocyte ratio of 1:1000 ($p<0.05$ in all instances except PHA-P). Lymphocytes appear to require macrophages to respond to both TI-1 (LPS, DS) and TI-2 antigens (poly[I][C]). Again these experiments were repeated on Balb/c mice and similar results were obtained (data not shown).

4.3.5 The Effect of Fixed Macrophages on Lymphocyte Responses

Having established the requirement for viable macrophages in lymphocyte responses to mitogen, paraformaldehye-fixed macrophages were added to macrophage-depleted lymphocyte cultures (1000 per well; 500 lymphocytes: 1 macrophage) after a one hour treatment with optimum concentrations of Con A, LPS, PWM, DS, Poly[I][C], OpZ, f-MLP and PMA with Ionomycin (as established by chemoluminescence in chapter 3). The lymphocytes were co-cultured with the six mitogens previously used, see Figure 4.5. In all instances (except in the response to Poly[I][C], Figure 4.5e) fixed, treated macrophages were unable to significantly restore lymphocyte responsiveness to any of the mitogens.

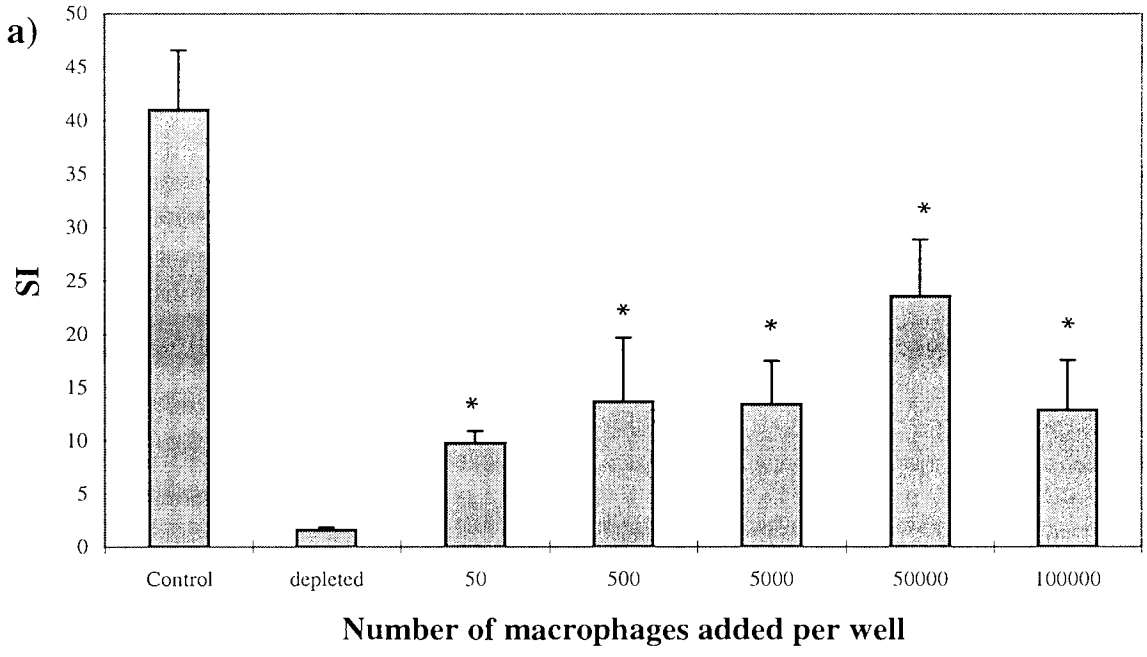
4.3.6 Lymphocyte Recruitment in Response to Mitogen

The effect of combinations of mitogens on lymphocyte proliferation was assessed to investigate whether any synergy was achieved (Figure 4.6). There appeared to be no synergy and only partial additive effects with combinations of two or three mitogens, in

comparison to the SIs produced with one mitogen (Figure 4.1). Thus, different mitogens do not recruit distinctly different sub-populations of lymphocytes to enter mitosis. Interestingly, DS appears to lower the proliferative effect of LPS or PWM incubated in combination with Poly[I][C].

The BrdU kit, which stains cells undergoing DNA synthesis (see section 2.4.5), was used to confirm that only a proportion of cells were stimulated to proliferate in response to mitogen. It demonstrated that between 30 and 50 % of cells were synthesising DNA in response to mitogen (data not shown). Therefore mitogens do not appear to activate all cells, but only a sub-set. However, this technique did not give good cell preparations due the large number of steps involved in the protocol, thus it was not totally reliable.

Figure 4.4 Splenocyte Proliferation with Varying Numbers of Macrophages (n=6)



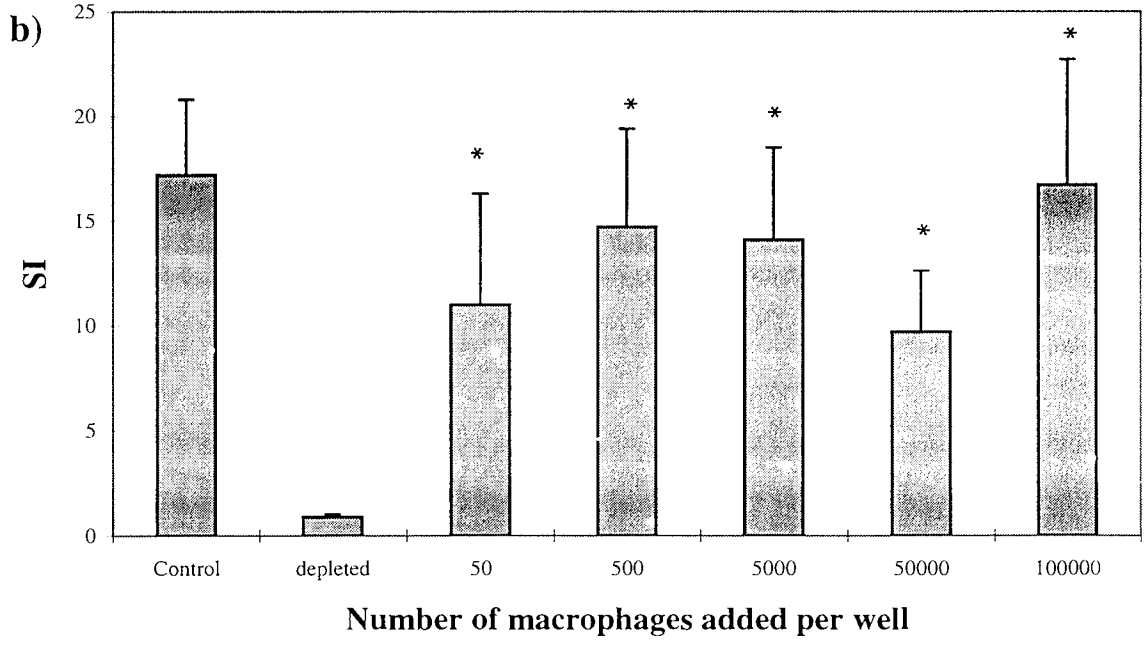
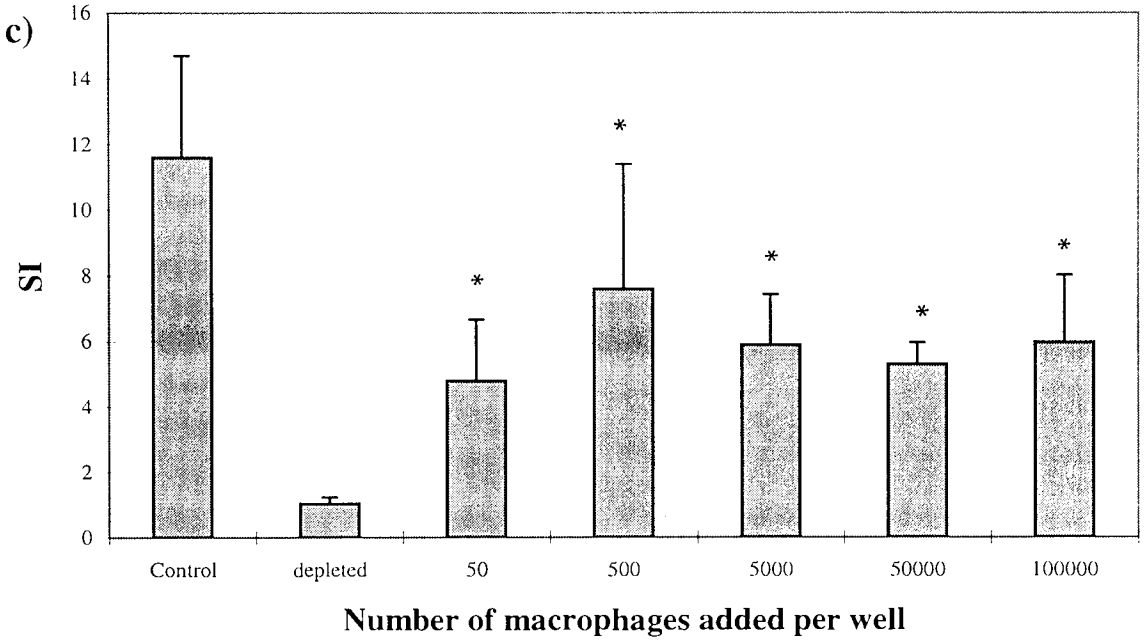


Figure 4.4 Splenocyte Proliferation with Varying Numbers of Macrophages (n=6)



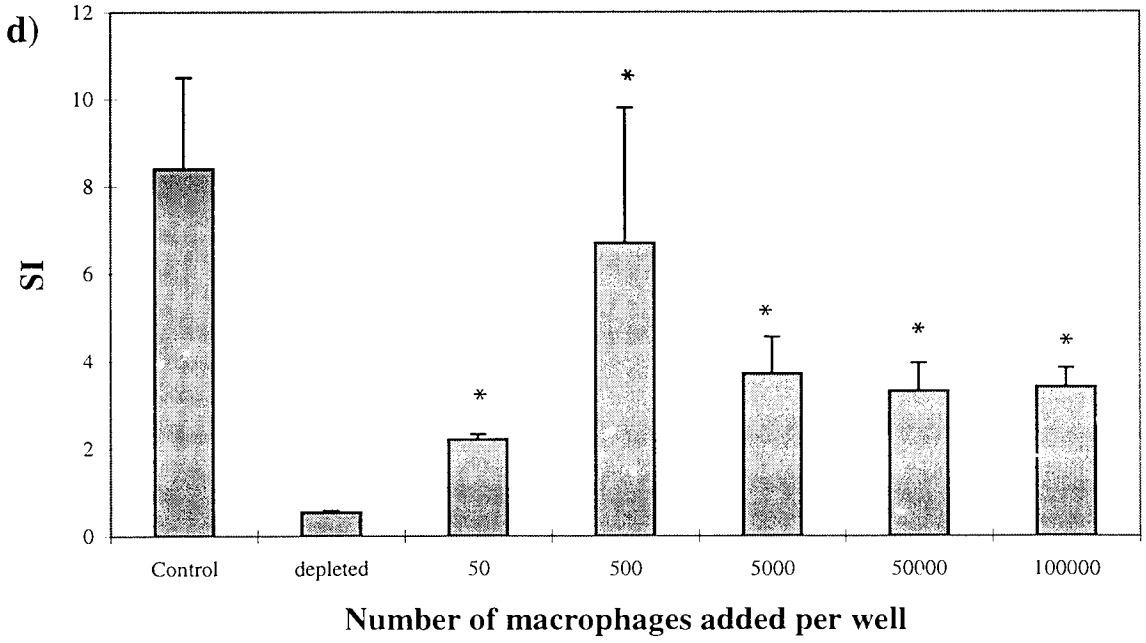
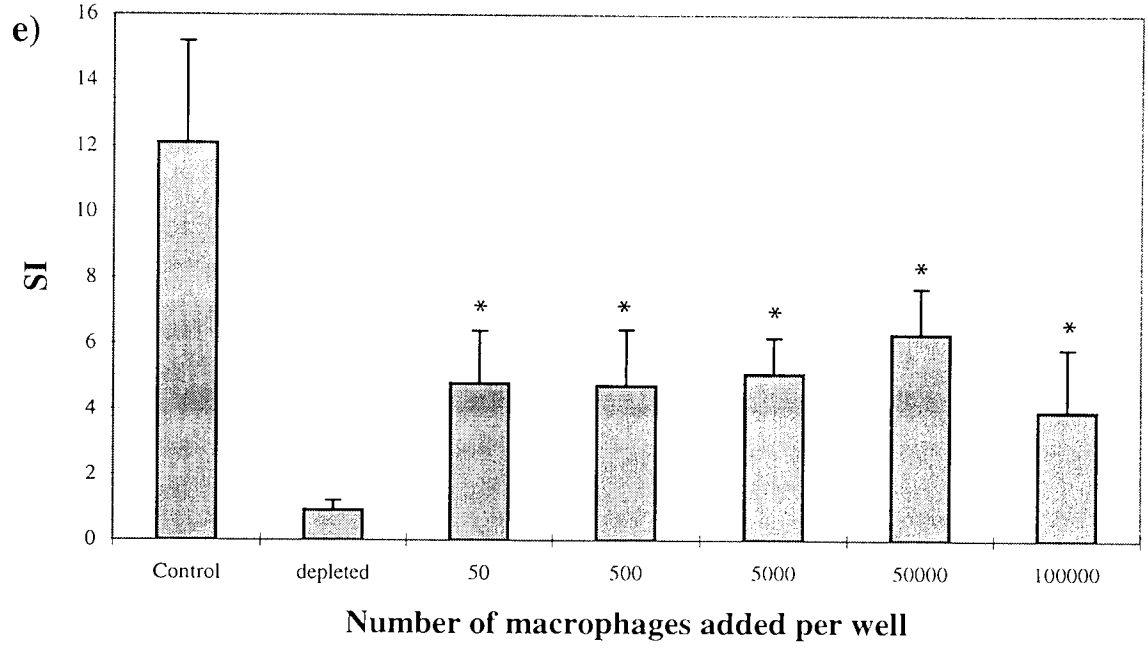
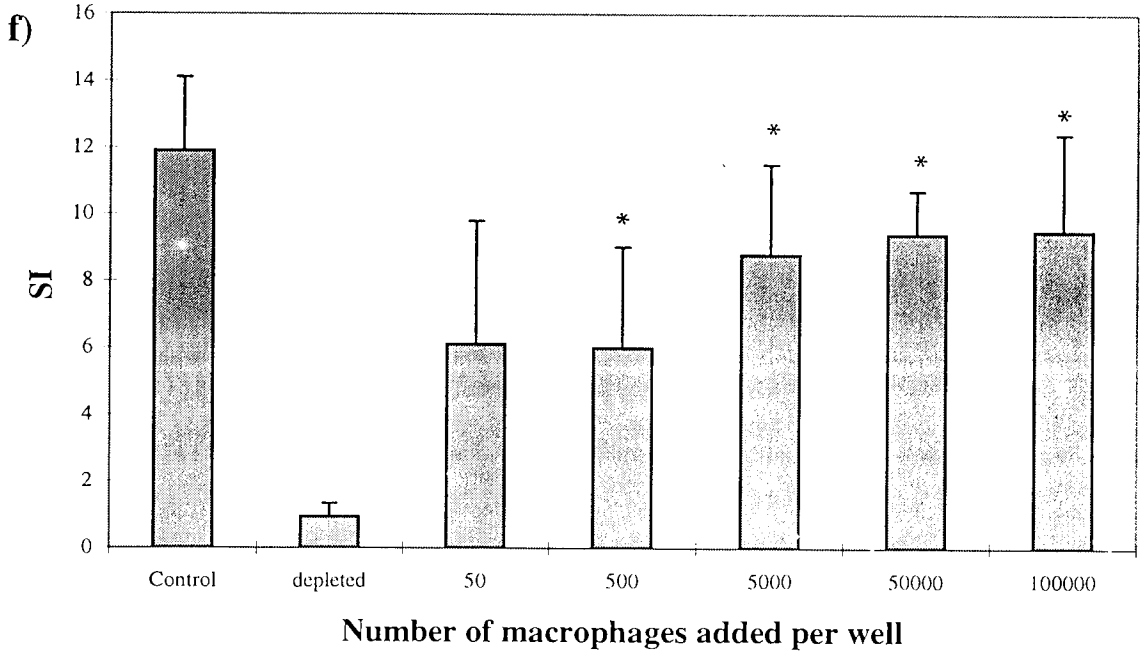


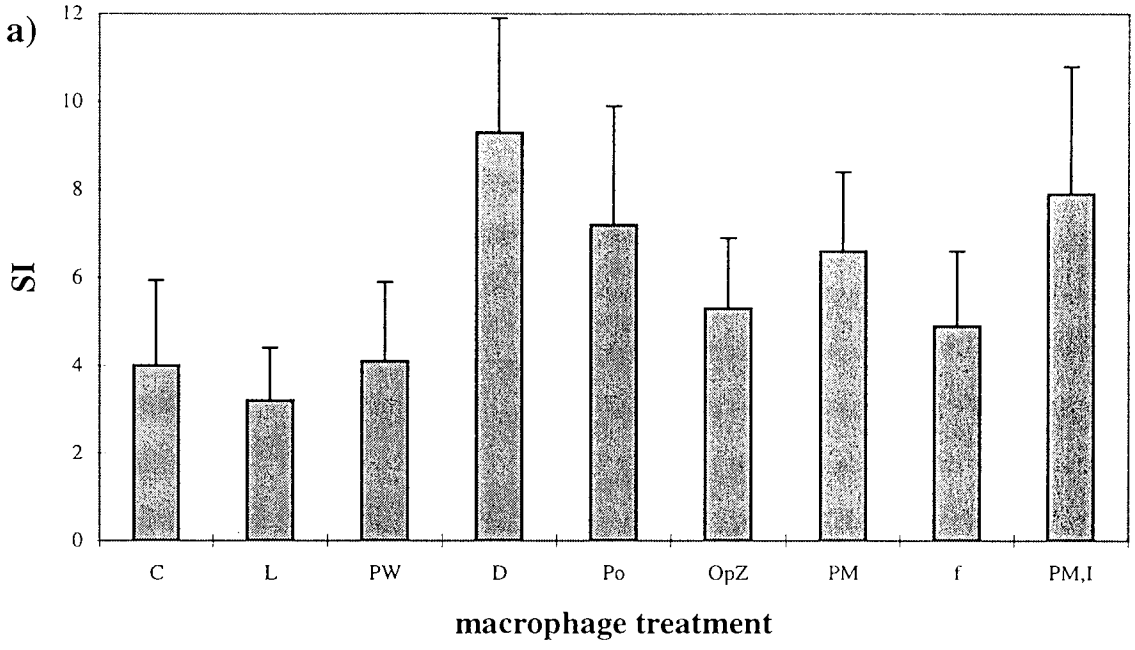
Figure 4.4 Splenocyte Proliferation with Varying Numbers of Macrophages (n=6)



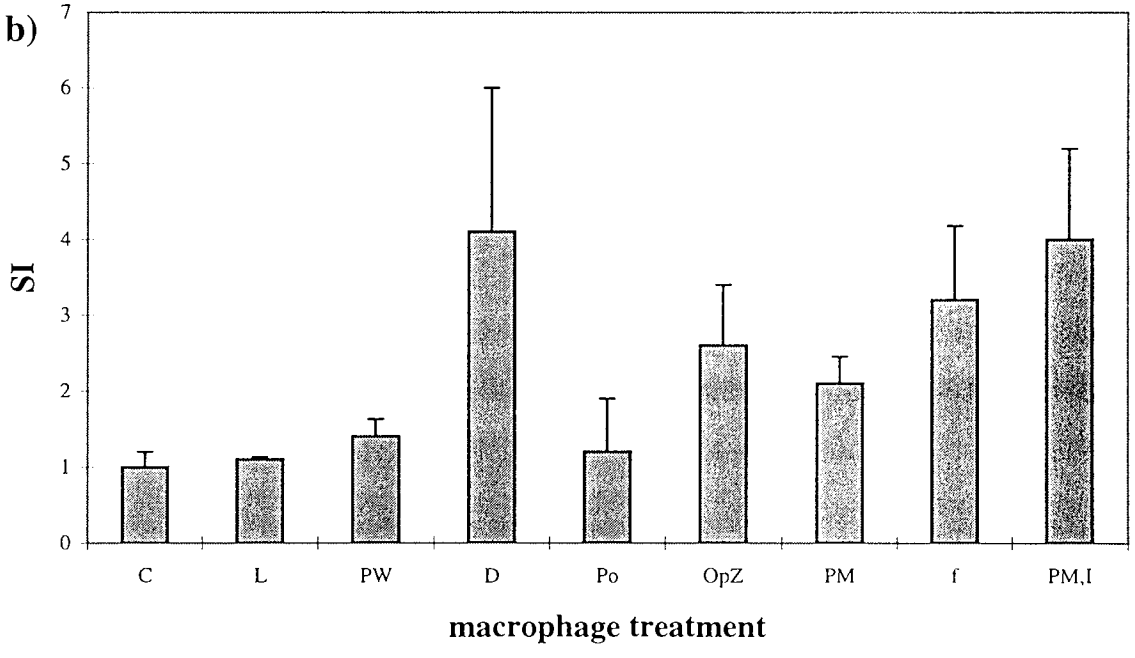


Splenocytes were cultured for 48 hours at a density of 5 x 10^5 cells per well with Con A (a), LPS (b), PWM (c), DS (d), Poly[I][C] (e) and PHA-P (f) and purified splenic macrophages over the range of 0 to 1 x 10^5 per well. The response to mitogen was partially restored with the addition of as few as 500 macrophages per well ($p<0.05$)*.

Figure 4.5 Splenocyte Proliferation with Activated-Fixed Macrophages (n=4)

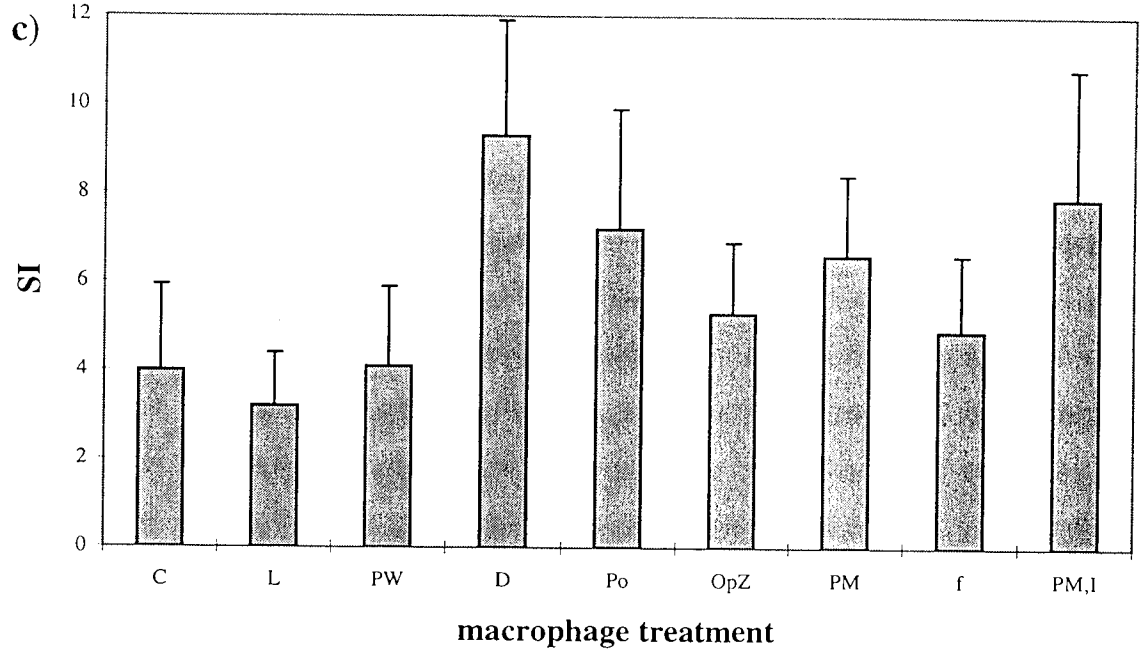


SI Controls: Mixed splenocyte culture = 61.1 +/- 11.3 Macrophage depleted = 14.6 +/- 3.2

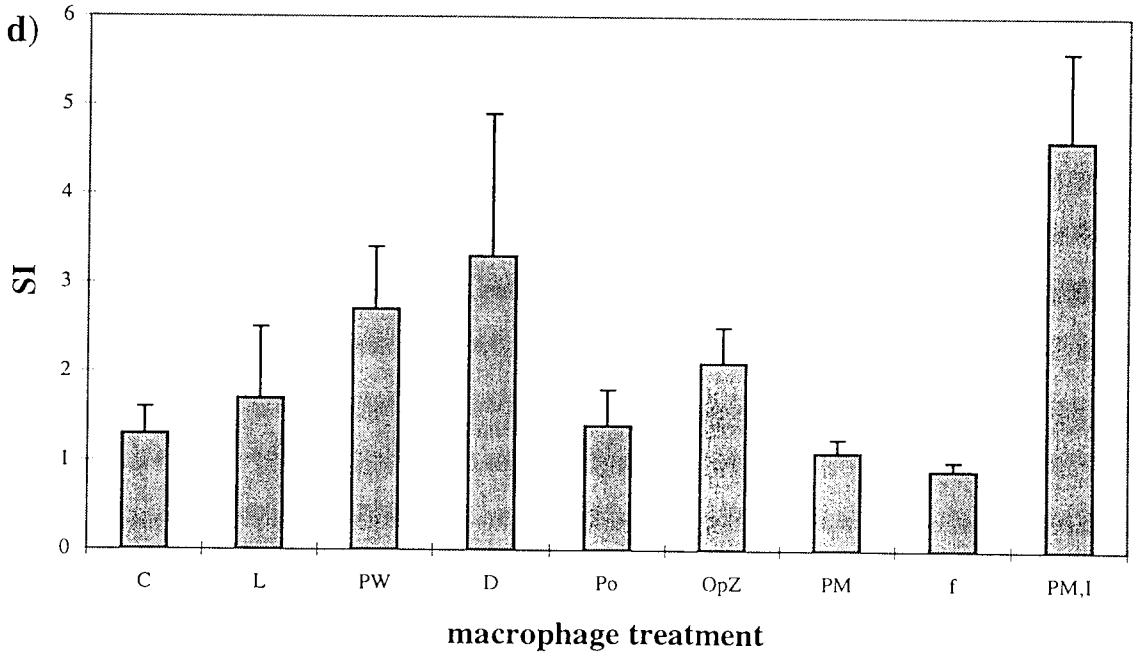


SI Controls: Mixed splenocyte culture = 16.8 +/- 4.4 Macrophage depleted = 3.2 +/- 1.3

