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B-1 AND B-2 B CELL RESPONSES TO
LIPOPOLYSACCHARIDE:
PUTATIVE ROLES IN THE PATHOGENESIS
OF PERIODONTITIS

Julia Rachel Philips
BSc (Hons)

A thesis submitted in fulfilment of the
requirement for the degree of Master of Science

Institute of Dental Research
Faculty of Dentistry
The University of Sydney
April 2006
Statement of Authorship

The experimental work described in this thesis was performed by the candidate except where stated otherwise. This work has not been submitted whole or in part for any other degree.

[Signature]

[Signature]
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Publications

The following papers contain material described in this thesis:


Philips JR and Hunter N. The murine B-1 cell LPS response. *In preparation*

Papers not related to thesis studies:


Conference presentations:

JR Philips, WLK Massey and N Hunter (1998) The effect of LPS stimulation on IgM production by murine B lymphocytes. Inaugural Research Conference, *College of Health Sciences*, University of Sydney, Australia

Philips JR, Massey WLK, and Hunter N (1999) Responses of murine CD5+ and conventional B cells to lipopolysaccharide. *International Association for Dental Research*, 77th General Session, Vancouver, Canada
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>2ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>AIHA</td>
<td>Autoimmune haemolytic anaemia</td>
</tr>
<tr>
<td>Azide</td>
<td>NaN3</td>
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<tr>
<td>B-1 cell</td>
<td>CD5⁺ B cell</td>
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<td>B-CLL</td>
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<td>BrMRBC</td>
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<tr>
<td>CFSE</td>
<td>5-carboxyfluorescein diacetate-succinimidyl ester</td>
</tr>
<tr>
<td>CH</td>
<td>Chapel Hill</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dToll</td>
<td><em>Drosophila melanogaster</em> Toll</td>
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<tr>
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<td>Human Toll</td>
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<tr>
<td>I/C</td>
<td>Isotype control</td>
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<tr>
<td>IDDM</td>
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<td>Lipopolysaccharide</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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mIgM  Membrane IgM
min   Minute
MOPS  3-[N-Morpholino] propanesulphonic acid
MTT   3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PBA   Polyclonal B cell activation
PBL   Peripheral blood lymphocyte
PBS   Phosphate buffered saline
PC    Phosphorylcholine
PE    Phycoerythrin
PFA   Paraformaldehyde
PHA   Phytohaemagglutinin
PI    Propidium iodide
PMA   Phorbol myristate acetate
PtC   Phosphatidyl choline
RF    Rheumatoid factor
RPMI  Roswell Park Medical Institute
RT    Room temperature
SA    Streptavidin
SA-CyChrome Streptavidin-CyChrome
s.e.m. Standard error of the mean
sheath fluid Osmosol
sIgM  Secreted IgM
SLE   Systemic lupus erythematosus
SPF   Specific pathogen free
SRBC  Sheep red blood cell
SSC   Side Scatter
TCR   T cell receptor
TD    T-dependent
tem   Transmission electron microscope
T_H   T helper
T_I   T-independent
TLR   Toll-like receptor

Note 1: The B-1 and B-2 descriptors were initially intended to be used to describe the cells arising from different lineages although they have been used by many investigators to describe the phenotype of the cells (Hayakawa et al., 2000). In this thesis, the terms will be used to describe the phenotypes of the cells as either CD5^+ B cells (B-1) or CD5^−/conventional B cells (B-2).

Note 2: Populations referred to as “control” in this thesis describe non-stimulated populations.
Abstract

Periodontal disease is one of the most widespread diseases in humans and is characterised by chronic gingival inflammation and B cell accumulation and resorption of the crest of alveolar bone with subsequent loss of teeth. Porphyromonas gingivalis has been identified as a putative aetiological agent for periodontitis.

The aim of the research presented in this thesis was to investigate, using in vitro systems, the responses of autoreactive B-1 and B-2 cells to enterobacterial and non-enterobacterial lipopolysaccharide (LPS) to shed light on the pathogenesis of chronic periodontitis and other diseases involving B cell accumulation and autoantibody production.

The hypotheses tested were:

1. B cells respond differently to enterobacterial and non-enterobacterial LPS.
2. B-1 cells are activated by a lower concentration of LPS than B-2 cells.
3. LPS stimulation results in preferential accumulation of B-1 cells.

Findings consistent with these hypotheses would provide new evidence for different roles for B-1 and B-2 cells in immune responses and that LPS stimulation could lead to B-1 cell accumulation in diseases thus characterised.

Initial experiments investigated the responses of representative B-1 (CH12) and B-2 (WEHI-279) cell lines to preparations of P. gingivalis and Salmonella enteritidis LPS utilising flow cytometric and quantitative molecular methods. The cell lines responded differently to the two LPS preparations. There were significant but limited effects on viability and proliferation in the WEHI-279 cell line, but no significant changes in mRNA expression levels for genes including Toll-like receptors (TLR2, TLR4, RP105), immunoglobulin (IgM), cytokines (IL-6, IL-10), co-stimulatory molecules (CD80, CD86), and regulators of apoptosis (Bcl-2, Bax). In the CH12 cell line however, LPS stimulation had greater effect. Addition of S. enteritidis LPS from a threshold level of 100ng/mL was found to rescue the cells from death, reflected by the percentage viability and proliferation. Stimulation of CH12 cells with S. enteritidis LPS also led to a
decrease in expression of RP105 mRNA, which may be part of a negative feedback loop.

Interestingly, stimulation with low concentrations *P. gingivalis* LPS appeared to inhibit proliferation but high LPS concentrations stimulated proliferation of CH12 cells, although no further significant effects were noted in other analyses. Evidence was found that CH12 cells have a high basal level of activation. This suggests that this line is constitutively activated. Stimulation with *P. gingivalis* or *S. enteritidis* LPS did not affect the level of CD80 mRNA expression. It is possible that the CH12 line constitutively expresses a maximal level of CD80 (and possibly CD86) and further stimulation will not cause any increase.

Since *S. enteritidis* LPS appeared to have more pronounced effects on both B cell populations, this LPS was used to further investigate B cell subset responses in a mixed splenocyte culture system. Experiments examining percentage viability and number of viable cells indicated that B-1 and B-2 B cells responded differently to LPS stimulation. A threshold level for B-2 cell response (significant increase in cell number) was found to be 100ng/mL LPS, in contrast to the B-1 B cell subset which were only significantly different to the unstimulated cells when stimulated with 50μg/mL LPS. By examining the expression of CD80, the majority of murine splenic B-1 cells were found to activated prior to any LPS stimulation in vitro. In contrast, the B-2 subset showed significant increase in CD80 expression only at high (≥10μg/mL) LPS concentrations. Studies of the division index of B-1 and B-2 cells showed a significant response in both subsets following stimulation with 1μg/mL and 10μg/mL LPS. However, overall, the results are inconsistent with LPS driving the preferential accumulation of B-1 cells in disease states.

These experiments provided useful evidence that supported the idea that B-1 and B-2 cells respond differently to LPS. However, these studies were unable to directly address the role of *P. gingivalis* LPS in periodontitis. It may be that *P. gingivalis* LPS could have different effects to *S. enteritidis* LPS on primary B cells. It is still possible that B-1 cells may be more sensitive to *P. gingivalis*, as opposed to *S. enteritidis* LPS.
Studies by other groups have suggested that the T\textsubscript{H}1/T\textsubscript{H}2 profile is skewed towards T\textsubscript{H}2 in chronic periodontitis and that \textit{P. gingivalis} may drive this shift via its ability to signal through TLR2 (and modulate TLR4 signalling). Further, recent studies in our laboratories have found that \textit{P. gingivalis} gingipains are able to polyclonally activate B cells and to break down both IFN\textsubscript{\gamma} and IL-12. Future studies should further examine the effects of B-1 and B-2 interactions in the mixed lymphocyte system together with subsequent studies utilising human periodontitis biopsies.

The results presented in this thesis, together with work undertaken by other investigators, suggests that LPS could perturb the normal homeostatic mechanisms of the B-1 B cell-subset and increase polyclonal activation therefore contributing to the genesis of pathologies such as chronic periodontitis.
Preface

Response to Examiners' Comments

This revised thesis has been prepared in response to comments from three examiners. The consensus points between the three examiners were the main drivers of revision work undertaken. These points were:

1) A greater alignment of the studies and theory towards chronic periodontitis. This included retiling the thesis to better reflect the material covered and the use of LPS from Porphyromonas gingivalis, an oral pathogen implicated in the pathogenesis of chronic periodontitis (Van Dyke and Serhan, 2003), in the cell line studies;

2) The inclusion of a B-2 (CD5+) B cell line for comparison with the CH12 CD5+ B cell line studies;

3) Reanalysis of previously presented data, where appropriate, to enhance the clarity of presentation; and

4) Further studies utilising purified murine B-1 and B-2 B cells, possibly from the spleens of foetal mice.

No issues arose in dealing with the first three points listed above, and these additional studies and reanalyses were undertaken as presented in this thesis. However, there were a number of problems regarding the animal studies suggested. Preliminary trials into isolation of B-1 cells using magnetic beads resulted in very low yields (see Appendix 2). Therefore a large number of mice would need to be sacrificed to gain sufficient cells for study and this could not be justified. Further, increasingly severe responses to mice and asthma affecting the candidate precluded further animal work. Additional molecular studies examining viability, activation and LPS receptors were undertaken to enhance the B-1 and B-2 cell line studies utilising Salmonella enteritidis and Porphyromonas gingivalis LPS.

An extensive reorganisation of the theoretical and experimental studies was also undertaken to better deal with the hypotheses in the light of the greater emphasis on chronic periodontitis.
**Problem Outline**

Periodontitis is a disease of the oral cavity occurring in approximately 10% of the population in early (Ripamonti, 1988) and modern humans (Clarke, 1990). Advanced/chronic human periodontitis is considered to be B lymphocyte and plasma cell lesion (Okada et al., 1983; Tew et al., 1989; Gemmell and Seymour, 1991). A large proportion of B lymphocytes found in the lesion are CD5⁺ (Sugawara et al., 1992).

B cells have been divided into two groups; B-1 and B-2 B cells. B-1 cells are CD5⁺, self-renewing in the periphery, producers of IgM polyreactive antibodies and concentrated in the peritoneal cavity. By virtue of these characteristics, B-1 cells have been postulated to play a major role in the first line of defence against pathogens.

Both B cell subsets produce and secrete antibodies; however the B-1 repertoire is restricted when compared with that of the B-2 subset. B-1 cells have been implicated in autoimmunity as B-1 cell derived IgM antibody is polyspecific and is able to bind to both self and non-self (bacterial) antigens. B-1 cells are generally relatively low in number in the peripheral blood, but accumulations of B-1 cells are found in a number of autoimmune diseases including Sjögren’s syndrome and rheumatoid arthritis and in diseases with postulated and established bacterial aetiology, including chronic periodontitis. The mechanisms for this accumulation are unknown at present.

Lipopolysaccharide (LPS) is a stimulus for polyclonal B cell activation, and stimulation with enterobacterial and non-enterobacterial LPS has been shown to have different effects on a number of different cell types. *Porphyromonas gingivalis* is a non-enterobacterial (*Bacteroides*) species which has been implicated in the pathogenesis of chronic periodontitis. Its LPS has been shown to be structurally different to that of enterobacterial LPS and to have lower biological activity in the majority of accepted functional assays.

The aim of the studies presented in this thesis is elucidation of the response of B-1 and B-2 cells to enterobacterial and non-enterobacterial LPS to shed light on the
pathogenesis of chronic periodontitis and other diseases involving B cell accumulations and autoantibody production.

To achieve this aim, B-1 and B-2 cell lines, together with purified murine splenic lymphocytes were utilised to examine the B cell response to enteric and non-enteric bacterial LPS.

The first chapter of the thesis contains a review of the relevant scientific literature and provides a background for the experimental work. The immune response to antigen, and particularly the roles of B cells and antibodies, is discussed. B cell development and activation and the use of cell lines to examine B cell function extend the B cell focus. This is followed by a brief discussion of selected diseases characterised by B-1 cell accumulation, including periodontitis. The final section of this chapter describes systems thinking and the application of the systems perspective to immunological studies and those presented herein. This provides an appreciation of the immune response as a system which is essential for an understanding of its roles and properties.

The experimental work is divided into four chapters. In the first of these (Chapter 2), the response of the CH12 CD5+ B cell line to *Salmonella enteritidis* and *Porphyromonas gingivalis* LPS is described. In the second experimental chapter (Chapter 3), the response of the WEHI-279 conventional B cell line to *S. enteritidis* and *P. gingivalis* LPS is described. Both of these chapters examine viability, proliferation and activation of the cell lines. The third experimental chapter (Chapter 4) utilises a simplified system approach and characterises a simple system to examine B cell responses to LPS. The final experimental chapter (Chapter 5) extends this work to examine whether the simplified system reflects the response patterns observed in the previously presented cell line studies. Each of the experimental chapters concludes with a brief discussion that draws together the main conclusions from the experiments in that chapter.

The final chapter contains a discussion of results from the experimental chapters and the relationship of the findings to the scientific literature. In addition, potential avenues for future work are discussed.
Specific hypotheses

A number of specific hypothesis were formulated regarding the response of B cell subsets to LPS:

$H_1$: B cell subsets respond differently to enteric and non-enteric LPS

$H_2$: B-1 cells are activated by a lower concentration of LPS than B-2 cells

$H_3$: LPS stimulation results in preferential accumulation of B-1 cells
1.1 Introduction

The goal of this chapter is to provide a background on the biology of different B cell populations and how they may be involved in disease processes. In particular, the literature concerning the response of B cells to the bacterial product lipopolysaccharide LPS is reviewed, and evidence is presented that a subpopulation of B cells (B-1 cells) may be particularly sensitive to LPS stimulation.

1.2 Overview

With more than a century of scientific research lying between our vantage point and that of the pioneers of immunology, it is curious to contemplate the 'sharp antagonism' that existed between the disciples of Paul Ehrlich and those of Elie Metchnikoff (Hektoen, 1909). Ehrlich's followers proposed that antibodies were the principal mediators of immunity, while those who subscribed to Metchnikoff's views held that phagocytic cells were paramount. The answer of course, is that both are important and work together as part of a complex, integrated system.

The complexity of the immune system is suitably illustrated by the biology of B cells. Since the discovery that a discrete population of cells produces antibodies (these cells were termed 'B' cells because of their origin in the bursa of Fabricius in birds) this population has been broken down into multiple subcategories; each having different origins and functions. The primary division of B cells is into B-1 and B-2 cells. Conventional (B-2) cells originate in the bone marrow in mammals and respond to protein and non-protein antigen after receiving help from T cells. In contrast, B-1 cells are self-replenishing and produce antibodies in the absence of help from T cells. Not only are the antigens recognised by these different B cell populations distinct, the antibodies produced by them may also have very different roles in immune responses.
The bacterial product LPS is an important immune activator and B cell antigen. LPS has been well described as being a mitogen for B cells. Despite considerable study on the mechanism of action of LPS, as well as its effects on diverse immune cells, it is unclear if there are differences in the ability of LPS to activate B-1 and B-2 cells. Furthermore, the LPS from individual species of bacteria, although sharing many structural similarities, may be significantly different in terms of biological activity. Little is known on how LPS derived from different species of bacteria affects B cells.

Given the wealth of evidence demonstrating the distinct behaviour and functions of B-1 and B-2 cells, it seems possible that these two cells types may have different sensitivities to LPS. If indeed this is case, differential sensitivity to LPS could have an impact on the way in which immune responses develop. Furthermore, inappropriate immune responses to LPS could lead to disease states. Periodontitis is potentially one such disease.

1.3 B cells and antibody in immune responses

The production of antibodies by B cells is one process by which the immune system can target effector mechanisms to specific antigens. Antigen specificity is provided by the V-region of the antibody, which potentially has extraordinary variability between molecules. This is then linked, in the same molecule, to an effector region (the Fc portion), which is able to direct conserved effector mechanisms to the antigen.

Antibodies do not simply direct effector mechanisms to antigen. The existence of different classes of immunoglobulin, with alternate conserved Fc regions, allows the antibody response to be directed towards functionally distinct effector mechanisms. This occurs when naïve B cells expressing IgM receive appropriate signals and switch their Fc region to alternate isotypes.

1.3.1 T-dependent responses

The antibody response to most proteins comes primarily from conventional (B-2) cells. These cells bind antigen via their B cell receptor (BCR) and present it to cognate T cells
in lymphoid tissues. The T cell then provides signals that cause the B cell to become activated, to proliferate and finally to differentiate into effector cells.

1.3.1.1 Initiation of immune responses

Presentation of antigen to naïve T cells is a critical step in the induction of a typical thymus-dependent antibody response. This is the checkpoint at which the ‘decision’ to respond to an antigen, or not, is made. The most important cell in this initial process is the dendritic cell (DC). These cells are very powerful initiators of T cell responses (McKeever et al., 1992). Upon contact with pathogens (De Smedt et al., 1996; Sparwasser et al., 1998) or ‘danger’ signals derived from the tissue (Gallucci et al., 1999), they become activated. Activation leads to the stable expression of antigen-MHC class II complexes on their surfaces (Cella et al., 1997), as well as the induction of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) (De Smedt et al., 1996; Gallucci et al., 1999). After activation, dendritic cells migrate to the T cell areas of lymphoid tissues (De Smedt et al., 1996) where they interact with B cells (Garside et al., 1998).

Not only do the initial responses of the immune system to an antigen/pathogen determine if an immune response will occur, they also influence the modality of the T cell (and therefore the overall) response. It has been recognised for decades that there is a division in the effector phases of immunity into cellular and humoral arms (Parish, 1972). The cellular basis of this dichotomy was explained in the late 1980s, by Mosmann et al., who identified two classes of T helper cell based on their secreted cytokine profiles (Mosmann et al., 1986). So-called T helper 1 (Th1) cells are primarily involved in cell-mediated immune responses and produce IFN-γ, TNF-β and IL-2. In contrast, Th2 cells stimulate the production of antibody by secreting IL-4, IL-5, IL-6 and IL-13. These two populations cross-regulate each other’s activity (Chretien et al., 1990; Hasbold et al., 1999). Th1 responses occur in response to intracellular pathogens (Hsieh et al., 1993) and are characterised by the activation of macrophages (Stout and Bottomly, 1989) and the development of delayed-type hypersensitivity (Cher and Mosmann, 1987). Infections with extracellular pathogens, however, result primarily in Th2 development and production of antibodies with isotypes such as IgG1 and, in extreme cases, IgE (reviewed in Sher and Coffman, 1992). This division of T cell
function into T\(_H_1/ T\_H_2\) is now regarded as overly simplistic (Kelso, 1995), however the classification is still useful when interpreting the development of many effector functions.

The development of T\(_H_1\) or T\(_H_2\) responses depends critically on the early immune response. During the initial phases of an immune response, antigen-specific T cells secrete a mix of T\(_H_1\) and T\(_H_2\) cytokines. These uncommitted cells are termed T\(_H_0\) cells (Street et al., 1990). Activation by DC, as well as the influence of early cytokines, in particular IL-12 and IL-4, causes differentiation of T\(_H_0\) cells into T\(_H_1\) and T\(_H_2\) cells respectively. If T\(_H_0\) cells are activated and exposed to IL-12, then T cells become committed to IFN-\(\gamma\) production (Seder et al., 1993). In contrast, early IL-4 production leads to T\(_H_2\) polarisation (Le Gros et al., 1990).

The ability of B-2 cells to produce antibodies of different isotypes is a crucial feature of the B cell response. Isotypes of different classes are able to link antigen to different effector mechanisms. For example, the production of IL-4 by T\(_H_2\) cells causes the production of IgE (Hasbold et al., 1998; Snapper & Paul, 1987). IgE binds specifically to Fc\(\varepsilon R\)I on mast cells and is involved in the response to parasites (Matsuda et al., 1990). Alternatively, T\(_H_1\) cell derived IFN-\(\gamma\), while weakly enhancing B cell proliferation, selectively drives IgG2a production (Hasbold et al., 1999; Snapper & Paul, 1987).

1.3.1.2 Foci, germinal centres and bone marrow

The initial interaction between T cells and B cells occurs in the T cell areas of the lymphoid tissue. In the spleen this is the periarteriolar lymphoid sheath (PALS) (Jacob et al., 1991), while in the lymph node this occurs in the medulla (Garside et al., 1998). After presentation of antigen, T cell help causes B cell division and subsequent differentiation to antibody production (Jacob et al., 1991; Jacob and Kelso, 1992).

Antibody forming foci dominate the early primary response. They are composed of oligoclonal aggregates of antibody-forming cells (AFC) in the PALS. The cells in these foci have V genes that are in germline configuration (Jacob et al., 1991; Jacob and Kelso, 1992; Jacob et al., 1993). Their main function is the immediate production of
antibody in an infection, and to allow antigen trapping by follicular dendritic cells (Song et al., 1998).

B cell affinity maturation occurs in the germinal centres. Germinal centres reach a peak in size around day 10-12 and remain after the foci disappear (Jacob et al., 1991). In the GC reaction, B cells undergo hypermutation of their V genes (Berek et al., 1991), and Darwinian selection for antigen that is bound to follicular dendritic cells (Berek and Ziegner, 1993). B cells that survive the germinal centre reaction leave and either become memory cells (Klaus, 1978; Smith et al., 1997) or AFC in the bone marrow (Dilosa et al., 1991; Smith et al., 1997).

1.3.2 T-independent responses

Subsets of B cells can also become activated and can produce antibody in the absence of T cell help. The role of this T-independent (TI) antibody is believed to be the provision of rapid protection from pathogens. There is more than one form of T-independent B cell response, and underlying each kind of response are different cellular pathways. B cells may become activated to secrete antibody either by stimulation with polyclonal mitogens (TI-1 responses) or by interaction with high molecular weight polymers (TI-2). Alternatively, ‘natural’ antibody is produced constitutively in vivo, and exists even in the absence of exogenous antigen.

1.3.2.1 TI-1 and TI-2 responses

T independent antigens are subdivided into two categories: T independent-1 (TI-1) and T Independent –2 (TI-2). TI-1 antigens are substances that are capable of directly causing the activation and proliferation of B cells, regardless of the B cell specificity. The best studied of these is LPS. LPS binds to germline-encoded Toll-like receptors on B cells and induces activation (see Section 1.5.2.1.3). In contrast, TI-2 antigens specifically activate B cells via the BCR, causing them to proliferate in the absence of T cell help (reviewed in Mond et al., 1995). TI-2 antigens have characteristic structural features; in particular they have repeated identical epitopes. The bulk of TI-2 antigens are polysaccharide chains with repeated motifs, and these are believed to activate B cells by extensive cross-linking of the BCR, probably with the help of cytokines derived from accessory cells ((see Mond et al., 1995), and Section 1.5.2.2).
The response to TI-2 antigens shows several noticeable differences when compared to the response to thymus-dependent B cell responses (previously discussed in section 1.3.1). In TI-2 responses in mice, there is minimal germinal centre activity (Weissman et al., 1976) and IgM and IgG3 are the primary isotypes produced (Perlmutter et al., 1978; McLay et al., 2002). The main site of this antibody production is in the extrafollicular foci of the spleen (Garcia de Vinuesa et al., 1999). Although in some experimental systems the absence of immunological memory was reported (Klaus and Humphrey, 1974), in other systems a significant anamnestic response can be demonstrated (Kolb et al., 1993).

The antibody produced upon TI-2 activation is important in the early response to encapsulated bacteria (McLay et al., 2002), and viruses that have capsids with repeated epitopes (Bachmann and Zinkernagel, 1997). In addition, it has recently been argued that TI-antibody aids the development of the T-dependent B-2 response (Baumgarth et al., 2000).

In contrast to TI-2 antigens, substances that stimulate a TI-1 response have the intrinsic ability to cause polyclonal B cell proliferation and antibody production. The best-characterised TI-1 antigen is LPS (Peavy et al., 1970; Brooks and Vitetta, 1987; Ogata et al., 2000). Despite the ability to non-specifically activate B cells, during the in vivo response to LPS, increased titres of anti-LPS antibody are produced (Wannemuehler et al., 1984). This LPS-specific antibody is important in the clearance of LPS and protection from endotoxic shock (Reid et al., 1997). The activation of B cells by LPS is further discussed in Section 1.5.2.1.