Figure 4.5 Splenocyte Proliferation with Activated-Fixed Macrophages (n=4)

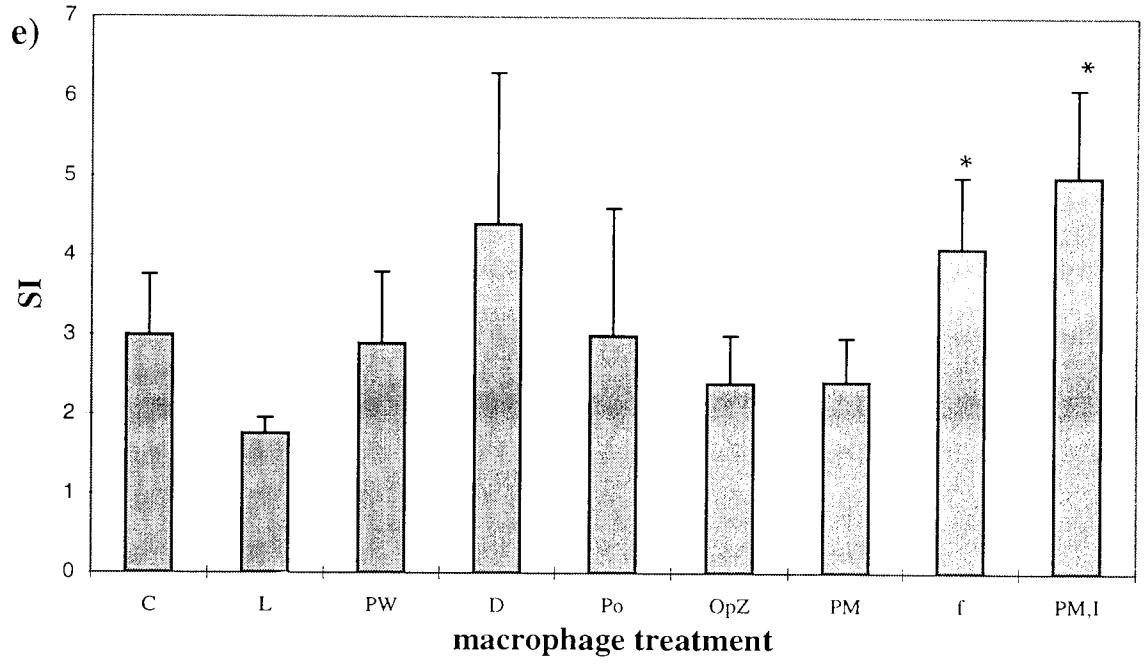


SI Controls: Mixed splenocyte culture = 11.7 +/- 3.0 Macrophage depleted = 3.3 +/- 1.2

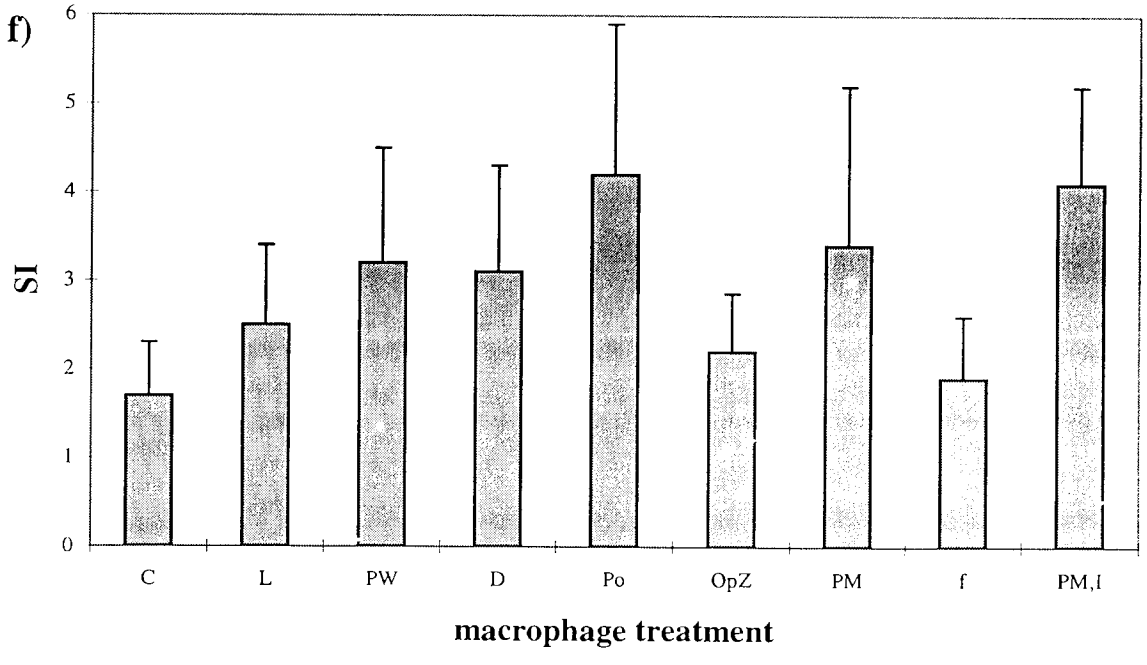


SI Controls: Mixed splenocyte culture = 9.4 +/-1.6 Macrophage depleted = 2.8 +/- 1.1

Figure 4.5 Splenocyte Proliferation with Activated-Fixed Macrophages (n=4)



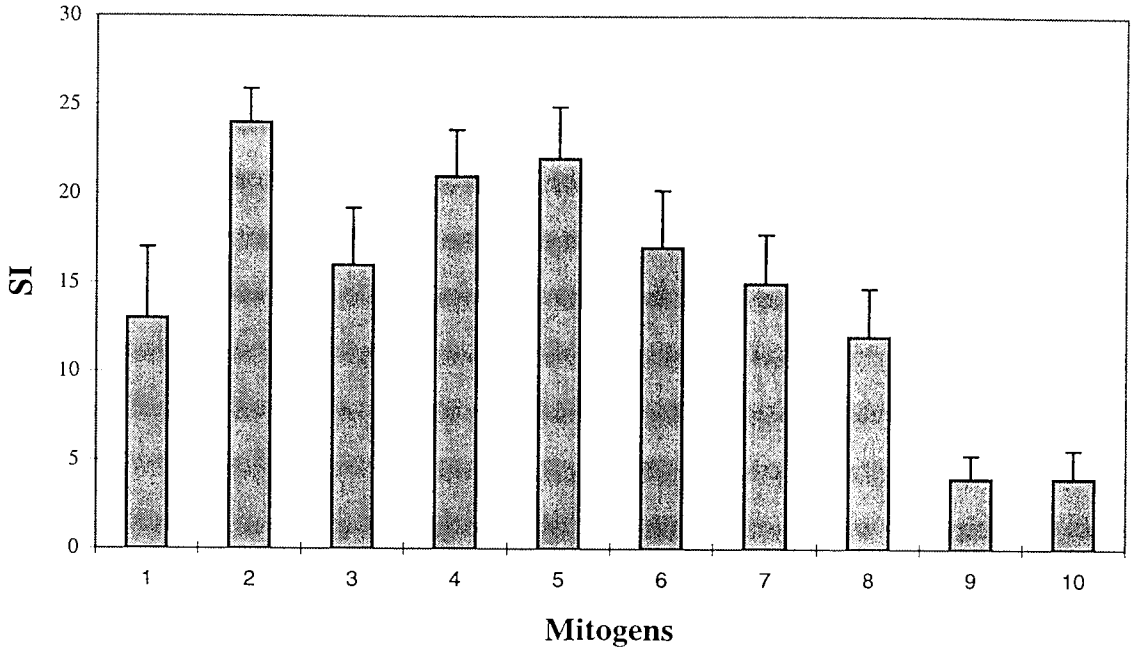
SI Controls: Mixed splenocyte culture = 11.6 +/- 2.3 Macrophage depleted = 1.9 +/- 0.6



SI Controls: Mixed splenocyte culture = 10.2 +/- 2.3 Macrophage depleted = 3.1 +/- 0.9

5 x 10^5 splenocytes and1000 fixed macrophages were cultured per well, with Con A (a), LPS (b), PWM (c), DS (d), Poly[I][C] (e) and PHA-P (f), $p<0.05$*. The macrophages had been pre-incubated for 1 h with optimum concentrations of Con A (C), LPS (L), PWM (PW), D (DS), Po (Poly[I][C], OpZ, PMA (PM), f (f-MLP) and PMA with Ionomycin (PM,I).

Figure 4.6 Effect ofCombinations of Mitogens on Splenocyte Proliferation (n=4)



5 x 10^5 splenocytes per well were cultured with a combination of mitogens (see Key) for 48 hours. Proliferation was assessed and expressed as an SI.

KEY:

1. LPS, PWM ; 2. LPS, DS ; 3. LPS, Poly[I][C] ; 4. PWM, DS ; 5. PWM, Poly[I][C] 6. DS, Poly[I][C] ; 7. LPS, PWM, DS ; 8. LPS, PWM, Poly[I][C] ; 9. LPS, DS, Poly[I][C] ; 10. PWM, DS, Poly[I][C].

4.4 Discussion

The mitogenic effects of Con A, LPS, PWM, DS, PHA-P and Poly[I][C] on T- and B-lymphocyte cultures was assessed by tritium incorporation. This method does accurately determine cell division over a short time period. The majority of the thymidine nucleotides entering DNA are derived from the endogenous pathway, however a constant proportion of tritiated thymidine does incorporate into the DNA. This may seem a dangerous assumption but there is considerable evidence to support this (Cooper *et al*, 1963). The most potent mitogen was the T-cell mitogen Con A. This was followed by the B-cell mitogen LPS which was only one third as potent. The T- and B-cell mitogen PWM was as potent as the B-cell mitogen Poly[I][C] and T-cell mitogen PHA-P and DS was only able to stimulate a nine-fold increase in cell proliferation (Figure 4.1). Mitogens therefore only activate a proportion of the cell population e.g. Con A is able to stimulate a larger percentage of T-cells than PWM. However different mitogens may activate the same sub-populations, as culturing with combinations of mitogens did not result in a cumulative proliferative response (Figure 4.9). These findings confirmed that the system employed was effective in determining cell responses to mitogen, as the data obtained corresponded with data from other sources (Rosenstreich *et al*, 1976; Rosenberg and Lipsky, 1979; Corbel and Melchers, 1983; Hu and Muller, 1994). This system could therefore be used to determine the requirement for the macrophage in T- and B-cell responses to the six mitogens investigated.

The depletion of macrophages from splenocyte cultures by adherence resulted in a weaker response to all six mitogens. However, replenishment with viable adherent cells did not regenerate the response, this could be due to the removal process affecting the cells in some way (Figure 4.2). This lack of responsiveness may also be attributed to changes in the differentiation state of adherent macrophages. Parkhouse and Dutton, 1966 described a similar phenomenon. They found that peritoneal macrophages isolated after adherence to plastic proved inhibitory with mouse splenocytes and PHA. Later experiments by Hibbs *et*

al, 1987, and Date *et al,* 1996 on splenocyte responsiveness to LPS with peritoneal macrophages isolated the 'macrophage suppressor factor' as an L-Arginine dependent mechanism that causes selective metabolic inhibition in target-cells. Thus macrophages in different states of differentiation may fulfill separate functions. It is therefore vital that the differentiation state of the macrophage is strictly controlled when investigating the influence they possess over lymphocyte responses to mitogen, hence macrophages allowed to adhere should not be used.

Macrophage depletion from lymphocyte cultures using the miniMACS separation technique also resulted in a significant reduction in the mitogenic responsiveness of purified T- and B-cells to the appropriate mitogens (Figures 4.3-4). Macrophages isolated by this method were able to replenish the response of T- or B-cells to mitogens, however a maximum response was not achieved when T-cells or B-cells alone were cultured with macrophages and their appropriate mitogens. This separation technique did not appear to alter the differentiation state of these macrophages compared to macrophages in control cultures, as they were both able to achieve lymphocyte activation in response to mitogen. The macrophages were separated from a single cell suspension of splenocytes immediately and not after a six to twentyfour hour incubation in a plastic petri dish which resulted in their differentiation to adherent cells. These results confirm that the macrophage is required by the lymphocyte in responses to mitogen and for a full response to occur T- and B-cells must be cultured together with macrophages.

Original work by Rosenstreich *et al,* 1976 demonstrated the need for macrophages in T-cell responses to Con A and PHA. In this situation there appeared to be two distinct mechanisms by which the macrophage functions, in the first the macrophage appeared to bind to the mitogen and then present it to the T-lymphocyte in a manner that induces cell activation. In the second the release of factor(s) from the macrophage then stimulated the activated T-cells. The requirement of macrophages in the lymphocyte response to Con A has also been confirmed by Oonishi *et al,* 1995. Their results also suggested that

macrophages play an important role in the proliferation of splenic lymphocytes and this was enhanced by the macrophages being pretreated with vitamin E. The aim of this study was to expand upon these findings by discovering how the macrophage was involved.

The involvement of macrophages in B-cell responses to LPS was first studied by Koenig *et al,* 1979 and Wetzel and Kettman, 1981 and later confirmed by Corbel and Melchers, 1983. Corbel and Melchers demonstrated that highly purified B-cells depleted of accessory cells do not respond to LPS, and accessory cells from different sources, such as peritoneal exudate, irradiated spleen cells, a bone marrow colony and the macrophage cell line P388 D1 all reconstituted LPS reactivity of the accessory cell-depleted B-cells. This study has also shown the need for splenic macrophages in response to other B-cell mitogens, such as PWM, DS and Poly[I][C] and that T-cells are not essential for B-cell responsiveness but are necessary to elicit an optimal proliferative response to mitogen. Similar findings were noted when T-lymphocytes were cultured with Con A, PWM or PHA-P and this abrogation of a response was again restored when macrophages were present. Due to the heterogeneity of macrophage populations only the requirement for splenic macrophages in response to splenic lymphocytes was investigated so that fair assumptions could be drawn related to the *in vivo* situation.

The number of macrophages required to restore lymphocyte responsiveness to the six mitogens was assessed in T- and B-cell cultures. T- and B-cells were cultured together as opposed to using purified T- or B-cell preparations, as a full response to mitogen was only achieved when all three cell types were present. The results obtained show that the addition of as few as 500 macrophages per 5×10^5 lymphocytes were able to partially restore responsiveness, which may correspond to a ratio of one macrophage to one thousand lymphocytes. In some instances the addition of only 50 macrophages per 5×10^5 lymphocytes achieved this. It is therefore unwise to presume that cell surface interactions between lymphocytes and macrophages are important in mitogenic responses. This finding was reinforced by the lack of restoration of a response when sufficient numbers of fixed

pre-treated macrophages were cultured with lymphocytes in the presence of mitogen. In the system examined by Corbel and Melchers between 30 and 100 macrophages per 5 x 10^4 B-lymphocytes were able to restore lymphocyte responsiveness to LPS (1 macrophage: 500 B-cells). The importance of using pure cultures of lymphocytes depleted of macrophages is paramount if conclusions are to be made from these results. FACS analysis confirmed that the relative purity of a culture separated by miniMACS was greater than 98% (see section 2.4.1). This may lead to a uniform error of up to 10,000 violating macrophages per 5 x 10^5 purified lymphocytes in a well. However even if this was the case and the macrophage: lymphocyte ratio was sufficient to allow cell surface interactions, the addition of 1000 fixed-treated macrophages per well could not effectively restore the lymphocyte response and this was at least double the number of viable-treated macrophages able to achieve this.

Many other studies have displayed the obligate need for macrophages in response to mitogens. Much research has focused on the T-Independent type-2 antigen TNP-Ficoll (Chused *et al,* 1976; Boswell *et al,* 1980; Claassen *et al*, 1986; Van den Eertwegh *et al,* 1992). These findings are of interest as not only do they demonstrate macrophage requirement *in vitro*, but *in vivo* experiments have demonstrated that the events take place in the marginal zone of the spleen, as TNP-Ficoll localizes in the MZ within 20 minutes of immunization (Van den Eertwegh *et al,* 1992). This provides further evidence that the MZ is highly important in the induction of humoral TI-2 immune responses. In this instance the role of the MZ macrophage appears to be primarily removal of particulate antigen as opposed to antigen presentation (indeed their study did not find a need for cell surface interactions), but there may be a further need for the release of a soluble mediator(s). The cells involved in the induction of immune responses to TI-2 antigens appear to be B-cells and accessory cells.

In summary, this chapter has demonstrated the requirement of splenic macrophages in splenic lymphocyte responsiveness to the six mitogens Con A, LPS, PWM, DS, PHA-P

and Poly[I][C]. The re-addition of macrophages to depleted cultures indicated that a ratio of one macrophage to one thousand lymphocytes was able to reconstitute the response at least partially if not completely in all cases. There appears to be a need for T-cells, B-cells and macrophages to be cultured together in order to achieve a maximum proliferative response to mitogen, even with T-Independent antigens. It may be merely coincidental that T-cells release mitogenic factors that the B-cells are able to respond to as T-cells are certainly not necessary for B-lymphocyte responsiveness to TI-1 antigens (the lack of requirement for T-cells in TI-2 responses is not so defined). The evidence provided in this chapter leads to the possibility that soluble mediators produced by the macrophage are essential for lymphocyte proliferation in response to mitogen. The following chapter examines the effect of macrophage products on lymphocyte responsiveness to mitogen.

5. EFFECT OF MACROPHAGE PRODUCTS ON LYMPHOCYTE PROLIFERATION

5.1 Introduction

The macrophage performs various functions in the induction of an immune response, central to this is the extensive secretory capability they possess. Over 100 hundred substances are secreted by macrophages, including cytokines, growth factors, complement components, enzymes, eicosanoids, ROIs and coagulation factors (Nathan, 1987). A single biological end point may reflect the cumulative action of many macrophage products and furthermore, few macrophage products are secreted solely by them. Some secretory products, such as complement components, lysozyme and apolipoprotein E are synthesized and secreted continuously, whereas others are only released upon appropriate stimulation.

The importance of putative macrophage products such as IL-1 and PAF in the lymphocyte response to TI antigens has been investigated (Hoffmann *et al,* 1979; Ryffel *et al,* 1997). There are a number of experiments in which supernatants have been taken from macrophages and these have subsequently been cultured with purified T- and B-cells to stimulate proliferation of these cells when mitogen is present (Corbel and Melchers, 1983; Endres *et al,* 1983). Thus, IL-1 is known to enhance the proliferation of helper T-cells, and can partially replace the need for macrophages in the B-cell response to antigen (Sinha *et al,* 1987; Goud *et al,* 1988; Oonishi *et al,* 1995). In addition IL-1, IL-10 and TNF may directly stimulate B-cell proliferation and IL-6 is a growth and differentiation factor for B-cells (Kehrl *et al,* 1987; Fluckiger *et al,* 1994; Takeuchi and Katayama 1994; Burdin *et al,* 1995; Bundschuh *et al,* 1997). The accessory cells or factors may also induce residual T-cells to secrete the factors neccessary for B-cell differentiation (Unanue and Allen, 1987). Thus, IL-5; a T-cell-derived factor substitutes for accessory cells in certain TI-2 antigen responses (O'Garra *et al,* 1986).

Therefore the macrophage secretes a number of products which could influence T- and B-cell responses. Certainly, evidence suggests that the macrophage does have a role in lymphocyte responsiveness to mitogen. The aims of this chapter are to investigate how important macrophage products are in lymphocyte responses and to establish the characteristics of any possible factor(s). For instance the conditions and time constraints under which macrophages produce such factor(s). TI-1 and TI-2 immune responses will also be compared, as will the importance of macrophage supernatants in T-cell responses to Con A, PWM and PHA-P. This chapter will also provide definitive proof as to whether macrophage-lymphocyte surface interactions are necessary for lymphocytes to proliferate in response to mitogen.

5.2 Experimental Design

Splenocytes, purified splenic T-cells, B-cells and macrophages were prepared as previously described in Chapter 2 and cultured at a density of 5 x 10^5 cells per well (50 µl aliquots) in 96 well flat bottomed microtitre plates for 48 hours in 5 CO_2, 95% air at 37°C. To some of these cultures optimum concentrations of the six mitogens Con A, LPS, PWM, DS, Poly[I][C] and PHA-P which were previously described in Chapter 3 were added in 10 µl aliquots.

Since macrophage depletion impairs responses to T- and B-cell mitogens, initial experiments investigated whether supernatants from various macrophage cultures could restore responsiveness. Macrophage supernatants (spts) were prepared by incubating 1 x 10^5 macrophages in one ml of supplemented medium with a range of activators. Initially, the ten activators investigated in Chapter 3; Con A, LPS, PWM, DS, Poly[I][C], PHA-P (all 1 µg/ml), OpZ (1 mg/ml), f-MLP (10^{-5}M), and a combination of PMA and Ionomycin (both 10^{-8}M) were used. These concentrations gave the maximum oxidative burst. The macrophages were incubated with these chemicals for 30 minutes, which was roughly at the peak of the oxidative burst and 90 minutes which was at the end of the burst activity. Later experiments focused on only three activators; Con A, OpZ and LPS and supernatants were obtained after 30, 60 and 120 minutes, and 24 hours. To eliminate any unbound Con A or LPS from supernatants in experiments where the mitogenic effect of the supernatants alone was assessed, methyl D mannopyranoside (10 µg/ml) or polymyxin B (5 µg/ml) was added to bind to free mitogen (Ballou and Raschke, 1974; Jacobs and Morrison, 1977). The opsonized Zymosan A particles were removed by micro-centrifugation. Control supernatants were obtained in two ways. Firstly by incubating macrophages alone for given time periods (these supernatants were used as controls in the following experiments) and secondly, by placing the macrophages with the activators and then centrifuging immediately so that the supernatants were the same as test supernatants i.e. contained residual amounts of the activators. When these supernatants (10 µl) were then added to the lymphocyte

cultures the final volume was made up to 100 µl. Therefore, there was a ten-fold dilution. Control supernatants prepared by either method were not found to be mitogenic (data not shown).

In all such experiments a positive control (splenocytes with all cell types present) and a negative control (splenocytes depleted of macrophages) were included. Any cultures that did not show a good separation were discarded (as assessed by their ability to respond to mitogen), as were cultures displaying <95% viability. This ensured that the effect of supernatants alone on lymphocyte responsiveness to mitogen was measured.