1.3.2.2 Natural antibodies

Natural antibodies are present in the serum of animals in the absence of antigenic stimulation (Bos et al., 1988; Diaw et al., 1997; Haury et al., 1997). They are produced by B cells, yet they are considered to be part of the innate immune system (Fearon and Locksley, 1996). They are polyclonal, and most are of the IgM isotype (Diaw et al., 1997). Despite its typical low affinity, due to its multimeric structure, IgM is able to bind with high avidity to polymeric antigens (Brewer et al., 1994).
In addition to recognising exogenous antigens, natural antibodies are also reactive with intracellular self-antigens (Bouvet and Dighiero, 1998). Natural antibodies found in normal human serum have been shown to react specifically with self-proteins including tubulin, actin, thyroglobulin, fetuin, albumin, transferrin, cytochrome c and collagen (Dighiero et al., 1985). B cells synthesizing polyspecific natural antibodies are present in neonatal nude, germ-free, and antigen-free mice (Avrameas, 1991), which suggests that the production of these antibodies is constitutive and does not rely on any external antigen stimulation for the initiation of production. This ability of natural antibodies to bind to intracellular compounds has been argued to fulfil a useful function as polyreactive antibodies bind to cellular debris from dying cells, aiding phagocytosis (reviewed in Potter and Melchers, 2000).

Natural antibodies which predominate early in life (Diaw et al., 1997; Bouvet and Dighiero, 1998), are encoded by immunoglobulin V genes under germinal, or non-rearranged, configuration (Diaw et al., 1997). An important peculiarity of natural antibodies is that they utilise selected V\textsubscript{H} gene segments, including V\textsubscript{H}III, V\textsubscript{H}IV and V\textsubscript{H}V. These gene segments seem to be most often in unmutated configuration (Casali and Notkins, 1989).

Natural antibodies can be considered to be a phylogenetically conserved first line of defence against pathogens (Avrameas, 1991; Diaw et al., 1997) and can lead to the enhancement of an ongoing specific immune response (Casali and Notkins, 1989). Natural antibody has been found to initiate complement activation (Borsos and Rapp, 1965), and to enhance monocyte phagocytosis (Avrameas, 1991) and neutrophil recruitment (Boes et al., 1998) in response to pathogens. As this “primordial antibody” system has persisted as a basic mechanism of immunity in higher vertebrates it may confer a survival advantage to the animal in the time when more specific defences are being initiated and prepared (Dighiero et al., 1985; Bouvet and Dighiero, 1998). In essence, it has been believed that natural antibody’s function is to ‘hold the fort’ in the stages of the immune response prior to the development of a T dependent immune response. Recently however, it has been shown that natural antibodies may play another role. In models of virus infection, natural IgM has been shown to be critical for the
development of a productive adaptive antibody response (Baumgarth et al., 2000). This is also consistent with studies in secretory IgM+ (sIgM+) mice, which showed that these mice mount defective T-dependent IgG responses (Boes et al., 1998; Ehrenstein et al., 1998), and that this deficit can be repaired by administration of normal serum IgM (Ehrenstein et al., 1998).

1.3.2.3 B-1 cells TI-1, TI-2 and natural antibodies

Discussion of the role of different populations of B cells in the production of T-independent or natural antibody presents certain difficulties. The definitions of different types of antigens are often hazy and many times they overlap. For example, antibodies produced to many TI-1 and TI-2 antigens are also included in ‘natural’ antibody. Alternatively, LPS, which is a prototypical TI-1 antigen, also has features of TI-2 antigens such as the presence of a polysaccharide chain with repeated epitopes (Paramonov et al., 2001; Parker et al., 2001).

B-1 cells (see Section 1.4.2 for a description of B-1 cells) are argued to be the major cell population responsible for the production of TI-1, TI-2 and natural antibodies.

The evidence for B-1 cells being the primary producers of natural antibody is based on studies that showed transfer of B-1 cells into irradiated recipients (Kroese et al., 1989) or neonatal mice (Forster and Rajewsky, 1987; Lalor et al., 1989) reconstitutes serum natural antibody levels. Furthermore, investigation of the B-1 repertoire has shown that antibodies produced by these cells recognise a range of natural IgM specificities. These include antibodies to phosphorylcholine (PC) (Masmoudi et al., 1990), phosphatidylcholine (PtC) (Hayakawa et al., 1984) and influenza virus (Baumgarth et al., 1999). Based on depletion experiments (Su et al., 1991), and ELISpot data (Reid et al., 1997), B-1 cells appear to be the major source of LPS-specific antibodies. This antibody is biologically relevant, as antibody deficient mice have an increased mortality in response to LPS when compared to controls (Reid et al., 1997).
1.4 B cell development

Results from many experimental systems have supported the division of B cells into two distinct populations. Conventional (B-2) cells predominate in secondary lymphoid tissue. They develop late in ontogeny, are derived from the bone marrow and require T cell help for antigen-specific proliferation and antibody production. During an immune response B-2 cells typically undergo affinity maturation in germinal centres and switch isotype. In contrast, B-1 cells are most common in the peritoneal and pleural cavities and are self-replenishing. They produce polyreactive IgM (see section 1.4.2), exhibit restricted V-gene usage and do not generally undergo somatic mutation (Brezinschek et al., 1997). Initially, B-1 cells were defined on their expression of CD5 (Hayakawa et al., 1983), a cell-surface marker previously associated with T cells. Subsequently it was shown that a CD5⁺ population exists, which has many of the characteristics of CD5⁺ B cells. This has led to the further subdivision of B-1 cells into B-1a (CD5⁺) and B-1b (CD5⁻) populations (Stall et al., 1992). Although uncertain, it seems likely that B-1 and B-2 cells represent distinct cell lineages.

1.4.1 Conventional (B-2) cells.

The development of B-2 cells is well characterised. Committed B cell precursors originate from the bone marrow (Li et al., 1996; Allman et al., 1999). There are three important phases in early B-2 cell development. Each of these is defined by characteristic steps in the development of the BCR. Initially, bone marrow precursor cells have germline heavy and light chain genes. These cells are termed pre-pro B cells and are identified by their expression of CD45R/B220 (Li et al., 1996; Ogawa et al., 2000). Using the products of recombination activating gene (RAG)-1 and RAG-2, pre-pro B cells rearrange their heavy chain genes and express a surface mu chain (Oettinger et al., 1990; Mombaerts et al., 1992). Cells at this stage are termed early B cells. B cells that fail to make productive rearrangements are deleted. Following heavy chain rearrangement, the mu chain is paired with a surrogate light chain (Karasyuk et al., 1990). It is believed that this is an important checkpoint in the development of the B cell and that cells with mu chains that are unable to productively pair with light chains are deleted (Gong and Nussenzweig, 1996). Finally, the B cell rearranges its light chain genes and pairs them with the heavy chain, thus making a functional BCR. It is at this
stage that the B cell is screened for reactivity to self-antigen. Evidence from a number of transgenic models suggests that cells that bind to membrane antigen with high affinity are deleted, while those cells binding with low affinity to membrane antigens, or to soluble antigens, either become anergic or undergo receptor editing (Goodnow et al., 1988; Nemazee and Burki, 1989; Hartley et al., 1991; Hartley and Goodnow, 1994; Lang et al., 1996; Retter and Nemazee, 1998).

1.4.2 B-1 cells

It is most likely that B-1 cells are derived from a separate lineage to conventional B-2 cells. The strongest evidence for this comes from studies examining the ability of different precursors to repopulate immunodeficient mice. Cells from foetal liver, with non-rearranged V-genes, preferentially give rise to B-1 cells, whereas those from adult bone marrow produced conventional B cells (Hardy and Hayakawa, 1991; Kantor et al., 1992). It has also been argued that CD5+ and conventional B cells might represent separate cell lineages much like that of αβ (conventional) and γδ T cells (DeFranco, 1992) in that these subsets show both characteristic repertoires and locations.

The V-gene usage of the different populations also provides evidence for the existence of distinct lineages. B-1 and B-2 cells show distinct patterns of V-gene usage, and this, in part, may reflect the origins (foetal liver versus bone marrow) of these cells. Human IgM⁺/CD5⁺ B cells have been found to contain significantly fewer hypermutated V_H chain genes than IgM⁺/CD5⁻ B cells (Brezinsicschek et al., 1997), and most B-1 cells in healthy humans carry unmutated V region genes (Fischer et al., 1997). However, CD5⁺ B cells can be somatically mutated (Brezinsichek et al., 1997).

The foetal and neonatal B cell repertoire differs qualitatively from that of the adult at both the molecular and cellular level (Cuisinier et al., 1989; Schroeder and Wang, 1990). Human foetal B-lineage cells rearrange a highly restricted set of V_H gene segments, and there is preferential rearrangement of certain antibody gene segments (Schroeder et al., 1987), including V_H6 and V_H5 (Cuisinier et al., 1989). Typical features of the early repertoire are the predominance of B-1 cells, characteristic restriction of V gene usage, and limited development of somatic diversity (Kearney et al., 1992). This can be seen at the Ig receptor level by the predominant expression of D
proximal $V_H$ genes in mice (Holmberg, 1987), and similar expression of $V_H$III family members in humans (Kearney et al., 1992). It has been suggested that this difference in repertoire could be due in part to the time constraint placed on the formation of the B-1 cell repertoire (this appears to be essentially fixed in mice by the onset of sexual maturity) (Lalor and Morahan, 1990).

Evidence from mice with altered BCR signalling pathways also supports the existence of separate lineages. For example, deletion of positive influences on BCR signalling such as CD19 (Rickert et al., 1995; Inaoki et al., 1997), Vav (Tarakhovsky et al., 1995) and Btk (Khan et al., 1995) leads to a relative decrease in B-1 cell numbers. In contrast, mutation of the negative regulator SHP-1 (Sidman et al., 1986) or overexpression of CD19 (Sato et al., 1996), leads to increased B-1 cell numbers in comparison to conventional cells. These results imply that BCR signalling has a positive effect on the development of B-1 cells, but not B-2 cells. Although it might be assumed that selection based on microbial antigens might be important for B-1 development, this appears not to be the case. A highly reactive B-1 cell repertoire develops in germ-free mice, demonstrating that the role of bacterial antigens, while possibly influencing the balance of the clones, is not the primary selective stimulus mediating formation of the unique, multi-reactive repertoire of this lineage (Lalor and Morahan, 1990; Haury et al., 1997). It has been argued that this positive signalling event is, in fact, selection for self-antigen (Hayakawa et al., 1999; Hayakawa and Hardy, 2000).

In contradiction to the model of B-1 and B-2 early lineage divergence, it has also been argued that this divergence occurs late; following V-gene rearrangement. This has been termed the 'induced differentiation hypothesis' (Clarke and Arnold, 1998). In this model, lineage commitment occurs on interaction with antigen, with B-1 cells committing to the B-1 lineage when they bind to T-independent antigen in the absence of T cell help (Haughton et al., 1993). It has been shown that conventional B cells, under certain circumstances, can express CD5 (Weichert and Schwartz, 1997). For example, CD5 expression is induced following challenge with TI-2 antigens such as anti-Ig (Cong et al., 1991; Wortis et al., 1995). In this model, the ability of the pro-B cell to become a B-1 cell is, under normal circumstances, restricted to a defined period
in development. Recognition of antigen during this period leads to upregulation of CD5 and commitment to the B-1a lineage.

### 1.4.2.1 Splenic and peritoneal B-1 cells

Studies (Wells et al., 1994; Fischer et al., 2001) have found that murine splenic B-1 cells, although similar in a number of respects, are different to peritoneal cavity B-1 cells. Peritoneal B-1 cells express CD11b (Mac-1) (Kantor et al., 1992; Stall et al., 1992), and some lack CD43, which is expressed on nearly all splenic B-1 cells (Wells et al., 1994). Splenic B-1 cells also differ from peritoneal B-1 cells in transcription factor (STAT3, CRE-binding activity, PU.1) and gene expression (IL-10) and in signalling for cell cycle progression (PMA response) (Fischer et al., 2001).

### 1.4.2.2 Homeostatic regulation of B-1 cells

B-1 cells appear to have no self/non-self discrimination. They are a ubiquitous population able to bind both self and foreign antigens (Casali et al., 1987; Murakami and Honjo, 1995; Diaw et al., 1997) and are self-renewing (Hayakawa and Hardy, 1988). Self-antigen has been shown to drive B-1 selection, activation, and possibly self-renewal (Carmack et al., 1990; Lalor and Morahan, 1990; Arnold and Haughton, 1992; Hardy, 1992; Chen and Kearney, 1996). This suggests that this subset would be highly regulated in vivo to prevent accumulation of these autoreactive cells.

There are a number of mechanisms by which control of B-1 cells is accomplished, although the level of redundancy of these mechanisms, in terms of control is unknown at present. Feedback regulation of B-1 cells has been suggested by Lalor et al. (Lalor et al., 1989), and this may be through binding of B-1 cell-derived IgM (O'Garra and Howard, 1992; Bandyopadhyay et al., 1995) or CD5/CD72 interaction (Van de Velde et al., 1991; Osman et al., 1992; Jamin et al., 1993; Lankester et al., 1994; Bikah et al., 1996; Jamin et al., 1996; Jamin et al., 1996; Van de Velde and Thielemans, 1996). Feedback regulation may be achieved in the same manner as quorum sensing in bacteria (reviewed in Withers et al., 2001); once a particular density of B-1 cells has been reached, micro-environmental concentrations of IgM or the number of CD5/CD72 interactions may reach a level which down-regulates self-renewal. Control of B-1 cells may also be achieved through cytokine interactions, as B-1 cells have been shown to be responsive to regulation by IL-10 (O'Garra et al., 1992; Velupillai et al., 1996) and
IFNγ (Chace et al., 1993; Abed et al., 1994). This regulation may have an autocrine component, as B-1 cells produce IL-10 (O'Garra et al., 1990; O'Garra et al., 1992). However, these control mechanisms can fail, leading to the accumulation of B-1 cells in pathologies such as Sjögren's syndrome (Plater-Zyberk et al., 1989) and chronic periodontitis (Sugawara et al., 1992). The role of bacterial mitogens, if any, in this breaking of control is not yet fully understood.

1.5 Pathways of B cell activation

Conventional B cells require T cell help to become activated (see Section 1.3.1). This linking of T cell and B cell antigen recognition provides an important checkpoint that prevents activation of autoreactive B cells. In contrast to the stereotypical B cell response to monomeric protein antigens, some B cells may become activated by cross-linking of their BCR in a manner that does not require T cell help (TI-2 antigens). This typically occurs in response to non-protein antigens, which have repeated epitopes (see Section 1.3.2). Further, some antigens such as LPS are capable of activating B cells in a non-clonal manner, by binding to germline-encoded receptors (TI-1 antigens, discussed below).

While the division of B cell responses into T-dependent, TI-1 and TI-2 is a useful categorisation, it must be recognised that it is a simplification. For example, viral capsid proteins can form multimeric arrays on the surface of viral particles, and this structure can cause T-independent activation of B cells (Bachmann and Zinkernagel, 1997). Alternatively, LPS may not only activate B cells via germline-encoded receptors, but by virtue of its multimeric structure it also signals via the BCR in specific cells.

1.5.1 CD40 ligation versus BCR cross-linking

B cells are able to integrate signals from a range of receptors (Wheeler et al., 1993; Snapper et al., 1995; Lam et al., 1999). The two best-characterised influences on B cell activation are B cell receptor (BCR) cross-linking and co-stimulation from T cells (via CD154/CD40L). At one end of the spectrum, in T-dependent responses, stimulation leading to activation and proliferation may be provided solely by CD154 on cognate T cells (Hasbold et al., 1998; Lam et al., 1999). Between these two extremes lies a
spectrum of stimuli that can lead to activation (Bachmann and Zinkernagel, 1997). Complex intracellular pathways are capable of integrating the signals resulting from various stimuli. This integration ultimately leads to different outcomes such as proliferation, rescue from apoptosis, isotype switching or tolerance.

The membrane protein CD154 (CD40L) is capable of causing activation and differentiation of B-2 cells. It is expressed on activated T cells and binds to CD40 on B cells (Hasbold et al., 1998; Lam et al., 1999). In most T-dependent responses where the antigen is not polyvalent, the BCR appears primarily to function as a means of focusing antigen into the MHC class II compartment, thus allowing increased presentation to T cells (Rock et al., 1984; Song et al., 1995; Cook et al., 1998). Cells that have the highest affinity for antigen are able to garner the most T cell help (Batista and Neuberger, 1998). Antigen presentation and co-stimulatory signalling via CD28 causes increased activation of the T cell. In turn, expression of CD154 on T cells is upregulated (Johnson-Leger et al., 1998; Yin et al., 1999), which further activates the B cell. The specificity of the interaction between antigen-presenting cells and T cells is achieved due to the polarised nature of the interaction, which has been termed the ‘immunological synapse’ (Grakoui et al., 1999).

In contrast to the typical T-dependent response, some B-2 cells are capable of being activated by BCR cross-linking alone (Monigini et al., 1992; Fehr et al., 1996; García de Vinuesa et al., 1999).

The response of B-1 cells to BCR ligation is very different to that which occurs in B-2 cells. Ligation of the BCR on B-1 does not induce proliferation (Rothstein and Kolber, 1988; Morris and Rothstein, 1993; Bikah et al., 1996; Wong et al., 2002), and may (Murakami et al., 1992) or may not (Morris and Rothstein, 1993) induce these cells to apoptosis.

1.5.1.1 BCR/CD40 intracellular signalling pathways
The signalling complexes of BCR and CD40 molecules belong to the family of receptors termed the ‘multi-chain immune recognition receptors’ (MIRR). Individual members of this family of receptors have multiple chains that associate together on the
surface of the cell to signal. The BCR, for example, is composed of an antibody-like antigen-binding domain that is complexed to two types of signalling chain, the α and β chains. These signalling chains have a characteristic amino acid motif, termed the immunoreceptor-tyrosine-based activation motif (ITAM). Receptor aggregation causes cytoskeletal interactions (Holowka et al., 2000) and translocation of the MIRR to discrete regions of the membrane, termed lipid rafts, which contain signalling kinases (Field et al., 1997; Cheng et al., 1999). These kinases phosphorylate tyrosine residues within the ITAM, which allows docking of intracellular signalling proteins that contain the Src-homology-2 (SH2) domain. Docking of the SH2 domain cause activation of multiple signalling pathways (Bevan and Baumgartner, 1996).

B cell receptor signalling may be modulated by co-ligation of different co-receptors. For example, co-ligation of the complement receptor 2 complex to the BCR decreases the activation threshold of the cell (Carter and Fearon, 1992; Dempsey et al., 1996). Alternately, co-ligation of the inhibitory FcγRIIB1 complex to the aggregated BCR increases the activation threshold; this is mediated by the recruitment of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) to the signalling complex (Sinclair, 2000). Phosphorylation of tyrosine residues on ITIMs causes the recruitment of inhibitory signalling molecules such as SHP-1 (Plas et al., 1996).

1.5.1.1 CD5 signalling in B cells

It appears likely that the CD5 expressed on B-1 cells is involved in negative regulation of B-1 cell activation. Splenic B-2 cells from mice deficient in CD5 proliferated normally in response to CD40 ligation, BCR cross-linking (anti-IgM) or LPS (Bikah et al., 1996). Peritoneal B-1 cells also proliferated normally in response to CD40 ligation or LPS stimulation. However, in response to BCR cross-linking, B-1 cells from CD5−/− mice not only failed to apoptose, but also underwent proliferation (Bikah et al., 1996). Further evidence for the role of CD5 comes from experiments involving co-ligation of CD5 and the BCR. In the presence of CD5, murine peritoneal cavity B cells do not proliferate in response to anti-IgM, but proliferate after cross-linking with anti-CD5 (Jamin et al., 1996).
The biological significance of CD5-mediated signalling appears to be the down-regulation of autoimmune B cell activation. In a system in which CD5⁺ mice were crossed with transgenic mice expressing both hen egg lysozyme (HEL) as well as HEL-specific B cells, CD5⁺ B cells were found to be anergic (Hippen et al., 2000).

The molecular mechanisms underlying CD5 inhibition of BCR-induced activation are not completely understood, and much of the evidence comes indirectly from studies of CD5 function in T cell biology. As with B cells (Lankester et al., 1994), CD5 in T cells associates with the antigen receptor (Burgess et al., 1992). In the absence of CD5, immature T cells are hyperresponsive to TCR stimulation (Tarakhovsky et al., 1995). Following TCR ligation, residues in an ITIM-like domain in the CD5 molecule become phosphorylated (Burgess et al., 1992), which presumably leads to recruitment of inhibitory molecules such as SHP-1. Co-ligation of the BCR and CD5 also leads to phosphorylation of the CD5 molecule, however neither SHP-1 nor SHIP becomes associated with the phosphorylated CD5 (Gary-Gouy et al., 2000). Whatever the details of the intracellular signalling cascade, it appears that CD5 may increase B-1 cell survival due to autocrine production of IL-10 (Gary-Gouy et al., 2002).

Jamin et al. postulated that, in association with CD4/CD29, CD5 is involved in CD5⁺B/CD5⁺ B cell interactions in non-organ-specific autoimmune diseases (Jamin et al., 1993). It seems likely that in CD5⁺ cells, interaction of CD5 with its ligand CD72 (Van de Velde et al., 1991) modulates signals transmitted through the BCR (Jamin et al., 1996). CD72 is a non-redundant regulator of B cell development and is a negative regulator of B cell activation. CD72⁺ B cells are hyperproliferative in response to various stimuli (Pan et al., 1999).

1.5.2 Polyclonal Activation

Polyclonal B cells activators (PBAs) are able to activate B cells without the requirement for T cell help, using non-Ig surface receptors (Clagett and Engel, 1978). These T-independent (TI) antigens include dextran sulphate, purified protein derivative of tubercle bacteria, Nocardia opaca water-soluble mitogen, and bacterial LPS in the murine system and Epstein-Barr virus (EBV) in the human system (Kettman and Wetzel, 1980; Casali and Notkins, 1989). LPS has also been found to be a polyclonal
activator for malignant (Baeker and Rothstein, 1986) and normal human B cells (Ringden et al., 1977; Hammarstrom et al., 1980; Matsushima et al., 1985; Gaidano et al., 1989; Settmacher et al., 1990). The effects of polyclonal activation include cytokine secretion, Ig production and differentiation (reviewed in Clagett and Engel, 1978). Polyclonal B cell activation could provide a selective advantage to the host as it provides a (non-specific) first line of defence against invading pathogens. In particular, the activation of B-1 cells that secrete polyreactive antibodies may be important. Polyclonal B cell responses may be protective in that polyvalent IgM may effectively bind to polymeric B cell activators of microbial origin and promote the activation of complement and the mobilisation of effector cells.

Although the activation of B-1 cells by PBAs may be beneficial in an immune response, long-term exposure to PBAs could result in the destruction of host tissues through uncontrolled polyclonal B cell responses and autoantibody production (Abed et al., 1994). For example, infection with Actinomyces viscosus is associated with plasma-cell rich inflammatory gingival disease and chronic inflammatory lesions of other soft tissues. A. viscosus produces a PBA that induces proliferation, Ig synthesis and differentiation of murine B cells into plasma cells (Clagett and Engel, 1978).

1.5.2.1 LPS signalling

Many of the components of the mammalian innate immune system are conserved across classes and even phyla. In Drosophila melanogaster, the protein encoded by the toll gene induces the innate anti-fungal immune response (Lemaitre et al., 1996). The plant tobacco mosaic virus resistance gene encodes a protein very similar to Toll, and it appears that the immune-response system mediated by Toll represents an ancient host defence mechanism (Medzhitov et al., 1997). The insect host defence involves synthesis of antimicrobial peptides by the fat body and secretion into the haemolymph. Drosophila melanogaster produces seven distinct antimicrobial peptides, allowing it to discriminate between various groups of microorganisms and to mount a response appropriate to the invading microorganism (Tauszig et al., 2000).

A human homologue of dToll (hToll) was cloned, expressed, and characterised by Medzhitov et al in 1997. It was shown to have homology with Drosophila Toll (dToll)
over the entire length of the protein chains (Medzhitov et al., 1997). The extracellular
domain of hToll contains 21 tandemly repeated leucine-rich motifs separated by a non-
leucine-rich repeat region (LRR), similar to dToll. At the N-terminal end of the LRR
domain there is a 31 amino acid long N-flanking region, while the C-terminal end of the
LRR domain is flanked by a cysteine-rich domain, which is also present in dToll. The
cytoplasmic domain is homologous to that of the human IL-1 receptor (Medzhitov et
al., 1997).

Although initially identified in the field of developmental biology for their role in
Drosophila dorsoventral patterning (Anderson et al., 1985), Toll-like receptors (TLR)
have subsequently been shown to have a critical role in the immune system. A number
of individual TLR have been identified, and each has a distinct specificity for pathogen-
derived products. These include flagellin (TLR5) (Hayashi et al., 2001), dsRNA (TLR3)
(Alexopoulou et al., 2001), lipopeptides (TLR2 homodimers) (Ozinsky et al., 2000),
peptidoglycan (TLR2/6 heterodimers) (Ozinsky et al., 2000), unmethylated CpG motifs
(TLR9) (Hemmi et al., 2000) and LPS (TLR4) (Poltorak et al., 1998; Poltorak et al.,
1998).

(For the purpose of continuity a more detailed discussion of TLRs in appended to this
document as Appendix 4.)

1.5.2.1.2 TLR4 is the major LPS receptor
As early as the 1960s, the C3H/Hej mouse strain was identified as being resistant to the
effects of LPS (Heppner and Weiss, 1965). This deficit was later attributed to a single
genetic locus, termed Lps (Watson and Riblet, 1974). In 1998, it was demonstrated that
TLR4 correlated with the Lps gene, and that TLR4 is primarily responsible for immune
responses to LPS (Poltorak et al., 1998; Poltorak et al., 1998; Hoshino et al., 1999). The
mammalian Tlr4 gene was identified as a candidate for Lps (Poltorak et al., 1998), and
it was later confirmed that Lps is identical to Tlr4 (Poltorak et al., 1998) and that TLR4
is the gene product that regulates LPS responses (Hoshino et al., 1999). Further studies
determined that TLR4 is the predominant receptor for LPS in human blood (Tapping et
al., 2000).
It is unclear at present whether LPS binds directly to TLR4, or whether it acts in concert with CD14. Early binding studies (Kirkland et al., 1990; Wright et al., 1990) and evidence from CD14 deficient mice (Haziot et al., 1996), implicated CD14 as the principal LPS binding protein. More recently, CD14 has been shown to co-localise with TLR4 following LPS binding (Jiang et al., 2000). However, it has also been suggested that TLR4 alone is capable of binding LPS (Poltorak et al., 2000).

1.5.2.1.3 TLR2 as an LPS receptor
The role of TLR2 in LPS signalling is unclear. Early studies using LPS derived from enterobacterial species suggested that TLR2 was a receptor for LPS (Kirschning et al., 1998). However, subsequent reports argued that this result was artifactual, and that TLR2-dependent LPS signalling results from “endotoxin protein” contamination in some LPS preparations (Hirschfeld et al., 2000). Further, work by Schwandner et al. showed that TLR2 is a receptor for soluble peptidoglycan and lipoteichoic acid (Schwandner et al., 1999; Yoshimura et al., 1999); both cell wall components of Gram-positive bacteria. More recently however, it has been found that highly-purified LPS from Porphyromonas gingivalis is capable of activating macrophages from C3H/Hej mice (Kirikae et al., 1999; Hajishengallis et al., 2002), and that differential signalling occurs in response to P. gingivalis, as opposed to TLR4-dependent E. coli LPS (Hirschfeld et al., 2001; Hajishengallis et al., 2002). Indeed, not only does P. gingivalis LPS appear to signal solely through the TLR2, it is able to antagonize signalling through TLR4 (Yoshimura et al., 2002).

1.5.2.1.4 RP105: A B cell-specific LPS receptor?
In addition to TLR2 and TLR4, it is appears that a third receptor, RP105, is important in the activation of B cells in response to LPS. RP105 is a cell surface protein belonging to the leucine rich repeat protein family (Miyake et al., 1995); the family to which TLR belong. The significance of RP105 in B cell activation/survival was first recognised when it was reported that B cells stimulated with anti-RP105 antibodies were resistant to irradiation- or dexamethasone-induced apoptosis (Miyake et al., 1994), but sensitised to anti-IgM induced apoptosis (Yamashita et al., 1996). Recently it was shown that B cells from mice deficient in RP105 respond poorly to E. coli LPS, which demonstrates that this RP105 is important in LPS signal transduction in B cells (Ogata et al., 2000).
However, it was also found that RP105-dependent signalling also requires the presence of TLR4 (Ogata et al., 2000) and the accessory molecule MD-1 (Miura et al., 1998; Nagai et al., 2002). This argues that RP105 and TLR4 work in concert in B cells to specifically recognise LPS. Interestingly, in contrast to cells of the monocyte/macrophage lineage, B cells express very low levels of TLR4 but high levels of RP105 (Akashi et al., 2000; Zaremb and Godowsdi, 2002).

1.5.2.1.5 Intracellular TLR signalling pathways

Toll-like receptors have considerable structural and functional similarities to the IL-1 receptor (O'Neill and Greene, 1998). As with the IL-1 receptor, TLR4 signalling is dependent upon association of the leucine-rich repeat domain with MD-2 (Shimazu et al., 1999). This interaction initiates recruitment of the adaptor proteins MyD88 and IRAK (Wesche et al., 1997; Medzhitov et al., 1998). Following this, a cascade, involving TRAF6 and NIK, culminates in NF-κB activation (Cao et al., 1996). The critical role of MyD88 in B cell activation in response to LPS has been demonstrated in mice deficient in this molecule. In knockout mice B cells both failed either to proliferate or to express increased levels of MHC class II in response to LPS (Kawai et al., 1999). TLR2 signalling pathways appear to be different to those activated by TLR4, with activation of NF-κB occurring via an alternate pathway involving Rac-1 (Arbibe et al., 2000).

1.5.2.1.6 Enteric and non-enteric LPS

It has been suggested that enteric and non-enteric LPS have different chemical properties (Kirikae et al., 1999) and can elicit different patterns of adaptive immunity (Pulendran et al., 2001). A number of groups have found that the LPS of P. gingivalis has a far more moderate endotoxic activity than the LPS from enterobacteria (Nair et al., 1983; Fujiwara et al., 1990). Tanamoto (1997) postulated that these differences in activity were due to differences in the lipid A moieties between P. gingivalis and enterobacteria; the lipid A of P. gingivalis was found to consist of unique branched fatty acids with longer carbon-atom chains than those of enterobacteria (Tanamoto et al., 1997)
1.5.2.1.7 Differential responsiveness of B-1 and B-2 cells?

It may also be that B-1 and B-2 cells differ in their responsiveness to PBAs, with B-1 cells reported to be much more sensitive to low concentrations of LPS (Chace et al., 1993). Since B-1 cells produce polyreactive antibodies that are involved in the early response to pathogens, preferential activation of these cells is likely to be an important protective response.

1.5.2.2 Cytokine control of polyclonal B cell activation

In addition to stimulation via the BCR or Toll-like receptors, B-1 cells are sensitive to the effects of numerous cytokines. Foremost among these is interleukin 10. Unstimulated peritoneal B-1 cells produce significant amounts of IL-10 (O'Garra et al., 1992). This possibly results from co-ligation of the BCR and CD5 (Gary-Gouy et al., 2002). Importantly, blocking of IL-10 by the administration of anti-IL-10 antibodies leads to an absence of peritoneal B-1 cells; however, it is not clear whether this is a entirely a direct autocrine activation effect of IL-10, as it is at least partly due to the removal of IL-10-mediated inhibition of IFN-γ (Ishida et al., 1992), and there are normal numbers of B-1 cells in IL-10 deficient mice (Kuhn et al., 1993). Interestingly, IL-10 expression has been reported to be higher in B-1 compared to B-2 cells (O'Garra et al., 1992; O'Garra and Howard, 1992). It has also been suggested that the IL-10 stimulating B-1a cells may come from other sources such as T cells or myeloid cells (Nisitani et al., 1998). Further, it is uncertain whether IL-10 acts directly on B-1 cells or makes them more sensitive to the actions of other cytokines (Rousset et al., 1992; Fluckiger et al., 1994; Balabanian et al., 2002).

Despite the uncertainty of its mechanism of action, IL-10 has been clearly implicated in B-1 cell activation and autoantibody production in a number of in vivo systems. In a transgenic anti-erythrocyte (self) mouse model, administration of exogenous IL-10 causes both activation of peritoneal B-1 cells and autoantibody production (Nisitani et al., 1995), while anti-IL-10 administration in Fas-deficient, anti-erythrocyte transgenic mice prevented autoantibody production (Watanabe et al., 2002). Moreover, IL-10 mediates expansion of the B-1 population in response to polysaccharide antigens (Velupillai et al., 1996).
T cells can influence the response of B cells to polyclonal activators as well as the production of T<sub>H</sub> cytokines – normal T cells can suppress the response to LPS and activated T<sub>H1</sub> clones also inhibit B cell antibody production. IFN<sub>γ</sub> is capable of modifying the B cell response to LPS and appears to act directly on B cells in regulating sensitivity to LPS. The effect of IFN<sub>γ</sub> on LPS-induced IgM secretion is most pronounced at sub-maximal concentrations of LPS and is not matched by an inhibitory effect on differentiation. It is therefore possible that IFN<sub>γ</sub> is important in regulating a subset of B cells that is extraordinarily sensitive to polyclonal activators (Cowdery and Fleming, 1992).

Several cytokines have actions that oppose that of IL-10 on B-1 cells. In particular, IFN<sub>γ</sub> is well described for its ability to inhibit the expansion of the B-1 subset in vivo. Examples include systems using saline-soluble egg antigens from Schistosoma mansoni or the polylactosamine sugar lacto-N-fucopentaose III (Velupillai et al., 1996). IFN<sub>γ</sub> may act as part of a negative feedback loop in the control of the B cell response to polyclonal activators as LPS upregulates the expression of the IFN<sub>γ</sub> receptor on LPS-activated B cells (Abed et al., 1994). Thus, as LPS activates B cells, it also renders them susceptible to inhibition by IFN<sub>γ</sub>. In systems driven by moderate concentrations of polyclonal activators, the major effect of IFN<sub>γ</sub> is to inhibit differentiation (Abed et al., 1994). IFN<sub>γ</sub> has been found to inhibit both conventional and CD5<sup>+</sup> B cell differentiation by decreasing the precursor frequency of IgM secreting cells (Chace et al., 1993). Activated peritoneal macrophages produce PGE<sub>2</sub>, which inhibits in vitro differentiation of peritoneal B-1 cells in a concentration-dependent manner (Chace et al., 1995).

1.6 Cell lines as models of B cell function

1.6.1 CH12 Cells

Studies of B-1 cells have been aided by the Chapel Hill (CH) series of lymphomas. These cell lines have been demonstrated to have many features associated with the B-1 B cell subset.
1.6.1.1 Phenotype

The CH12 B lymphoma cell line arose as a peritoneal ascites and disseminated lymphoma in a $2^{a4^b}$ mouse 310 days after intra-peritoneal inoculation of $2 \times 10^7$ splenocytes and 20μL of serum from a syngeneic donor previously immunised with sheep red blood cells (SRBC) at 35, 14, and 7 days before transfer of spleen cells (Arnold et al., 1983).

The phenotype of CH12 cells is that of a relatively mature B cell. CH12 lacks detectable Fc receptors, but expresses CD5 (Arnold et al., 1983). The cell line binds both sheep red blood cells (RBC) and chicken RBC, but not human, burro, horse, mouse, or rabbit RBC to surface Ig (Arnold et al., 1983).

CH12 is not permanently frozen at one specific step of differentiation. Under the influence of changing growth conditions, either in vivo or in vitro, it can be induced to differentiate and secrete Ig. CH12 bears surface IgM indicating that secondary, class switching, heavy chain gene rearrangement has not occurred. Under normal conditions of growth less than 3% of CH12 cells differentiate to Ig secretion (Arnold et al., 1983).

In humans, B chronic lymphocytic leukaemia (B-CLL) tumours present phenotypic characteristics similar to those of the CH series of murine lymphomas. Approximately 20% of B-CLL patients develop autoimmune haemolytic anaemia, consistent with an anti-phospholipid specificity of the monoclonal antibody produced by the tumour (Mercolino et al., 1986). The normal CD5+ B cell precursors of B cell chronic lymphocytic leukaemia cells may play a role in other autoimmune diseases, eg human systemic lupus erythematosus (SLE) (Mercolino et al., 1986).

1.6.1.2 Repertoire

Lord and Dutton (1975) suggested that the spontaneous plaque-forming cells found in normal spleens are representative of an autoimmune response against antigenic determinants to which the animal is continually exposed. They suggested that the determinant recognised on BrMRBC becomes exposed in vivo on effete erythrocytes, providing a means for their recognition and elimination (Lord and Dutton, 1975).

Many of the CH lymphomas bear cell surface immunoglobulin that binds to SRBC. The hapten recognised by SRBC-binding CH lymphomas is phosphatidyl choline (PtC) (Mercolino et al., 1986). The reactive hapten can be exposed on various erythrocytes by treatment with the proteolytic enzyme, bromelain. Cells that express this specificity can
be found in the spleens of many normal mice, but their number is not increased by immunising with SRBC (Cunningham, 1976). These normal, spontaneous plaque-forming cells (PFC) belong to the CD5$^+$ B cell lineage, as do all the CH lymphomas of similar specificity. Reactivity toward phosphorylated autoantigens may be characteristic of normal and aberrant CD5$^+$ B cells, and it appears that the CH series of B cell lymphomas are neoplastic analogues of normal CD5$^+$ B cells (Mercolino et al., 1986). The polyspecificity of many antibodies produced by the CD5$^+$ B cell lineage results from the similarity in chemical structure of the antigens recognised. For example, repeating membrane phospholipids are similar to nucleic acids (Mercolino et al., 1986). CD5$^+$ B cells are responsible for the production of several commonly studied IgM autoantibodies. A relationship between anti-DNA and spontaneous anti-erythrocyte specificities is suggested. Polyspecificity of antibodies produced by CD5$^+$ B cells for structurally related antigens is known in other systems as well. Anti-DNA antibodies from SLE patients can recognise cardiolipin (Colaco and Elkon, 1985).

1.6.1.3 Response to Stimulation
As described in Section 1.6.1.1, CH12 is an inducible monoclonal population with known antigen specificity for senescent autologous erythrocytes. Studies in vitro have shown that during logarithmic growth in culture, at cell concentrations lower than $6 \times 10^5$/mL, the frequency of detectable plaque-forming cells (PFC) remains well below 1%. As the cell concentration exceeds this level and the growth rate slows, there is a concomitant increase in the proportion of PFC, up to about 3%. Culture of CH12 cells for 24-48 hr in the presence of 50μg/mL LPS causes a modest increase above the intrinsic high rate of proliferation and also a 10 to 20-fold increase in the proportion of antibody secreting cells (Haughton et al., 1986). After LPS stimulation CH12 cells increase in size, and proliferation is increased. A large proportion (at least 50%) of CH12 cells differentiate into antibody-secreting cells. The fraction of antibody-secreting cells is similar to the proportion of B cells within individual B cell clones that can be driven to secrete antibodies. The striking similarities between the responses of CH12 cells and normal splenic B cells to LPS stimulation have been argued to make this an appropriate model system in which to study surface molecule expression during B cell differentiation (Ovnic and Corley, 1987).
In addition to antibody production, CH12 cells produce a range of cytokines including neuroleukin and IL-1 mRNA and high levels of IL-4 mRNA (O'Garra et al., 1989). The IL-4 mRNA is functional. CH12 cells also express high affinity receptors for IL-4. CH12 cells express IgM (O'Garra et al., 1989). All murine B lymphomas of the CH series (to date) are characterised by the expression of the CD5 surface antigen. It is believed that B-1 cells represent their normal counterpart. CH12 constitutively express RNA encoding TGFβ and IL-10 and produce varying levels of IL-3, IL-4, and GM-CSF, but not IL-5 and IFNγ (O'Garra et al., 1990). The data show a similarity in cytokine production by the CD5+ lymphomas and LPS-stimulated B-1 and B-2 normal cells: both populations express IL-6, TNFα, TNFβ, and IL-10; neither population expresses IL-1, IL-2, IL-5, IL-7, or IFNγ (O'Garra et al., 1990).

1.6.2 WEHI-279

WEHI-279 originated in the NZC inbred mouse strain derived from an (BALB/c x NZB) FI mouse (Warner et al., 1975; Sibley et al., 1980). WEHI-279 cells have Fc receptors, high levels of mlgM molecules, and secrete negligible amounts of IgM molecules (Warner et al., 1975; Paige et al., 1982). WEHI-279 resemble normal resting B cells and can be induced to mature to the state of active Ig secretion (Paige et al., 1982). WEHI-279 expresses Fas and CD40 and CD40 ligation increases Fas sensitivity in WEHI-279 (Koizumi et al., 1996).

This cell line has been used to study in the effects of IFNγ (Wong et al., 1983; Reynolds et al., 1987; Ben Jilani et al., 2001; Yoshikawa et al., 2001), B cell maturation factor (Sidman et al., 1984), surface presentation of IgM (Haustein and Von der Ahe, 1986), FceR (Waldschmidt et al., 1988), IL-6 production and effects (Hobbs et al., 1991), Fas-mediated apoptosis (Koizumi et al., 1996; Wang et al., 2000), CD80 and CD86 function (Suvas et al., 2002) in B cells.

1.7 B-1 cell involvement in disease

An increased number of B-1 cells has been shown to be an important characteristic of many autoimmune and chronic inflammatory diseases such as rheumatoid arthritis (Section 1.7.1), Sjögren's syndrome (Section 1.7.2), and periodontitis (1.7.3) (Hayakawa
et al., 1983; Kipps, 1989; Sugawara et al., 1992). Other autoimmune diseases are also associated with increased accumulation of B-1 cells, as are immunodeficiency states including AIDS, chronic lymphocytic leukaemia, and patients immuno-suppressed to receive bone marrow transplants. Autoimmune and immunodeficient mice also have elevated levels of B-1 cells (Talal et al., 1992). The exact role of this subset in pathogenesis has yet to be established, but the production of autoantibodies by these cells may play a role in the pathogenesis and continuity of such chronic conditions.

1.7.1 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory autoimmune disease characterised by an accumulation of B cells in the synovial tissue (Berek and Kim, 1997), with large numbers of B cells, including B-1 cells, present in these infiltrates (Plater-Zyberk et al., 1989). Another feature of rheumatoid arthritis is the presence of inflammatory foci within synovial tissue, and these foci have been identified as ectopic germinal centre-like structures (Berek and Kim, 1997). Also characteristic of the disease is the presence of autoantibodies. These antibodies are produced by both conventional and CD5+ B cells.

In patients with rheumatoid arthritis, CD5+ B cells are strikingly increased in number (Hardy et al., 1987; Dauphinee et al., 1988; Plater-Zyberk et al., 1989), accounting for 50% or greater of the circulating B cells (Casali and Notkins, 1989). B-1-derived antibodies binding to ssDNA and the Fc fragment of IgG are characteristic of rheumatoid arthritis (Casali et al., 1987), and this subset appears to be largely responsible for rheumatoid factor (RF) secretion (Hardy et al., 1987). Interestingly, B-1 cell-derived IgM RF autoantibodies have been shown to undergo an antigen-directed affinity maturation process (Mantovani et al., 1993).

1.7.2 Sjögren's syndrome

Primary Sjögren's syndrome is an autoimmune disease characterised by dry eyes and mouth resulting from lymphocyte infiltration of lacrimal and salivary glands (Celenligil et al., 1990; Talal et al., 1992) and the production of typical autoantibodies (Dorner and Lipsky, 2002). The major autoantibodies associated with Sjögren's syndrome are rheumatoid factor, anti-Ro (SS-A), and anti-La (SS-B). Autoantibodies against salivary
duct antigens are also found in the serum (MacSween et al., 1967) and, recently, 95% of primary Sjögren's syndrome patients were reported to have serum antibodies against α-fodrin (Stott et al., 1998).

B-cell clonal expansion is a key feature of Sjögren's syndrome (De Vita et al., 2001) and CD5⁺B cells are expanded in the peripheral blood of patients with rheumatoid arthritis and primary Sjögren’s syndrome (Dauphinee et al., 1988; Plater-Zyberk et al., 1989).

1.7.3 Periodontitis

1.7.3.1 Human disease

Periodontal disease is one of the most widespread diseases in humans. For example, it is estimated that at least 35% of the dentate U.S. adults aged 30 to 90 have periodontitis, with 21.8% having a mild form and 12.6% having a moderate or severe form (Albandar et al., 1999). Periodontitis is characterised by chronic gingival inflammation and resorption of the crest of alveolar bone with subsequent loss of teeth (Lally et al., 1980; Clarke, 1990; Aramaki et al., 1998). The different forms of the process are characterised by deterioration of the connective tissue and alveolar bone with resultant loss of support, tooth mobility, gingival pockets, haemorrhage, and local pain (Liebana and Castillo, 1994).

Advanced/progressive human periodontitis is considered by many to be a B lymphocyte and plasma cell lesion (Okada et al., 1983; Tew et al., 1989; Gemmell and Seymour, 1991). Gemmell and Seymour examined the phenotype of B-lymphocytes in periodontitis and found that a majority of these cells were activated (Gemmell and Seymour, 1991); T cells in periodontitis lesions are also active (Yamazaki et al., 1993). The periodontitis lesion in the adult periodontitis patients contains a substantial number of B cells, of which 30% demonstrated autoreactive features (Berglundh et al., 2002). Up to 38% of B cells from inflamed gingival tissues were determined to be CD5⁺ and many of these cells produced anti-collagen (auto-) antibodies (Sugawara et al., 1992). B-1 cells are activated in the inflamed gingival tissues of adult periodontitis patients (Aramaki et al., 1998). An increase of CD5⁺ B cells was also found in the peripheral blood of periodontitis patients (Afar et al., 1992). B-1 cells in gingival tissue may be
regarded as the first line of defence as they produce polyreactive antibodies to phosphorylcholine, LPS, and other commensal bacterial antigens (Aramaki et al., 1998).

Active periodontal disease is associated with a shift in bacterial composition to Gram-negative bacteria dominated in most cases by anaerobic bacteria (Dahlen, 1993), e.g. *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Fusobacterium nucleatum ss vincentii* (Bragd et al., 1987; Socransky and Haffajee, 1992; Socransky et al., 1998).

### 1.7.3.2 Porphyromonas gingivalis

*Porphyromonas gingivalis* can be found in the gingival tissues and can pass through the epithelial barrier. In deeper epithelial layers *P. gingivalis* can actively invade endothelial cells and replicate intracellularly (Deshpande et al., 1998).

*P. gingivalis* is a non-fermentative pigmented *Bacteroides* species, isolated principally from the human gingival sulcus and differs from *B. asaccharolyticus* by the production of phenylacetic acid, haemagglutination of sheep erythrocytes, trypsin-like activities, and antigenicity (reviewed in van Winkelhoff et al., 1988).

*P. gingivalis* strains degrade almost all proteins in guinea pig serum, including the C3 protein. Black-pigmented *Bacteroides* bacteria can produce leukotoxic substances such as low molecular weight fatty acids which directly inhibit polymorphonuclear leukocyte chemotaxis. Black-pigmented *Bacteroides* also inhibit the phagocytic killing of facultative bacteria in a mixed bacterial population by a mechanism which depends on serum. The LPS of black-pigmented *Bacteroides* species is different from the LPS of Gram-negative facultative anaerobic rods (reviewed in van Winkelhoff et al., 1988). *P. gingivalis* LPS has much less potency than does enterobacterial LPS in some endotoxic activities, such as pyrogenicity, chicken embryo lethality, and lethal toxicity to mice (Mansheim et al., 1978; Isogai et al., 1996). However, *P. gingivalis* LPS acts as a mitogen and polyclonal activator of B cells (Koga et al., 1985; Isogai et al., 1988; Fujiwara et al., 1990).
1.7.3.3 Porphyromonas gingivalis related effects in host response

Tew suggested that a suppression of the spontaneous proliferation response in lymphocytes from untreated severe periodontitis patients may reflect changes in regulatory T cell function induced by a state of active disease (Tew et al., 1983). A shift from a T helper 1 to a T helper 2 response has been observed in chronic diseases and is implicated in chronic periodontal inflammation (Gemmell and Seymour, 1994; Rook et al., 1994; Gemmell et al., 2001).