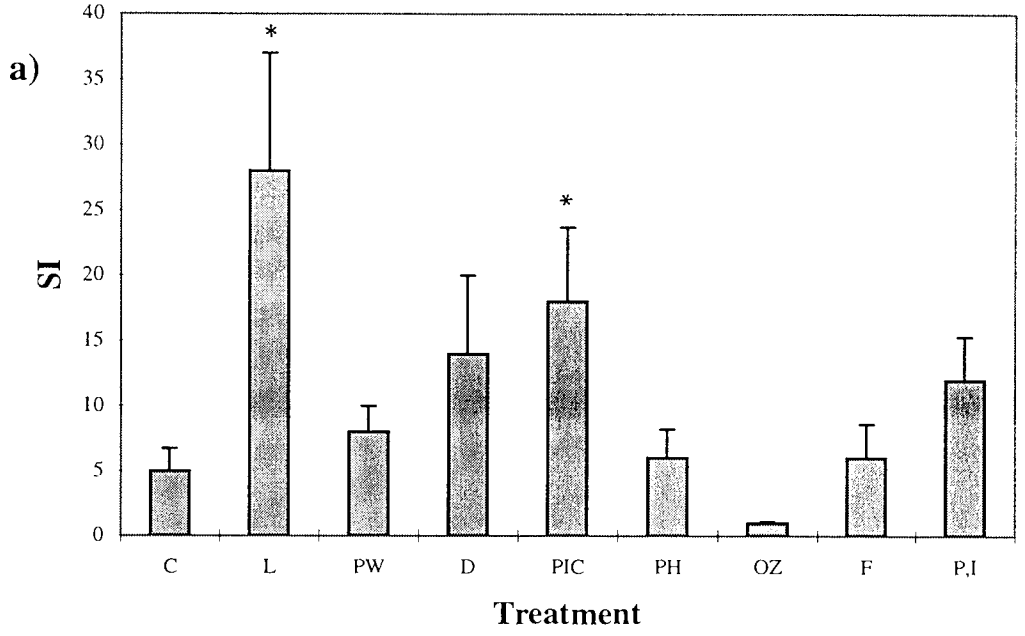
5.3 Results

5.3.1 Effect of Macrophage Supernatants on Lymphocyte Responses to Mitogen

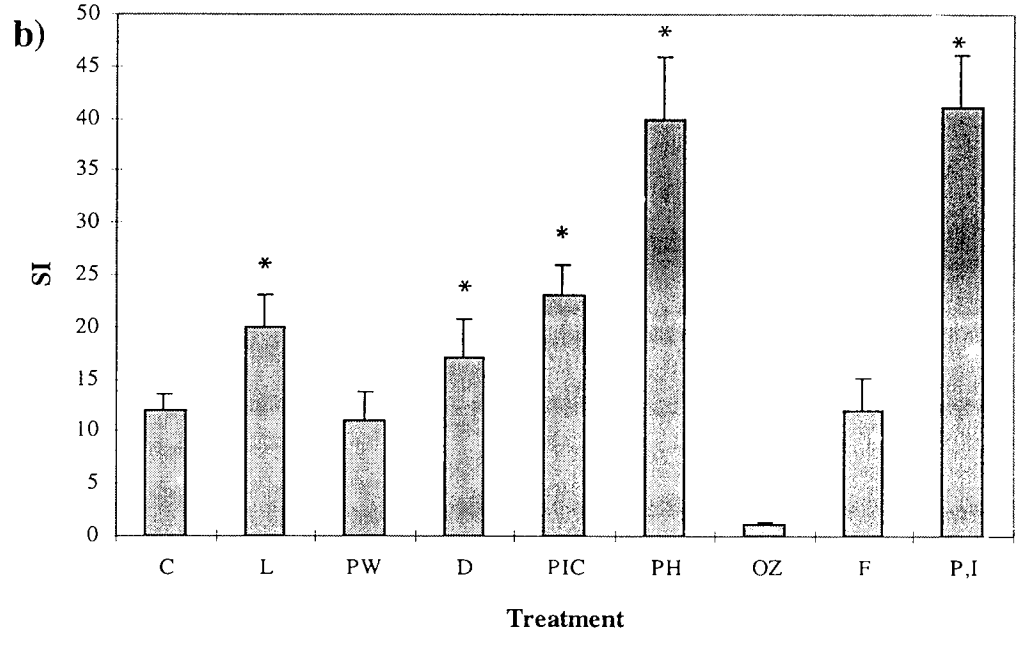
When splenocyte cultures containing macrophages were treated with Con A for 48 hours the SI was 48.2 +/- 12.9, whereas if these cultures were depleted of macrophages the SI was only 7.1 +/- 2.4. Supernatants from macrophages treated for only 30 minutes with some of the activators were able to give a partial restoration (Figure 5.1a). Thus, supernatants from a macrophage culture treated with LPS enhanced the SI to 27.2 +/- 8.7 ($p<0.05$). Supernatants from macrophages treated with PWM, DS, Poly[I][C] also gave some restoration. Treatment of macrophages with Con A, PHA-P, f-MLP and OpZ gave only a very modest restoration. By 90 minutes all of the putative macrophage activators with the exception of OpZ now gave significant restoration. Clearly the additional incubation time with PMA and the PMA/Ionomycin mixture was required for the release of material(s) which facilitated the mitogenic effect of Con A. Although it may be argued that the combination of PMA and Ionomycin could be mitogenic in its own right (Guy *et al*, 1985), concentrations were very low (10^{-9}M) and indeed were the same in Figures 5.1a and 5.1b.

Similarly macrophage supernatants had varying effects on the ability of LPS to promote mitogenesis in macrophage-depleted splenocytes. Once again only a 30 minute incubation period was required for LPS and DS to cause release of soluble co-mitogenic factors from the macrophage ($p<0.05$). Con A, PWM, f-MLP and PMA/Ionomycin seemed to require longer incubation times and generally were less effective (Figure 5.2a and 5.2b). Although OpZ could generate the most powerful oxidative burst, it did not generate any co-mitogenic substances to promote the effects of either Con A or LPS in splenic lymphocytes. When PWM, DS, Poly[I][C] and PHA-P were used as the mitogens a similar pattern of events were obtained (data not shown).

Figure 5.1 Effect of Supernatants on Lymphocyte Responsiveness to Con A (n=4)

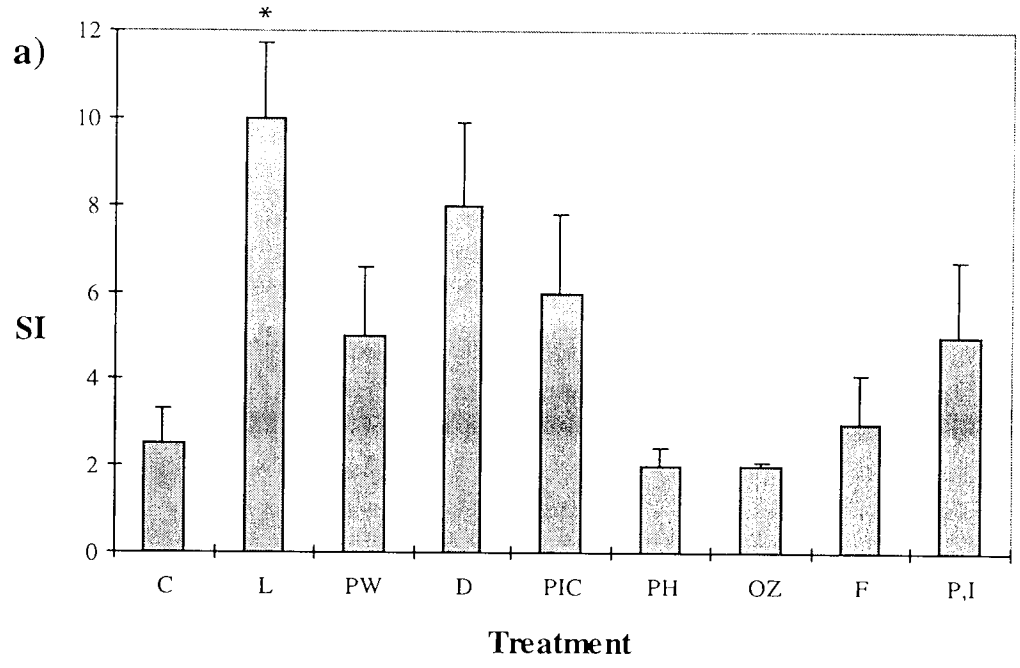


SI Controls: Mixed splenocyte culture = 48.2 +/- 12.9 Macrophage depleted = 7.1 +/- 2.4

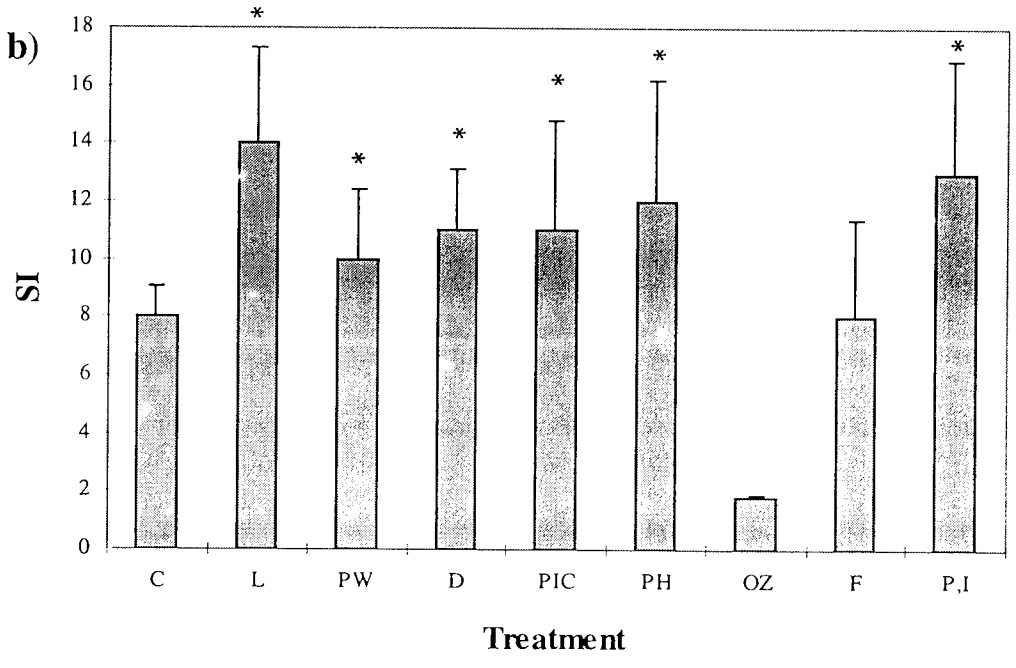


5 x 10^5 macrophage-depleted splenocytes were cultured with macrophage spts taken after an a) 30 minute or b) 90 minute incubation with Con A (C), LPS (L), PWM (PW), DS (D), Poly[I][C] (PIC), PHA-P (PH), OpZ (OZ), f-MLP (F) and PMA with Ionomycin (P,I), $p<0.05$*.

Figure 5.2 Effect of Supernatants on Lymphocyte Responsiveness to LPS (n=4)



SI Controls: Mixed splenocyte culture = 18.4 +/- 3.9 Macrophage depleted = 4.6 +/- 1.3



5 x 10^5 macrophage-depleted splenocytes were cultured with macrophage spts taken after an a) 30 minute or b) 90 minute incubation with Con A (C), LPS (L), PWM (PW), DS (D), Poly[I][C] (PIC), PHA-P (PH), OpZ (OZ), f-MLP (F) and PMA with Ionomycin (P,I), $p<0.05$*.

5.3.2 Effect of Macrophage Supernatants on Lymphocyte Responses to Mitogen

Further experiments were then carried out using only three macrophage activators: Con A, OpZ and LPS, to confirm the observations found previously (Figures 5.3a and b). The macrophages were incubated for 30 minutes, 1, 2 and 24 hours. Supernatants derived from Con A treated macrophages were able to partially restore responsiveness after one hour and this continued up to 24 hours ($p<0.05$). However, the supernatants from macrophages incubated with LPS produced a significant response ($p<0.01$) that was up to six-fold greater than that of Con A and this was achieved after only 30 minutes. The factor(s) must be metabolised or degraded after a longer time period as the activity diminishes over a 24 hour incubation. OpZ supernatants were only able to achieve a partial increase in proliferation after 24 hours.

Although supernatants from Con A- and LPS-treated macrophages were able to restore mixed splenic lymphocyte responsiveness to both B- and T-cell mitogens they were not found to be mitogenic in their own right (Figure 5.4a-c). In fact in nearly all cases a SI of less then 2 was achieved and this was not significantly different to control values. Thus, macrophage derived factors are also required for B- and T-lymphocytes to enter DNA synthesis in response to mitogenic signals.

5.3.3 Effect of Macrophage Supernatants on B-Cell Responsiveness to Mitogen

When highly purified B-cells were cultured in the presence of LPS for 48 hours there was no significant stimulation. Supernatants from macrophages treated with LPS and Con A ($p<0.05$) but not OpZ allowed some mitogenic effect to be manifest (Figure 5.5), with LPS this was achieved after only 30 minutes. The low stimulation indices reflect the difficulty in culturing a pure population of B-cells.

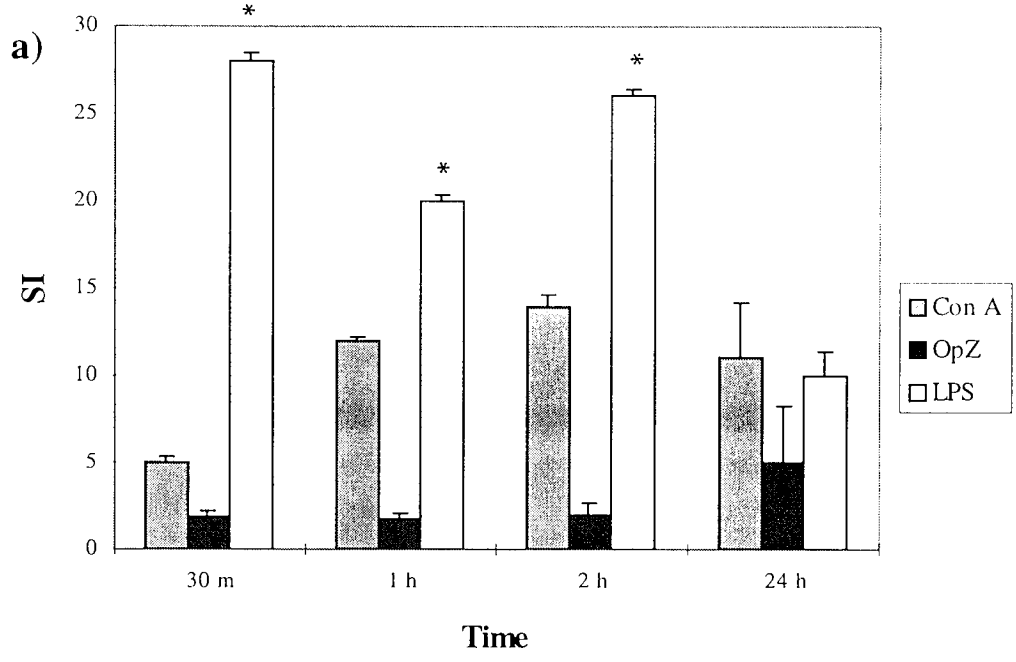
5.3.4 Effect of Macrophage Supernatants on T-Cell Responsiveness to Mitogen

Similarly highly purified T-cells only responded to Con A if supernates from macrophages treated with LPS or Con A were also present, $p<0.05$ (Figure 5.6). Once again LPS appeared to promote secretion faster than Con A and the material exhibited some lability over a 24 hour incubation period. Alternatively this could be the late release of an inhibitory factor(s).

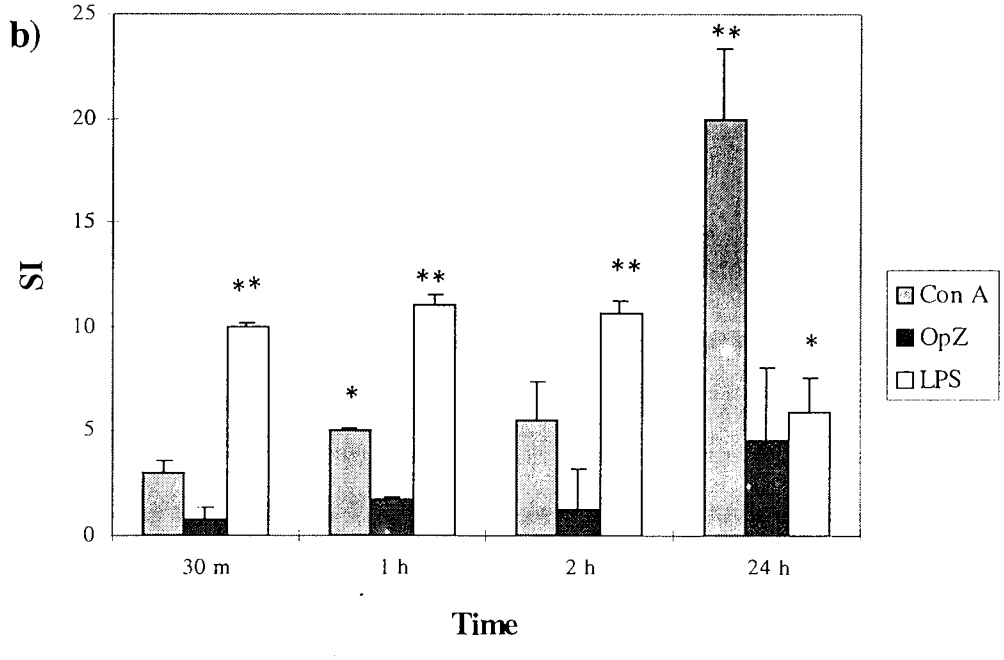
It should be noted that SI's achieved with LPS and Con A in these highly purified B- and T-cell preparations was very much less than when mixed cultures were employed, even though cell viabilities were very similar. This perhaps suggests the existence of several mutually cooperative stimuli operating between all three of the cell types in question.

In Figure 5.7 the response to the mitogens Con A and LPS was examined in purified cultures of T- or B-cells and in co-cultures involving both cell types. In some instances purified macrophages, or supernatants from LPS-treated macrophages were added to the lymphocyte cultures. It must be emphasised that only autologous cell mixtures were used. In all cases the ability of lymphocytes to respond to the mitogens was profoundly impaired in the absence of macrophages. The addition of 1000 macrophages to 5×10^5 lymphocytes stimulated proliferation 4- to 6-fold in all instances. Supernatants from macrophage cultures which had been exposed to LPS for 2 hours partially restored T-cell responsiveness to Con A and almost completely restored B-cell responsiveness to LPS. These experiments also revealed that T-cells responded better to Con A if B-cells were also present. Likewise B-cells showed better responses to LPS if co-cultured with T-cells.

Figure 5.3 The Effect of Supernatants on Lymphocyte Responses to Mitogen (n=8)



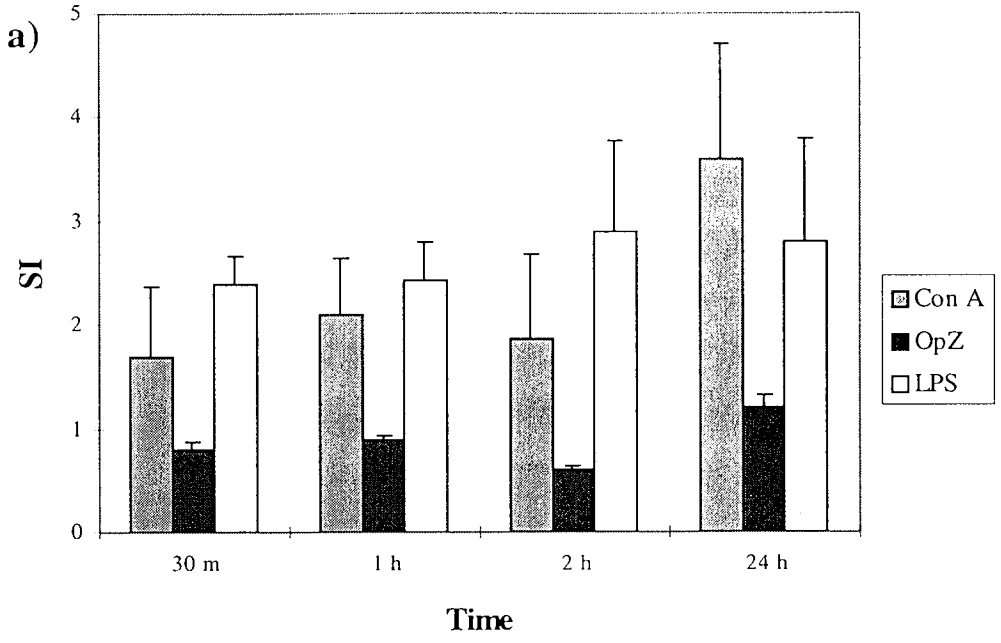
SI Controls: Mixed splenocyte culture = 79.9 +/- 15.3 Macrophage depleted = 11.4 +/- 4.2



SI Controls: mixed splenocyte culture = 12.1 +/- 1.3 Macrophage depleted = 2.2 +/- 0.4

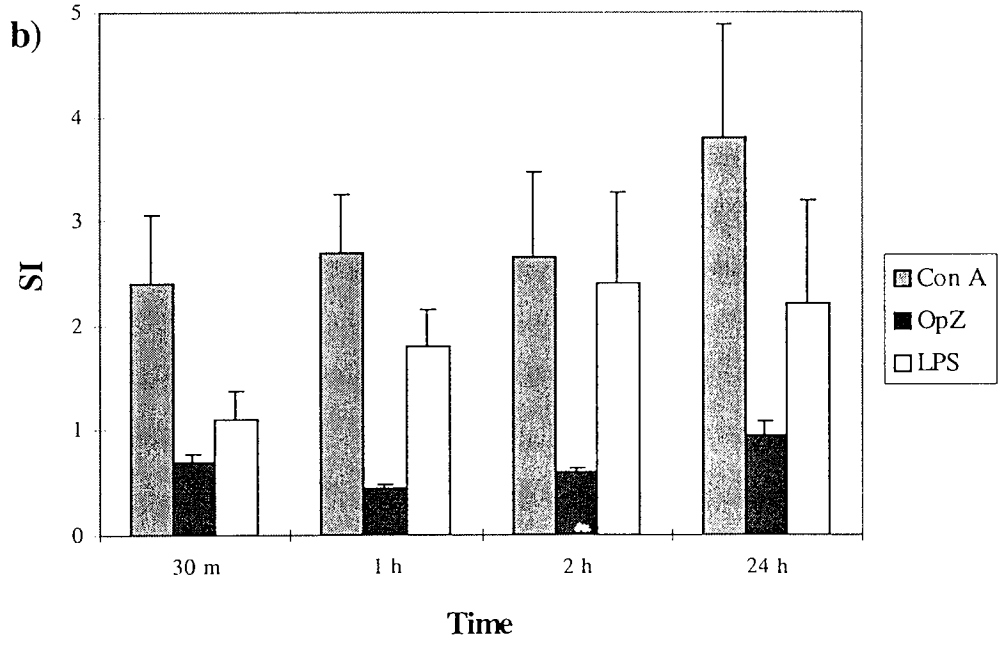
5 x 10^5 macrophage-depleted splenocytes were cultured with Con A (a) and LPS (b) plus, macrophage supernatants taken after a 30 min, 1, 2, and 24 hour incubation with Con A, LPS or OpZ, $p<0.05$*, $p<0.01$**.

Figure 5.4 Effect of Macrophage Supernatants Alone on Lymphocyte Proliferation (n=4)



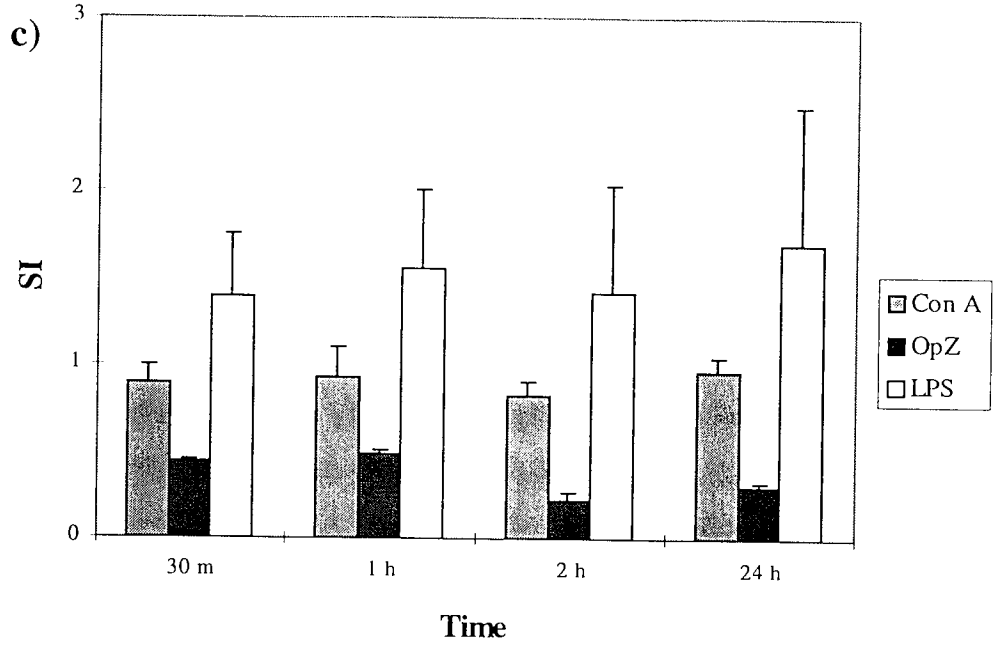
SI Controls (Con A): Mixed splenocyte culture = 49.6 +/- 5.8 Macrophage depleted = 6.4 +/- 1.3

SI Controls (LPS): Mixed splenocyte culture = 13.8 +/- 1.9 Macrophage depleted = 2.5 +/- 0.9



SI Controls (Con A): Splenic T-cell culture = 22.0 +/- 4.3 Macrophage depleted = 4.1 +/- 2.7

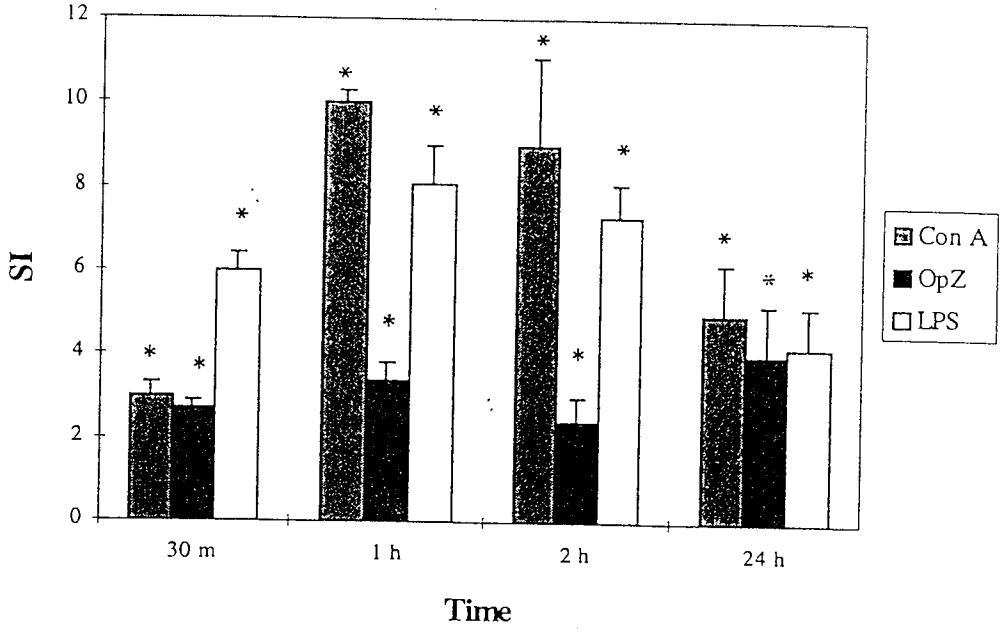
Figure 5.4 Effect of Macrophage Supernatants Alone on Lymphocyte Proliferation (n=4)



SI Controls (LPS): Splenic B-cell culture = 6.1 +/- 0.8 Macrophage depleted = 1.7 +/- 0.5

5 x 10^5 macrophage-depleted splenocytes (a), purified T-cells (b) and purified B-cells (c) were cultured with macrophage supernatants obtained after a 30 min, 1, 2, and 24 hour incubation with Con A, LPS or OpZ.