Gemmell and Seymour (Gemmell and Seymour, 1998) have shown that P. gingivalis induces IL-1 production by B cells rather than monocytes in periodontitis patients. This suggests that lymphocytes (B cells) may be a major source of IL-1 in periodontitis. Macrophage-derived IL-1 is a necessary co-factor for LPS-induced polyclonal activation (Bucala, 1992). P. gingivalis cysteine proteinases (gingipains) have been found to cleave IFNγ (Yun et al., 1999), hydrolyse IL-12 and reduce the IL-12-induced IFNγ production from CD4+ T cells (Yun et al., 2001). LPS upregulates expression of IFNγR on LPS-activated B cells and induces production of IFNγ (Abed et al., 1994). IFNγ produced by T\(_H\)1 and NK cells activates monocytes and macrophages and inhibits IgM secretion and B cell activation. IFNγ may therefore be part of a negative feedback loop that regulates B cell response to polyclonal activators (Abed et al., 1994). Decrease in IFNγ may permit increased sensitivity to B cell PBA (Chace et al., 1995). Macrophages produce IFNγ (reviewed in Gessani and Belardelli, 1998), and the paucity of macrophages and their lack of activation within the periodontal tissue (Chapple et al., 1998), coupled with the cleavage of IFNγ by gingipains could result in unregulated B cell activation by periodontal pathogens.

Periodontal diseases are infectious diseases. The aetiology is specific Gram negative organisms, such as P. gingivalis and Bacteroides forsythus. While the aetiology of periodontitis is bacterial, it is becoming clear that the pathogenesis of disease is mediated by the host response (Van Dyke and Serhan, 2003).
1.8 In vitro models of complex systems

The reductionist approach to biological problems has resulted in many important insights (Pollard, 2000). The challenge now, however, is to understand how cells and organisms as a whole survive and reproduce (Hartwell et al., 1999).

1.8.1 Systems thinking

"Systems thinking is a discipline for seeing wholes"

(Senge, 1994).

Skyttner defines a system as a "set of interacting units or elements that form an integrated whole intended to perform some function" (Skyttner, 1996). To call something a "system", the parts must be connected together to perform a transformation process (or processes). There must be a boundary, inputs and outputs. There must be emergent properties, i.e. the whole must provide something that is not available from the unconnected parts, output that requires interconnection and interrelationships (Harrington et al., 1999). Most systems are collections of parts that form "natural wholes" - obvious entities that clearly function to produce outputs that cannot be produced by the parts until they are "joined together" into an organised system. The essential or defining properties of any system are the properties of the whole which none of its parts have, because properties of a system derive from the interaction of its parts (Skyttner, 1996; Gruska, 2000). The performance of the system therefore depends on how the parts interact, not how they act separately. The essential properties of the system are lost when it is disassembled into unconnected parts (Gruska, 2000).

In a systems perspective one should be careful to consider the system in the context of the environment and not as an isolated entity. Thus one should include the interactions and relationships between the system and the environment (Bar-Yam, 2000). In one sense, a system is defined by its inputs and outputs, and how outputs feed back to be inputs. These are the system's relationship with its environment.

Skyttner lists properties which together comprise a general systems theory. A non-exhaustive list includes:

- Unrelated and independent elements do not constitute a system.
Holistic properties impossible to detect by analysis should be possible to define in the system.

Systemic interaction must result in some goal or final state to be reached or some equilibrium point being approached.

All systems, if they are to attain their goal, must transform inputs into outputs.

The interrelated objects constituting the system must be regulated (detection and correction of deviations) so that its goals can be realised. Feedback is therefore a requisite of effective control.

Systems are generally complex wholes made up of smaller subsystems.

In complex systems, specialised units perform specialised functions. This is a characteristic of all complex systems.

Open systems have equally valid alternative ways of attaining the same objectives (divergence) or, from a given initial state, obtain different, and mutually exclusive, objectives (convergence) (Skyttnner, 1996).

1.8.2 Application to current research

"A system-level understanding requires a shift in our notion of "what to look for" in biology. While an understanding of genes and proteins continues to be important, the focus is on understanding a system's structure and dynamics" (Kitano, 2002).

An examination of the dynamic interaction between individual components of a system is required to understand how a particular system functions (Kitano, 2002). Complex systems are characterised by networks and the immune system is an example of a complex system. Through reductionism, much is now known about the components of this system and how they interact which each other. However, knowledge of how networks are formed within the immune system and how properties emerge from these networks at the level of the system as a whole is still in its early stages (Efroni and Cohen, 2002).

Diseases can also be examined using the systemic approach. An understanding of the contribution of individual cells and molecules has been developed through the
reductionist approach, however the full scale of interactions between these components is yet to be recognised.

A full understanding of the complex system that is the immune system and its B cell component is beyond the scope of this thesis. However, recognition of the benefits of the systemic approach and the contribution of knowledge gained through the reductionist approach to systemic understanding drives the studies presented here. The reductionist approach is reflected in the parallel cell line studies examining the effects of LPS-stimulated on individual cell types whilst the systemic approach is reflected in the mixed splenic lymphocyte cultures. The mixed culture studies presented are initial characterisations of a simple system which can provide understanding of the interactions between the B-1 and B-2 cell subsets and also their interaction with T cells. (Note: T cell involvement is not examined as the focus of the studies presented in this thesis are B cell subsets.)
Chapter 2 Response of a B-1 cell line to lipopolysaccharide

2.1 Introduction

As described in section 1.5.2.1, the response of B-1 cells, as opposed to B cells, to LPS is poorly understood. B-1 cells produce antibodies that participate in early protection from infection, and/or may influence the development of the B-2 response (see section 1.3.2.2). In addition, while some studies have investigated the response of non-B cell populations to LPS preparations derived from non-enteric bacteria (see section 1.5.2.1.6), there is a paucity of studies examining how B cell subsets respond to these types of LPS. It was postulated that, in comparison with B-2 cells, very low concentrations of LPS may activate B-1 cells, thereby leading to B-1 cell accumulation in diseases such as periodontitis. It was therefore important to investigate the response of CD5⁺ cells to different concentrations and types of LPS.

In order to investigate the LPS responsiveness of B-1 cells, the murine B-1 derived cell line (CH12 see section 1.6.1) was used. Initial studies used CH12 cells cultured in the presence of relatively high concentrations of foetal calf serum (FCS), however the effects of LPS stimulation were obscured under these conditions. To avoid this problem, the response to LPS was examined in cells that were cultured under serum-limiting conditions. CH12 cells were cultured with varying concentrations of either Salmonella enteritidis or Porphyromonas gingivalis LPS and the viability, proliferation and activation were assessed by a variety of techniques. Moderate concentrations of both S. enteritidis and P. gingivalis LPS led to increased survival and proliferation of CH12 cells. The expression patterns of mRNA for Toll-like receptors were examined by quantitative PCR (qPCR). In addition, changes in mRNA levels of cytokines known to be involved in B-1 cell activation and survival, as well as mRNA for the apoptotic regulators Bcl-2 and Bax, were evaluated (Other regulators of cell behaviour such as cyclins, kinases, cell cycle inhibitors and other members of the bcl-2 family of pro/anti-apoptotic molecules were not examined due to time and budgetary constraints). Despite
the array of parameters examined, no clear picture of the mechanisms underlying the patterns of proliferation and survival was found.

2.2 Materials and Methods

2.2.1 CH12 cell line

The IgM-producing CH12 B lymphoma cell line was a gift of Dr Kenneth W. Beagley (Discipline of Pathology, Faculty of Medicine and Health Sciences, University of Newcastle, Australia). The cells were cultured in RPMI 1640 medium supplemented with 10% FCS (JRH Biosciences, Brooklyn, VIC), 5 x 10⁻⁵M 2ME (Sigma, St Louis, MO, USA), and antibiotic mixture (penicillin 50IU/mL and streptomycin 50μg/mL; Trace Biosciences, Castle Hill, NSW).

2.2.2 Thawing of cells

Vials of cells was removed from liquid nitrogen storage and placed into a covered water bath at 37°C. When the cells were almost thawed they were transferred to a 15mL centrifuge tube and 1mL FCS was added drop-wise, followed by 10mL RPMI/20% FCS drop-wise, whilst the tube was shaken gently. The cell suspension was centrifuged at 4°C for 10 min at 300g, the supernatant was decanted and the cells resuspended in residual medium. Five to 10mL RPMI/20% FCS was added and the concentration and viability of cells was determined using Trypan Blue exclusion. The cells were placed in a 25cm² flask and incubated at 37°C and 5% CO₂.

2.2.3 Freezing of cells

Cells were passaged 24hr prior to freezing. The cells were harvested from the culture flask and centrifuged 10 min, 4°C at 300g, the supernatant was decanted and the cells resuspended in the residual medium. The cells were diluted to 4 x 10⁶ cells/mL in RPMI/20% FCS and placed into a beaker of ice and water. An equal volume of cold freezing medium (RPMI/20% FCS/20% DMSO) was added drop-wise to the cells with gentle shaking. The cells were mixed with a pipette and 1mL aliquots were transferred to precooled cryovials (Nunc, Roskilde, Denmark). The cells were placed in a −70°C freezer for three days and the transferred to liquid nitrogen storage.
2.2.4 Passaging of CH12

The cells were determined to have a doubling time of approximately 12hr, and hence were passaged 1:4 for confluence after 24hr of culture, or 1:8 for confluence after 48hr of culture. Passaging at lower than 1:10 resulted in very slow growth, and often death of cells in the culture (results not shown).

Initial studies utilised CH12 cells cultured in medium supplemented with 10% FCS and further studies used serum-limiting conditions (3% or 2% FCS depending on batch of serum) to slow the doubling time of the line (Zetterberg and Larsson, 1985) and induce apoptosis (Dean et al., 1986; Howard et al., 1993; Shichiri et al., 1993; Fang et al., 1995; Jayadev et al., 1995) in order to examine aspects of the response which could not be studied satisfactorily in cultures supplemented with 10% FCS.

2.2.5 Seeding of culture vessels

Cells were treated with mitogens before seeding in complete medium at 1.56x 10^5 cells/mL in 96 well flat-bottomed microtitre plates (Sarstedt, Ingle Farm, SA) to a final well volume of 100μL. Eight replicate wells were prepared for each time point, and control (non-stimulated), and mitogen-stimulated test population were utilised.

Cells used for flow cytometric analysis were seeded at 1.56x 10^5 cells/mL in 25cm^2 cell-culture flasks (Sarstedt) to a final volume of 5mL. Control and mitogen-stimulated populations for all time points were cultured for this analysis.

2.2.6 Mitogens

Salmonella enteritidis LPS, prepared by a phenol-water extraction procedure and chromatographically purified, was obtained from Sigma. Note: S. enteritidis LPS was used as the work presented in this thesis was initially a continuation of that started by Dr WLK Massey at the IDR utilising S. enteritidis LPS.

Porphyromonas gingivalis LPS (protein free) was prepared (see Appendix 1, section “LPS extraction for P. gingivalis”) and provided by Ky-Anh Nguyen of the IDR.

2.2.7 MTT colourimetric assay

Ten microlitres 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) 5mg/mL was added to each cell-containing well of a 96-well microtitre plate
and incubated for 3 hr at 37°C. The plates were centrifuged for 10 min at 300g and 18°C. The supernatant was decanted from the wells and 100μL absolute ethanol was added to each well and mixed with a pipette. Absorbance was read at 690 and 540nm using a Titertek Twinreader Plus (Flow Laboratories, McLean, VA, USA) and change in absorbance was calculated to determine biomass (Mosmann, 1983; Loveland et al., 1992).

2.2.8 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) staining of cells

The method of Lyons and Parish (1994) was used in CFSE staining of cells: 1 x 10^8 cells were harvested from culture and washed twice with serum-free RPMI 1640. The cells were resuspended at a concentration of 5 x 10^7/mL in serum-free RPMI 1640 and warmed for 5 min at 37°C. 1μL of 1mM CFSE (Molecular Probes, Eugene, OR, USA) was added for each mL of cell suspension to give a final CFSE concentration of 1μM. The cells were incubated for 10 min at 37°C and inverted every 3 - 4 min. The reaction was stopped with several volumes of cold RPMI/FCS and the cells were washed twice with RPMI/FCS prior to addition of mitogens and seeding.

2.2.9 Determination of division index in CFSE-stained B cell subsets

The division index gives a measure of the overall mitotic activity and was determined using the method of Angulo and Fulcher (Angulo and Fulcher, 1998).

The division index = (100 - Y)/Y

Where Y(%) = X_0 + X_1/2 + X_2/4 + X_3/8 + X_4/16 + X_5/32 + X_6/64 + X_7/128

X_0 is the percentage of undivided cells

X_1 to X_7 are those cells in CFSE division gates

Two cells of given CFSE intensity arose from the single mitosis of a cell possessing CFSE intensity immediately greater. A division index of 1 means 1 mitosis for every cell added to the culture, an increase in the value means more mitotic activity. Cells dying in culture are ignored by this analysis. If these are a significant proportion of mitotic cells then an underestimate of the actual extent of cell division will result.
2.2.10  **Flow cytometric analysis of CFSE stained cells**
Cultured cells were washed twice with PBS/2% FCS/0.1% NaN₃ and were resuspended to 2 x 10⁶ cells/mL in sheath/PFA prior to flow cytometric analysis on a FACScan (Becton Dickinson, San Jose, CA, USA). Thirty thousand (30,000) cells were counted for each sample.

2.2.11  **Cell cycle analysis**
Cells were harvested from culture and 10⁶ – 10⁷ cells were resuspended in 5mL PBS in a centrifuge tube. Cells were centrifuged for 6 min at 4°C and approximately 200g and resuspended in 500μL PBS using a Pasteur pipette. The cell suspension was transferred to FACS tubes containing ice cold 4.5mL 70% ethanol and incubated for approximately 2hr on ice. Ethanol suspended cells were centrifuged for 5 min, 4°C, 200g and then the ethanol was thoroughly decanted. The cell pellet was resuspended in 5mL PBS, incubated for 1 min and then centrifuged for 5 min, 4°C, 200g. The cell pellet was resuspended in 1mL PI/Triton X-100 staining solution with RNase A and analysed within 30 min of staining.

2.2.12  **Antibodies for flow cytometry**
PE-conjugated anti-murine Bcl-2 (clone 3F11), purified anti-Bax (clone 6A7), and the corresponding isotype-matched controls were purchased from Pharmingen (San Diego, CA, USA) (Except Mouse IgG1 which was purchased from Sigma). Fc Block (anti-murine CD16/CD32, clone 2.4G2) (Pharmingen) was used to prevent non-specific binding of murine monoclonals to Fcγ receptors on the cell surface.

2.2.13  **Staining of CH12 cells for Bcl-2 and Bax by flow cytometry**
The cells were harvested and washed twice with PBS/2% FCS/0.1% Azide and resuspended to 1 x 10⁶ cells/mL. One million (1 x 10⁶) cells were placed in analysis tubes and 875μL cold PBS was added to the cells and mixed gently. One hundred and twenty five microlitres of cold fixation solution was added and the cells were mixed again. The cells were incubated for 1hr, 4°C and then centrifuged for 5min, 300g, 4°C.
The supernatant was removed and 1mL permeabilisation solution was added to the resuspended pellet and mixed gently. The cells were then incubated 15min at 37°C, 1mL PBS was added and then the cells were centrifuged for 5min, 300g, 4°C and the supernatant removed. One hundred microlitres 1° antibody (anti-Bcl2-PE and anti-Bax) in permeabilising solution was added to the pellet and mixed well. The cells were incubated 30min at 4°C, 1mL washing buffer was added and the cells were centrifuged 5min, 300g, 4°C. The supernatant was removed and the pellet washed again with 1mL washing buffer. One hundred microlitres 2° antibody (FITC anti-mouse-IgG F(ab')₂) in permeabilising solution was added to the pellet, mixed well, and then incubated for 30min, 4°C. One millilitre of washing buffer was added and the cells centrifuged for 5min, 300g, 4°C. The supernatant was removed and the pellet washed again with 1mL washing buffer and centrifuged for 5min, 300g, 4°C. The supernatant was removed and the cells resuspended to 10⁶ cells/mL in staining buffer and analysed on a FACScan.

Isotype matched controls, PE-conjugated for the anti-Bcl-2-PE control and unconjugated for the anti-Bax control (together with the 2° FITC-antibody) were used in all experiments.

2.2.14 Quantitative PCR

Quantitative PCR protocols were obtained from Dr H Ball (Department of Pathology, University of Sydney).

Cells were harvested from plate wells and spun down in 2mL microfuge tubes. The supernatant was discarded and the cells were resuspended in 1mL Tri-reagent (Sigma) and then vortexed. Samples were stored at -70°C until required.

2.2.14.1 RNA Isolation

Samples were thawed and incubated at room temperature for 5 minutes. Chloroform (0.2mL per mL of Tri-reagent originally used) was added to each sample. Samples were shaken vigorously for 15 seconds and incubated for 2-15 minutes at room temperature. The mixture was centrifuged at 12000g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube and 0.5mL isopropanol per mL of Tri-reagent used in sample preparation was added to each sample and mixed. The samples were incubated for 5-10 min at room temperature and then centrifuged at 12000g for 10 minutes at 4°C. The
supernatant was removed and the RNA pellet washed by adding 1mL (minimum) of 75% ethanol per 1mL of Tri-reagent used in sample preparation. The samples were vortexed and centrifuged at 7500g for 5 min. The supernatant was removed by decanting and pipette. The RNA pellet was briefly dried for 5-10 minutes by leaving the eppendorf tubes open on the bench at room temp. The pellet was redissolved in 50μL water (dH₂O; all RNase free). Samples were stored at -70°C until required.

2.2.14.2 DNase treatment

DNase treatment was undertaken according to instructions included with the Ambion (Austin, Texas) DNA-free™ kit. Briefly, a master mix of 0.1 volume DNase I buffer and 1μL. DNase I per sample was made up and 5.5μL of this mix was added to each sample. The samples were mixed and spun down gently and then incubated for 20-30min at 37°C. DNase inactivation reagent (0.1 volume) was added to each sample which was then thoroughly mixed. Samples were incubated for 2min at room temperature and spun down at 10,000 rpm for approximately 1 min. Aqueous RNA was pipetted into a new tube and stored at -70°C until required.

2.2.14.3 Determination of RNA concentration

RNA concentration in each sample was determined by optical density measurement at 260 nm using a BioRad Ultramark Microplate Reader (Hercules, CA) using Microplate Manager v5.1 build 75 and comparing with a standard curve.

2.2.14.4 Reverse transcription of RNA

Individual samples were prepared in eppendorf tubes, each with 2μg RNA and 1μl of oligo dT (0.1μg/μl; Geneworks, Rundle Mall, SA) in a total volume of 11μl, in dH₂O (RNA plus water to 10μl). Samples were heated to 70°C for 5-10 minutes and then placed on ice and spun briefly to collect contents. A mastermix was made up, with a total volume of 9μL per sample:
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Details</th>
<th>µL/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x RT buffer</td>
<td>Invitrogen (Carlsbad, CA, USA)</td>
<td>4</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>Invitrogen</td>
<td>2</td>
</tr>
<tr>
<td>dNTP</td>
<td>Stock with 5µg/µl of each nucleotide in dH₂O</td>
<td>2</td>
</tr>
<tr>
<td>RNase inhibitor</td>
<td>Invitrogen</td>
<td>0.5</td>
</tr>
<tr>
<td>MMLV-RT</td>
<td>Invitrogen</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>9µl</strong></td>
</tr>
</tbody>
</table>

Mastermix, 9µL, was added to each tube containing RNA. The samples were mixed and incubated for 1 hour at 37°C. The samples were then incubated for a further 3-5 minutes at 92°C. RNase-free water (480µL) was added to each sample and cDNA samples were stored at -20°C until required.

### 2.2.14.5 Primers

Primers were designed using Primer Express (Applied Biosystems, Foster City, CA). Default settings for real time primer design were used. Target sequences used were sourced from the Entrez Nucleotides Database (http://www.ncbi.nlm.nih.gov). Potential cross-reactivity of primers with other mRNA species was assessed by using the Basic Local Alignment Search Tool (BLAST) and the Entrez Nucleotides Database. Primers were purchased from Geneworks and were designed as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>GCTTTCCCTGTTAGCGAGGATACA</td>
<td>CAAACTGTCTCGGAATTTCAATCTC</td>
</tr>
<tr>
<td>CD80</td>
<td>AATGGCAGCCAAAATTTCTCAAC</td>
<td>TGGTTCTGAGAGCACCCACATA</td>
</tr>
<tr>
<td>CD86</td>
<td>TATCTGCGCTGGCCAGATTACA</td>
<td>TGTCGCCAATAAGTGCTCGTAC</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>GGCATCTCCACACTTGGAT</td>
<td>AACCCAGACTCTACATCACC</td>
</tr>
<tr>
<td>Bax</td>
<td>GCCCTTTTGTGCTACAGGGTT</td>
<td>GCTCAGGTGTCTCCCCAGC</td>
</tr>
<tr>
<td>TLR2</td>
<td>AAGATGGCCTTCCTGAAATTGC</td>
<td>CCCGCTCCTGAGGAATGCA</td>
</tr>
<tr>
<td>TLR4</td>
<td>CATGGAACACATGGCTGC</td>
<td>GGTAATTTATATATCCCTGGAAGG</td>
</tr>
<tr>
<td>RP105</td>
<td>CCGATGTAGCAGCTATCTCTGATGT</td>
<td>CAAAATTAGCAAACAGAAAGGAAT</td>
</tr>
<tr>
<td>IgM</td>
<td>AGCTCTCTACAGCGAGAAAGATATGT</td>
<td>CAGTCAGGATGCTGTTGGTAAA</td>
</tr>
<tr>
<td>IL-6</td>
<td>ACAAGTCGGGAGAGAGAAGGAGCTTC</td>
<td>CGATTCTCCCAGAGAAACATGTTG</td>
</tr>
<tr>
<td>IL-10</td>
<td>GCCCTTTTGTGCTATGGTGCTCTTT</td>
<td>TGAAGCTGCTGAGGAATGATC</td>
</tr>
</tbody>
</table>

The primers were optimised for the PCR conditions described in Section 2.2.14.7.
2.2.14.6 **Real time PCR set-up using Sybr green**

For each gene tested a master mix of Sybr green mix plus primers was prepared:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Reagent details</th>
<th>μL/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR green master mix</td>
<td>Invitrogen</td>
<td>10</td>
</tr>
<tr>
<td>Rox dye</td>
<td>Invitrogen</td>
<td>1</td>
</tr>
<tr>
<td>Primer mix</td>
<td>Forward and Reverse in dH₂O each at 2μM</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>12μl</strong></td>
</tr>
</tbody>
</table>

The SYBR green/primer master mix was aliquoted into wells of real time PCR plate (12μL per well; Applied Biosystems, Foster City, CA, USA). cDNA was added to each well (8μL per well) and the plate was heat-sealed. The plate was briefly spun in a centrifuge to ensure solutions were at bottom of plate.

Positive and negative controls were included for each gene on each plate (see Appendix 3 for a sample plate layout showing samples and controls).

2.2.14.7 **Real time PCR run**

PCR was performed in an Applied Biosystems Real Time PCR instrument (AB7700) using the following conditions with a combined annealing and extension step:

- 50°C for 2min
- 95°C for 10min
- 95°C for 15sec and 60°C for 1min (repeated 40 times).

The data was collected at 60°C.

2.2.14.8 **Dissociation curve analysis**

Dissociation analysis was performed for every plate in an Applied Biosystems Real Time PCR instrument (AB7700) using the following conditions:

- Denaturation step at 95°C for 15 sec
- Annealing step at 65°C for 15 sec
- Ramp from 65°C (5 minute ramp time) to 95°C (15 sec)

Data was collected from the ramp step.

According to Sambrook and Russell (2001):

"The shape of the melting curve indicates whether the amplified products are homogenous and the Tₘ (melting temperature) provides reassurance that the correct product has been specifically amplified. Primer-dimers...generally
denature at much lower temperatures and can easily be distinguished from the amplified target DNA" (p8.94-8.95).

2.2.14.9 **Determination of relative levels of expression**
mRNA expression of selected genes was determined using real-time PCR normalised to hypoxanthine phosphoribosyltransferase (HPRT).