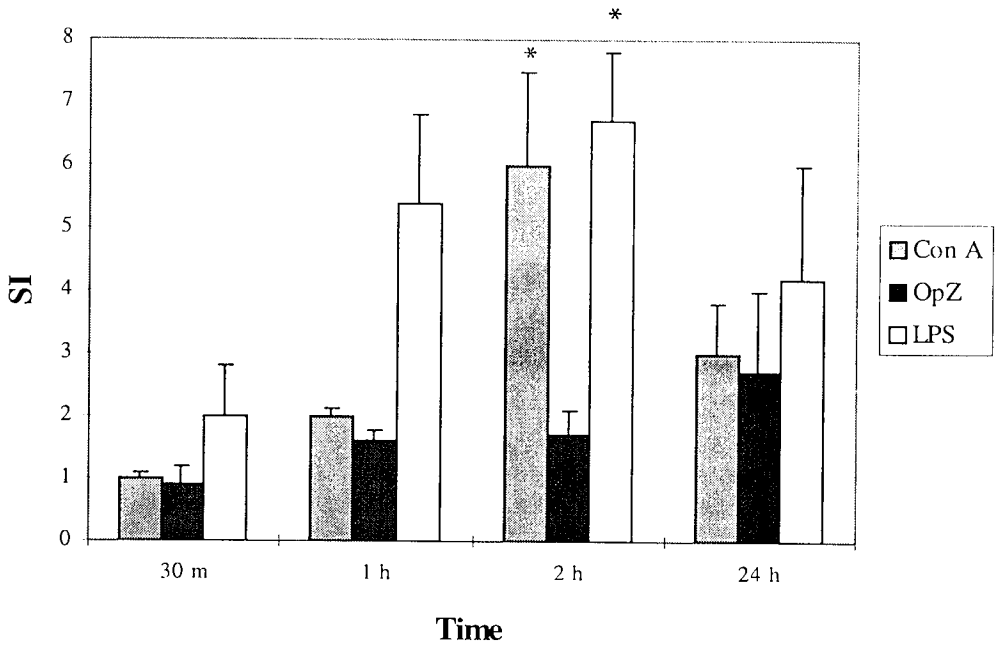
Figure 5.5 Effect of Macrophage Supernatants on B-Cell Responsiveness to LPS (n=4)



SI Controls: Splenic B-cell culture = 5.6 +/- 1.1 Macrophage depleted = 0.7 +/- 0.1

5×10^5 purified B-cells were cultured with mitogen and macrophage supernatants taken after a 30 minute, 1, 2, and 24 hour incubation with Con A, LPS or OpZ, $p<0.05$*.

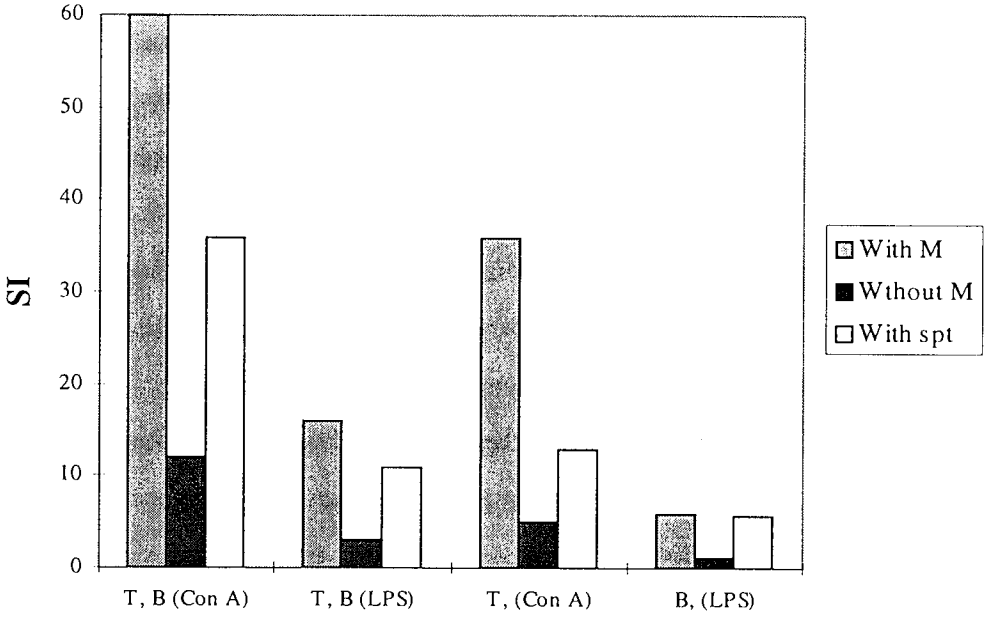
Figure 5.6 Effect of Macrophage Supernatants on T-Cell Responsiveness to Con A (n=4)



SI Controls: Purified T-cell culture = 21.0 +/- 3.6 Macrophage depleted = 3.2 +/- 0.7

5 x 10^5 purified T-cells were cultured with mitogen and macrophage supernatants taken after a 30 minute, 1, 2, and 24 hour incubation with Con A, LPS or OpZ, $p<0.05$*.

Figure 5.7 Proliferation of T- and B-Cells In Response to Con A and LPS With or Without Macrophages or Their Supernatants (n=3)

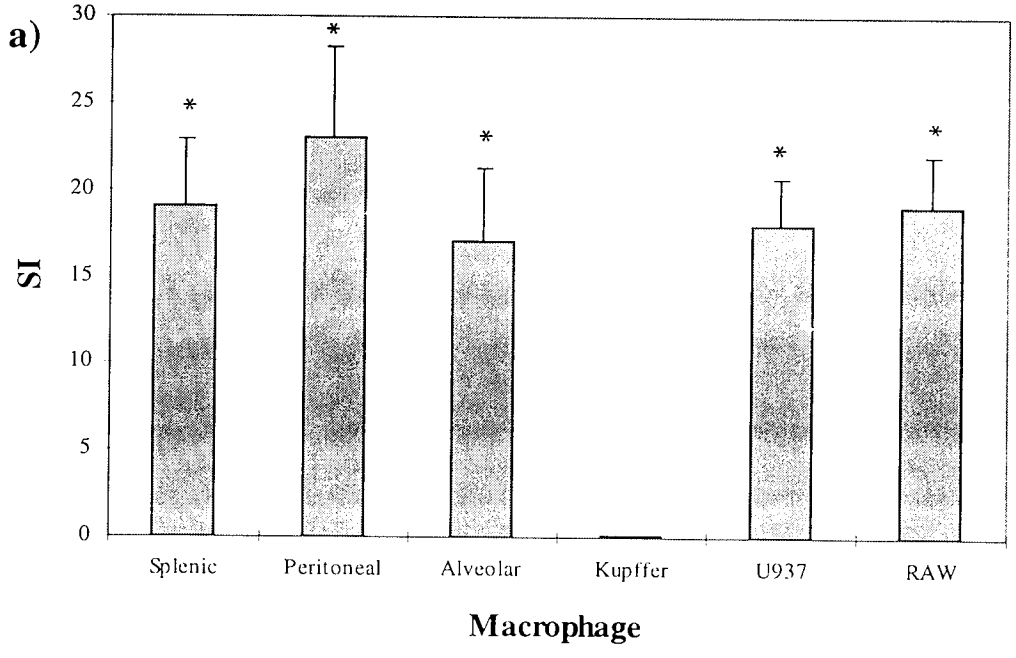


5 x 10^5 purified T- and B-cells (T,B), T-cells (T) and B-cells (B) were cultured with Con A and LPS as shown. Some cultures were replenished with 1000 macrophages per well (With M) and others with supernatants obtained from macrophages treated with LPS for 2 hours (With spt). Residual LPS was removed from the supernatants with polymyxin B. The remaining lymphocytes were incubated without macrophages or supernatants (Without M).

5.3.5 Effect of Supernatants From Different Macrophage Populations on Lymphocyte Responses to Mitogen

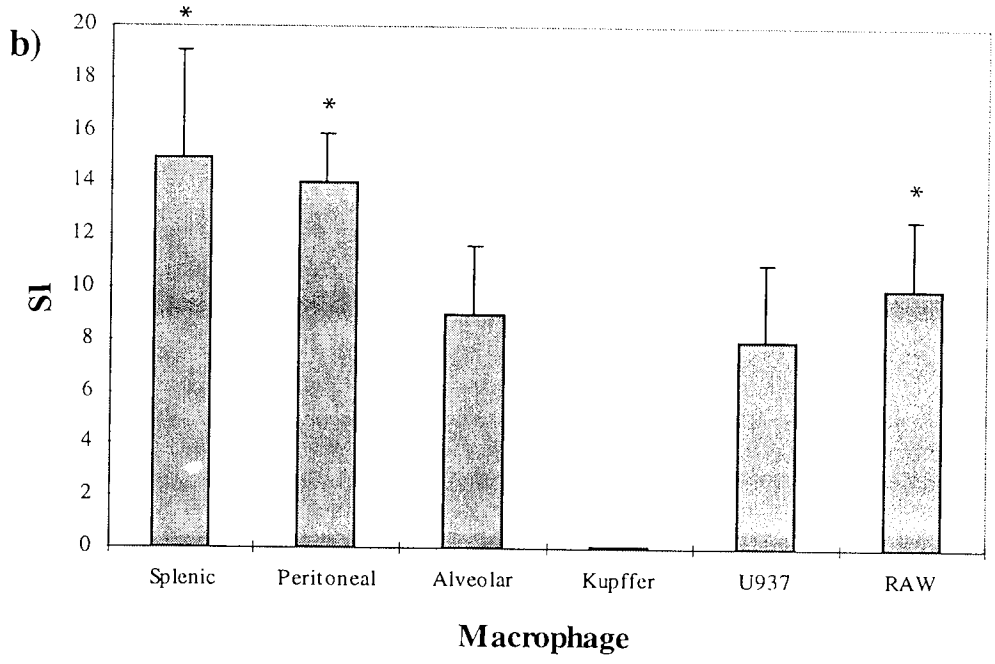
Macrophages derived from the spleen, peritoneum, lung and liver and the RAW 264.7 cell line and the myelomonocytic cell line, U937 were incubated for two hours with LPS. LPS was chosen, as the supernatants from LPS-treated macrophages were the most effective in restoring proliferation at this time point. Supernatants from splenic, peritoneal and alveolar macrophages, and the RAW 264.7 and U937 cells were all able to significantly restore macrophage-depleted splenocyte responsiveness to the mitogens Con A and LPS (Figures 5.8a and b) ($p<0.01$). Kupffer cells were unable to achieve this. The supernatants from LPS-treated splenic, peritoneal, alveolar and RAW 264.7 macrophages did partially stimulate proliferation when mitogen was not present, but in all instances this did not reach significance (Figure 5.8c).

Figure 5.8 Effect of Supernatants From Different Macrophage Populations on Lymphocyte Responses to Mitogen (n=4)

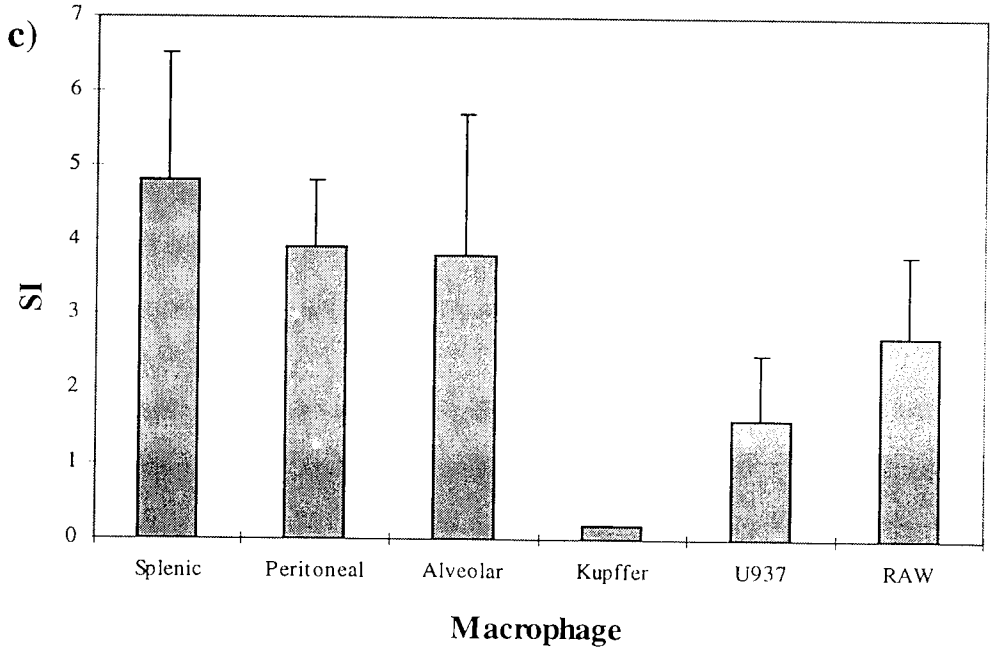


SI Controls: Mixed splenocyte culture (Con A) = 50.8 +/- 7.7 Macrophage depleted = 5.3 +/- 1.4

Figure 5.8 Effect of Supernatants From Different Macrophage Populations on Lymphocyte Responses to Mitogen (n=4)



SI Controls: Mixed splenocyte culture (LPS) = 16.5 +/- 3.9 Macrophage depleted = 4.1 +/- 1.8



SI Control: macrophage spts after no LPS treatment, macrophage depleted = 1.9 +/- 1.5

5 x 10^5 T- and B-cells were cultured with a) Con A, b) LPS, or c) No mitogen, and macrophage supernatants taken after a 2 hour incubation with LPS, $p<0.01$*.

5.4 Discussion

These experiments have demonstrated that certain macrophage supernatants can at least partially restore lymphocyte responsiveness to mitogen without the actual need for macrophages to be present. This confirms that the mitogen response is not dependent on cell surface (cognate) interactions between lymphocytes and macrophages, but there is a strong requirement for soluble products which can be provided by the macrophage.

Supernatants obtained from macrophages incubated with the mitogens LPS, PWM, DS, Poly[I][C] and PHA-P for only 30 minutes were able to partially restore lymphocyte responsiveness to Con A and LPS, this effect was further improved when macrophages were incubated with these chemicals for 90 minutes (Figures 5.1 and 5.2). These results have demonstrated that lymphocytes require a soluble product(s) secreted by the macrophage in order to respond to mitogen. Hence after a 90 minute incubation macrophages were able to release greater quantities of the factor(s) which were able to restore the immune response. LPS-stimulated macrophage supernatants were the most effective at restoring responses to LPS, whereas PHA-P supernatants were for Con A. These findings suggest that T- and B-cells may require a different macrophage-derived factor(s) to enable them to respond to the appropriate mitogen. This research has elaborated on work initially carried out by Corbel and Melchers, 1983. In which they demonstrated that supernatants obtained from macrophages treated with LPS were able to restore B-lymphocyte responses to LPS. This study has investigated the need for macrophages (or their supernatants after an extensive range of treatments) in purified T- and B- cell cultures, as well as mixed cultures, in response to six different mitogens which include TI-1 (LPS) and TI-2 (Poly[I][C]) antigens.

When macrophages were incubated with Con A and f-MLP, stimulatory material only appeared in the supernatants after 90 minutes (Figures 5.1-5.3). This indicates that it takes longer for these compounds to stimulate the secretion of the required product(s), perhaps they activate a different cell signalling mechanism to that of the agents previously mentioned or perhaps they stimulate the production of different mediators.

Although OpZ was a potent stimulator of the oxidative burst in macrophages it was unable to induce the secretion of the factor(s) necessary for lymphocyte proliferation when incubated with mitogen (Figure 5.3). During the oxidative burst an array of reactive oxygen and nitrogen intermediates are formed, some of which are known to inhibit cell growth, for example nitric oxide (Eisenstein *et al,* 1994; Liew 1995). Other inhibitory factors may also be released in response to OpZ, such as arachidonate metabolites (Burch, 1987). LPS in contrast had very little capacity to stimulate the release of ROIs but was most effective in the production of the co-mitogenic factor. It appears that different receptors may employ different signal transduction mechanisms to direct function towards ROI production or cytokine secretion. Environmental niches also direct macrophage differentiation resulting in functional heterogeneity. This phenomenon has been demonstrated, as macrophages from different micro-environments were not equally able to release a stimulatory co-mitogenic factor(s) (Figure 5.8). In fact, Kupffer cells were unable to produce any of the relevant factor(s). These cells possess very specific functions within the liver. They are involved in the clearance of particulate and soluble substances and express Fc and other receptors, such as the mannosyl-fucosyl receptor (MFR), CD14 and CD33. It is believed that the response of Kupffer cells to LPS and other gut-derived stimuli may be important in their interactions with hepatocytes (Fuller *et al*, 1987). Indeed these cells may be at least partially responsible for regulating the acute phase response in injury and malignancy by producing IL-6 which appears to trigger the altered metabolism associated with the acute phase response.

Macrophage supernatants after co-incubation with PMA and Ionomycin for 90 minutes (more effective than 30 minutes) also restored responsiveness. It might be argued that PMA and Ionmycin would be transferred to the lymphocyte cultures within the supernatants and could themselves be directly mitogenic (Guy *et al*, 1985). However final concentrations of these in the lymphocyte cultures would be reduced to 10^{-9}M which our own experiments (Griffin and Perris, unpublished) have shown are without effect. Guy *et al* also found that these mitogenic effects were at 10^{-7}M.

T-cells exhibit a greater ability to undergo cell division after treatment with Con A than the same number of B-cells do with LPS and there is increased DNA synthesis when T- and B-cells are cultured together with macrophages or their supernatant in response to mitogen (Figures 5.5-5.7). Factor(s) secreted by the macrophage in response to LPS and Con A were not significantly mitogenic (Figure 5.4). They do however provide a vital second signal or accessory signal which facilitates proliferation when the mitogens bind to the lymphocyte surface.

Having demonstrated the ability of the macrophage to produce a factor(s) which is essential in lymphocyte responses to mitogens the next step is to investigate what this factor(s) could be. The macrophage produces over 100 different compounds it would therefore be impossible to screen each one of these in this study. However, this chapter has helped to isolate the possible candidates by investigating some of the characteristics of this product(s). The factor(s) must be secreted after only a short period of time (as LPS stimulated the macrophage to produce this factor(s) after only 30 minutes). It must have growth promoting characteristics and be able to influence lymphocyte development, but it must not be able to sustain cell division in its own right. It therefore seems logical to investigate cytokine production by the macrophage i.e. IL-1, IL-10, IL-12, IFN-α and TNF-α for growth promoting properties and for the early phase factors such as eicosanoids and PAF. Unfortunately, it is very hard to measure PAF due to its lack of immunogenicity. It would also be interesting to measure the production of oxidative metabolites to discover whether they may be the cause of OpZ's inhibitory effect on proliferation.

6. MACROPHAGE PRODUCTS

6.1 Introduction

As discussed previously in chapter 5, the macrophage secretes a diverse range of products, some of which may be required by the lymphocyte in response to mitogen. The factor(s) in question must be released quickly (after only 30 minutes in the case of LPS, see Figure 5.3). Possible candidates may therefore include eicosanoids and reactive oxygen intermediates (ROI). Cytokines which in other investigations have been found to stimulate DNA synthesis, must also be examined. The cytokines IL-1, IL-10, IFN-α and TNF-α were chosen as they are known from other studies (Kehrl *et al,* 1987; Fluckiger *et al,* 1994; Takeuchi and Katayama 1994; Trubiani *et al,* 1994; Burdin *et al,* 1995; Bundschuh *et al,* 1997) to have some effect on lymphocyte proliferation.

IL-1 is produced predominantly by macrophages and mediates activation of both T-cells and B-cells, it does stimulate proliferation in the presence of mitogen but this action is weaker than that of the T-cell derived factors IL-2 and IL-4 (Takeuchi and Katayama 1994). IL-10 is produced by Th2 cells, monocytes, macrophages and B-cells (O'Garra, *et al,* 1990; Fine *et al,* 1994). It displays potent stimulatory effects towards B-cells (Fluckiger *et al,* 1994; Burdin *et al,* 1995). Quiescent B-cells respond to IL-10 by the up-regulation of class II MHC antigen expression, which could facilitate cell surface interactions between the B-cell and macrophage. It promotes the proliferation and differentiation of B-cells pre-activated with either anti-Ig or a combination of anti-CD40 and IL-4 (Jumper *et al,* 1995). IFN-α is produced by a wide range of cell types including macrophages and lymphocytes (Mosmann and Coffman, 1989). It can potentiate the stimulation caused by anti-Ig of B-cells resulting in proliferation and promotes Ig secretion, but in this case the effect of mitogen-driven Ig secretion was concentration-dependent, low concentrations providing a stimulus and high being inhibitory (Trubiani *et al,* 1994). There are two forms of TNF (TNF-α and β). In B-cell assays both forms show positive effects on lymphocyte proliferation and synergise with

IL-2, IL-4 and IFN-γ in supporting B-cell proliferation Kehrl *et al,* 1987; Bundschuh *et al,* 1997).

Macrophages are a major source of eicosanoids and their release constitutes an major aspect of macrophage activation, they are involved in the inflammatory response and can feedback on the macrophage to alter its function (De Rose *et al,* 1997). Eicosanoids include ThromboxaneB_2, ProstaglandinE_2 and LeukotrieneB_4. They are all derived from arachidonate, which is converted into Leukotrienes by Lipoxygenase and Prostaglandins or Thromboxanes by Cyclo-Oxygenase. Their action is highly localised because they are short lived. Some examples of the actions of eicosanoids are the inhibition of lipolysis, stimulation of inflammation, regulation of blood flow, control of ion transport across membranes, modulation of synaptic transmission and inhibition of tumour cytolysis. The complexity of their interactions with cells is illustrated by their ability to stimulate adenylate cyclase in some cells and inhibit it in others (Marshall *et al,* 1994).

During phagocytosis reactive oxygen intermediates are formed, this is known as the oxidative or respiratory burst and results in the reduction of oxygen to the superoxide anion O_2^-. Other reactive oxygen intermediates which are extremely toxic are then produced (Johnston *et al,* 1985). The oxidative burst also generates other powerful oxidising agents, including reactive nitrogen intermediates. Some examples of these are nitrogen oxide (NO), nitrogen dioxide and nitrous acid, these combine with the superoxide anions to produce extremely potent anti-microbial substances directed against fungal, helminthic, bacterial and protozoal pathogens (Bhagat and Vallance, 1996). Nitric oxide has also been shown to inhibit proliferation of T- and B-cells (Eisenstein *et al,* 1994; Liew 1995).

Having described the actions of a number of possible macrophage products which may be important in lymphocyte proliferation in response to mitogen, macrophage supernatants were collected after various treatments and the levels of IL-1, IL-10, IFN-α, TNF-α, prostaglandin E_2, leukotriene B_4, thromboxane B_2 and nitric oxide were measured.

6.2 Experimental Design

To obtain macrophage supernatants, 1 x 10^5 splenic macrophages separated using miniMACS were suspended in 1 ml of supplemented RPMI 1640 medium with or without optimum concentrations of Con A, OpZ or LPS (as assessed in chapter 3). The macrophages were only used if they displayed a viability of greater than 95%. Supernatants were collected after a 30 minute, 1, 2, 5 and 24 hour incubation in 95% air, 5% CO_2 at 37°C. After collection they were stored at -70°C until required (this was for no longer than one week). A proliferation assay was also set up to ensure that the supernatants had the capacity to restore mitogenic responses in macrophage-depleted cultures. Indeed supernatants from all LPS- and Con A-treated macrophages (treated for 2 hours) gave a partial restoration of the mitotic response (data not shown) confirming earlier experiments (Figures 5.1-5.7). The assay was performed in the same manner as those in the previous chapter.

ELISAs were performed according to the manufactures' protocol (see chapter 2). ELISAs analysing cytokine concentrations found in the supernatants were all from R & D Systems (section 2.5.2.1) and eicosanoids were measured using kits from Cayman Chemical Company (section 2.5.2.2), as was nitric oxide (section 2.5.2.3).

The expression of the early activation marker CD69 was measured on macrophages using a FACScan to investigate how quickly the macrophage responded to LPS and OpZ. The macrophages were dual labelled with an antibody to murine CD11b conjugated to FITC, and an antibody to murine CD69 conjugated to R-PE after being incubated for 30 minutes and 2 hours with optimum concentrations of OpZ and LPS as well as just medium. The dual labelling ensured that only the expression of CD69 on macrophages was measured. For a full explanation of the FACScan see section 2.6.3.