2.2.15 **Statistical Analyses**
Control and LPS-stimulated values were compared for all experiments. Two-way ANOVA to compare differences at particular time points between unstimulated and stimulated populations, with Tukey's multiple comparison post test to compare differences between time points in a population, and one-way ANOVA to test differences overall between unstimulated and stimulated populations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All data shown are pooled means from a number of experiments. All error bars shown are the standard error of the mean.

2.3 **Results**

2.3.1 **Response of CH12 cells to Salmonella enteritidis LPS**

2.3.1.1 **CH12 cells cultured in RPMI/10%FCS do not show any response to S. enteritidis LPS stimulation**

In initial experiments, CH12 cells were cultured in medium containing 10% foetal calf serum (FCS) and varying concentrations of *Salmonella enteritidis* LPS. All cultures showed an increase in biomass over the culture period (Figure 2.1) and there were no significant differences between any of the LPS-stimulated cultures and corresponding unstimulated controls at any timepoint. Analysis of the viability (Figure 2.2A) and cell numbers (Figure 2.2B) mirrored this result. None of the LPS-stimulated cultures were significantly different to the control at the corresponding timepoint.

To further characterise the response of CH12 cells to LPS in serum rich conditions, activation was determined by measuring CD80 expression by flow cytometry. As shown
in Figure 2.3, CH12 cells were gated using forward and side scatter to select the major population cluster, and then viable cells from this population were selected for further analysis by gating on 7AAD fluorescence levels. The M1 (negative) and M2 (positive) regions for anti-CD80-FITC staining were determined using an isotype-matched control. The percentage of CD80$^+$ cells was then determined for the viable population (Figure 2.4). The percentage of CD80$^+$ CH12 cells was very high for all populations, including 95.4% (± s.e.m. 2.3%) of cells at 0hr. Further, the percentage of CD80$^+$ cells did not change significantly over the culture period.

2.3.1.2 Serum limitation slows doubling time and reduces viability

In order to slow the doubling time of the cell lines, the concentration of FCS in the culture medium was reduced to a level that slowed proliferation and reduced viability (Zetterberg and Larsson, 1985). In medium supplemented with 10% FCS, CH12 cells double approximately every 12 hours (author’s preliminary studies, data not shown). An initial range of concentrations between 1 and 5% was established from a preliminary study (Figure 2.5A), and this was further broken down as shown in Figure 2.5B. Supplementation with 3% FCS slowed the doubling time of the cells to 24hr, but the viability still remained high. All serum limitation studies included a 10% FCS unstimulated control.

Studies undertaken with *S. enteritidis* LPS in the CH12 cell line utilised 3% FCS for the serum limitation studies. However, a new and different batch of serum (from the same manufacturer, JRH Biosciences) was utilised for the *P. gingivalis* studies in CH12 and all studies in the WEHI-279 cell line. It was found that the use of 2% FCS resulted in similar patterns of proliferation and viability to that of 3% from the alternate serum batch. The results of experiments utilising 3% FCS from the new batch were not significantly different to those of utilising 10% FCS from this new batch (data not shown).

2.3.1.3 *S. enteritidis* LPS stimulation ‘rescues’ CH12 cells from death in serum-limited cultures

When cultured under serum-limiting conditions, the viability of CH12 cells decreased over the course of the experiment. However, addition of moderate concentrations of *S. enteritidis* LPS significantly diminished this loss of viability (Figure 2.6A). The
percentage viability in the cultures stimulated with 100ng/mL, 1µg/mL and 10µg/mL LPS were significantly different to the non-stimulated culture overall. All of the 3% FCS cultures were significantly different to the control 10% FCS culture. The concentration of viable cells (Figure 2.6B) remained low but stable in the control 3% FCS and 1pg to 1ng/mL LPS-stimulated cultures. An increase in cell concentration in the 10ng/mL LPS culture is noted, but this is not significantly different to the control culture. The concentration of cells in the cultures stimulated with 100ng/mL, 1µg/mL and 10µg/mL LPS were significantly different to the control culture overall.

2.3.1.4 S. enteritidis LPS stimulation affects population dynamics in CH12 cells in serum-limited cultures

It was important to evaluate the ability of different LPS concentrations to induce proliferation in CH12 cells. Cell division was measured by two methods: CFSE dye dilution (Lyons and Parish, 1994) and cell cycle analysis.

For CFSE dye dilution experiments, cells were gated by forward and side scatter and 7AAD fluorescence to select the viable population, and CFSE fluorescence was measured over the culture period (Figure 2.7). Figure 2.7A is an overlay of the 24, 48 and 72hr histograms shown as B, C and D. In all cultures the division index increased over the culture period (Figure 2.8). All 3% FCS cultures (except 100ng/mL LPS) are significantly different to the 10% FCS control (data not shown). The 10ng/mL, 100ng/mL, 1µg/mL and 10µg/mL LPS-stimulated cultures are significantly different to the 3% FCS control culture.

For cell cycle analysis, cells were gated on forward and side scatter and Propidium iodide (PI)-Area and Width to gate out doublets (Figure 2.9). The restricted range of LPS concentration and time points were chosen for this experiment as they were deemed to be the concentrations and time points in which any LPS effects were easily discernable (given the results of the previous experiments shown in Figure 2.6 and Figure 2.8). Ten nanogram/mL and 100ng/mL LPS were threshold concentrations for LPS effects on division index and viability in 3% FCS cultures, and stimulation with 1µg/mL LPS results were significantly different to 3% FCS control results in the previous experiments. As shown in Figure 2.10, at both time points, G0/G1 phase percentages in the LPS-stimulated and 10% FCS control cultures were significantly lower than the non-stimulated control. S phase percentages were different to 3% FCS
control in the 100ng/mL and 1µg/mL LPS-stimulated cultures. The 10% FCS unstimulated culture was only significantly different to the 1µg/mL culture at the 48hr time point. For cells in the G2/M phase, the 10% FCS unstimulated control was different to the 3% FCS control at both time points and was also significantly different to the 24hr 1µg/mL LPS and 48hr 100ng/mL LPS cultures.

2.3.1.5 Effects of S. enteritidis LPS on CH12 activation

To assess changes in the activation state of CH12 cells in response to S. enteritidis LPS, levels of CD80, CD86 and IgM mRNA were determined using qPCR (for an example of typical qPCR raw data, see Figure 2.11). The restricted range of LPS concentration and time points were chosen for this experiment as they were deemed to be the concentrations and time points in which any LPS effects were easily discernable (given the results of the previous experiments shown in Figure 2.6 and Figure 2.8). No significant differences were found between control and LPS-stimulated cultures for either CD80 (Figure 2.12A) or CD86 (Figure 2.12B). When changes in the mRNA expression of sIgM were examined, however, significant differences were found. The 1µg/mL LPS-stimulated culture was significantly different overall to the control culture. In the 100ng/mL and 1µg/mL LPS-stimulated cultures the 48hr timepoint was significantly different to the 0 and 24hr timepoints (Figure 2.13).

2.3.1.6 Investigation of molecular events associated with S. enteritidis LPS stimulation

Potential molecular mechanisms underlying the effects of S. enteritidis LPS on CH12 cells were examined. Bcl-2 and Bax protein levels were measured simultaneously in CH12 cells stimulated with S. enteritidis LPS using flow cytometry and RT-qPCR, while levels of mRNA for relevant Toll-like receptors and cytokines were examined by RT-qPCR. The restricted range of LPS concentration and time points were chosen for this experiment as they were deemed to be the concentrations and time points in which any LPS effects were easily discernable (given the results of the previous experiments shown in Figure 2.6 and Figure 2.8).
2.3.1.6.1 Changes in Bcl-2 and Bax expression

For determination of Bcl-2 and Bax protein expression, cells were stained for intracellular Bcl-2 and Bax simultaneously (Figure 2.14). No statistically significant differences were found for Bcl-2/Bax ratios between the serum-limited non-stimulated culture and the corresponding LPS-stimulated cultures (see Figure 2.15). In the 3% FCS control culture the 48hr ratio was significantly higher than the 24hr ratio – none of the other cultures showed a significant difference between the 24 and 48hr values. However, overall, the 48hr cultures were significantly different to the 24hr cultures. To further examine the Bcl-2/Bax ratio, changes in the ratio of Bax and Bcl-2 mRNA was determined using RT-qPCR. The ratio of Bax and Bcl-2 expression was calculated (Figure 2.16) and no significant differences were found between control and LPS-stimulated cultures.

2.3.1.6.2 Changes in Toll-like Receptor mRNA expression

Levels of mRNA expression of TLR2 (Figure 2.17A) and TLR4 (Figure 2.17B) were determined using RT-qPCR. No significant differences overall were found between control and LPS-stimulated cultures. At the 24hr timepoint, the level of expression of TLR2 in the 100ng/mL and 1μg/mL LPS-stimulated cultures was lower than that of the control culture. In contrast to the expression of TLR2 and TLR4, mRNA levels of RP105 were significantly affected by *S. enteritidis* LPS. All LPS-stimulated cultures were significantly different overall to the control culture (Figure 2.18). In the 10ng/mL LPS-stimulated culture, the 48hr timepoint was significantly lower than both the 0 and 24hr timepoints. In the 100ng/mL LPS-stimulated cultures the 24 and 48hr timepoints were lower than the 0hr timepoint, whilst in the 1μg/mL LPS-stimulated culture only the 48hr timepoint was significantly different to the 0hr culture. In both the 100ng/mL and 1μg/mL LPS-stimulated cultures the 24hr timepoint was different to the control.

2.3.1.6.3 Changes in IL-6 and IL-10 mRNA expression

As both IL-6 and IL-10 have been shown to affect B-1 cell activation and survival (see section 1.5.2.2), changes in the mRNA of these cytokines were investigated using RT-qPCR. There were no significant differences in IL-6 mRNA expression between control and LPS-stimulated cultures (Figure 2.19A). In contrast, CH12 cells showed a
significant increase in IL-10 message at both 24 and 48 hours in cells stimulated with 100ng/ml *S. enteritidis* LPS (Figure 2.19B). However, the magnitude of this increase was small (1.5 fold increase at 24 hours compared with control.)

### 2.3.2 Response of CH12 cells to Porphyromonas gingivalis LPS

#### 2.3.2.1 *P. gingivalis* LPS affects proliferation but not viability in CH12 cells

The response of CH12 cells to *P. gingivalis* LPS was investigated in serum-limited cultures. There were some difficulties interpreting the results of these experiments, as it was necessary to use a different batch of FCS to that used in the *S. enteritidis* studies (section 2.3.1.2). Although the batch was re-titrated to determine appropriate serum-limiting conditions (data not shown), there was some proliferation of cells seen in non-LPS-stimulated cultures (Figure 2.20). Nevertheless, all 2% FCS cultures were significantly different to the 10% FCS unstimulated control culture.

In contrast to the pattern of increased survival seen in *S. enteritidis* LPS-stimulated cultures (see section 2.3.1.3), viability in *P. gingivalis* LPS-stimulated cultures was not significantly different to unstimulated control cultures (Figure 2.20A).

While the concentration of cells in all groups increased over the culture period (Figure 2.20B), *P. gingivalis* LPS did appear to affect the cell numbers in culture in a complex dose-dependent manner. When present at high concentrations (100ng/mL and 10μg/mL), *P. gingivalis* LPS caused increased numbers of cells in culture. However, low doses (1pg/mL and 10pg/mL) actually resulted in a significantly decreased number of cells in culture (Figure 2.20B). This response pattern was supported by CFSE proliferation data. By this measure, low concentrations of *P. gingivalis* LPS led to a decreased proliferation, while high concentrations caused increased proliferation at 24 hours, but a slight decrease at 72 hours of culture (Figure 2.21).

#### 2.3.2.2 Effects of *P. gingivalis* LPS on activation

As with the response to *S. enteritidis* LPS (section 2.3.1.5), the activation of CH12 cells was assessed by examining changes in the expression of CD80, CD86 and sIgM. There
were no significant changes in the expression of either CD80 (Figure 2.22A), or CD86 (Figure 2.22B) between control and LPS-stimulated cultures over the culture period. However, the levels of sIgM mRNA in the 1µg/mL stimulated cultures were significantly lower than the control at 24 and 48hrs (Figure 2.23).

2.3.2.3 Investigation of molecular events associated with P. gingivalis 

LPS stimulation in CH12 cells

Potential mechanisms underlying the effects of P. gingivalis LPS on the activation and differentiation of CH12 were investigated by determining changes in the expression of a range of genes that have shown to be important in the regulation of B cell survival and growth. Bcl-2 and Bax mRNA levels were measured simultaneously in CH12 cells stimulated with P. gingivalis LPS using RT-qPCR, while levels of mRNA for relevant TLR and cytokines were also examined.

2.3.2.3.1 Stimulation with P. gingivalis LPS does not affect the Bax/Bcl-2 ratio

Levels of mRNA of the apoptosis regulatory genes Bax and Bcl-2 were determined by RT-qPCR and the ratio of their expression was calculated (Figure 2.24). No significant differences were found between control and LPS-stimulated cultures.

2.3.2.3.2 Stimulation with P. gingivalis LPS does not affect level of expression of Toll-like receptors

The mRNA expression of relevant TLR was determined in culture using RT-qPCR. There were no significant changes in the expression of either TLR2 (Figure 2.25A) or TLR4 (Figure 2.25B) mRNA. There was a significant increase in Rp105 expression in the 1µg/ml culture (Figure 2.26), however the magnitude of the increase was very small (1.5 fold).
2.3.2.3.3 Stimulation with *P. gingivalis* LPS does not cause changes in cytokine mRNA levels

Changes in the levels of IL-6 and IL-10 mRNA in response to *P. gingivalis* LPS were investigated by RT-qPCR (Figure 2.27). There were no significant changes over the course of the culture period.
Figure 2.1 Effect of *S. enteritidis* LPS on biomass in 10% FCS cultures

CH12 cells were cultured in medium containing 10% FCS and varying concentrations of LPS. Biomass was measured using an MTT-formazan assay (see section 2.2.7). There were no statistically significant differences (p<0.05) overall, as determined by two-way ANOVA, between the control and LPS-stimulated cultures. Data shown are the means and s.e.m. of six pooled experiments.
Figure 2.2 Cell viability and concentration (cells/mL) of CH12 cells in 10% FCS culture

The viability (A) and concentration (B) of CH12 cells for all concentrations of *S. enteritidis* LPS and all time points was determined using Trypan Blue exclusion. There were no statistically significant differences, as determined by two-way ANOVA, between the control and LPS-stimulated cultures at specific timepoints. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the non-stimulated control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.3 Representative flow cytometric plots for analysis: 7AAD/CD80

CH12 cells were gated using forward and side scatter to select the population and then further gated for viable cells in this population using 7AAD/side scatter. Levels of non-specific staining were determined using fluorescently-conjugated isotype-matched controls for anti-CD80-FITC (M1 is the negative region and M2 is the positive region). Viable cells were examined for CD80 staining characteristics. Data shown are from a single representative experiment.
Figure 2.4 Changes in CD80 expression in response to *S. enteritidis* LPS

CH12 cells were cultured in medium containing 10% FCS and varying concentrations of *S. enteritidis* LPS. The percentage of viable CH12 positive for CD80 was determined by flow cytometry. There were no statistically significant differences as determined by two-way ANOVA with Tukey’s multiple comparison post-test and one-way ANOVA. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.5 Effect of serum supplementation on CH12 viability and proliferation

CH12 cells were cultured in different concentrations of FCS and the cell number and viability determined at 24, 48 and 72 hours. The optimal concentration to FCS to slow the doubling time yet retain viability was determined in two stages (bars denote percentage viability and lines denote cell numbers). The first stage (A) selected the general range of the new lower FCS concentration between 1% and 5% FCS, whilst the second stage (B) identified the optimal concentration as 3% FCS. The CH12 cells cultured in medium supplemented with 3% FCS showed a concentration profile not significantly different (p=0.1736) to that of marine splenic B-1 cells in control cultures. Data shown are from representative experiments. Note: 2% FCS was utilised for the P. gingivalis and qPCR studies as the serum was from a different batch and was retitrated to ensure comparable viability and doubling times.
Figure 2.6 Effect of *S. enteritidis* LPS on viability and cell number under limited serum conditions

CH12 cells were cultured in 3% FCS in the presence of different concentrations of *S. enteritidis* LPS. The viability (A) and concentration (B) of CH12 cells was determined using haemocytometer count and Trypan Blue exclusion. Statistically significant differences (p<0.05) between the non-stimulated control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Statistically significant differences (p<0.05) between the 10% FCS and 3% FCS cultures, as determined by two-way ANOVA, are denoted by a # above the relevant time point. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the non-stimulated control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.7 Representative FACScan plots for flow cytometric analysis: 7AAD/CFSE

CH12 cells were gated using forward and side scatter and 7AAD prior to analysis of CFSE staining. (A) is an overlay of the CFSE histograms for (B) 24hr, (C) 48hr, (D) 72hr of culture. M1 indicates cells that have divided once, M2 indicates cells that have divided twice, M3 indicates cells that have divided three times and M4 indicates cells that have divided four or more times. Data shown are from a single representative experiment.
Figure 2.8 Division index of CH12 cells cultured in RPMI/3% FCS and stimulated with S. enteritidis LPS

CH12 cells were cultured in the presence of different concentrations of S. enteritidis LPS and the division index (see section 2.2.9) was determined at 24, 48 and 72 hours. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Statistically significant differences (p<0.05) between the 10% FCS and 3% FCS cultures, as determined by two-way ANOVA, are denoted by a # above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the category name on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.9 Representative cell-cycle analysis histograms

CH12 cells were gated using forward and side scatter and FL2-W/FL2-A to exclude doublets. Propidium iodide was used to stain DNA to determine cell cycle stages (see section 2.2.11). M1 indicates cells in G0/G1, M2 indicates cells in S and M3 indicates cells in M phase. Data shown are from a single representative experiment.
Figure 2.10 Cell cycle in CH12 cells cultured in RPMI/3% FCS

The percentage of viable cells in each phase of the cell cycle for control and test samples was determined at 24 and 48hr by propidium iodide staining and flow cytometric analysis. For this set of experiments a two-way ANOVA was used to determine significant differences; Culture (control, *S. enteritidis* LPS-stimulated etc: \( p < 0.0001 \)) and time (\( p < 0.05 \)) were both shown to have significant effects overall on the percentage of cells in G0/G1. For cells in S phase, culture (\( p < 0.05 \)) and time (\( p < 0.0001 \)) were also shown to have significant effects on the percentage of cells in this phase. There were no significant differences due to culture or time in the G2/M-phase populations. Statistically significant differences (\( p < 0.05 \)) between the control and LPS-stimulated cultures, as determined by one-way ANOVA and post-hoc Tukey’s multiple comparison test, are denoted by a * above the relevant data point. Statistically significant differences (\( p < 0.05 \)) between the 10% FCS and 3% FCS cultures, as determined by two-way ANOVA, are denoted by a # above the relevant time point. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.11 Representative RT-qPCR amplification and melting curves

Typical amplification curves for each well of a sample set for the housekeeping gene HPRT is shown in panel A. Relative quantities of target genes were determined by normalisation to HPRT using the delta-delta Ct method (see section 2.2.14.9). Product purity for all PCR runs was determined by melting curve analysis (B). Data shown are from a single representative experiment.
Figure 2.12 Changes in CD80 and CD86 mRNA expression in response to S. enteritidis LPS

The relative level of expression of the CD80 (A) and CD86 (B) genes as compared with HPRT was determined using RT-qPCR for control and S. enteritidis LPS-stimulated populations. There were no statistically significant differences as determined by two-way ANOVA with Tukey’s multiple comparison post-test for any cultures.
Figure 2.13 Changes in sIgM mRNA expression in response to *S. enteritidis* LPS

The relative level of expression of secretory IgM was determined using RT-qPCR for control and *S. enteritidis* LPS-stimulated populations. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † alongside the culture descriptor on the X axis. Significant differences between timepoints within a culture are denoted by lines connecting the columns. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.14 Representative FACScan plots for flow cytometric analysis: Bcl-2/Bax

CH12 cells were gated using forward and side scatter and stained with fluorescent dye-conjugated antibodies to either Bcl-2 or Bax. Isotype controls were used to establish negative (M1) and positive (M2) regions. Data shown are from a single representative experiment.
Figure 2.15 Changes in Bcl-2 and Bax expression in CH12 cells in response to S. enteritidis LPS

CH12 cells were cultured in the presence of varying concentrations of S. enteritidis LPS and Bcl-2 and Bax protein expression was determined by flow cytometry. The Bcl-2/Bax expression ratio was determined using the percentage of cells positive for each stain at each S. enteritidis LPS concentration and time point. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Overall, the Bcl-2/Bax ratios for the 48hr cultures are significantly different to those for the 24hr cultures, denoted by the † on the X axis, as determined by one-way ANOVA. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.16 Changes in the ratio of Bax and Bcl-2 mRNA in response to \textit{S. enteritidis} LPS

CH12 cells were cultured in the presence of varying amounts of \textit{S. enteritidis} LPS and the ratio of mRNA for BAX and Bcl-2 was determined by RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.17 Effect of *S. enteritidis* LPS on TLR2 and TLR4 mRNA expression

CH12 cells were cultured with *S. enteritidis* LPS at varying concentrations and the expression of TLR2 (A) and TLR4 (B) mRNA was examined by RT-qPCR. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by one-way ANOVA and post-hoc Tukey's multiple comparison test, are denoted by a * above the relevant data point. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.18 Effect of *S. enteritidis* LPS on RP105 mRNA expression

CH12 cells were cultured with *S. enteritidis* LPS at varying concentrations and the expression of RP105 mRNA was examined by RT-qPCR. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † alongside the culture descriptor on the X axis. Significant differences between timepoints in a culture are denoted by the lines connecting the columns. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.19 Effect of *S. enteritidis* LPS on IL-6 and IL-10 mRNA expression

CH12 cells were cultured with *S. enteritidis* LPS at varying concentrations and the expression of IL-6 (A) and IL-10 mRNA were examined by RT-qPCR. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † alongside the culture descriptor on the X axis. Significant differences between timepoints in a culture are denoted by the lines connecting the columns. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.20 Effect of *P. gingivalis* LPS on CH12 viability and cell number.

CH12 cells were cultured, under serum-limited conditions, with *P. gingivalis* LPS at varying concentrations and the viability (A) and concentration (B) of cells was determined using Trypan Blue exclusion. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.21 Division index of CH12 cells cultured in RPMI/2% FCS and stimulated with *P. gingivalis* LPS

CH12 cells were cultured, under serum-limited conditions, with *P. gingivalis* LPS at varying concentrations and the division index of viable CH12 cells was determined using CFSE. Statistically significant differences (p<0.05) between the control and test cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and test cultures are denoted by a † below the category name on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.22 Effect of *P. gingivalis* LPS on CD80 and CD86 mRNA expression

CH12 cells were cultured with *P. gingivalis* LPS at varying concentrations and changes in the levels of CD80 (A) and CD86 (B) mRNA were determined using RT-qPCR. Statistically significant differences (p<0.05) between the control and test cultures at a particular time point, as determined by two-way ANOVA, are denoted by a * above the relevant time point.
Figure 2.23 Effect of *P. gingivalis* LPS on sIgM mRNA expression

CH12 cells were cultured with *P. gingivalis* LPS at varying concentrations and changes in the levels of sIgM mRNA were determined using RT-qPCR. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and test cultures are denoted by a † below the category name on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.24 Effect of *P. gingivalis* LPS on BAX/Bcl2 mRNA ratio

CH12 cells were cultured with *P. gingivalis* LPS at varying concentrations and changes in ratio of BAX to Bcl-2 mRNA was determined using RT-qPCR. The Bax/Bcl-2 mRNA expression ratio was determined using the relative levels of expression of each gene (as compared with HPRT) for each *P. gingivalis* LPS concentration and time point. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.25 Effect of *P. gingivalis* LPS on TLR2 and TLR4 mRNA expression

CH12 cells were cultured with *P. gingivalis* LPS at varying concentrations and changes in the levels TLR2 (A) and TLR4 (B) mRNA were determined using RT-qPCR. There are no statistically significant differences between the control and test cultures, as determined by two-way ANOVA. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.26 Effect of *P. gingivalis* LPS on RP105 mRNA expression

CH12 cells were cultured with *P. gingivalis* LPS at varying concentrations and changes in the levels of CD80 (A) and CD86 (B) mRNA were determined using RT-qPCR. There are no statistically significant differences between the control and test cultures, as determined by two-way ANOVA. Significant differences between timepoints in a culture are denoted by the lines connecting the columns. Data shown are the means and s.e.m. of three pooled experiments.
Figure 2.27 Effect of *P. gingivalis* LPS on IL-6 and IL-10 mRNA expression

The relative level of expression of IL-6 (A) and IL-10 (B) as compared with HPRT was determined using quantitative PCR for control and *P. gingivalis* LPS-stimulated populations. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
2.4 Discussion

The aim of the studies presented in this chapter was to investigate the response of the B-1 cells, using the immortalised CH12 cell line, to enteric (*S. enteritidis*) and non-enteric (*P. gingivalis*) LPS. Initial efforts at developing a culture system foundered when it became apparent that changes in proliferation and activation were obscured in cultures containing high concentrations of FCS. Subsequently, a serum-limiting culture system was developed that enabled the response to LPS to be examined.