6.3 Results

6.3.1 Cytokine Production by Macrophages

Macrophage production of IL-1β increased over the first five hours, Figure 6.1. LPS was the most potent and rapid stimulator, increasing secretion six-fold after 5 hours. This was followed by Con A which stimulated a three-fold increase in IL-1β release and lastly OpZ, only increasing secretion two-fold. There were only transient changes in IL-10 production. Con A induced a 1.5-fold increase after 5 hours and OpZ doubled the amount released, however LPS had no effect (Figure 6.2). A two hour treatment with both LPS and Con A caused a four-fold increase in IFN-α (Figure 6.3). Once again LPS seemed to be the faster acting secretagogue. There was no increase in IFN-α release in OpZ-treated macrophages. As with IL-1, the most potent inducer of TNF-α release was LPS, with a five-fold increase being achieved over 24 hours, Con A and OpZ both stimulated a two to three fold increase during this time period (Figure 6.4).

6.3.2 Eicosanoid Production by Macrophages

Con A was the only activator able to stimulate increases in ProstaglandinE_2 (PGE_2) release (Figure 6.5). LeukotrieneB_4 (LTB_4) secretion however did increase in response to Con A, OpZ and LPS, although controls were quite variable (Figure 6.6). LPS and Con A both induced a five- to six-fold increase in LTB_4 and OpZ a three-fold increase. The basal levels of ThromboxaneB_2 (TXB_2) were approximately 25 times larger than that of PGE_2 and LTB_4 (Figure 6.7). OpZ and Con A stimulated a marked increase in TXB_2 production (approximately double), whereas LPS did not cause any substantial increase in release.

Figure 6.1 IL-1β Production by Macrophages (n=2)

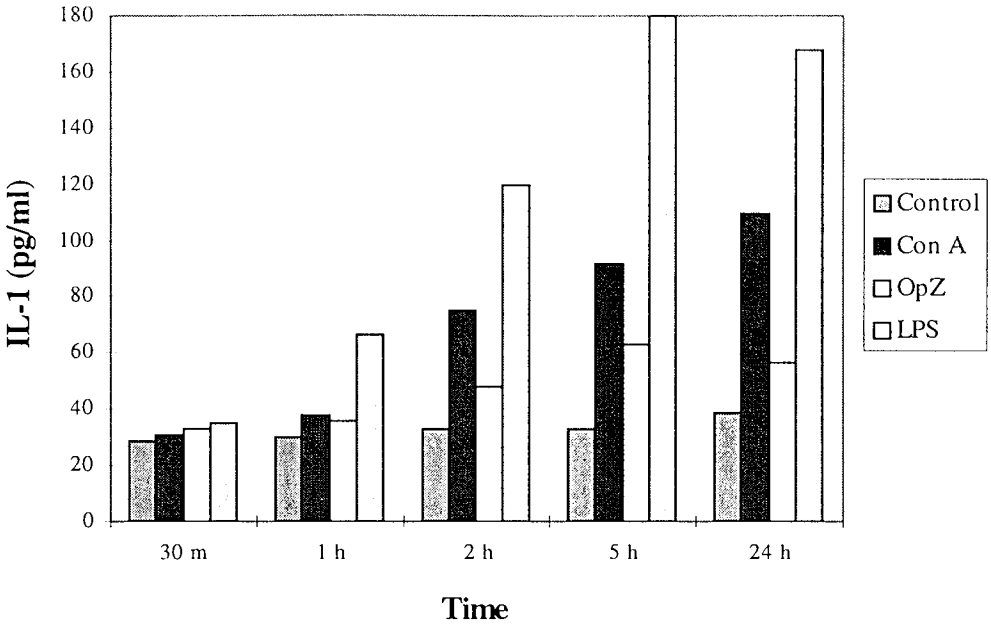
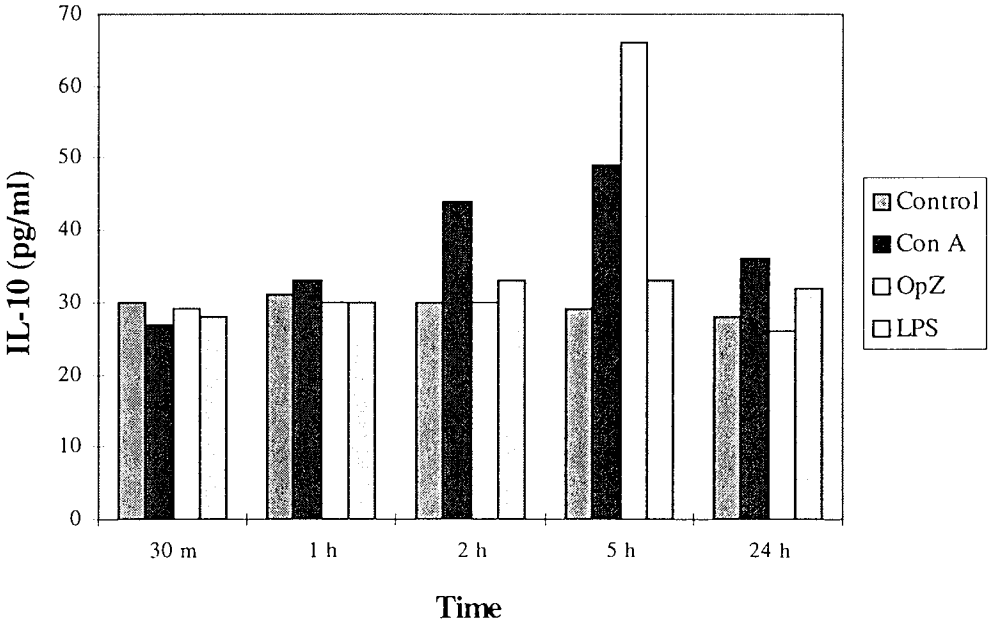


Figure 6.2 IL-10 Production by Macrophages (n=2)



1 x 10^5 macrophages were incubated at 37°C for 30 min, 1, 2, 5 and 24 h with Con A (1 μg/ml), OpZ (I mg/ml) and LPS (1 μg/ml) in 1 ml of supplemented medium. ELISA kits (R & D Systems) were used to measure IL-1β and IL-10 levels.

Figure 6.3 Production of IFN-α by Macrophages (n=2)

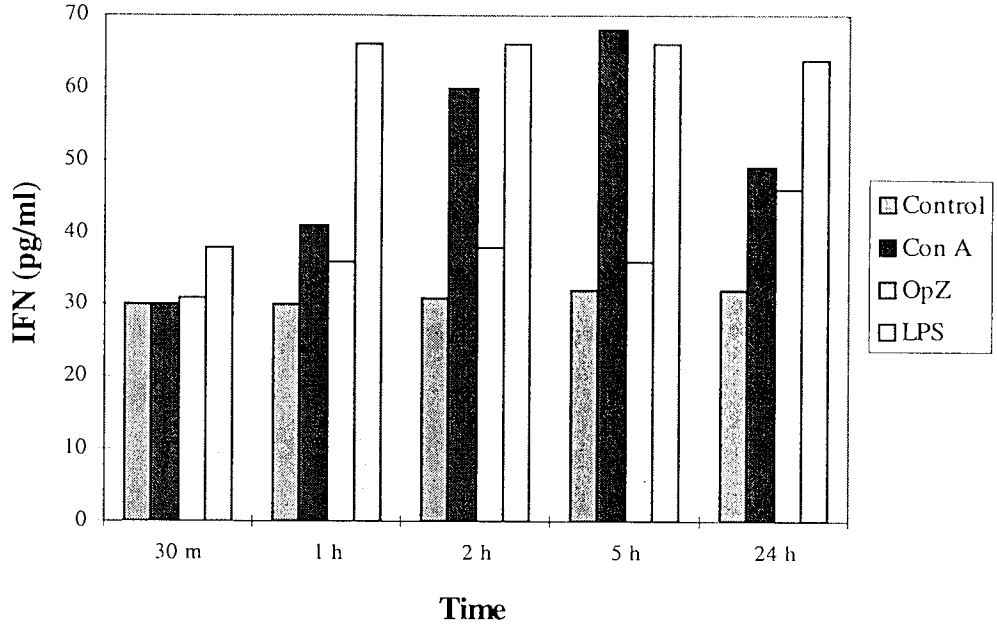
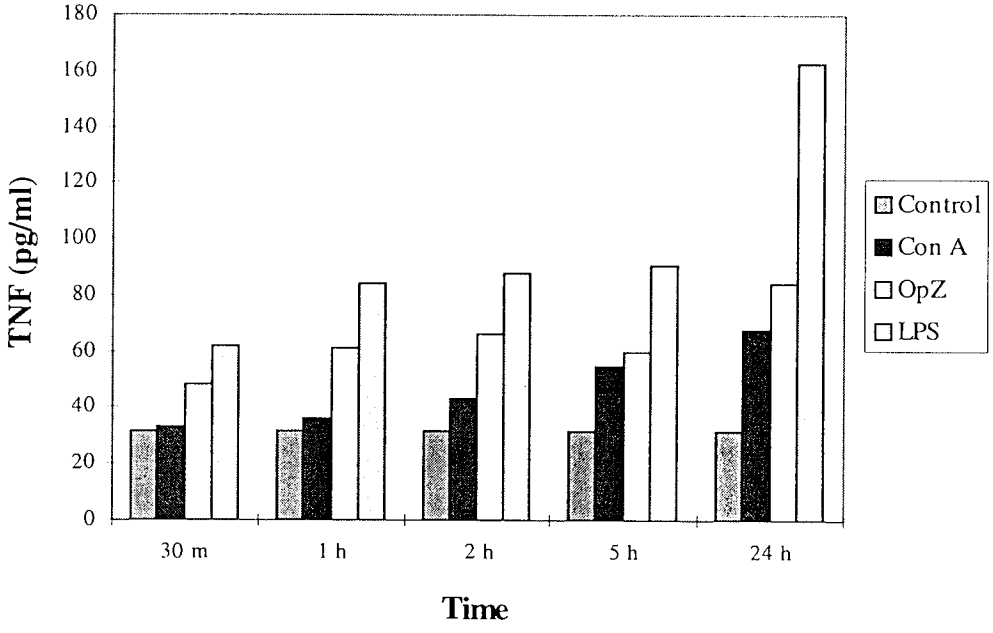


Figure 6.4 Production of TNF-α by Macrophages (n=2)



1 x 10^5 macrophages were incubated at 37°C in 95 % air, 5 % CO_2 for 30 min, 1, 2, 5 and 24 hours with Con A (1 μg/ml), OpZ (I mg/ml) and LPS (1 μg/ml) in 1 ml of supplemented medium. ELISA kits (R & D Systems) were used to measure IFN-α and TNF-α levels.

Figure 6.5 Production of PGE_2 by Macrophages (n=2)

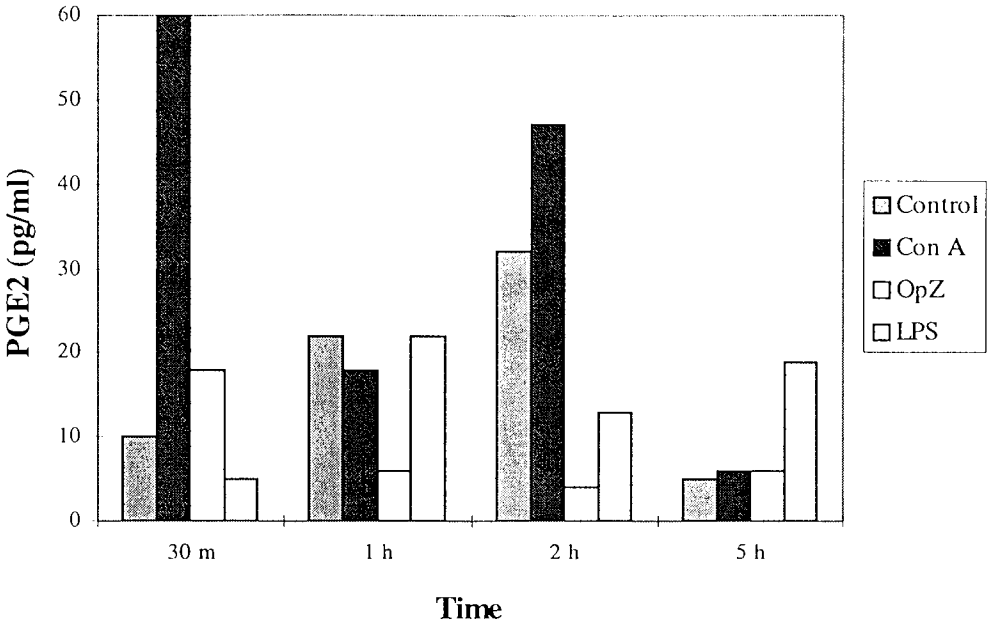
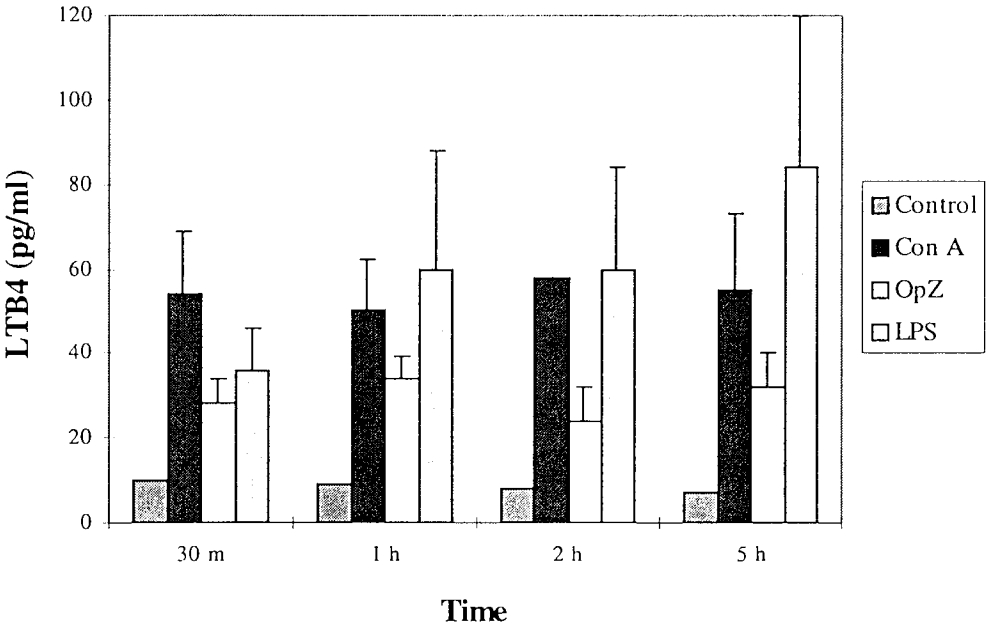
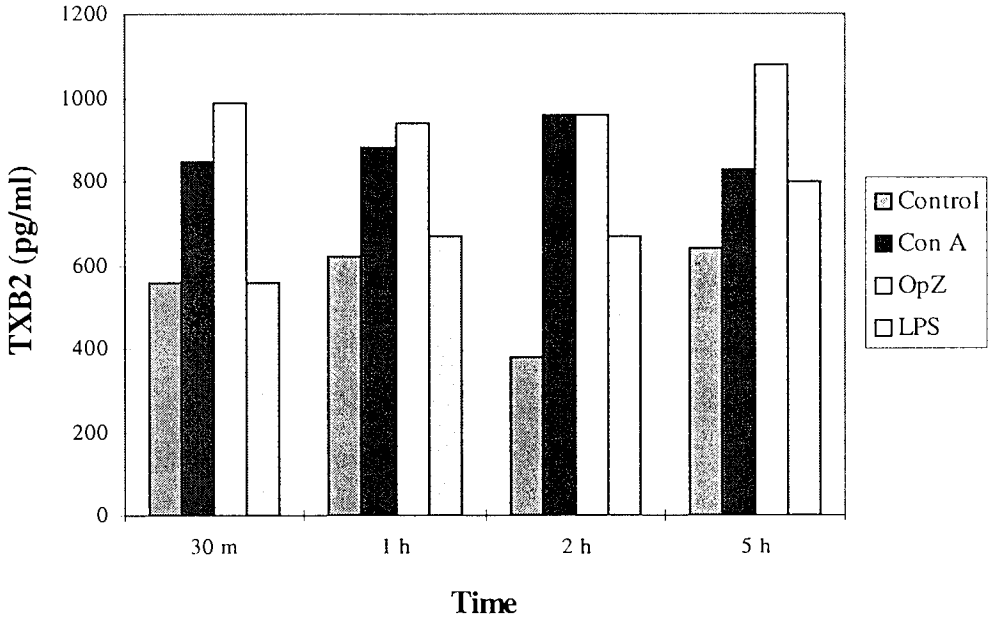


Figure 6.6 Production of LTB_4 by Macrophages (n=3)



1 x 10^5 macrophages were incubated at 37°C in 95 % air, 5 % CO_2 for 30 min, 1, 2, 5 and 24 hours with Con A (1 μg/ml), OpZ (I mg/ml) and LPS (1 μg/ml) in 1 ml of supplemented medium. ELISA kits (Cayman Chem. Co.) were used to measure PGE_2 and LTB_4 levels.

Figure 6.7 Prodcution of TXB_2 by Macrophages (n=2)

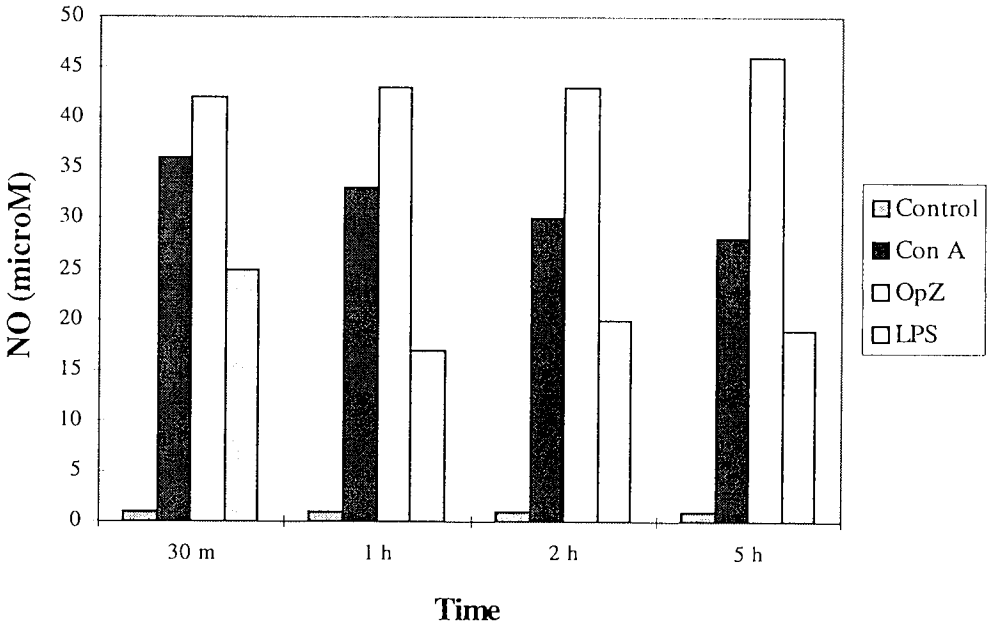


1 x 10^5 macrophages were incubated at 37°C in 95 % air, 5 % CO_2 for 30 min, 1, 2, 5 and 24 hours with Con A (1 μg/ml), OpZ (I mg/ml) and LPS (1 μg/ml) in 1 ml of supplemented medium. ELISA kits (R & D Systems) were used to measure TXB_2 levels.

6.3.3 NO Production by Macrophages

OpZ was the most potent stimulator of Nitric Oxide. The basal concentration remained at 1 μM throughout the 5 hours examined and this increased to approximately 45 μM by OpZ at 5 hours, see Figure 6.8 below. This was closely followed by Con A (approximately 32 μM) and LPS (approximately 22 μM). A summary of these several macrophage-derived secretory products is given in Table 6.1.

Figure 6.8 Production of NO by Macrophages (n=2)

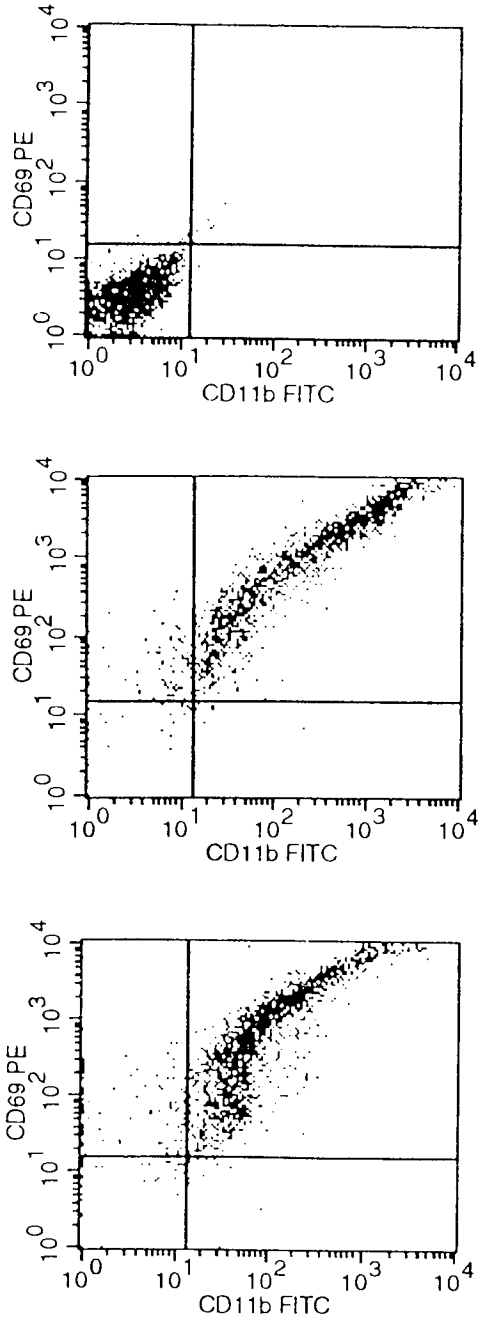


1 x 10^5 macrophages were incubated at 37°C in 95 % air, 5 % CO_2 for 30 min, 1, 2 and 5 hours with Con A (1 μg/ml), OpZ (1 mg/ml) and LPS (1 μg/ml) in 1 ml of supplemented medium. The NO assay kit utilising the Griess reaction from Cayman Chemical Company was employed.

6.3.4 CD69 Expression on Macrophages

Analysis of CD11b bearing cells revealed that expression of the early phase cell activation marker CD69 increased from 1 % to over 90% after a 30 minute incubation with LPS and similar findings were achieved with OpZ, see Figure 6.9.

Figure 6.9 The Expression of CD69 on Macrophages



1 x 10^5 macrophages (top) were treated with LPS (1 μg/ml) for 30 minutes (middle) and 2 hours (bottom) and fixed in paraformaldehyde, washed and resuspended in 1 ml of PBS 'A'. They were then labelled with anti-C11b-FITC antibody and anti-CD69-R-Pe and the expression of CD11b and CD69 was measured. Similar results were found with OpZ (1 mg/ml) data not displayed.

Table 6.1 Summary of Macrophage Products

Factor	Concentration in Supernatant after 2 hour Treatment with:			Mitogenic Potential
	LPS	Con A	OpZ	
IL-1β	++	+	-	B- and T-cell
IL-10	—	+	—	B-cells
IFN-α	+	+	—	-
TNF-α	++	—	+	B-cells
PGE_2	--	+	--	Inhibitory
LTB_4	++	++	+	-
TXB_2	+	++	++	-
NO	+	++	++	Inhibitory

++ : large increase + : increase — : no change - : decrease --:large decrease

6.4 Discussion

The data for supernatants from Con A- and LPS-treated macrophages did give an overall picture of altered cytokine profiles, however OpZ only appeared to partially increase levels of IFN-α (after 24 h) and TNF-α. Although OpZ was a powerful macrophage activator for ROIs (NO) it did not have a significant effect on T- and B-cell mitogenesis. Given that NO has been shown to inhibit proliferation (Eisenstein *et al,* 1994; Liew 1995), this may have overcome the stimulatory action of TNF-α.

LPS stimulated the most profound increase in the production of IL-1 by the macrophage even after only one hour which could have facilitated T- and B-cell proliferation in response to mitogen. A study by Unanue and Allen, 1987, did demonstrate the ability of macrophages and lymphocytes to modify each others' behaviour and this in part was due to the release of IL-1 and IFN-γ. B-cell responsiveness to TI antigens can be enhanced by the addition of the Th1-cell-derived cytokine IL-2 (Endres *et al,* 1983; Mond *et al,* 1987) and IL-1 stimulates Th1 cells to produce IL-2 (Abbas *et al*, 1991). Perhaps the release of IL-1 from the macrophage stimulates IL-2 production in the Th1 cell. This could account for the much greater response to mitogen when T-cells, B-cells and macrophages are all present in the culture and LPS-treated macrophage supernatants alone could not completely restore lymphocyte responses to mitogen. Th1 cells activate macrophages and this further activation could explain the enhanced proliferation displayed when macrophages (rather than their supernatants) are cultured with lymphocytes. To gain a full insight into which is the dominant T helper cell subset in these experiments the lymphocyte cultures could be analysed for the presence of IFN-γ and IL-2 (Th1) or IL-10 and IL-4 (Th2).

There appeared to be a transient increase in IL-10 production by macrophages incubated with OpZ and to a lesser extent Con A. Recently IL-10 which is produced by type 2 helper T-cells, B-cells, macrophages and monocytes has been found to possess macrophage deactivator characteristics, very similar to that of TGF-β. IL-10 and TGF-β appear to inhibit the release of ROIs, NO, PAF and the inflammatory cytokines IL-1, IL-8 and TNF

from macrophages in response to LPS (Bogdan *et al,* 1992; Gazzinelli *et al,* 1992; Cassatella *et al,* 1993; Bussolati *et al,* 1997). This demonstrates the complexity of interactions occurring between the three cell types both *in vitro* and *in vivo.* The amount of IL-10 released into the LPS and Con A-treated macrophage supernatants was clearly not sufficient to prevent the release of IL-1 and TNF-α under these conditions. However, perhaps IL-10 was inhibiting the cytokine production in OpZ-treated macrophages (especially after 5 hours) and this could be one explanation for the lack of responsiveness in cultures containing OpZ-treated macrophage supernatants.