Reduction of the percentage of FCS in culture decreased both the viability and the division index of CH12 cells. For unstimulated cells in *S. enteritidis* LPS experiments, CH12 viability decreased over the course of the culture period, and at 72hr viability was significantly different to the starting viability (Figure 2.6A), while the concentration of viable cells remained relatively stable (Figure 2.6B). This balance was presumably maintained by the interacting effects of a low level of proliferation (Figure 2.8 and Figure 2.10) offset by the well-described pro-apoptotic effects of serum deprivation (Grimm *et al.*, 1996; Hasan *et al.*, 1999). A similar pattern was seen in the *P. gingivalis* LPS experiments, although the picture was not as clear-cut as the growth, and viability inhibition that occurred with this particular batch of serum was not as marked. Nevertheless, serum limitation led to decreased viability (Figure 2.20A) and proliferation (Figure 2.20B and Figure 2.21) in comparison to 10% FCS control cultures. As uncontrolled proliferation of immune cells, such as B cells, could potentially have dire consequences for organisms, it has been argued (Allen *et al.*, 1993; Gordon *et al.*, 1993) that the default program for cell survival in most immune cells is, in fact, to apoptose. The apoptosis regulatory pathways in CH12 cells are obviously compromised, as the cell is immortal. Nevertheless, limitation of serum allowed the pro- and anti-apoptotic effects of different LPS preparations to be examined.

Under serum-limiting conditions, the addition of *S. enteritidis* LPS at 100ng/ml and above led to the rescue of CH12 cells from death (Figure 2.6A), stimulated them to enter the cell cycle (Figure 2.10) and caused them to proliferate (Figure 2.8). In contrast, *P. gingivalis* LPS at high concentrations appeared to have no effect on viability (Figure 2.20A), but did lead to proliferation. The threshold dose for this effect on proliferation
was unclear as increased cell numbers in culture were seen for doses of LPS from 100ng/ml and above (Figure 2.20). However, using the division index as a measure, several 72-hour timepoints for different LPS concentrations, and also including the 10% FCS control, actually showed a slightly decreased proliferation compared with the unstimulated timepoint controls. The fact that the division index of the unstimulated control was higher than the 10% FCS control suggests that the unstimulated control at this timepoint was artifactually high, possibly being obscured by the relatively high background level of proliferation in this series of experiments. Nevertheless, at 24 hours of culture, *P. gingivalis* LPS at a concentration of 10μg/ml caused significant proliferation of CH12 cells by this measure.

Despite a series of investigations, the cellular mechanisms underlying the anti-apoptotic/pro-proliferative effects of the different LPS preparation remained unclear. The relative levels of Bcl-2 and Bax have been shown to be important in regulating cell survival (see section 2.1). The Bcl-2/Bax ratio did not change in the *P. gingivalis* LPS challenged cells (Figure 2.24), which was in keeping with the lack of cell death seen in these cultures. However, in response to *S. enteritidis* LPS, which at high concentrations rescued CH12 cells from death, neither the protein (Figure 2.15) nor mRNA (Figure 2.16) ratios of these mediators correlated with protection. Although Bcl-2 has been implicated in the rescue of many cell types from apoptosis initiated by various stimuli, including serum-deprivation (Grimm et al., 1996), it may be that Bcl-2 expression is already abnormally high in CH12 cells, and that other parallel pathways are responsible for the observed protection. Supporting this argument is evidence that high endogenous levels of Bcl-2 are seen in a variety of B-1 lymphomas (Gottardi et al., 1996) and in CH12 cells in particular (Brown and Phipps, 1996). Regulation of apoptosis is complex and incompletely understood. Other apoptosis regulators such as anti-apoptotic Al/Bfl-1, Bcl-w, Bcl-xL, Boo/Diva/Bcl-B, and Mcl-1 and pro-apoptotic Bak, Bok/Mtd, Bcl-x, and Bcl-GL (reviewed in Marsden and Strasser, 2003) may also play a role in B cell survival and death; however, it is beyond the scope of this thesis to further discuss these alternative regulators.

The autocrine stimulation of B cells by a range of cytokines, in particular IL-10, has also been implicated in promoting B cell survival and proliferation (see section 1.5.2.2).
CH12 cells have previously been shown to produce both IL-6 and IL-10 (O'Garra et al., 1990), so it was possible that an increased production of either of these cytokines may have been responsible for the enhanced proliferation seen in LPS simulated cultures. This, however, did not appear to be the case. No significant changes were seen in the mRNA levels of IL-6 in response to either LPS preparation. Although there was a significant increase in IL-10 mRNA in CH12 cells cultured with 100ng/ml of S. enteritidis LPS, this increase was small in magnitude and not seen in other stimulated cultures. In addition, no significant changes were noted in cultures stimulated with P. gingivalis LPS.

Increased activation of CH12 cells by the LPS preparations was not seen. The co-stimulatory molecules CD80 and CD86 are typically upregulated in activated B cells (Ding and Shevach, 1996; Kozono et al., 1998; Nagai et al., 2002), however neither CD80 nor CD86 mRNA levels were increased following incubation with either LPS preparation (Figure 2.12 and Figure 2.22). Although not directly tested in serum-limited cultures, under non serum-limiting conditions the majority of non LPS-stimulated CH12 cells expressed high levels of CD80 (Figure 2.4), which suggests that these cells have a high level of basal activation. In contrast to the lack of effect of LPS on co-stimulatory molecule expression, high concentrations of S. enteritidis LPS did cause an increase in secretory IgM mRNA (Figure 2.13). This is consistent with previously published reports that have demonstrated either increased sIgM mRNA or IgM secretion by CH12 cells in response to enteric (E. coli) LPS (Stockdale et al., 1987; Arnold et al., 1988; Bost et al., 1990). Interestingly, P. gingivalis LPS did not affect sIgM mRNA levels (Figure 2.23), which suggests that this LPS may act via alternative signalling pathways.

As described in section 1.5.2.1.2, the primary LPS receptor on macrophages is TLR4, while in B cells the predominant receptor appears to be RP105. Alternatively, some have argued that TLR2 is important in the recognition of LPS derived from non-enteric bacteria such as P. gingivalis. Although it was beyond the resources available to this project to investigate the Toll-like receptor signalling pathways in detail, it was practical to investigate changes in the levels of mRNA for the major TLR that could be involved in the response to enteric and non-enteric LPS. In response to P. gingivalis LPS, there were no changes in any of the TLRs examined (Figure 2.25 and Figure 2.26). While
there were no changes in the expression of either TLR2 or TLR4 (Figure 2.17) in response to *S. enteritidis* LPS, there was a clear decrease in RP105 expression over the course of culture (Figure 2.18). Down-regulation of TLR4 mRNA has been reported in macrophage lines in response to enterobacterial LPS (Poltorak *et al.*, 1998; Medvedev *et al.*, 2000; Nomura *et al.*, 2000), while the level of TLR2 mRNA was decreased in response to bacterial lipoprotein challenge (Wang *et al.*, 2002). Such regulation of expression has been argued to contribute to the phenomenon of endotoxin tolerance, which is believed to be a mechanism to limit the deleterious effects of macrophage-derived products on the organism (Medvedev *et al.*, 2000; Nomura *et al.*, 2000; Wang *et al.*, 2002). The biological significance of the down-regulation of RP105 seen in the present experiments is unclear, but may represent an analogous B cell regulatory mechanism.

Whatever the overall biological outcome of changes in RP105 expression, it appears that *S. enteritidis* and *P. gingivalis* LPS have differential effects on RP105 regulation. It is possible that this reflects differential use of TLR in responding to the two LPS preparations. It has been suggested that *P. gingivalis* LPS signals through TLR2 (see section 1.5.2.1.3). However, the lack of changes in TLR2 expression in response to either LPS preparation makes it difficult to assess if *P. gingivalis* LPS is signalling via this pathway or through some other pathway. However, enterobacterial LPS is argued to signal via TLR4, which is associated with RP105 in B cells (see section 1.5.2.1.4). It seems likely that *S. enteritidis*, but not *P. gingivalis* LPS is acting via this pathway, as in the present experiments, RP105 expression was down-regulated by *S. enteritidis*, but not *P. gingivalis* LPS.

Taken together, these results suggest that LPS from the enteric and non-enteric bacteria may have different effects on the activation of B-1 cells. While LPS from the enteric pathogen *S. enteritidis* leads to B-1 cell proliferation and production of antibody at moderate concentrations, highly purified LPS derived from the periodontal pathogen *P. gingivalis* had less marked effects. Moreover, the results presented here are consistent with at least partially independent signalling pathways between the two types of LPS.
Chapter 3 Response of a B-2 cell line to lipopolysaccharide

3.1 Introduction

The experiments described in the previous chapter found that a B-1 cell line responded differently to Salmonella enteritidis and Porphyromonas gingivalis LPS. Both LPS preparations caused proliferation at moderate to high concentrations, while S. enteritidis LPS also increased viability of cells and enhanced IgM secretion. As it was postulated that B-1 cells are more sensitive to the effects of LPS than B-2 cells, it was also important to examine the response of B-2 cells to LPS.

In order to achieve this aim, the WEHI-279 cell line was used as a model for studying B-2 cells as this line is responsive to LPS stimulation (Kleine et al., 1985; Hobbs et al., 1991). As described in section 1.6.2, WEHI-279 cells have Fc receptors, high levels of mlgM molecules, and secrete negligible amounts of IgM (Warner et al., 1975; Paige et al., 1982). WEHI-279 resembles normal resting B cells and can be induced to mature to the state of active Ig secretion (Paige et al., 1982). As with studies in the previous chapter, serum-limiting conditions were used in order to slow the doubling time of the line and to enable comparisons to be made between aspects of the B-1 and B-2 cell responses. These aspects included proliferation in response to LPS (utilising CFSE staining), analysis of cell cycle (using PI), analysis of activation (CD80 and CD86 expression) and the ratio of the levels of the cell-death related proteins Bcl-2 and Bax (using flow cytometry and quantitative real-time PCR).

3.2 Methods

3.2.1 WEHI-279

The WEHI-279 cell line was purchased from ATCC (catalogue number CRL-1704; Manassas, VA, USA).
3.2.2 Cell Culture

WEHI-279 cells were cultured in DMEM supplemented with 4mM glutamine, 1.5g/L sodium bicarbonate, 4.5g/L glucose, 1mM sodium pyruvate, 0.05mM 2-mercaptoethanol, antibiotic mixture (penicillin 50IU/mL and streptomycin 50μg/mL) and 10% FCS.

(See Sections 2.2.2 Thawing of cells, 2.2.3 Freezing of cells, and 2.2.4 Passaging of CH12 as the same methods were utilised for WEHI-279.)

3.2.3 Mitogens

See Section 2.2.6 Mitogens

3.2.4 Flow cytometry

See Sections 2.2.8 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) staining of cells, 2.2.9 Determination of division index in CFSE-stained B cell subsets, and 2.2.10 Flow cytometric analysis of CFSE stained cells.

LPS concentrations for further analysis were chosen on the basis of significant differences in the viability and cell number data to that of the control population.

3.2.5 Quantitative PCR

See Section 2.2.14 Quantitative PCR and sub-sections.

3.2.6 Statistical Analyses

Control and LPS-stimulated values were compared for all experiments. Two-way ANOVA to compare differences at particular time points between unstimulated and stimulated populations, with Tukey's multiple comparison post test to compare differences between time points in a population, and one-way ANOVA to test differences overall between unstimulated and stimulated populations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All data shown are pooled means from a number of experiments. All error bars shown are the standard error of the mean.
3.3 Results

3.3.1 Studies utilising S. enteritidis LPS

3.3.1.1 High concentrations of S. enteritidis LPS enhance viability but do not induce proliferation

The effect of S. enteritidis LPS on the viability and proliferation of WEHI-279 cells was examined by culturing the cells under serum-limiting conditions in the presence of varying concentrations of LPS. In the absence of S. enteritidis LPS, the viability WEHI-279 cells decreased over the course of the experiment (Figure 3.1A). While low concentrations of LPS did not have any effect on cell viability, some higher concentrations did protect the cells from death. Cells cultured in with 10ng/ml LPS showed significantly increased viability at 24 hours in comparison to the unstimulated control at the same timepoint. However, there was no clear trend, as most of the higher doses tested did not appear to protect the cells from death. Nevertheless, the highest concentration of LPS used, 10µg/ml, did show significantly increased viability at both 24 and 48 hours in comparison to non-stimulated controls (Figure 3.1A).

Despite the decrease in viability for the whole cell populations, cells did in fact appear to be proliferating within the cultures. There were modest increases in cell concentrations in the non-stimulated controls and all of the test cultures, although the magnitude of this increase was much less than that seen in the 10%FCS control (Figure 3.1B). Perhaps surprisingly, S. enteritidis LPS did not appear to induce further proliferation of WEHI-279 cells, as none of the LPS-stimulated cultures were significantly different to the control 2% FCS unstimulated culture at the same timepoint (Figure 3.1B). These findings were confirmed when proliferation was examined using CFSE (Figure 3.2). While the division index increased over the culture period in individual cultures, suggesting proliferation was occurring, there did not appear to be an induced proliferation in the presence of LPS. In fact, at the 72-hour timepoints in several of the cultures, a decreased division index was noted (Figure 3.2).
3.3.1.2 *S. enteritidis* LPS does not affect CD80, CD86 or sIgM mRNA levels

In addition to investigating the effects of *S. enteritidis* LPS on the survival and proliferation of WEHI-279 cells, potential activation effects were also gauged by examining changes in the expression of molecules associated with B cell activation. Changes in mRNA levels of the co-stimulatory molecules CD80 and CD86, as well as the expression of the constant chain of secretory IgM, were determined by RT-qPCR in high dose LPS cultures. Neither CD80 (Figure 3.3A), nor CD86 (Figure 3.3B) changed significantly over the course of the experiment. Also, sIgM mRNA levels did not change with LPS stimulation suggesting that *S. enteritidis* LPS did not induce increased antibody production (Figure 3.5).

3.3.1.3 Investigation of molecular events associated with *S. enteritidis* LPS stimulation

In order to further investigate the molecular basis of *S. enteritidis* LPS effects on WEHI-279 cells, changes in the mRNA levels of different TLRs, as well as those of the apoptosis regulators Bax and Bcl-2, were determined by RT-qPCR in cultures stimulated with LPS. Neither the Bax/Bcl-2 mRNA ratio (Figure 3.4), nor the expression levels of TLR2 (Figure 3.6A), TLR4 (Figure 3.6B), or RP105 (Figure 3.7) in LPS-stimulated cultures were significantly different to unstimulated timepoint controls.

3.3.1.4 Stimulation with *S. enteritidis* LPS affects cytokine mRNA levels

Changes in the levels of IL-6 and IL-10 mRNA in response to *S. enteritidis* LPS were investigated by RT-qPCR (Figure 3.8). While there were no significant changes over the course of the culture period in IL-6 mRNA expression apart from an increase at 8hr in the 1μg/mL culture, the level of IL-10 mRNA expression in the 10μg/mL culture increased significantly over the culture period (Figure 3.8B).
3.3.2 P. gingivalis LPS studies

3.3.2.1 Low concentrations of P. gingivalis LPS enhance but high concentrations inhibit WEHI-279 proliferation

The effects of *P. gingivalis* LPS on the viability and proliferation on WEHI-279 cells was investigated by culturing cells in serum-limited conditions in the presence of varying concentrations of LPS. As with the experiments described in section 3.3.1.1, in the absence of LPS WEHI-279 cells in serum-limited cultures decreased in viability over the timecourse of the experiment (Figure 3.9A). In contrast to *S. enteritidis* LPS however, none of the doses of *P. gingivalis* LPS were associated with changes in viability compared to non-stimulated controls at the corresponding timepoints (Figure 3.9A).

The effects of *P. gingivalis* LPS on the proliferation of WEHI-279 cells were complex. There was some proliferation evident in the non-stimulated control group, as well as in most of the LPS-stimulated controls, over the course of the experiment. However, when changes in cell number were used to assess proliferation, low doses of LPS (10pg/mL and 100pg/mL) caused small, but significant, increases in cell number when compared to non-stimulated timepoint controls (Figure 3.9B). High doses of LPS (1μg/mL, 10μg/mL) however, were associated with reduced cell numbers at 48 and/or 72 hours (Figure 3.9B). When proliferation was measured by CFSE dye dilution, significantly increased proliferation in response to low dose *P. gingivalis* was not seen, however cells cultured with either 10ng/mL and 1μg/mL LPS clearly showed decreased levels of proliferation at both 48 and 72 hours when compared to non-stimulated timepoint controls (Figure 3.10).

3.3.2.2 P. gingivalis LPS does not affect CD80, CD86 or sIgM mRNA levels

Since there appeared to be differential effects of *P. gingivalis* LPS on cellular proliferation, both low and high doses of LPS were chosen for further study of activation markers. As was found with *S. enteritidis* LPS (section 3.3.1.2), and despite the significant differences in proliferation observed in response to *P. gingivalis* LPS, no
changes were found in mRNA levels for CD80 (Figure 3.11A), CD86 (Figure 3.11B) or sIgM (Figure 3.13) in response to different LPS concentrations.

3.3.2.3 Investigation of molecular events associated with P. gingivalis LPS stimulation

As with the response of WEHI-279 cells to S. enteritidis LPS, the molecular basis of the effects of P. gingivalis LPS on WEHI-279 cells was investigated. Changes in the mRNA levels of different TLR, as well as those of the apoptosis regulators Bax and Bcl-2, were determined by RT-qPCR in cultures stimulated with either low or high concentrations of LPS. Neither the Bax/Bcl-2 ratio (Figure 3.12), nor the expression levels of TLR2 (Figure 3.14A), TLR 4 (Figure 3.14B), or Rp105 (Figure 3.15) in LPS-stimulated cultures were significantly different to unstimulated timepoint controls. While there was an apparent decrease in the Bax/Bcl-2 ratio in the LPS-stimulated cultures over time, this was not statistically significant (p>0.05 for all timepoints compared with t=0).

3.3.2.4 Stimulation with P. gingivalis LPS does not cause changes in cytokine mRNA levels

Changes in the levels of IL-6 and IL-10 mRNA in response to P. gingivalis LPS were investigated by RT-qPCR (Figure 3.16). The only significant change in cytokine mRNA expression was for IL-6 between the 24 and 48hr time points in the 1μg/mL culture.
Figure 3.1 Effect of *S. enteritidis* LPS on the viability and concentration of WEHI-279 cells in serum-limited cultures

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the viability (A) and concentration (B) of cells was determined using Trypan Blue exclusion. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.2 Division index of WEHI-279 cells stimulated with *S. enteritidis* LPS

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the division index of viable WEHI-279 was determined using CFSE dilution and flow cytometry. Statistically significant differences (p<0.05) between the control and test cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and test cultures are denoted by a † below the category name on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.3 Effect of *S. enteritidis* LPS on expression of CD80 and CD86 mRNA

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the relative level of expression of the CD80 (A) and CD86 (B) genes as compared with HPRT was determined using RT-qPCR. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.4 Effect of *S. enteritidis* LPS on Bax and Bcl-2 relative expression ratio

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the Bax/Bcl-2 mRNA expression ratio was determined using the relative levels of expression of each gene by RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.5 Effect of *S. enteritidis* LPS on expression of slgM mRNA

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the relative level of expression of mRNA for slgM was determined using RT-qPCR. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.6 Effect of *S. enteritidis* LPS on expression of TLR2 and TLR4 mRNA

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the relative level of expression of TLR2 (A) and TLR4 (B) mRNA was determined by RT-qPCR. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.7 Effect of *S. enteritidis* LPS on expression of RP105 mRNA

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the relative level of expression of RP105 mRNA was determined using RT-qPCR. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.8 Effect of *S. enteritidis* LPS on expression of IL-6 and IL-10 mRNA

WEHI-279 cells were cultured in serum-limited conditions with *S. enteritidis* LPS at varying concentrations and the relative level of expression of IL-6 (A) and IL-10 (B) mRNA was determined using RT-qPCR. Statistically significant differences (p<0.05) between the control and test cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Significant differences between time points at a particular LPS concentration are denoted by bars joining the relevant columns. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.9 Effect of *P. gingivalis* LPS on the viability and concentration of WEHI-279 cells in serum-limited cultures

The viability (A) and concentration (B) of WEHI-279 cells for all concentrations of *P. gingivalis* LPS and all time points with 2% FCS was determined using Trypan Blue exclusion. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), as determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.10 Division index of WEHI-279 cells cultured in RPMI/2% FCS and stimulated with *P. gingivalis* LPS

WEHI-279 cells were cultured in serum-limited conditions with *P. gingivalis* LPS at varying concentrations and the relative level of expression of the division index of viable WEHI-279 cells was determined using CFSE dye dilution and flow cytometry (see section 2.2.9). Statistically significant differences (p<0.05) between the control and test cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and test cultures are denoted by a † below the category name on the X axis. In some cases differences between control and test were not able to be tested and these are denominated by “n.d.” under the appropriate column. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.11 Effect of P. gingivalis LPS on expression of CD80 and CD86 mRNA

WEHI-279 cells were cultured in serum-limited conditions with P. gingivalis LPS at varying concentrations and the relative level of expression of CD80 (A) and CD86 (B) mRNA was determined RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.12 Effect of *P. gingivalis* LPS on Bax/Bcl2 mRNA ratio

WEHI-279 cells were cultured in serum-limited conditions with *P. gingivalis* LPS at varying concentrations and the ratio of Bax/Bcl-2 mRNA was determined using RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.13 Effect of *P. gingivalis* LPS on expression of sIgM mRNA

WEHI-279 cells were cultured in serum-limited conditions with *P. gingivalis* LPS at varying concentrations and the relative level of expression of sIgM mRNA was determined using RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.14 Effect of \textit{P. gingivalis} LPS on expression of TLR2 and TLR4 mRNA

WEHI-279 cells were cultured in serum-limited conditions with \textit{P. gingivalis} LPS at varying concentrations and the relative level of expression of TLR2 (A) and TLR4 (B) mRNA was determined using RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.15 Effect of *P. gingivalis* LPS on expression of RP105 mRNA

WEHI-279 cells were cultured in serum-limited conditions with *P. gingivalis* LPS at varying concentrations and the relative level of expression of RP105 mRNA was determined using RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey's multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
Figure 3.16 Effect of *P. gingivalis* LPS on expression of IL-6 and IL-10 mRNA

WEHI-279 cells were cultured in serum-limited conditions with *P. gingivalis* LPS at varying concentrations and the relative level of expression of IL-6 (A) and IL-10 (B) was determined using RT-qPCR. There are no statistically significant differences between the different cultures at each time point as determined by two-way ANOVA with Tukey’s multiple comparison post test. Data shown are the means and s.e.m. of three pooled experiments.
3.4 Discussion

In this chapter, the response of a B-2 cell line (WEHI-279) to both enteric (*S. enteritidis*) and non-enteric (*P. gingivalis*) LPS was examined. As a model for the general B-2 response to different forms of LPS, the WEHI-279 line provides initial information regarding the response, and allows comparison with a B-1 cell line (Chapter 2). In addition, as the species of origin of the LPS may be important in determining B cell proliferation, differential activation by different LPS preparations was able to be assessed.

A serum-limiting culture system was used in order to decrease cellular proliferation and survival. This also allowed comparison to the results of investigations using the CH12 cell line in a similar serum-limiting culture system (Chapter 2). As was seen with CH12 cells, there appeared to be a balance between cellular proliferation and apoptosis induced by serum limitation. Culture of WEHI-279 cells under serum-limited conditions resulted in a reduction of viability over the culture period (Figure 3.1 and Figure 3.9). However, in contrast to its effects on CH12 cells (Figure 2.6), *S. enteritidis* LPS only increased cell viability at high concentrations (10µg/ml). In addition, and again in contrast to its effects on CH12 cells, *S. enteritidis* LPS appeared to have no positive effect on cell proliferation. There were no significant changes in the number of cells in culture in response to any LPS concentration (Figure 3.1B), while there actually appeared to be a decrease in proliferation at 72 hours in several of the LPS-stimulated cultures when this was assessed by CFSE dye dilution (Figure 3.2). The effects of *P. gingivalis* LPS on WEHI-279 viability and proliferation are not simply explained. As with *S. enteritidis* LPS, there was no effect of *P. gingivalis* LPS on viability (Figure 3.9). At low concentrations, culture with *P. gingivalis* LPS led to increased numbers of cells in culture, while high concentrations led to decreased numbers (Figure 3.9). The high dose inhibitory effect was confirmed using CFSE dye dilution (Figure 3.10). However, the low dose enhancement of proliferation was not evident by this measure, which questions the biological significance. It therefore appears that the WEHI-279 cells are less sensitive than CH12 cells to the pro-survival and proliferative effects of both enteric and non-enteric LPS. It may also be that, in contrast to *S. enteritidis* LPS, *P. gingivalis* LPS actively inhibits WEHI-279 proliferation. These observations suggest
that B-2 cells may be less sensitive to the anti-apoptotic and pro-proliferative effects of LPS than B-1 cells and, further, that B-2 proliferation may be actively inhibited by high concentrations of non-enteric LPS.

As there were few changes seen in viability of WEHI-279 cells in response to *P. gingivalis* LPS, it was expected that there would be little or no change in the expression of apoptosis regulators in these cultures. This was indeed the case. There was no difference in the Bax/Bcl-2 ratio of any of the test cultures when compared with timepoint controls (Figure 3.12). In contrast to *P. gingivalis* LPS-stimulated cultures, cells stimulated with 10μg/ml *S. enteritidis* LPS did have slightly enhanced viability (Figure 3.1), however there was no difference between the Bax/Bcl-2 mRNA ratio of these cultures and those of the controls (Figure 3.4). The reason for this is unclear, but since the magnitude of viability change is not large, the lack of change in the Bax/Bcl-2 ratio could simply reflect but this. However, it could also indicate that these molecules are not important in the regulation of viability of the WEHI-279 cell line.

There were effects of *P. gingivalis* - and possibly *S. enteritidis* - LPS, on proliferation, however these effects did not correlate with changes in mRNA levels of IL-10 or IL-6. In response to *S. enteritidis* LPS stimulation, changes in proliferation were equivocal, as described above. However, there were no significant differences in either IL-10 or IL-6 mRNA levels between *S. enteritidis* LPS-stimulated and unstimulated cultures. In contrast to the proliferative changes seen in response to *S. enteritidis* LPS, there was clear inhibition of the proliferation of WEHI-279 cells by *P. gingivalis* LPS. This response was not accompanied by any changes in the either IL-10 or IL-6 mRNA (Figure 3.16). This data supports the argument that removal of autocrine cytokine stimulation is not responsible for the inhibition of WEHI-279 proliferation by *P. gingivalis* LPS. The exact mechanism remains unclear.

In contrast to the effect of LPS on TLR mRNA levels in CH12 cells (see section 2.4), there were no effects of either *S. enteritidis* LPS (Figures 3.6 and 3.7), or *P. gingivalis* LPS (Figure 3.14 and Figure 3.15) on TLR mRNA levels. As described in section 1.5.2.1, changes in TLR mRNA levels may be involved in the down-regulation of responsiveness of cells to LPS. The lack of changes in TLR mRNA levels in response to
LPS means that regulation of TLR mRNA levels is unlikely to account for the inhibition of proliferation seen in cultures.

Although enhancement of survival and proliferation are critical features of the B cell response to LPS, it was also important to determine changes in the activation state of cells. In response to LPS, there were no differences in the mRNA levels of the co-stimulatory molecules CD80 and CD86 between LPS-stimulated and control cultures (Figure 3.3 and Figure 3.11). This argues that not only does LPS not stimulate these cells to divide, but it also does not activate them. The absence of changes in sIgM mRNA (Figure 3.5 and Figure 3.13) also supports a lack of activation/differentiation by either LPS preparation.

Taken together, these observations argue that the response of WEHI-279 to LPS is not dramatic, but what response there is may differ between LPS from enteric and non-enteric bacteria. *S. enteritidis* LPS appears to enhance proliferation at very high concentrations, while *P. gingivalis* LPS may, in fact, inhibit proliferation at similar concentrations. Further, there was no apparent activation of these cells as measured by co-stimulatory molecule or sIgM expression. The relevance of these results to the generalised B-2 response to LPS is unclear. Although the WEHI-279 cell line can be thought of as an immortalised ‘typical’ B-2 cell line (see section 1.6.2), immortalised cell lines may show certain characteristics that do not reflect the population from which they are drawn.

The studies presented in this chapter, together with those dealing with B-1 cells in Chapter 2, begin to develop a picture of B cell subset responses to enteric and non-enteric LPS. However, as the studies were carried out separately in unmixed populations, cross-subset effects cannot be examined. A mixed system containing both subsets in the same culture presents an opportunity for studying B cell subset interactions. Work examining such a system is presented in Chapters 4 and 5 of this thesis.
Chapter 4 Effects of a high concentration (50µg/mL) of lipopolysaccharide on CD5+ and conventional murine splenic lymphocytes

4.1 Introduction

The two main aims of the studies presented in this thesis are firstly to compare the responses of B-1 and B-2 cells to LPS, and secondly to compare the B cell responses to LPS preparations derived from enteric (S. enteritidis) and non-enteric (P. gingivalis) species. The results of the preceding experimental chapters, which used cell lines as model systems, suggested that B cells may not only respond differently to LPS from different bacteria, but that there may be differences between the responses of B-1 and B-2 cells to LPS derived from a single species. Although there were differential effects of P. gingivalis LPS on the responses of both cell lines, the effect was most marked in response to S. enteritidis LPS. In particular, it appeared that the B-1 cell line (CH12), was rescued from cell death, proliferated and differentiated in response to moderate concentrations of S. enteritidis LPS, whereas the proliferation of the B-2 cell line (WEHI-279) appeared to be actively inhibited by similar LPS concentrations. Although examination of the response of individual cell lines is a useful first step for investigating B cell responses, the response of a cell line may not necessarily reflect that of the original cell population. In addition, interactions between different cell types (including the interaction between B-1 and B-2 cells) may be important in regulating the response of B cell subpopulations to LPS. Therefore, a simplified mixed splenocyte culture system was developed to contemporaneously examine the response of B-1 and B-2 cells to LPS.

As S. enteritidis LPS was shown to cause more marked differences in activation of B-1 and B-2 cell lines (Chapters 2 and 3), this LPS preparation was chosen for use in the
development of the splenocyte culture system. A high concentration of 50μg/mL LPS was chosen to ensure that the conventional B cell subset would be appropriately stimulated, and to enable the comparison of the CD5⁺ B cell response to a “normal” polyclonal B cell response (Melchers et al., 1975). Young mice (4-6 weeks old) were used as the source of splenocytes as the percentage of CD5⁺ B cells in the spleen decreases with age. At this age, the spleens were large enough for good yields of cells, but the proportion of CD5⁺ B cells was still high enough for this population to be accurately identified in the flow cytometric analyses.

The activation and proliferation of B-1 and B-2 cell subsets, as well as overall apoptosis were assessed after culture with LPS. Activation of stimulated and unstimulated cells was identified through the combination of morphological and MTT colourimetric assay studies. The activation of B cell subsets was determined using a combination of 7AAD, PE-anti-CD5 and FITC-anti-CD80. Activated B cells express CD80 (B7-1) (Hathcock et al., 1994; Ding and Shevach, 1996), and the selective gating by forward and side scatter of lymphocytes together with the staining levels of the anti-CD5 and anti-CD80 antibodies on viable cells allowed the identification of activated CD5⁺ and CD5⁻ B cells. Viability status – viable, apoptotic, or degenerate – of B cell subsets was determined using 7-aminoactinomycin D (7AAD). Since analyses of tissues from patients with chronic periodontitis suggested that many of the B cells within gingival tissues were activated (Gemell and Seymour, 1991; Afar et al., 1992) and that many others were degenerate (Joachim et al., 1990), morphology and patterns of death of splenic lymphocytes were examined using electron microscopy. Unfortunately, resources did not allow immunogold labelling to identify B cell subsets harvested from the in vitro cultures, although these studies would be useful in supporting the flow cytometric analyses of stimulated and unstimulated cells.

### 4.2 Materials and Methods

#### 4.2.1 Mice

Balb/c mice 4-6 weeks of age were obtained from the Blackburn Animal House Facility, University of Sydney, and from the Institute of Dental Research, Sydney. All mice were
allowed commercial pellets and water *ad libitum* until sacrifice by inhalation of Fluothane (Zeneca, Cheshire, UK).

### 4.2.2 Mitogens

*Salmonella enteritidis* LPS, prepared by a phenol-water extraction procedure and chromatographically purified, was obtained from Sigma (St Louis, MO, USA) and diluted to 50μg/mL.

### 4.2.3 Splenocyte preparation

Murine splenocyte suspensions were prepared in RPMI 1640 medium (ICN, Costa Mesa, CA, USA) supplemented with 10% foetal bovine serum (FCS; Trace), 5 x 10^{-5}M 2-mercaptoethanol (2ME) (Sigma), and antibiotic mixture (penicillin 50IU/mL and streptomycin 50μg/mL; Trace) (RPMI/10% FCS). Spleens were removed from the mice and placed into RPMI 1640 medium on ice. The spleens were placed into a petri dish in the laminar flow cabinet and mashed using the plunger of a 10mL syringe. A 20mL syringe and 18-gauge needle were used to create a single cell suspension, and this was then passed through nylon mesh of 200μm pore size (Australian Filter Specialists, Blacktown, NSW, Australia) into a 50mL centrifuge tube. The cells were then washed twice with RPMI/10% FCS (centrifuge 10 min, 4°C, 300g) and the supernatant discarded. The pellet was resuspended in ammonium chloride erythrocyte lysing buffer (5mL per spleen used, to a total of 45mL) and incubated for 5 min at room temperature, with occasional agitation of the tube. RPMI/10% FCS was added to fill the tube and it was centrifuged for 10 min, 4°C at 200g, using a TJ-6 refrigerated centrifuge (Beckman-Coulter, Palo Alto, California). The pellet was washed again and resuspended in RPMI/10% FCS. The cell suspension was poured into a 50mL centrifuge tube precoated with FCS and underlaid with an equal volume of Histopaque-1083 (Sigma). The tubes were centrifuged for 30 min, 18°C at 400g with no brake to allow preservation of layers formed after centrifugation. The buffy layer at the interface of the Histopaque-1083 and the medium was collected and added to 40mL RPMI/10% FCS and centrifuged 10 min, 4°C at 300g. The supernatant was discarded and the pellet resuspended in RPMI/10% FCS and the cell concentration and viability determined,
using Trypan Blue staining, prior to seeding and addition of LPS (see Appendix A for more detail regarding splenocyte preparation protocols).

4.2.4 **Seeding of culture vessels**

Cells were treated with mitogens before seeding in complete medium at $2 \times 10^6$ cells/mL in 96 well flat-bottomed microtitre plates (Sarstedt) to a final well volume of 100μL. Eight replicate wells were prepared for each time point, and control (unstimulated), and mitogen-stimulated test population were utilised. Cells used for flow cytometric analysis were seeded at $5 \times 10^6$ cells/mL in 25cm$^2$ cell-culture flasks (Sarstedt) to a final volume of 5mL. Control and mitogen-stimulated populations for all time points were cultured for this analysis.

4.2.5 **Depletion of adherent cells**

Adherent cells were depleted after 24hr of culture at 37°C and 5% CO$_2$ in a humidified atmosphere. The supernatant containing the non-adherent cells was removed and placed into a fresh flask or well. The efficacy of depletion was confirmed by flow cytometry using Phycoerythrin (PE) conjugated anti-mouse CD11b (Pharmingen) to stain the adherent cells before and after depletion, and visual analysis of the cultures.

4.2.6 **Preparation of cells for microscopy**

Method as follows from the Electron Microscopy Unit, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, Westmead, NSW, Australia, and adapted from Lazzaro (Lazzaro, 1983).

4.2.6.1 **Primary fixation**

1 $\times 10^7$ cells were collected and spun for 5 min, 4°C at 300g. The supernatant was decanted and the pellet resuspended in PBS and placed into an Eppendorf tube. The cells were washed twice with PBS, resuspended in 1mL Karnovsky’s fixative (see Appendix B for details) and incubated 4 to 24h at 4°C. The cells were centrifuged for 5 min, resuspended in 1mL PBS and stored at 4°C for up to 1 week.
4.2.6.2  Secondary fixation and staining
The fixed cells were spun down and resuspended in a buffered solution of osmium tetroxide for 3hr at room temperature. The cells were rinsed with distilled water, 2% uranyl acetate was added to cover the sample, and this was then incubated 1hr at room temperature whilst mixing continuously.

4.2.6.3  Dehydration
Tubes were one-quarter filled for each step and incubated for 10min at room temperature;
50% EtOH + 0.1% NaCl, followed by 70% EtOH + 0.1% NaCl, then 95% EtOH + 0.1% NaCl, and finally two steps of 100% EtOH.

4.2.6.4  Acetone
Two acetone steps each comprising quarter filling the tubes with 100% Acetone (dry) and incubating for 10 min at room temperature.

4.2.6.5  Infiltration with acetone/resin
Acetone/resin mixture (1:1) was added to the tubes and incubated for 1hr, followed by three changes of resin at 70°C, each incubated for 10 min.

4.2.6.6  Embedding and Polymerisation
The samples were embedded in gel caps and the resin allowed to polymerise at 70°C for 10hr.
Sections were further processed for light and electron microscopy by staff of the Electron Microscopic Unit, ICPMR, Westmead Hospital.

4.2.7  Transmission electron microscopy
Sections were cut at 70nm on a Reichert Ultracut ultramicrotome and stained with alcoholic uranyl acetate (Fluka, Milwaukee, WI, USA) and Reynold's lead citrate. Sections were examined at 80kV in a Philips EM400 electron microscope (Philips Electron Optics, Eindhoven, Netherlands) and photographed on Kodak EM film.
4.2.8 **MTT colourimetric assay**

See section 2.2.7 MTT colourimetric assay

4.2.9 **Antibodies for flow cytometry**

PE-conjugated anti-murine CD5 (clone 53-7.3; PE-anti-CD5), FITC-conjugated anti-mouse B220 (RA3-6B-2; FITC-anti-B220), and FITC-conjugated anti-mouse CD80/B7-1 (16-10A1; FITC-anti-CD80), and the corresponding isotype-matched controls (I/C) were purchased from Pharmingen. Fc Block (anti-murine CD16/CD32, clone 2.4G2), also from Pharmingen, was used to prevent non-specific binding of murine monoclonals to Fcγ receptors on the cell surface.

4.2.10 **Flow cytometric analysis**

Cultured cells were washed twice with PBS containing 2% FCS and 0.1% NaN₃ (PBS/FCS/Azide) and incubated with 0.25μg Fc Block/10⁶ cells (5μL of 0.05mg/mL working solution) for 3 min at 4°C. The cells were then incubated with 0.25μg/10⁶ cells of 7-Aminoactinomycin D (7AAD; Sigma) and 1μg/10⁶ cells of PE-anti-CD5 (25μL of 0.04mg/mL working solution) and FITC-anti-B220 (20μL of 0.05mg/mL working solution) and corresponding I/C at the same concentration were added to I/C tubes, or PE-anti-CD5 and FITC-anti-CD80 (20μL of 0.05mg/mL working solution) and corresponding I/C at the same concentration were added to I/C tubes, for 30 min at 4°C. The cells were then washed twice with PBS/FCS/Azide and then resuspended to 2 x 10⁶ cells/mL in Osmosol sheath fluid (Lab Aids, Narrabeen, NSW, Australia) with 2% paraformaldehyde (sheath/PFA) prior to flow cytometric analysis on a FACSScan (BDIS).

4.2.11 **Statistical analysis**

Control and LPS-stimulated values were compared for all experiments. Two-way ANOVA to compare differences at particular time points between unstimulated and stimulated populations, with Tukey's multiple comparison post test to compare differences between time points in a population, and one-way ANOVA to test differences overall between unstimulated and stimulated populations were performed.
using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statview v4.5 (Abacus Concepts Inc, Berkeley, CA, USA) was used to assess correlations for Figure 4.6. All data shown are pooled means from a number of experiments. All error bars shown are the standard error of the mean.

4.3 Results

4.3.1 Derivation of lymphocytes: spleen versus peritoneal cavity

The aim of experimental work presented in this chapter was to develop a culture system that allowed the investigation of both B-1 and B-2 cell responses to LPS within the one culture. It was important to develop a method to isolate large numbers of B-1 cells as efficiently as possible from mice. Two anatomical locations were identified in the mouse as being potentially suitable for the harvesting of B-lymphocytes: the spleen and the peritoneal cavity. The peritoneal cavity is a better source for B-1 cells than the spleen as 10 – 20% of total cells able to be harvested in the peritoneal cavity are B-1 (Hayakawa et al., 1983; Hayakawa et al., 1986), compared to approximately 2 - 5% of cells in the spleens of adult mice. (Hayakawa et al., 1983; Hayakawa et al., 1986). Younger mice have a greater proportion of CD5⁺ B cells than older mice (Hayakawa et al., 1986), therefore 3-5 week old mice were used to maximise total splenocyte yields and percentage of CD5⁺ B cells. However, preliminary experiments showed that total cell yields were greater using spleens than peritoneal washes. Peritoneal washes were more time consuming and this led to decreased viability of harvested cells prior to seeding into flasks for experiments. Given this, it was determined that the spleen was the better source of B cells for the purposes of the experimental work to be performed.

4.3.2 Efficacy of adherent cell depletion

Non-lymphocyte CD11b⁺ cells were 5.5% of the cells in spleen cultures prior to depletion of adherent cells, and 3.7% of cells after the depletion step (data not shown). This finding was supported by morphological analysis of cultures both pre- and post-depletion. Numerous adherent cells and very few degenerate cells were present in the culture flasks before depletion, but fewer than ten adherent cells per 25cm² culture flask
and a greater than ten-fold increase in the number of degenerate cells were found after depletion.

4.3.3  Overall view of the splenic lymphocyte response to LPS

An increase in cell number and size can be seen in the LPS-stimulated culture (Figure 4.1 F-J) compared to the control (Figure 4.1 A-E). This is apparent after 24hr of culture, and by 48hr of culture the field of view is almost full of cells, compared to the sparser population in the control flask at the same time point. The cells in the LPS-stimulated cultures form clumps/islands from about 48hr of culture onwards.

4.3.4  Ultrastructural analysis of splenic lymphocyte cultures

Representative transmission electron micrographs of control (Figure 4.2) and LPS-stimulated (Figure 4.3) cultures add more detail to the data presented in Figure 4.1. Figure 4.2a shows lymphocytes at the 0hr time point. The lymphocytes are resting as the cytoplasm is relatively scanty and the endoplasmic reticulum (ER) is not easily discernable. After 24hr of culture, some apoptotic and degenerate cells, as well as some viable, resting lymphocytes, are found in the control culture (Figure 4.2b). These non-viable cells are characterised by the lack of recognisable features found in viable cells, and by the appearance of vacuoles within the cell. More apoptotic and degenerate cells are found after 48hr of culture (Figure 4.2c), although some viable cells persist. Cellular debris is also present in this image. A higher magnification of three of the cells in Figure 4.2c is shown in Figure 4.2d. The cell in the lower half of the image is most likely undergoing apoptosis; the mitochondria are intact but the ER has become distended and chromatin is packing inside the nucleus. By 72hr most of the cells in the control culture are no longer viable. A late stage apoptotic cell can be seen at the middle right (Figure 4.2e). The cell has lost its structure and has formed apoptotic bodies. Cellular debris and other degenerate cells are also present. Figure 4.2f is a magnified image of the late stage apoptotic cell described in Figure 4.2e. The apoptotic bodies are more clearly seen in this image, and some organellar fragments, particularly mitochondria, are found. After 96hr of culture, virtually all the cells in the culture are dead. Some longer-lived, and perhaps relatively hardier, cells are still viable, but most cells display signs of apoptosis or secondary necrosis (Figures 4.2g and 4.2h).
In contrast to the viability of the control cultures, which decreased continuously from the initial seeding value, the ultrastructure of LPS-stimulated cultures showed a markedly different response. Figure 4.3a shows an activated lymphocyte after 24hr of culture with 50μg/mL LPS. The cytoplasm is greatly increased compared with resting cell below it, and the ER has become distended, suggesting that protein synthesis has been upregulated. At 48hr of culture, more activated cells are found. The cells in Figure 4.3b and 4.3c are highly activated. They have a high cytoplasm to nucleus ratio and the ER and ribosomes are distinct. Vesicles budding from the Golgi apparatus are also visible. Many apoptotic and degenerate cells are found by 72hr of culture, as seen in Figure 4.3d. Activated and resting cells (lower half 4.3d as well as 4.3e) are still present, but the constituents of the culture change, with more apoptotic cells appearing (Figure 4.3f). At 96hr of culture most of the cells are apoptotic or degenerate (Figure 4.3g). Figure 4.3h is a higher magnification of the early apoptotic cell in the centre of Figure 4.3g. As described above for apoptotic cells in control cultures, the mitochondria are still active, but the ER is distended and the chromatin is clumped (mostly) around the edges of the nucleus.

4.3.5 MTT assay of cultured splenic lymphocytes

The MTT assay of cellular biomass (proliferation and increase in size due to activation) was utilised to examine the effects of 50μg/mL LPS on murine splenic lymphocytes (Figure 4.4). Δ absorbance, reflecting change in biomass, in the control culture decreased until the 48hr time point, and remained stable for the duration of the culture period (although these changes were not significant). The biomass of the LPS-stimulated culture increased significantly until 72hr of culture and then decreased significantly by 96hr.

4.3.6 Viable LPS-stimulated cells increase until 48hr and then decrease

The number of viable and non-viable cells in control and LPS-stimulated cultures was determined by Trypan Blue exclusion as presented in Figure 4.5. The number of viable cells in the control cultures (A) decreased significantly over the culture period, and the number of non-viable cells increased significantly. In the LPS-stimulated cultures (B),
the number of viable cells did not change significantly until 96hr. Non-viable cell numbers did not increase until 72hr of culture. Viable cell numbers in the LPS-stimulated culture were significantly different to the control culture at the 48 and 72hr timepoints.

4.3.7 Correlation between Trypan Blue and 7AAD viability data

In order to reduce the effects of non-specific staining of apoptotic and degenerate cells on the results, 7AAD was used to stain the cells prior to flow cytometric analysis. This enabled viable, apoptotic and degenerate populations to be identified during the acquisition and analysis of the cultured splenic lymphocytes. To obtain absolute counts for B-1 and B-2 cells, there had to be a correlation between the Trypan Blue and 7AAD percentage viability data. Culture viability as measured by Trypan Blue and 7AAD (in the same cultures) is presented in Figure 4.6A. The only significant difference between the Trypan Blue and 7AAD values is at 24hr in the control culture. The Trypan Blue and 7AAD values were shown to correlate (Figure 3.6B and C), and therefore the absolute numbers of B-1 and B-2 cells can be calculated by combining Trypan Blue absolute counts and subset data generated by flow cytometry.