LPS clearly stimulated a rapid release of TNF-α confirming studies by Tracey *et al,* 1989; Blasi *et al,* 1994 and Bundschuh *et al,* 1997. However, the speed at which it was released must be noted, after only half an hour there was roughly twice as much TNF-α in the supernatants (61 pg/ml, n=2). It may be that there is a small amount of TNF-α stored in vesicles within the cell that can be quickly exocytosed or some cell surface TNF-α can be cleaved upon activation. However mRNA for TNF appears after only one minute after LPS addition so this may be enough time for the first products to be secreted (Coffey *et al,* 1992). The release of TNF-α continued to increase up to 24 hours with all three macrophage treatments which may be due to its positive autocrine control on TNF-α release (Blasi *et al,* 1994), it is also PKC dependent so similar results would probably have occurred after treatment with PMA (Chapter 5). In lymphocyte cultures endogenous TNF-α produced by these activated macrophages would directly stimulate T- and B-cells to proliferate, since anti-TNF-α antibody inhibits proliferation and TNF-α is an autocrine growth factor in activated purified B-cells augmenting further proliferation (Boussitis *et al,* 1994). This might imply a potential role for the cytokine in polyclonal B-cell expansion and malignancies. TNF-α is therefore a very strong candidate as the macrophage-derived co-mitogenic factor under investigation.

Splenic macrophages secrete IFN-α in response to LPS and by 24 hours concentrations were approximately twice that of basal values. The literature suggests that in addition to

possessing a marked anti-viral, increasing expression of MHC Class II antigens and NK cell activity (Arenzana-Seisdedos *et al,* 1985; Pestka *et al,* 1987), it also can actually inhibit proliferation (Trubiani *et al,* 1994). This suggests that the amount of IFN-α in the supernatants was not significant enough to impair lymphocyte proliferation, or that its actions were counteracted by a stronger mitogenic signal.

Although LPS provoked only a minimal oxidative burst (Figure 3.5) it was the most potent activator of cytokine secretion. The macrophage was thus activated to become a 'secretory' cell in response to LPS. The range of cytokines released by a macrophage in response to LPS include IL-1, IL-6, IL-8, IL-12, IFN-α, G-CSF, GM-CSF, M-CSF, PDGF, TGF-β and TNF-α (Auger and Ross, 1992). There must therefore be complex interactions occurring between these cytokines and T- and B-cells present in the culture. These interactions lead to the release or inhibition of a number of other cytokines, for example PDGF induces the production of IL-1, IFN-β and IFN-γ, whereas TGF-β inhibits the effects of IL-2 (Abbas *et al,* 1991). It has also been shown that in mixed splenocyte cultures T-cell cytokines control the balance of functionally distinct macrophage populations (e.g. stimulatory, suppressor or phagocytic macrophages) (Tormey *et al,* 1997). These authors investigated the influence the T-cell derived cytokines IL-2, IL-4, IL-10 and IFN-γ have on the balance of sub-populations of mature macrophages. IL-4 and IFN-γ were seen to increase the capacity of the macrophage pool to stimulate T-cell proliferation. IL-10 promoted the differentiation of monocytes to mature phagocytes and suppressive macrophages, whereas IL-2 had no effect on the macrophage. Their studies concluded that the mature macrophage is 'plastic' and under the control of T-cell derived mediators. This again demonstrates the diverse range of actions cytokines have on cells and thus the complexity of lymphocyte responses to stimuli. It is therefore very difficult to isolate the specific mechanism by which the macrophage interacts with T- and B-cells.

Raised levels of PGE_2 were discovered when macrophages were cultured with Con A for 30 minutes and to a lesser extent 2 hours (Figure 6.5). PGE_2 has an inhibitory effect on

cell proliferation (Date *et al.* 1996), and this may account for the inability of Con A-treated macrophage supernatants obtained after 30 minutes to restore lymphocyte responsiveness to mitogen (Figure 5.3). Other studies have shown that PGE_2 is released by Kupffer cells and alveolar macrophages in response to LPS (Brouwer *et al,* 1995; Chong-Jeh *et al,* 1996 respectively). It may be that splenic macrophages behave differently in their release pattern of PGE_2, as there is great heterogeneity in macrophage populations. All three activators induced an increase in the secretion of LTB_4 and TXB_2. LPS was the most potent stimulator of LTB_4 release, raising levels eight-fold after 5 hours, this was closely followed by Con A and lastly OpZ. These results confirm studies by Sun and Funk, 1996. LTB_4 could therefore be a candidate for the co-mitogenic factor produced by macrophages, as LPS-treated macrophage supernatants displayed the greatest co-mitogenic ability. The production of TXB_2 was stimulated by OpZ and to a lesser extent Con A. Splenic macrophages appear to produce significantly more TXB_2 than PGE_2 and LTB_4. In summary, LTB_4 appears to be an important candidate for the co-mitogenic factor produced by macrophages. However, due to the pronounced production of TXB_2 in splenic macrophages it cannot be ruled out and the production of PGE_2 by Con A-treated macrophages may account for their inability to restore proliferation.

Macrophages produced high levels of Nitric Oxide after only 30 minutes, especially when incubated with OpZ. This was not surprising as the oxidative burst produced by OpZ-treated macrophages was significantly large and NO is a bi-product of this. NO is considered an important intra- and intercellular regulatory molecule exhibiting functions as diverse as vasodilation, neural communication, host defense and immunoregulation (Bosca *et al,* 1995). NO is known to be anti-proliferative (Eisenstein *et al, 1994*; Takagi *et al,* 1994; Bhagat, 1996; Liew 1997) and this may be one reason why OpZ-treated macrophage supernatants do not possess the ability to restore responsiveness to mitogen. OpZ-treated macrophages also failed to release significant amounts of IL-1 and TNF-α which appear to be important in the lymphocyte response to mitogen.

Macrophages express the early activation marker CD69 in response to LPS (Ziegler *et al,* 1994) and this was confirmed in this study. The expression of CD69 was displayed after a 30 minute incubation with LPS and OpZ, emphasising the speed with which macrophages are able to respond to these chemicals. Further studies could investigate how early CD69 is expressed after treatment with LPS, Con A or OpZ and thus how quickly they are able to respond to such activators. This method could also provide useful information on the sensitivity of different macrophage populations to compounds such as LPS.

This chapter has given an insight into the possible macrophage mediators involved in lymphocyte mitogenesis, namely IL-1, TNF-α and LTB_4. TXB_2, IL-6 and possibly IL-12 may also be involved and PGE_2, IL-10 and NO appear to be inhibitory. However it would be naive to assume that these were the only products involved, as all of the cytokines/chemokines/factors produced by the macrophage have not been measured. It appears that the macrophage produces a co-mitogenic factor that is essential for lymphocyte proliferation in response to mitogen. Research has shown that IL-1 and IL-2 are important in T- and B-cell responsiveness to mitogen (Endres *et al,* 1983; Mond *et al,* 1987), therefore the release of IL-1 by the macrophage could be the stimulus required to activate the production of IL-2 by T-cells and thus drive proliferation. This could also explain the immense increase in the synthesis of DNA seen when T- and B-cells are cultured together. TNF-α is an autocrine growth factor in activated purified B-cells (Boussitis *et al,* 1994) and due to its rapid release from the macrophage after stimulation with LPS, it also is a strong candidate for the macrophage-derived co-mitogenic factor under investigation. The next phase is to inhibit the release of certain factors in LPS or Con A-treated macrophage supernatants (e.g. protein (cytokines) using Cylcoheximide or eicosanoids using Dexamethasone). In this way the importance of either cytokines or eicosanoids in B- and T-cell responses to mitogen can be determined.

7. EFFECT OF INHIBITORS ON LYMPHOCYTE PROLIFERATION

7.1 Introduction

Previous chapters identified a number of potential candidates in macrophage supernatants capable of restoring lymphocyte responses to a range of mitogens. This chapter attempts to selectively remove individual factors to ascertain their relative importance in the mitogenic response. In addition potential factors were added to macrophage-depleted splenocyte cultures and the effect this had on responses to mitogen was also investigated. The products analysed were cytokine proteins, the arachidonate metabolites and also TNF-α, TGF-β, PAF and TXB_2. Their actions were inhibited by Cycloheximide, Dexamethasone, an MMPI, Hexanolamine PAF and anti-TXB_2 neutralising antibody respectively.

Cycloheximide is a glutarimide antibiotic that inhibits protein biosynthesis (Obrig *et al,* 1971). The inhibition has been shown to involve one or more steps in the reaction sequence by which amino acids are transferred from aminoacyl transfer RNA into nascent peptides on ribosomes. The glucocorticoid Dexamethasone is a potent inhibitor of arachidonate metabolism, it also affects cytokine/chemokine production (Manz *et al,* 1983; Shappiro *et al,* 1991). Hexanolamine PAF C16 is an inhibitor of platelet activating factor (PAF) (Grigoriadis and Stewart *et al,* 1991). It is an analogue of PAF that inhibits phospholipid turnover and secretion.

The matrix metalloproteinases are a family of zinc-dependent enzymes that degrade all of the major components of the extracellular matrix. They are potentially very damaging enzymes and their activity is closely regulated. Expression is under tight control by pro- and anti-inflammatory cytokines and/or growth factors and, once produced, the enzymes are usually secreted as inactive zymogens. Upon activation they are subject to local control by inhibitors called tissue inhibitors of metalloproteinases

(TIMPS). These inhibitors have great therapeutic potential and are the subject of intense research, especially for the treatment of certain cancers, multiple sclerosis and rheumatoid arthritis. These metalloproteinases cleave many products from the cell surface, for example TGF-β, TNF-α and Fas ligand (Tanaka *et al*, 1996). The use of an inhibitor can prevent this cleavage and therefore stop secretion of these cytokines. This could have many consequences including the prevention of disease states such as hairy cell leukaemia and endotoxic shock (Gearing *et al,* 1994, McGeehan *et al,* 1994, Mohler *et al,* 1994; Williams *et al,* 1996). The broad spectrum MMPI (BB3103) used in this research was a generous gift from British Biotech Ltd.

7.2 Experimental Design

The splenocytes prepared as described previously in Chapter 2, were cultured at a density of 5 x 10^5 cells in 50 µl of supplemented RPMI 1640 medium per well in 96 well flat bottomed microtitre plates. Prior to seeding into wells macrophages could be removed where required using the miniMACS separation technique. Optimum concentrations of Con A (1 µg/ml) or LPS (20 µg/ml) were added to the wells (10 µl), as well as macrophage supernatants (10 µl). In each experiment splenocytes and mitogen were cultured with or without macrophages to ensure that the cells were able to respond to mitogen.

Supernatants were prepared by incubating 1 x 10^5 macrophages in one ml of supplemented medium with Con A (1 µg/ml), OpZ (1 mg/ml) and LPS (1 µg/ml) and a range of inhibitors. Supernatants were collected after 30, 60 and 120 minutes. The inhibitors were used at concentrations found to be effective in other studies. They included: Cycloheximide (10 µg/ml, Obrig *et al,* 1971), Dexamethasone (3 µg/ml, Shappiro *et al,* 1991), an MMPI, BB3301 (10 µM, Layton *et al*, unpublished), a PAF antagonist (1 µM, Grigoriadis *et al,* 1991) and a TXB_2 neutralising antibody (100 ng/ml, Sigma). Macrophages were pretreated with Cycloheximide (10 µg/ml) for two hours and then washed prior to incubation with Con A, OpZ or LPS, therefore the supernatants did not contain Cycloheximide which was found to be a very potent inhibitor of proliferation (data not shown). When macrophage supernatants were added to lymphocyte cultures any inhibitor still present was diluted ten-fold. The direct effect of inhibitors at these final concentrations on the proliferation of splenocytes in response to Con A and LPS was assessed. At a concentration of 1 µM the MMPI did reduce proliferation with both mitogens by approximately 35% (data not shown). This could not be avoided as the MMPI could not be easily removed from the supernatants; this factor will therefore be taken into account when analysing the results. All of the other inhibitors did not significantly affect proliferation as these final concentrations.

The effect of IL-1 and TNF-α on macrophage-depleted T and B-cell responses to Con A (1 μg/ml) and LPS (20 μg/ml) was assessed. Optimum concentrations of the cytokines (10 ng/ml) were used. The lymphocytes were seeded at a density of 5 x 10^5 in 50 μl of supplemented medium per well, to this the cytokines (10 μl) and mitogens (10 μl) were added. Cultures were incubated for 48 hours in 5% CO_2, 95% air at 37°C.

7.3 Results

7.3.1 Effect of Cycloheximide

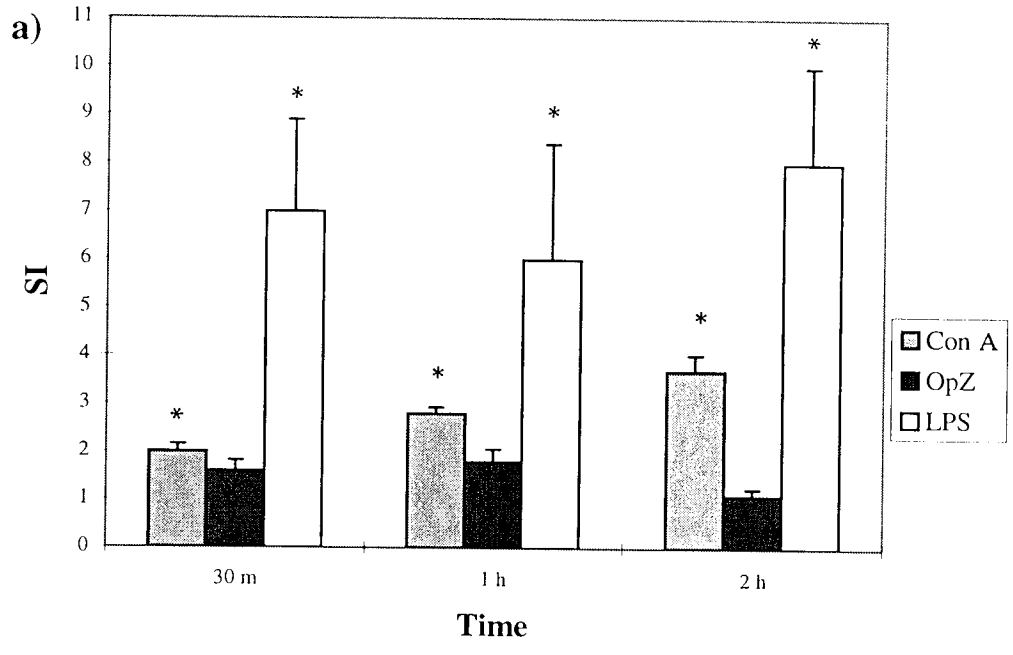
Supernatants from Con A- or LPS-treated macrophages in which protein synthesis had been inhibited by a 2 hour pre-treatment with Cycloheximide, showed ~1/2 the ability to restore macrophage-depleted lymphocytes responses to Con A and LPS, ($p<0.05$), in comparison to control LPS- or Con A- treated-macrophage supernatants, Figure 7.1a and b.

Analysis of LPS-treated macrophage supernatants after a 2 hour pre-treatment with Cycloheximide revealed significantly decreased production of IL-1 and TNF-α (approximately half that of control LPS-treated macrophage supernatants), Figure 7.2. The production of IL-10 and IFN-α was also slightly reduced, Figure 7.2. IL-1 and TNF-α could partially restore lymphocyte responsiveness to Con A and LPS in the absence of macrophages ($p<0.05$), and when the cytokines were combined the response was additive, Figure 7.3.

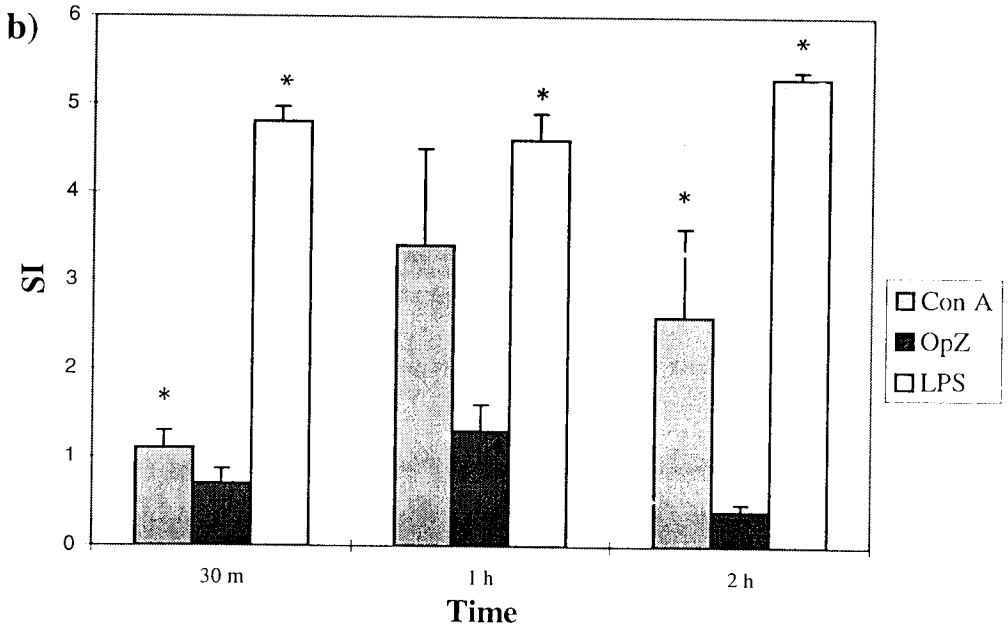
7.3.2 Effect of Dexamethasone

Supernatants from macrophages incubated with LPS and Dexamethasone for between 1 and 2 hours did partially restore the lymphocyte response to Con A and LPS ($p<0.05$), however supernatants harvested after 30 minutes could not achieve this (Figure 7.4a and b). Restoration of the lymphocyte response to Con A was approximately half of that achieved with control supernatants. Splenocyte proliferation in response to LPS was reduced by three-fold when Dexamethasone-treated macrophage supernatants were employed compared to control LPS-treated macrophage supernatants. When macrophages were incubated with Con A in the presence of Dexamethsone supernatants still retained the ability to enhance the response of lymphocytes to mitogen. Thus, Dexamethasone prevents the release of a secretory co-mitogenic factor in LPS-treated macrophages.

Figure 7.1 Effect of Cycloheximide on the Ability of Macrophage Supernatants to Restore Lymphocyte Responsiveness to Con A and LPS (n=4)



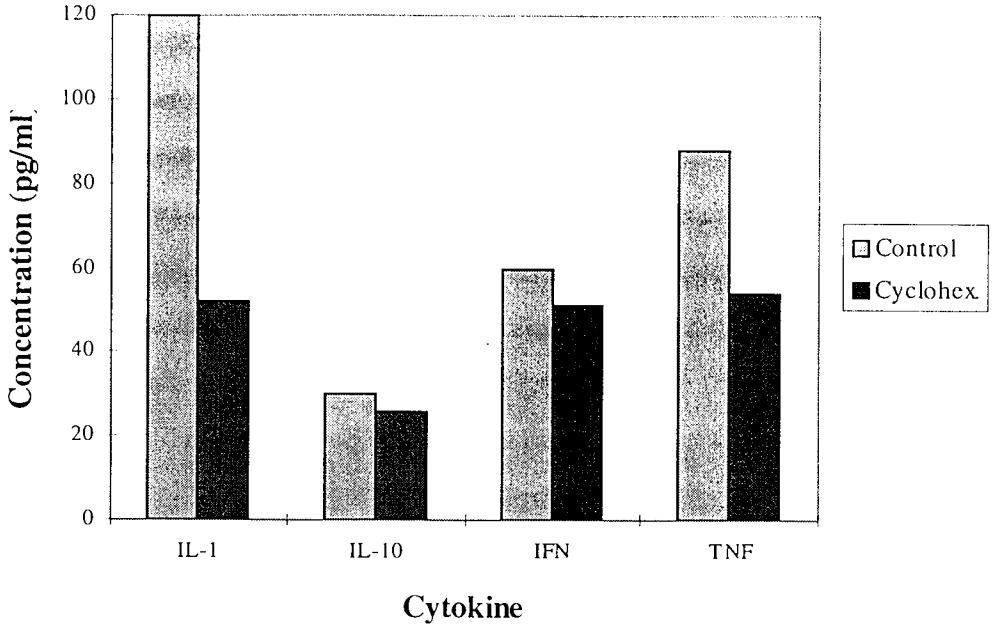
Controls: Mixed splenocyte culture = 43.2 +/- 7.1; Con A 2h spt = 13.9 +/- 3.1; LPS 2h spt = 22.7 +/- 4.4



Controls: Mixed splenocyte culture = 16.7 +/- 3.8; Con A 2h spt = 6.1 +/- 1.6; LPS 2h spt = 10.5 +/- 3.3

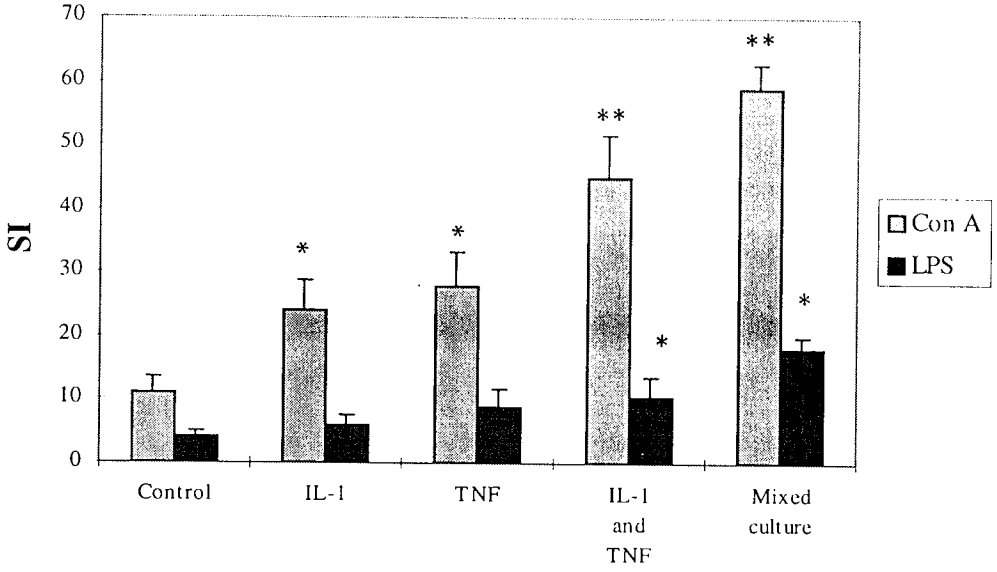
5 x 10^5 macrophage-depleted splenocytes were incubated with a) Con A (1 µg/ml) or b) LPS (20 µg/ml) and supernatants from Con A (1 µg/ml), or OpZ (1 mg/ml), or LPS (1 µg/ml) -treated macrophages pre-treated with Cycloheximide (10 µg/ml), $p<0.05$*.

Figure 7.2 Effect of Cycloheximide on Cytokine Secretion by Macrophages (n=2)



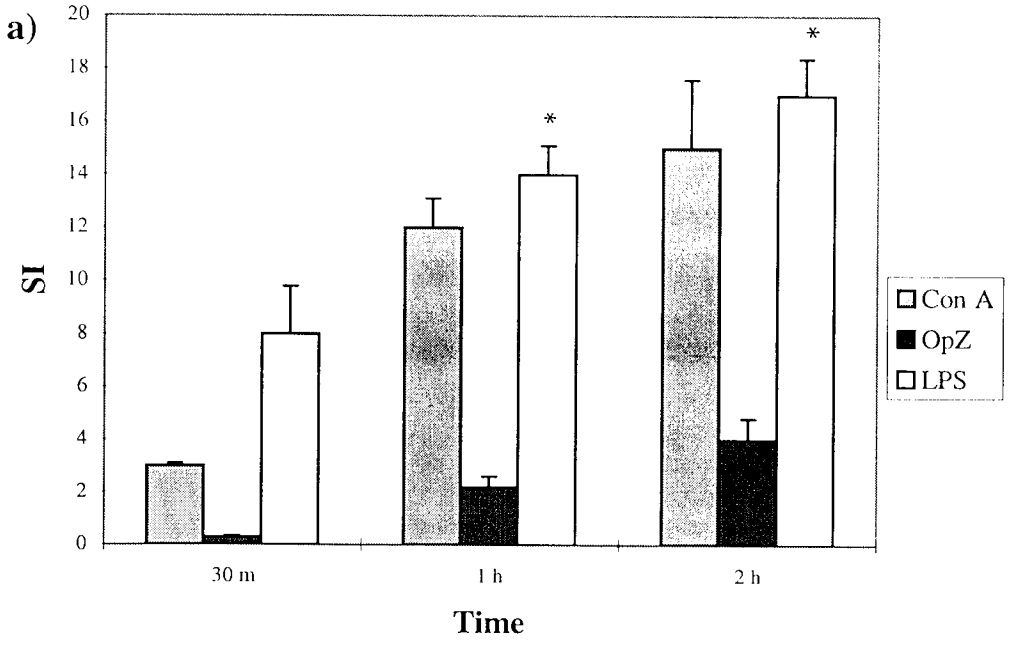
1×10^5 purified macrophages were incubated with LPS (1 μg/ml) for 2 hours. Prior to this some macrophage preparations had been pre-treated for 2 hours with Cycloheximide (10 μg/ml) to inhibit protein synthesis. ELISA kits (R & D Systems) were used to measure the production of IL-1β, IL-10, IFN-α and TNF-α.

Figure 7.3 Effect of IL-1 and TNF-α on the Restoration of Immune Responses by Macrophage-Depleted Splenocytes to Mitogen (n=4)

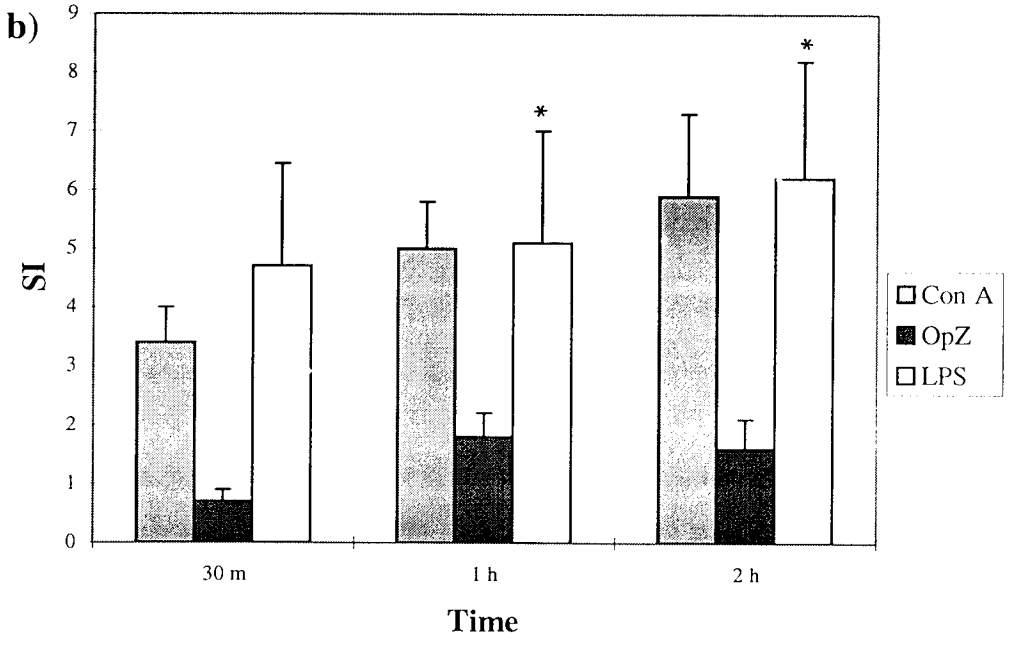


5 x 10^5 macrophage-depleted splenocytes were incubated with IL-1 and/or TNF-α (both 10 ng/ml) and Con A (1 μg/ml) or LPS (20 μg/ml) for 48 h, $p<0.05$*, $p<0.01$**.

Figure 7.4 Effect of Dexamethasone on the Ability of Macrophage Supernatants to Restore Lymphocyte Responsiveness to Con A and LPS (n=4)



Controls: Mixed splenocyte culture = 48.9 +/- 4.4; Con A 2h spt = 18.4 +/- 2.8; LPS 2h spt = 26.2 +/- 3.9



Controls: Mixed splenocyte culture = 18.9 +/- 3.1; Con A 2h spt = 8.8 +/- 1.9; LPS 2h spt = 15.8 +/- 2.7

5 x 10^5 macrophage-depleted splenocytes were incubated with a) Con A or b) LPS and supernatants from Con A/OpZ/LPS-treated macrophages incubated +/- Dexamethasone (3 μg/ml), $p<0.05$*. Positive controls are displayed underneath the figures.

7.3.3 Effect of MMPI

Supernatants from macrophages treated with LPS (particularly) and (Con A) partially could enhance the ability of splenic lymphocytes to respond to mitogenic stimuli in the form of Con A and LPS (Figures 5.1-5.3). In contrast OpZ-treated macrophages did not yield co-mitogenic supernates. When purified macrophages were incubated with LPS or Con A in the presence of MMPI for up to one hour resultant supernatants had a markedly reduced capacity to act as a potentiator of the mitogenic response. Cultures incubated for 2 hours could partially restore responsiveness to Con A and LPS, Figures 7.5a and b ($p<0.05$). However in all instances the response achieved was less than half that of Con A or LPS-treated macrophage supernatants incubated without the MMPI. Inevitably in these experiments there was carry over of MMPI in to the splenocyte cultures (final concentration 1 μM) and this might directly reduce proliferation by 35%. However it appeared that macrophages treated with OpZ in the presence of MMPI produced a supernatant with enhanced capacity to act as a co-mitogen (Figure 7.5) compared to when incubated without MMPI (Figure 5.1-5.3). Perhaps the MMPI prevents cleavage and release of a mitogenic inhibitor under these circumstances (e.g. TGF-β). TNF-α release was markedly reduced in macrophage cultures treated with MMPI. The cleavage of TNF-α was prevented to some degree after only 30 minutes and after 2 hours TNF-α release was reduced by approximately 60 %, see Figure 7.6. Thus as with Figures 7.2 and 7.3 there is strong supportive evidence that TNF-α is at least one of the co-mitogenic factors released by LPS-treated macrophages.

7.3.4 Effect of PAF-16 Antagonist

Supernatants obtained from macrophages incubated with LPS and a PAF antagonist for between 30 minutes and 2 hours did partially restore the proliferative response by lymphocytes to Con A and LPS. This restoration was not significantly different to that achieved by LPS-treated macrophage supernatants, indicating that PAF secretion has no effect on the ability of macrophages to produce the co-mitogenic factor(s) in question

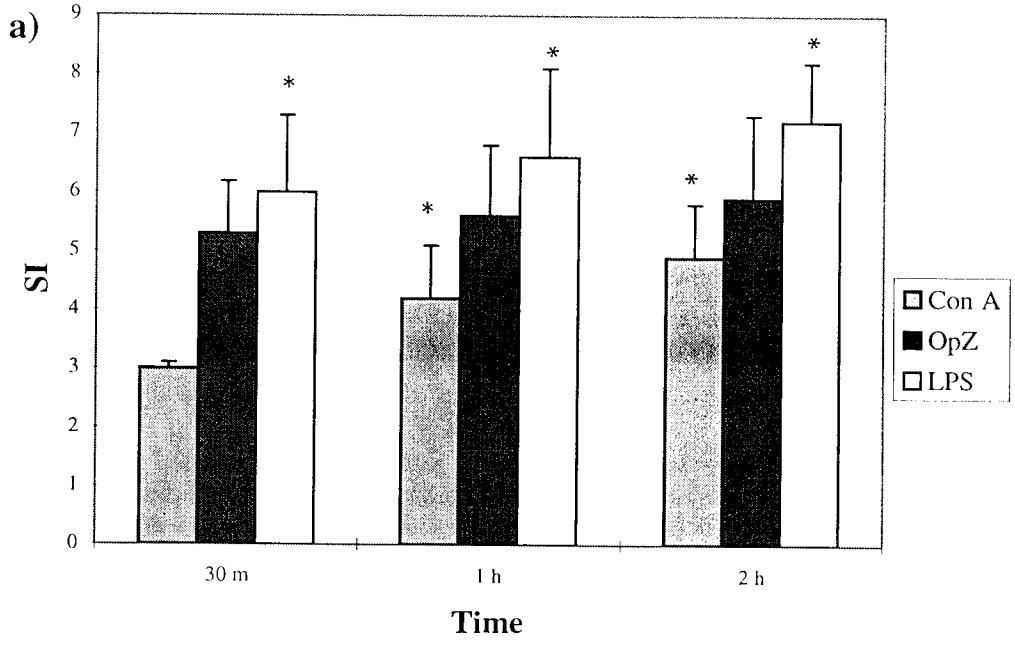
(Figure 7.7a and b respectively). Similar results were found with Con A, however, OpZ supernatants stimulated no proliferation at all.

7.3.5 Effect of anti-TXB_2 Neutralising Antibody

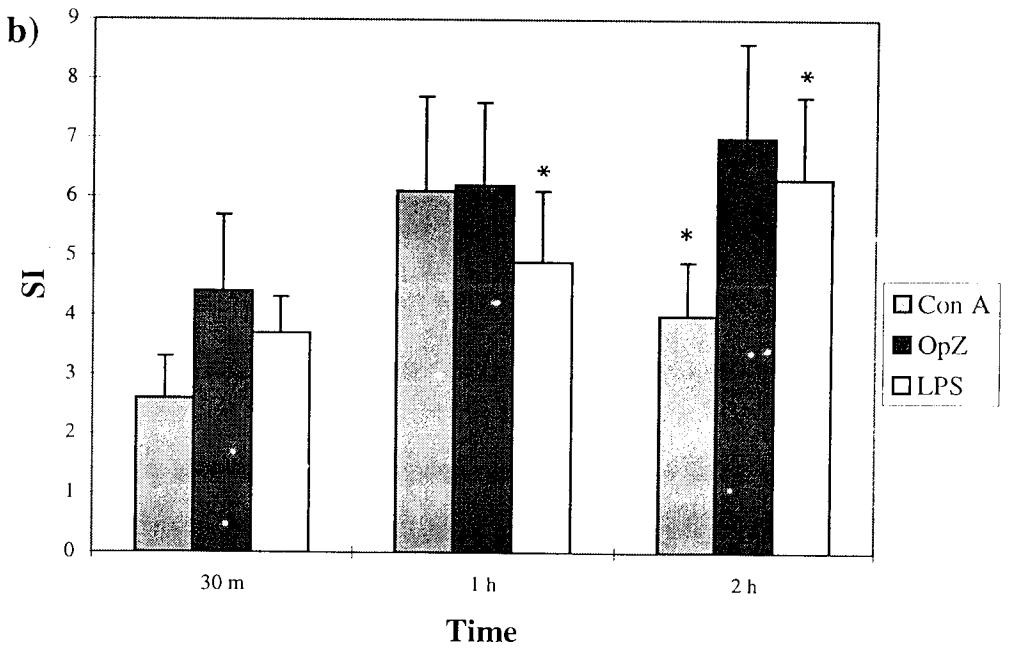
When macrophages were incubated with either LPS or Con A in the presence of anti-TXB_2 antibody the supernatants still retained the ability to enhance the mitogenic response of macrophage-depleted splenic lymphocytes to the two standard agonists, Figure 7.8a and b.

Figure 7.9 summarises the effects of all of the inhibitors on the ability of macrophage supernatants to restore lymphocyte proliferation in response to Con A and LPS. Supernatants were obtained after a one hour incubation with LPS and the appropriate inhibitor. There was a significant reduction in the ability of Cycloheximde, Dexamethasone and MMPI treated macrophage cultures to restore lymphocyte responses to mitogen.

Figure 7.5 Effect of an MMPI on the Ability of Macrophage Supernatants to Restore Lymphocyte Responsiveness to Con A and LPS (n=4)



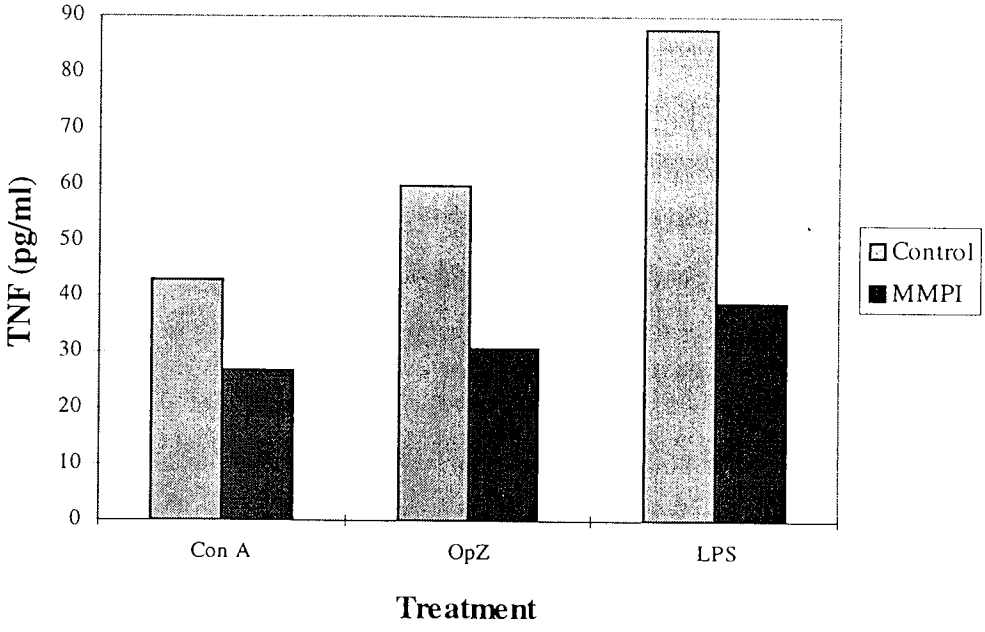
Controls: Mixed splenocyte culture = 49.1 +/- 4.1; Con A 2h spt = 15.6 +/- 3.2; LPS 2h spt = 21.6 +/- 3.8



Controls: Mixed splenocyte culture = 14.9 +/- 3.5; Con A 2h spt = 8.2 +/- 1.6; LPS 2h spt = 12.4 +/- 3.9

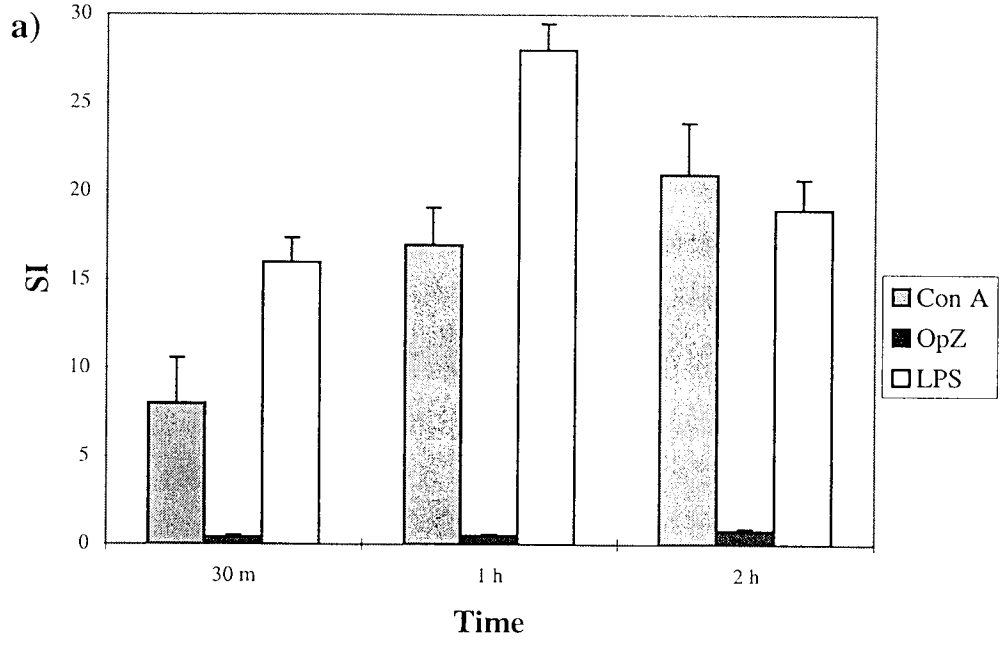
5 x 10^5 macrophage-depleted splenocytes were incubated with a) Con A (1 µg/ml) and b) LPS (20 µg/ml) and supernatants from Con A/OpZ/LPS treated macrophages incubated +/- MMPI (10 µM), $p<0.05$*.

Figure 7.6 Effect of MMPI on TNF-α Secretion by Macrophages (n=2)

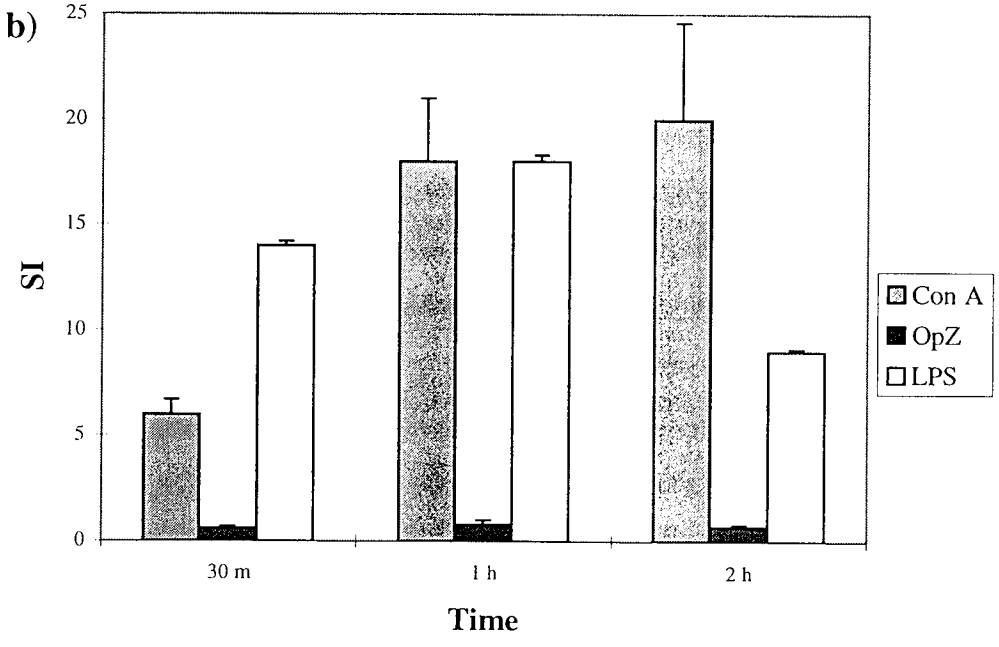


1 x 10^5 macrophages were incubated for 2 hours with LPS , Con A (both 1 μg/ml) or OpZ (1 mg/ml) +/- MMPI (10 μM). ELISA kits (R & D Systems) were used to measure TNF-α secretion.

Figure 7.7 Effect of Hexanolamine PAF on the Ability of Macrophage Supernatants to Restore Lymphocyte Responsiveness to Con A and LPS (n=4)



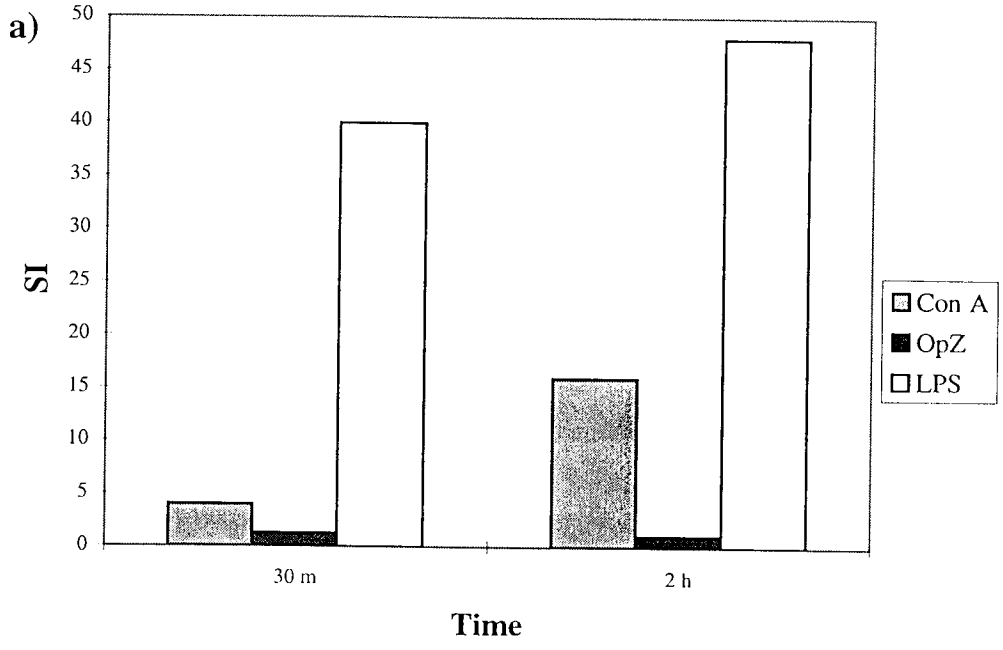
Controls: Mixed splenocyte culture = 38.7 +/- 2.6; Con A 2h spt = 16.5 +/- 4.1; LPS 2h spt = 18.9 +/- 4.3



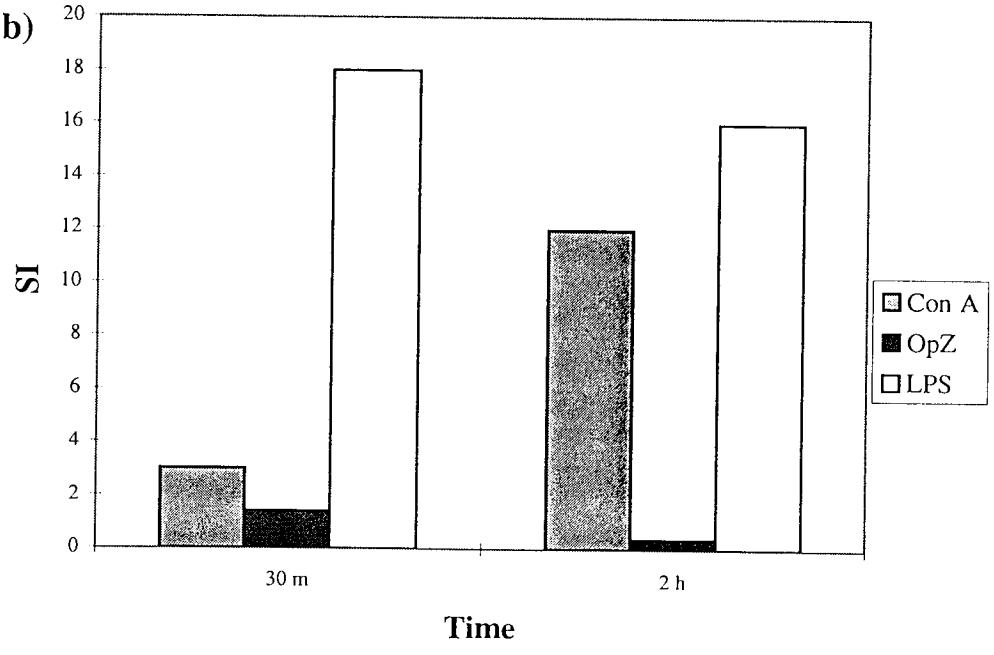
Controls: Mixed splenocyte culture = 14.7 +/- 3.5; Con A 2h spt = 14.4 +/- 4.2; LPS 2h spt = 12.5 +/- 3.9

5 x 10^5 macrophage-depleted splenocytes were incubated with a) Con A (1 μg/ml) and b) LPS (20 μg/ml) and supernatants from Con A/OpZ/LPS-treated macrophages incubated +/- C16 Hexanolamine PAF(1 μg/ml).

Figure 7.8 Effect of TXB_2 Neutralising Antibody on the Ability of Macrophage Supernatants to Restore Responsiveness to Con A and LPS (n=4)



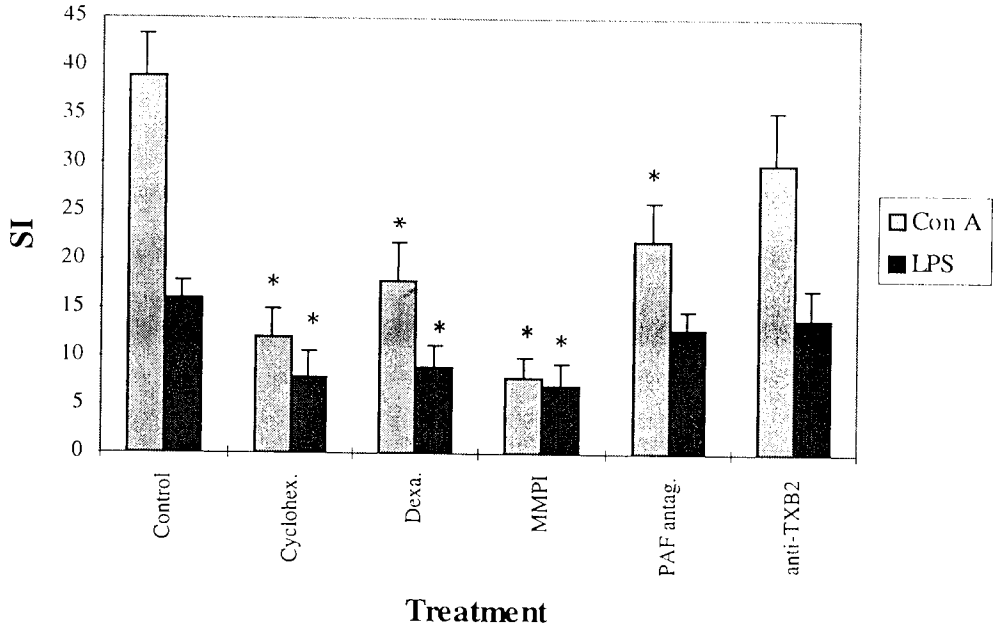
Controls: Mixed splenocyte culture = 56.7 +/- 6.4; Con A 2h spt = 19.3 +/- 4.0; LPS 2h spt = 45.5 +/- 7.3



Controls: Mixed splenocyte culture = 22.3 +/- 4.7; Con A 2h spt = 11.3 +/- 2.5; LPS 2h spt = 16.1 +/- 3.8

5 x 10^5 macrophage-depleted splenocytes were incubated with a) Con A (1 µg/ml) and b) LPS (20 µg/ml) and supernatants from Con A/OpZ/LPS-treated macrophages incubated +/- anti-TXB_2 antibody(100 ng/ml).

Figure 7.9 Summary of Effects of a Range of Inhibitors on Lymphocyte Responses to Mitogen



5 x 10^5 macrophage-depleted splenocytes were cultured for 48 hours with Con A and LPS. Added to this were supernatants obtained from macrophages incubated for 1 hour with LPS (1 μg/ml) and either Dexamethasone (3 μg/ml), MMPI (10 μM), PAF antagonist (1 μM) or anti-TXB_2 neutralising antibody (100 ng/ml) or a pre-treatment with Cycloheximide (10 μg/ml). Control wells contained supernatants from Macrophages treated for 1 hour with LPS alone. There was a significant reduction in the ability of Cycloheximde, Dexamethasone and MMPI treated macrophage cultures to restore lymphocyte responses to mitogen ($p<0.01$)*.

7.4 Discussion

The use of inhibitors has given an overall picture of which classes of compound produced by the macrophage are important in lymphocyte responses to mitogen. The factor(s) acts as an early signal to stimulate proliferation and is not mitogenic in its own right, it merely activates the cells to become responsive to mitogen. One part of this signal appears to be protein based, as the lack of protein in macrophage supernatants after incubation with cycloheximide resulted in the inability of these supernatants to restore lymphocyte responses to Con A and LPS. Therefore, it may be assumed that a protein cytokine(s) is an essential part of this early signal.

Analysis of cytokine secretion by macrophages incubated with LPS and Cycloheximide revealed lower levels of IL-1 and TNF-α than supernatants from macrophages incubated with LPS alone, however there was no alteration in the amount of IL-10 and IFN-α secreted. These results indicate that IL-1 and TNF-α may be important early signals in lymphocyte responses to mitogens as reduced levels in the supernatants resulted in the lack of restoration of a response and indeed IL-1 has been shown to be able to partially restore responsiveness by lymphocytes (Figure 7.3). Oonishi *et al,* 1995 showed that vitamin E (VE) in splenocyte cultures enhanced proliferation in response to Con A, but this only occurred in the presence of macrophages. It was subsequently shown that VE stimulated IL-1 release by the macrophage. Our own studies (Figures 7.1-7.3 & 7.5-7.6) certainly confirm the importance of IL-1 and further suggest that TNF-α could contribute to the macrophage-dependent response. Other protein products may also be required which have not been investigated, for example IL-6 and IL-12. Although we found that IFN-α release from the macrophage was also enhanced by LPS and Con A it is unlikely to contribute to mitogenicity since other studies have demonstrated anti-proliferative properties (Trubiani *et al,* 1994). Likewise IL-10 (Jumper *et al,* 1995) cannot be implicated and we found only very transient changes in the concentration in macrophage supernates after mitogen application (Figures 6.2-6.3).

Supernatants from macrophages incubated with LPS and MMPI, which prevents protease-mediated cleavage of TNF-α and TGF-β (So *et al,* 1992; McGeehan *et al,* 1994; Mohler *et al,* 1994) from the surface of the macrophage did have profound effects on lymphocyte proliferation in response to both Con A and LPS. TNF-α was produced by macrophages in response to LPS (Figure 6.4) and the amount in the supernatant was halved by the MMPI (Figure 7.6). These results are difficult to interpret due to the direct inhibitory action of the MMPI on cell proliferation, however they do indicate that TNF-α from the macrophage may be important in stimulating the cells to divide in response to mitogen. It could be presumed that if the amount of TNF-α found in the supernatants was halved the same would be true of TGF-β which is known to inhibit proliferation (Abbas *et al,* 1991). The lack of this inhibitory cytokine in the supernatants may have served to promote lymphocyte proliferation. Indeed surprisingly supernatants from macrophages treated with OpZ and MMPI could partially restore lymphocyte responses to mitogen (Figure 7.5). It would therefore appear that this cytokine was involved in the inhibition mitogenic responses associated with OpZ-treated macrophage supernatants. The importance of TNF-α in lymphocyte responses could be further investigated by noting the effects Cyclosporin A has on this response, as it is known to inhibit TNF-α production in B-cells (Smith *et al,* 1994). A study by Ryfell *et al,* 1997 has shown that TNF-α is vital in TI-2 response to DNP-alanyl-glycyl-glycyl-Ficoll and TI-1 response to TNP-LPS, as these responses were essentially absent in TNF-LTα-deficient mice, as assessed by IgM and IgG production. These findings further enhance the assumption that TNF-α is important in the macrophage-dependent response investigated in this study.

PAF is a potent mediator of hypersensitivity and inflammatory reactions (Honda *et al,* 1991). Supernatants from macrophages incubated with LPS and Hexanoleamine PAF did not affect the ability of them to restore mitogen responsiveness by T- and B-cells (Figure 7.7). Therefore surprisingly PAF was not found to be required in the lymphocyte response to Con A and LPS, although it does stimulate lymphocyte proliferation and mitogen-activated protein kinase (Honda *et al,* 1994). Perhaps the

antagonist did not completely prevent PAF release. It would be very hard to test this, as PAF cannot be measured easily using an ELISA due to its lack of immunogenicity. The importance of PAF was assessed as it is released by monocytes and macrophages upon activation with LPS after only 30 minutes due to a direct stimulation via the mCD14 receptor (Bussolati *et al,* 1997), thus it may have been the 'early phase factor' required by lymphoyctes. PAF is synthesized in a novel biphasic manner as it also has a later sustained peak after 6 hours which is protein-dependent and regulated by the cytokines IL-1β, TNF-α and IFN-α (Valone & Epstein, 1988). This again demonstrates the complexity of cytokine interactions.

There does appear to be a requirement for a compound(s) from the arachidonate metabolism in lymphocyte responses to LPS, but this is not the case in all Con A responses (see Figure 7.4). This finding demonstrates a difference between lymphocyte responses to Con A and LPS, and indicates that T-cells do not require eicosanoids in order to respond to Con A, whereas they are essential in B-cell responses to LPS. Although eicosanoids in general may be needed for a full response to mitogen it seems that TXB_2 is not a major factor (Figure 7.8). The effect of an anti-TXB_2 antibody was assessed due to the substantial amounts that were produced by splenic macrophages in this system. Another eicosanoid must therefore be important, it may be LTB_4, as raised levels were discovered when macrophages were incubated with LPS. LTB_4 certainly has an effect on B-cells especially in host defense and inflammatory responses (Lewis *et al,* 1990; Garrelds *et al,* 1994).

In summary this study has demonstrated the importance of IL-1 and TNF α in the induction of lymphocytes to proliferate in response to mitogen. Kehrl *et al,* 1987 have shown that these cytokines do stimulate cell division in B-cells and Salkowski *et al,* 1995 revealed that mRNA for TNF-α is expressed as little as 30 minutes after injection with LPS in mice. It therefore may be feasible that TNF-α can be secreted at an early phase. PAF does not seem to be important in this early signal, however this evidence is not conclusive as the amount of PAF released was not measured. An Eicosaniod(s) also appears to be required but it is not TXB_2.

8. GENERAL DISCUSSION

The lack of an immune response to TI antigens in the young where the mature macrophage/lymphocyte architecture is not yet established suggests such interactions could be important (Timens *et al,* 1989). The very close physical proximity between macrophages and B-cells in the marginal zone where TI antigens are sequestered likewise hints at possible functional interactions (Claassen *et al,* 1986; Buiting *et al,* 1996).

The overall findings in this study have demonstrated that the macrophage is indeed required in lymphocyte responses to mitogen. Soluble factors released from activated macrophages seem particularly important. Macrophage activation was initially measured by their production of reactive oxygen intermediates in response to OpZ, PMA, Ionomycin, f-MLP and mitogens (Figures 3.2-3.8). PMA and Ionomycin were not as effective as OpZ in eliciting an oxidative burst therefore other second messenger pathways as well as PLC and DAG are important. The oxidative burst and phagocytosis can be dissociable (Chateau and Caravano, 1993) so care must be taken when investigating macrophage activation using this method. The macrophages may also have been stimulated in other ways for example to release factors other than ROIs or to differentiate into adherent cells. The nature of the macrophage response is dependent on the second messenger cascade which is activated. To demonstrate this there are four cascades regulating two forms of macrophage activation; activation for tumour cytolysis and activation for antigen presentation (Adams *et al*, 1990). This study has demonstrated the diverse range of functions the macrophage possesses and its function is dependent on its state of differentiation, the nature of the activation stimulus, its anatomical location and the isolation technique employed. For example, splenic macrophages left to adhere, differentiated into 'suppressor' macrophages (Parkhouse and Dutton, 1966; Kirchner *et al,* 1976), whereas miniMACS-isolated macrophages were able to restore lymphocyte proliferation in response to a range of T- and B-cell mitogens (Figures 4.2 and 4.3 respectively). Peritoneal macrophages were able to release substantially more ROIs in response to opZ than U937 and RAW 264.7 cells and

splenic macrophages demonstrating macrophage heterogeneity (Figure 3.10-3.11). Also, supernatants obtained from LPS-treated-macrophages taken from different anatomical sites displayed varying abilities to restore lymphocyte responses to mitogen (Figure 5.8). The differentiation state of the macrophage must be taken into account when investigating possible activation protocols. For instance, primed U937 cells (with PMA or IFN-γ) were substantially more responsive to OpZ than un-primed cells and, although LPS could not induce a significant oxidative burst in macrophages it was able to stimulate the release of a factor(s) that could restore lymphocyte responsiveness to mitogen. However the reverse was true of OpZ.

Con A displayed the greatest ability to stimulate lymphocyte proliferation and this was followed by LPS, PWM, Poly[I][C], PHA and lastly Dextran sulphate (Figure 4.1). This demonstrated that different mitogens recruit varying numbers of lymphocytes into mitosis. In fact even though PWM stimulates both T- and B-cells the total number of cells in the S phase of the cell cycle is less than in the lymphocyte response to Con A which only stimulates T-cell proliferation. Results obtained using the BrdU kit to stain dividing cells also showed a lower percentage of cells were stained after PWM-treatment than after Con A and LPS. The simultaneous presence of T-cells enhanced the induced DNA synthesis of B-cells stimulated with LPS and the same was seen when T-cells were cultured with B-cells in response to Con A (Figure 5.7). This may be due to the production of potent growth factors such as IL-2 and IL-4. The removal of macrophages from cultures did prevent a full proliferative response to mitogen from occurring therefore both T- (Rosenstreich *et al,* 1976; Oonishi *et al,* 1995) and B-cells (Hoffmann *et al,* 1979; Boswell *et al,* 1980; Corbel and Melchers, 1983) are dependent on macrophages (Figures 4.2-4.4). This could have been due to macrophages presenting mitogen to the lymphocyte or releasing factors essential to drive proliferation. It appeared to be the release of a factor(s) which was the most important. As few as five hundred macrophages per fifty thousand lymphocytes were able to restore responsiveness and this represents one macrophage to one hundred lymphocytes. It may be that only a very small amount of the product(s) is required due to the close proximity of cells within the well. However fixed-macrophages did show a slight

ability to restore lymphocyte responsiveness to mitogen (Figure 4.5), therefore cell-cell interactions do help. These interactions could be mediated via the CD22 adhesion molecule which is expressed on B-lymphocytes and mediates the adhesion of erythrocytes, T- and B-lymphocytes, neutrophils and monocytes, theCD22 ligand remains incompletely characterized (Engel *et al,* 1993). It may also be via CD21/CR2 (C3d receptor) or CD23 associations between MZ B-cells and MZ macrophages (Carter *et al,* 1988 & 1989; Timens *et al*, 1989). Indeed the synergistic signalling of the complement receptor and mIg could provide an additional activation signal for B-cells and TI-2 antigens are able to activate complement via the alternative pathway (Griffeon *et al,* 1991). Claassen, 1986 demonstrated that marginal zone macrophages were directly involved in the processing and presentation of TI-2 antigens. This was shown by injecting mice with dichloromethylene diphosphate encapsulated in liposomes to eliminate MZ macrophages. Subsequent immunization with TNP-Ficoll resulted in a strong decrease in the antibody response in macrophage-depleted animals. They concluded that MZ macrophages were indispensable for the formation of anti-TNP AFC after immunization with a TI-2 antigen. Chao and Macpherson, 1990, have also demonstrated that MZ macrophages were able to selectively take up TI antigens and that they may present these antigens to the B-cell. The ligand specificity showed cross-reactivity with the mannosyl-fucosyl receptor with high affinity for mannan and zymosan. They therefore suggest that the TI antigen uptake system may also mediate phagocytosis.

Supernatants from macrophages treated with PHA, LPS, PWM, DS, PIC, PMA and Ionomycin (Con A and f-MLP after a longer incubation period) could restore lymphocyte responses to mitogen. One interesting finding was that although f-MLP elicited a very quick oxidative burst in macrophages, f-MLP-treated macrophages do not release the product(s) essential for proliferation until after a much longer time period. Perhaps f-MLP stimulates the release of activators in two phases, early factors promote chemotaxis (Polla *et al,* 1989) and the production of ROIs and later factors being co-mitogenic materials. Macrophage supernatants contain a range of products which could provide the required signal. These include the cytokines IL-1, IL-6, IL-10,

IFN-α, PAF, TNF-α (Salkowski *et al.* 1995) and the eicosanoids Prostaglandin E_2, Leukotriene B_4, Thromboxane B_2 which may be important due to their quick release (Marshall *et al*, 1994). PAF may also play some role as it too is released after a very short time course and has numerous effects on the immune system (Valone and Epstein, 1988; Bussolati *et al*, 1997) but this was not seen. LPS-stimulated macrophage supernatants contained raised levels of IL-1, TNF-α, LTB_4, TXB_2 and Nitric oxide. Early phase Con A-treated macrophage supernatants also contained raised amounts of PGE_2 and this may explain why Con A supernatants do not restore proliferation at an early stage, because PGE_2 has an inhibitory effect on cell proliferation (Date *et al,* 1996). The inability of OpZ-treated macrophage supernatants to restore proliferation was probably due to the large amounts of NO released which also inhibits cell division (Hibbs *et al,* 1987; Adams *et al*, 1990; Eisenstein *et al*, 1994; Liew 1995). This study demonstrated that the factor(s) was not mitogenic but was required to prime the lymphocyte to subsequently respond to mitogen and IL-1 and TNF-α were isolated as the macrophage products which were required by lymphocytes, LTB_4 may also be important.

Figure 8.1 shows the regulatory network of cytokines produced by macrophages, T-cells and B-cells in response to mitogen. The MMPI prevented the release of TNF-α (Figure 7.6) and possibly TGF-β (Gearing *et al*, 1994). TGF-β inhibits lymphocyte proliferation (Bogdan *et al*, 1992), whereas TNF-α stimulates B-cell proliferation (Kehrl *et al,* 1987; Bundschuh *et al,* 1997). The decreased release of TNF-α and TGF-β by LPS-treated macrophage supernatants treated with MMPI resulted in their inability to restore lymphocyte responses to mitogen. However LPS-treated macrophage supernatants are capable of producing the co-mitogenic factor, hence the stimulatory actions of TNF-α are much stronger than the inhibitory actions of TGF-β. TNF-α has been implicated in TI antigen B-cell responses, as TNF-α deficient mice could not respond to the TI type 2 antigen DNP-alanyl-glycyl-glycyl-Ficoll and their response to the TI type 1 antigen TNP-LPS was also significantly reduced (Ryffel *et al*, 1997). Another study has shown that TNF-α produced by murine macrophages in response to LPS potentiated the cells' production of IL-1 (Blasi *et al*, 1994). IL-1 could therefore

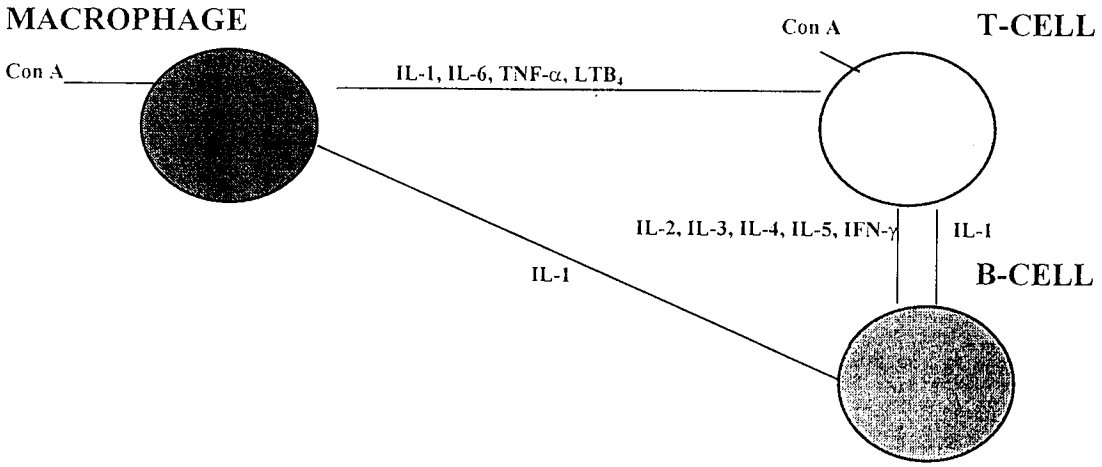
heighten their ability to stimulate T- and B-cells to respond to mitogen. Research by Sorimachi *et al*, 1995 has shown that TNF-α is rapidly secreted by the macrophage in response to a lignin derivative and peaks after only one hour. It is therefore feasible that TNF-α is one of the factors required by lymphocytes in mitogenic responses and that LPS can also stimulate this very quick release of TNF-α .

The cytokine profile in lymphocyte cultures is worthy of investigation, as it could further increase the understanding of events taking place in mitogenic responses. The most dominant T helper cell subset could be elucidated by measuring the concentration of IL-2 and IL-4 in cultures of T-cells, B-cells, macrophages and mitogen. These cytokines are produced predominantly by Th1 cells and Th2 cells respectively, thus the subset could be defined. This division within the subsets is clearly defined in the mouse. The subset involved may be Th1 as the mitogen response appears to be dependent on IL-2 (Endres *et al,* 1983; Mond *et al,* 1987) and IL-1 (Garg *et al,* 1996), which will also further stimulate the release of IL-2. However, IL-4 in combination with anti-Ig is a potent stimulator of quiescent (G_0 and G_1) B-cells and IL-4 primed B-cells make a more vigorous response upon stimulation by anti-Ig or LPS (Stack *et al,* 1994).

Histo-cytochemical staining using antibodies to IL-1, IL-2, IL-4, TNF-α and possibly IL-6 of spleen sections could help to confirm which cytokines are important. Although IL-6 is a macrophage-derived cytokine (Van Snick, 1990) it shows poor growth promoting activity in mice for this reason the presence of IL-6 in activated macrophage supernatants was not determined. However it can synergise with anti-Ig, particularly if IL-1 is present (Kishimoto *et al*, 1992) and so could actually be important. The use of gene 'knock-out' mice may give further enlightenment for a role of IL-1 and TNF-α in responses to TI antigens. Knock-out mice are clearly an informative way of understanding the role of the gene in normal life. For example, knock-out mice lacking the gene encoding IL-2 suffer from lymphoproliferation and autoimmunity, therefore a role for IL-2 *in vivo* appears to pro-apoptotic (Robins and Todd, 1998). IL-6 has been shown to be a crucial mediator of the acute-phase protein reaction and TNF/TNFR1

have been found to be essential for immune responses against *Listeria monocytogenes* using of knock-out mice (Bluethmann *et al*, 1994). However, there are limitations to using knock-out mice as a role of a given gene may vary among tissues, e.g. the mutant phenotype of mice lacking the gene for TGF-α is limited to wavy hair and curly whiskers (Mann et al, 1993).

Figure 8.1a Model Of Lymphocyte Response To Con A; Importance Of The Macrophage Revealed

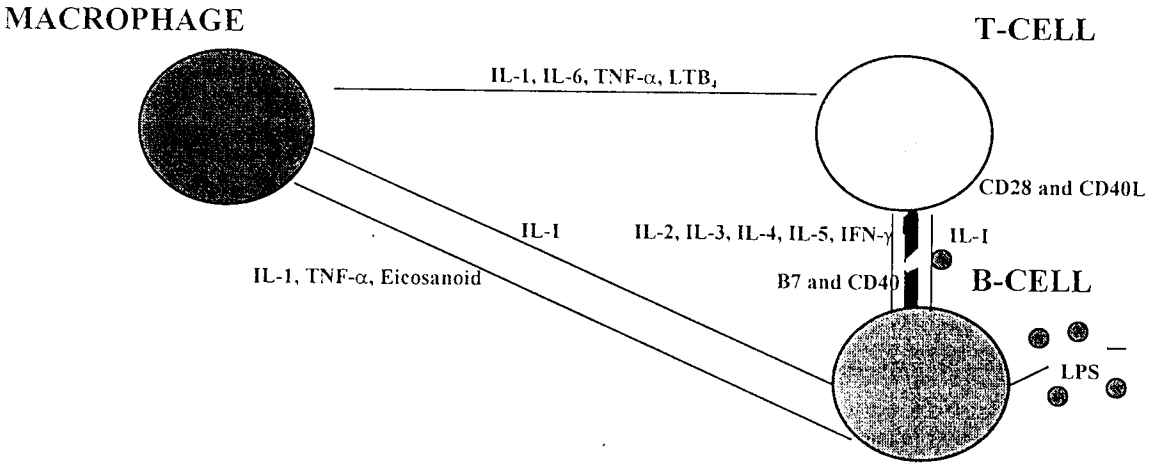


Signal 1 IL-1 (Figure 6.1), TNF-α (Figure 6.4), LTB_4 (Figure 6.7) and possibly IL-6 (Van Snick, 1990) are released by the activated macrophage which stimulates the T-cell in the presence of Con A (Figures 5.1, 5.3, 5.4, 5.6 and 5.7). The stimulated T-cell may release IL-4 (Stack *et al*, 1994) and this may activate the B-cell to release IL-1 (Takeuchi and Katayama 1994). These cytokines could then cause enhanced release of IL-1 by the macrophage.

Signal 2 The T-cell releases IL-2, IL-3, IL-4, IL-5, IFN-γ (O'Garra *et al*, 1986; Mosmann and Coffman, 1989; Moreau *et al*, 1995) in response to Con A. There is also increased expression of the IL-2 receptor on the T-cell to enhance its responsiveness further (Hermann *et al*, 1985).

Signal 3 This stimulates the B-cell to release more positive growth factors such as IL-1 (Scala *et al*, 1984) resulting in the enhanced T-cell proliferation seen when T-cells, B-cells and macrophages were cultured together (Figure 5.7).

Figure 8.1b Model Of Lymphocyte Response To LPS; Importance Of The Macrophage Revealed



Signal 1 LPS binds to the B-cell via the CD14 molecule (Wright et al, 1990) and the B-cell moves from G_0 to G_1. The B-cell may associate with the macrophage via the CD22 adhesion molecule (Engel *et al*, 1993) or CD21/CR2 (C3d receptor) or CD23 (Carter *et al,* 1989).

Signal 2 IL-1 (Figure 6.1), TNF-α (Figure 6.4 and 7.5) and an eicosanoid (Figures 6.6-6.8 and 7.4) are released by the macrophage they are referred to as the 'alpha factors'. This results in the B-cell proceeding to the S phase of the cell cycle and eventually to the G_2 phase (Melchers and Anderson, 1984) (Figures 5.2-5.3, 5.5 and 5.7). These events may stimulate the B-cell to produce IL-1 (Scala *et al*, 1984) which could further stimulate the macrophage to release more co-mitogenic factors.

Signal 3 Through ligation of the B7-CD28 complex and the binding of CD40 to CD40L the T-cell releases IL-2, IL-3, IL-4, IL-5 and IFN-γ (Van den Eertwegh *et al*, 1993 and 1994). Their production may also be enhanced by the macrophage-derived factors IL-1 and possibly IL-6. These are known as the 'beta factors' (Melchers and Anderson, 1984). This results in the enhanced proliferation seen in lymphocyte responses to LPS when all three cell types are cultured together (Figure 5.7). The B-cell has firmly progressed to the M phase.

This model shows that the macrophage is undoubtedly important in lymphocyte responses. This could explain the lack of a good immune response in the young to highly virulent bacteria such as *Neisseria meningitidis*, *Haemophilus Infuenzae* type b or *Streptococcus pneumoniae* (Timens *et al*, 1986). At this stage their MZ, which contains large numbers of B-cells in close association with macrophages is highly immature, and its maturity correlates with the ability to mount an effective immune response to these polysaccharide encapsulated bacteria. With a greater understanding of events taking place in the MZ perhaps therapies which mimic the beneficial actions these macrophages have could be found. For example the spleen might be targeted with low doses of IL-1 and TNF-α. This could also help splenectomised patients that could not have an immunisation program before splenectomy e.g. car accident victims. This work may also have some implications in lymphocyte growth and control. TNF-α is an autocrine growth factor for B-cells (Boussiotis *et al*, 1994) and B-cell lymphomas. The use of an MMPI could therefore control cell growth which could be of great therapeutic benefit in certain cancers such as Hairy Cell Leukaemia.

In summary this study has attempted to unravel the nature of the relationship between macrophages and lymphocytes as measured by their response to a range of T-dependent and T-independent antigens. It has demonstrated that the macrophage is essential in lymphocyte responses to a range of T- and B-cell mitogens, TI-1 and TI-2 antigens by the release of essential factors namely IL-1, TNF-α and perhaps LTB_4. In discovering this it has raised a multitude of questions and opened up many possible avenues of investigation.

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