4.3.8 Viability of B cell subsets after LPS stimulation

The viability of B cell subsets was examined using flow cytometry with the combination of CD5-PE, B220-FITC and 7AAD to determine cell subsets and viability respectively. Representative flow cytometry data is shown in Figure 4.7. Cells were first gated by size and internal complexity, using forward and side scatter (A). This gating strategy removes most of the debris as well as a proportion of dead cells from further analyses, and also serves to discriminate between lymphocytes and other cells that remained despite the enrichment procedure (for example, macrophages and eosinophils). After gating on forward and side scatter, cells were gated for viability using 7AAD versus side scatter (B). Viable cells show low 7AAD fluorescence, apoptotic cells intermediate, and degenerate/late stage apoptosis/necrotic showed high 7AAD fluorescence. Cells are gated according to their level of 7AAD fluorescence and further analysed for division into the B-1 and B-2 cell subsets. Quadrants are set for
positive staining using fluorescently conjugated isotype controls (C). A representative plot for PE-CD5 and FITC-B220 staining after gating for viable lymphocytes is shown in (D). T cells (CD5+/B220+) are seen in the upper left quadrant, and the percentage of these cells remains relatively stable over the culture period. B-1 cells are CD5lo/B220+ and are 5-10% of the viable lymphocyte population at initial seeding. B-2 cells are CD5- /B220+, and are approximately 28% of the viable lymphocyte population at 0hr. A large debris plume appeared over the culture period, and this plume consisted of dead and dying cells and cellular debris (data not shown). The plume increased in size, as there were virtually no phagocytic cells available in the cultures to remove this debris.

Figure 4.8 shows cell numbers for B-2 (A) and B-1 (B) B cell subsets in control and LPS-stimulated cultures. Conventional B cell numbers decreased over the culture period in the control cultures, and were significantly lower than the 0hr value at 24, 48, and 72hr. In the LPS-stimulated culture there was a trend for an increase in conventional B cell numbers, with a peak at 48hr followed by a decline at 72 and 96hr, although the differences were not significant. A difference in conventional B cell number between the control and the LPS-stimulated cultures was found at 24 and 48hr of culture, although in the later stages of culture there was no difference between the control and the LPS-stimulated cultures. B-1 cells (B) were strongly represented and numbers did not change significantly over time in the control cultures. An increase in the number of CD5+ B cells up until 72hr of culture when stimulated by LPS culture was noted, although this increase is not statistically significant. There were no significant differences between the control and LPS-stimulated B-1 cells.

The percentage of viable B cells in control cultures (Figure 4.9A) decreased significantly after 24hr of culture compared to the 0hr value over the culture period and the percentage of apoptotic cells increased significantly after 24hr compared to the 0hr value. In the LPS-stimulated culture (Figure 4.9B) the percentage of viable cells remained stable for the first 48hr of culture but then decreased significantly over the remaining time. The percentage of apoptotic and degenerate cells increased significantly only at 72 and 96hr of culture as compared to the 0hr value.
4.3.9 Activation of B cell subsets after LPS stimulation

Figure 4.10 shows the gating scheme used for the CD80 and I-A expression experiments. The data shown is for one representative experiment utilising CD80-FITC staining together with CD5-PE and CD19-CyChrome, however, the same protocol was used for cells stained with I-A-FITC, CD5-PE and CD19-CyChrome.

The activation of B-2 (A) and B-1 (B) cells was measured using CD80 (B7-1) as a marker of activation (Figure 4.12). When stimulated with LPS a significantly increased percentage of B-2 cells became activated at 48, 72 and 96hr of culture when compared to the control culture. At the later stages of culture (48 and 72hr), a significantly greater percentage of the LPS-stimulated B-1 cells were activated than in the control cultures. Overall, the percentage of CD80\(^+\) B-1 cells was significantly different to that of B-2 cells for both control and LPS-stimulated populations.

Activation was also measured using I-A expression (Figure 4.12). Overall, the percentage of B-2 cells expressing I-A was significantly increased in LPS-stimulated cells as compared to the control cells. In the control cultures, the percentage of B-2 cells expressing I-A was significantly different over the culture period to the initial level after harvesting from murine spleens. At 24 and 96hr the percentage of B-1 cells expressing I-A was significantly higher in the in LPS-stimulated culture than in the control culture.
Figure 4.1 Photographs of cultures cells *in vitro*

Control and 50μg/mL *S. enteritidis* LPS stimulated cells were cultured in 25cm² tissue culture flasks.

The cultures were photographed before the cells were harvested for staining at each time point (0hr was photographed after seeding). (20x magnification)

(a) Control 0hr  
(b) Control 24hr  
(c) Control 48hr  
(d) Control 72hr  
(e) Control 96hr  
(f) LPS (50μg/mL) 0hr  
(g) LPS 24hr  
(h) LPS 48hr  
(i) LPS 72hr  
(j) LPS 96hr
Figure 4.2 TEM images of control splenic lymphocyte cultures

Cells from control cultures were harvested and processed for transmission electron microscopy. Bars are 1 µm.

(a) 0hr  13.2k x magnification  
(b) 24hr  10.4k x magnification  
(c) 48hr  31.0k x magnification  
(d) 48hr  13.2 x magnification  
(e) 72hr  10.4k x magnification  
(f) 72hr  42.0k x magnification  
(g) 96hr  13.2k x magnification  
(h) 96hr  23.0k x magnification
Figure 4.3 TEM images of *S. enteritidis* LPS Stimulated splenic lymphocyte cultures

Cells from 50μg/mL *S. enteritidis* LPS stimulated cultures were harvested and processed for transmission electron microscopy. Bars are 1μm.

(a) 24hr  17.8k x magnification  (e)  72hr  17.8k x magnification
(b) 48hr  23.0k x magnification  (f)  72hr  17.8k x magnification
(c) 48hr  10.4k x magnification  (g)  96hr  13.2k x magnification
(d) 72hr  10.4k x magnification  (h)  96hr  31.0k x magnification
Figure 4.4 Change in biomass over the culture period in control and *S. enteritidis* LPS-stimulated splenic lymphocyte cultures

The MTT-formazan assay was used to examine the change in biomass in control and 50μg/mL *S. enteritidis* LPS-stimulated splenic lymphocyte cultures. This was measured by determining the difference in absorbance between the yellow soluble substrate (540nm) and the purple insoluble product (690nm). Statistically significant differences (p<0.05) between the control and LPS stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS stimulated cultures are denoted by a † next to the culture descriptor on the X axis. Data shown are the pooled means and s.e.m. of five experiments.
Figure 4.5 Viable and non-viable cells in control and *S. enteritidis* LPS-stimulated cultures

The numbers of viable and non-viable cells in control (A) and *S. enteritidis* LPS-stimulated (B) cultures were determined using Trypan Blue exclusion. Statistically significant differences (p<0.05) between the control and LPS stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Data shown are the means and s.e.m. of five pooled experiments.
Figure 4.6 Viability of cultures determined by Trypan Blue and 7-
aminoactinomycin D exclusion

The percentage viability determined using Trypan Blue exclusion and 7AAD was compared to
ascertain whether the two methods resulted in the same values (A), and hence whether the two
results could be combined to derive cell numbers of flow cytometrically determined subsets.
The correlation matrix (B) and p-value (C) for the two methods are also shown. Data shown are
the means and s.e.m. of five pooled experiments.
Figure 4.7 Representative FACScan plots for flow cytometric analysis
A specific gating strategy was utilised in order to examine only lymphocytes from the splenocyte cultures. Cells were first gated by forward (FSC) and side (SSC) scatter to select cells of the correct size and internal complexity (A). Cells meeting these criteria were further gated by their 7AAD fluorescence and side scatter to determine viable and non-viable cells (B). Apoptotic and degenerate cells are also able to be identified using 7AAD. Fluorescently-labelled isotype controls were used to set the quadrant parameters for positive and negative events, using only those cells that were gated as viable lymphocytes (C). Identification of subsets of viable lymphocytes was then able to be performed using, for example, anti-CD5-PE and anti-B220-FITC (D) to identify B-1 cells (CD5+/B220−, upper right quadrant), B-2 cells (CD5+/B220+, lower right quadrant) T cells (CD5−/B220−, upper left quadrant) and non T or B cells (CD5−/B220+, lower left quadrant). Data shown is from a single representative experiment.
Figure 4.8 Absolute cell counts for B-2 and B-1 cells
The numbers of viable B-2 (A) and B-1 (B) cells in control and S. enteritidis LPS stimulated cultures were determined by multiplying the percentage of cells classed as B-1 cells (CD5^+/B220^+) or B-2 cells (CD5^+/B220^-) by the number of viable cells counted by haemocytometer. Statistically significant differences (p<0.05) between the control and LPS stimulated cultures at particular time points, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS stimulated cultures are denoted by a † next to the culture descriptor on the X axis. Data shown are the means and s.e.m. of five pooled experiments.
Figure 4.9 Viable and non-viable B cells in Control and LPS-stimulated cultures
The percentage of total B cells (B-1 + B-2) in Control (A) and S. enteritidis LPS-stimulated (B) cultures that were viable or non-viable was determined by 7AAD staining levels of CD5⁺/B220⁺ and CD5⁻/B220⁺ cells. Statistically significant differences (p<0.05) between the control and LPS stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Data shown are the means and s.e.m. of five pooled experiments.
Figure 4.10 Representative FACScan plots for CD80 and I-A flow cytometric analysis

A specific gating strategy was utilized in order to examine only lymphocytes from the splenocyte cultures. Cells were first gated by forward (FSC) and side (SSC) scatter to select cells of the correct size and internal complexity. Cells meeting these criteria were further gated by their CD5 PE and CD18 Cy-Chrome fluorescence and side scatter to determine B-1 and B-2 cell subsets. Fluorescently-labelled isotype controls were used to set the quadrant parameters for positive and negative events, using only those cells that were selected from the FSC/SSC gates. The level of expression of CD80 (as shown here) or I-A was determined for each subset using the level of CD80-FITC (or I-A-FITC) fluorescence. Data shown is from a single representative experiment.
Figure 4.11 Activation of B cell subsets: CD80 expression

Activation of control and *Salmonella enteritidis* LPS stimulated B-2 (A) and B-1 (B) cells was examined using the CD80 activation marker. Data are presented as the percentage of each subset expressing CD80*. Statistically significant differences (p<0.05) between the control and LPS stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS stimulated cultures are denoted by a † next to the culture descriptor on the X axis. The control and LPS stimulated B-1 populations are significantly different overall to the control and LPS stimulated B-2 populations. Data shown are the means and s.e.m. of five pooled experiments.
Figure 4.12 Activation of B cell subsets: I-A

Activation of control and *S. enteritidis* LPS stimulated B-2 (A) and B-1 (B) cells was examined using the I-A activation marker. Data are presented as the percentage of each subset expressing I-A⁺. Overall significant differences (p<0.05), determined by one-way ANOVA, between the Control and LPS stimulated cultures are denoted by a † next to the culture descriptor on the X axis. Statistically significant differences (p<0.05) between the 0hr and other time points, as determined by one-way ANOVA, are denoted by a * above the relevant time point. Data shown are the means and s.e.m. of four pooled experiments.
4.4 Discussion

The results presented in this chapter demonstrate that it was possible to use a non-fractionated splenocyte culture system to simultaneously investigate the responses of B-1 and B-2 cells to LPS. By using flow cytometry to identify B-1 and B-2 cells, it was possible to dissect these subpopulations out of the overall responding cells. A range of parameters, such as viability and activation, could then be investigated for each population.

The overall activation of cells in culture was examined by phase contrast microscopy and electron microscopy. Using these techniques, it was evident that LPS caused significant activation and proliferation of cells in cultures (Figures 4.1, 4.2 and 4.3). This was confirmed by the more objective MTT assay (Figure 4.4), and by quantitation of viable cells by Trypan Blue exclusion (Figure 4.5). Together, these results indicated that in unstimulated cultures, biomass decreased as cells apoptosed, while in LPS-stimulated cultures, cells became activated and proliferated. Interestingly, by both MTT assay and viability counts, it appeared that at 96 hours there was a decrease in the number of viable cells in stimulated cultures. A decline in $^3$H-thymidine uptake by mitogen-stimulated splenic cultures by 72 hours has been noted previously (Dziarski, 1982a). The decline has been ascribed to several possible factors, and a number of investigations (Dziarski, 1980; Dziarski, 1982a; Dziarski, 1982b) have led to the interpretation that the substantial fall in activity is related to the terminal differentiation of B lymphocytes. Certainly, published kinetics of the plaque forming cell (PFC) response in mitogen-stimulated cultures are consistent with a switch from proliferation to differentiation around 48 hours. Further supporting this are functional and morphological studies of mitogen-stimulated populations in vivo and in vitro have confirmed a switch to immunoglobulin secretion and plasmacytoid-like morphology (Dziarski, 1982a). However, a decline in [3H]-thymidine incorporation has also been shown by Mamchak and Hodgkin to be caused by LPS-induced B cell proliferation depletion of essential nutrients from the growth medium (Mamchak and Hodgkin, 2000).
In addition to enabling the morphological investigation of the activation of the overall cultures, electron microscopy also allowed apoptosis to be assessed. A consistent finding was the marked dilation of the Golgi vesicles in activated cells at 72 hours after stimulation. There appeared to be a rapid progression of these cells to death as few transitional forms were observed. Dilation of the Golgi apparatus and of the endoplasmic reticulum has been noted in apoptotic thymocytes but it occurs after the characteristic nuclear changes (Wylie, 1988). The relation of the possible accumulation of secretory products to the initiation of a programmed death sequence in B cells is unknown. In this context, there is evidence that sialyltransferases, located primarily within the Golgi membranes, have an important role in the glycosylation of secreted protein (Paulson and Colley, 1989) and of B cell differentiation antigens (Kepler et al., 1992). Disturbance of this function could explain the accumulation of putative secretory proteins, particularly immunoglobulin, in the dying cells. There is the additional possibility of disruption of the tubulin network in relation to the location of the organising centrioles in proximity to the Golgi complex. These changes could either be part of the initiation or the later stages of the death process.

As shown in figure 4.5, there was a discrepancy between the dramatically decreasing numbers of viable cells in unstimulated cultures and the less-marked increase in non-viable cells. This difference could possibly be explained by two mechanisms: firstly, that the dead cells were removed from the culture by the very small population of macrophages remaining after the purification and depletion steps, and secondly, that the dead cells fragmented rapidly into sub-cellular sized debris.

Flow cytometric studies of cell death utilising 7AAD as a marker correlated significantly with the Trypan Blue staining of the cultures, allowing for more detailed analyses. The different gating patterns shown in Figure 4.7 enabled greater characterisation of the responses of the different lymphocyte subsets in combination with the Trypan Blue studies.

The CD5+ B cell population was protected from death by the addition of 50µg/mL LPS to the culture, but did not become increasingly activated until the very late stages of culture. Activation was measured by CD80 expression, and detection of IgM produced
by this subset would be useful in further examining the function of the CD5+ B cell in the disease state. CD5+ B cells respond differently to conventional B cells when stimulated with LPS. The response is later in terms of proliferation (as determined by absolute counts shown in Figure 4.8) and activation (as determined by the ratio of CD80+ cells to the 0hr value shown in Figure 4.11). An examination of the dose response to LPS would allow further dissection of the role of the CD5+ B cell in chronic periodontitis and other pathologies with low mitogenic load where an accumulation of these cells is found.

Results indicate that 50μg/mL LPS has a protective as well as a proliferative effect on conventional and possibly CD5+ B cells. CD5+ B cell numbers remain constant despite the increase in the percentage of non-viable cells in both the control and the LPS-stimulated cultures. CD5+ B cells are known to be self-renewing in the periphery (Hardy and Hayakawa, 1986) and this self-renewal is clearly shown in the unstimulated cultures where viable cell numbers remain constant over the culture period (Figure 4.8). Addition of LPS interferes somehow in this balance between self-renewal and death by preventing death in cells that would otherwise die in this process. It can be postulated that during the self-renewal process only a certain number of these CD5+ B cells survive, perhaps only one of the two daughter cells produced after mitosis. The perturbation of this state could lead to an increase in the number of these cells. Such an imbalance can be argued to result in an accumulation of this subset in pathologies such as chronic periodontitis (Sugawara et al., 1992). Stimulation with 50μg/mL of LPS resulted in an increase in activation, as measured by CD80 (B7-1) expression, of conventional and CD5+ B cells.

The evolutionary advantage of such polyclonal B cell reactivity to ubiquitous bacterial products such as LPS and peptidoglycan are recognised, but the subsequent activation of a programmed death sequence would protect the host from multiple autoreactive antibodies (Freitas et al., 1986). In this context the nuclei of splenic B cells, but not T cells, contain large amounts of endogenous endonuclease (Cohen et al., 1992). This is compatible with populations primed for rapid activation of programmed death.
Chapter 5 Effects of an 8-log range of lipopolysaccharide on CD5+ and conventional murine splenic lymphocytes

5.1 Introduction

The results of experiments that used B cell lines (Chapters 2 and 3) suggested that LPS from different bacterial species might have differing effects on B cell activation. Furthermore, it appeared that B-1 and B-2 subsets might respond differentially to the one LPS preparation. Such differential sensitivity to the proliferative and activating effects of LPS could explain the selective accumulation of B-1 cells in a number of disease states (see section 1.7). In the preceding chapter, a culture system was developed that allowed the responses of both B-1 and B-2 cells to be studied simultaneously. Using S. enteritidis LPS, it was shown that the viability, proliferation and activation of the two subsets, in response to S. enteritidis, could be followed. The results of these experiments were also consistent with the cell line studies in that B-1 and B-2 cells appeared to respond differentially to LPS, however the sensitivity of the subsets to LPS at different concentrations was not examined. It was therefore important to investigate the effect of differing concentrations of LPS on B-1 and B-2 cells.

The culture system developed in Chapter 4 was used to investigate the responses of B-1 and B-2 cells to LPS. Although understanding the response of B cells to P. gingivalis LPS was a major original focus for the project, S. enteritidis LPS had a more marked effect on B cell activation and proliferation and was therefore used in this series of experiments. Splenic lymphocytes were stimulated with an 8-log range of LPS concentrations and compared to non-stimulated control cultures. Changes in biomass were determined by MTT colourimetric assay. The viability, activation, IgM production, cell cycle and division index of B-1 and B-2 B cell subsets were examined by flow cytometry.
5.2 Materials and Methods

5.2.1 Mice
BALB/c mice 3-5 weeks of age were obtained from Gore Hill Research Laboratories, Gore Hill, NSW, Australia. All mice were allowed commercial pellets and water ad libitum until sacrifice by inhalation of Fluothane (Zeneca).

5.2.2 Mitogens
See 4.2.2 Mitogens

5.2.3 Splenocyte preparation
See 4.2.3 Splenocyte preparation

5.2.4 Depletion of adherent cells
Adherent cells were depleted after 1hr of culture at 37°C and 5% CO₂. The supernatant containing the non-adherent cells was removed and placed into a fresh flask or well.

5.2.5 Seeding of culture vessels
See 4.2.4 Seeding of culture vessels

5.2.6 MTT colourimetric assay
See 2.2.7 MTT colourimetric assay

5.2.7 Antibodies for flow cytometry
PE-conjugated anti-murine CD5 (clone 53-7.3), FITC-conjugated anti-mouse CD19 (1D3), biotin-conjugated anti-mouse CD19 (1D3), unlabelled- and FITC-conjugated anti-mouse IgM (II/41), the corresponding isotype-matched controls, and streptavidin-CyChrome (SA-CyChrome) were purchased from Pharmingen. Fc Block (anti-murine CD16/CD32, clone 2.4G2) (Pharmingen) was used to prevent non-specific binding of murine monoclonals to Fcγ receptors on the cell surface.
5.2.8 Flow cytometric analysis

Cultured cells were washed twice with PBS containing 2% FCS and 0.1% NaN₃ (PBS/FCS/Azide) and incubated with 0.25µg Fc Block/10⁶ cells for 3 min at 4°C. The cells were then incubated with 0.25µg/10⁶ cells of 7-Aminoactinomycin D (7AAD; Sigma)(Schmid et al., 1992) and 1µg/10⁶ cells of PE-anti-CD5 and FITC-anti-CD19 for 15 min at 4°C. The cells were then centrifuged for 3min at 4°C and 1500g, and then resuspended to 2 x 10⁶ cells/mL in Osmosol sheath fluid (Lab Aids) with 2% paraformaldehyde (sheath/PFA) prior to flow cytometric analysis on a FACScan (BDIS). 30,000 viable lymphocytes were analysed for each sample.

5.2.9 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) staining of cells

See 2.2.8 5-carboxyfluorescein diacetate-succinimidyl ester (CFSE) staining of cells

5.2.10 Determination of division index in CFSE-stained B cell subsets

See 2.2.9 Determination of division index in CFSE-stained B cell subsets

5.2.11 Flow cytometric analysis of CFSE stained cells

Cultured cells were washed twice PBS/FCS/Azide and incubated with 0.25µg Fc Block/10⁶ cells for 3 min at 4°C. The cells were then incubated with 1µg/10⁶ cells of PE-anti-CD5 and biotin-anti-CD19 for 15 min at 4°C. The cells were then centrifuged for 3min at 4°C and 1500g, and the supernatant discarded. The cells were incubated with 0.15µg/10⁶ cells of SA-CyChrome for 15 min at 4°C and then centrifuged for 3min at 4°C and 1500g, and the supernatant discarded. The cells were resuspended to 2 x 10⁶ cells/mL in sheath/PFA prior to flow cytometric analysis on a FACScan (BDIS). 30,000 lymphocytes were counted for each sample.
5.2.12 *IgM production in B-1 and B-2 cells*

Cells were harvested from murine spleens as per Section 4.2.3 and then cycloheximide (Sigma) was added to the cells, to a final concentration of 10μg/mL. The cells were incubated for 6hr at 37°C in 5% CO₂ in the presence of cycloheximide to inhibit protein synthesis (including IgM). The cells were harvested and then washed cells twice with RPMI/FCS. Cells were seeded and stimulated with LPS as mentioned previously, with the addition of 1μg/mL Brefeldin A (Sigma) to prevent secretion of proteins (cells were incubated for 0-3hr for these experiments).

Blocking of surface IgM was undertaken using an unlabelled anti-IgM antibody. The same clone was used in labelled form to detect intracellular Ig.

5.2.13 *Flow cytometric analysis of IgM production by splenic B lymphocyte subsets*

Cells were harvested, washed twice with PBS/FCS/Azide and resuspended to 1 x 10⁶ cells/mL. The cells were then incubated with 0.25μg Fc Block/10⁶ cells for 3 min at 4°C, 1μg/10⁶ cells of PE-anti-CD5 and biotin-anti-CD19 and 2μg/10⁶ cells unlabelled anti-IgM (clone II/41; to block surface IgM) was added and the cells incubated a further 15 min at 4°C. The cells were then centrifuged for 3min at 4°C and 1500g, and the supernatant discarded. The cells were incubated with SA-CyChrome (0.15μg/10⁶ cells) for 15 min at 4°C and then centrifuged for 3min at 4°C and 1500g, and the supernatant discarded. 875μL cold PBS was added to each tube containing a pellet of 10⁶ stained cells, and mixed gently, followed by 125μL cold fixation solution. The cells were incubated for 1hr at 4°C and then centrifuged for 5 min, at 4°C and 300g. The supernatant was removed and 1mL cold PBS was added to each tube. The cells were then centrifuged for 5 min, at 4°C and 300g, and the supernatant removed. 1mL permeabilisation solution (PBS/0.2% Tween 20) was added and the cells incubated 15min at 37°C. 1mL PBS was added and the cells centrifuged for 5 min, at 4°C and 300g. The supernatant was carefully removed and 3μg/10⁶ cells FITC-anti-IgM (clone II/41) in permeabilisation solution was added to the pellet and incubated 30min at 4°C. 1mL washing buffer (PBS/0.1% Tween 20) was added and the cells centrifuged for 5 min, at 4°C and 300g. The supernatant was removed and the cells washed again with
1mL washing buffer. The cells were resuspended to $2 \times 10^6$ cells/mL in PBS/FCS/Azide and analysed within 2hr using a FACScan (BDIS). Thirty thousand lymphocytes were analysed for each sample.

### 5.2.14 Cell cycle analysis of B cell subsets

Cells were harvested, washed twice with PBS/FCS/Azide and resuspended to $1 \times 10^6$/mL. Fc Block (0.25µg /$10^6$ cells) was added and the cells incubated for 3 min at 4°C. PE-anti-CD5 and FITC-anti-CD19 (and appropriate isotype controls; 1µg/$10^6$ cells) were added to tubes and incubated 15 min at 4°C. Tubes were centrifuged for 3 min at 1500g and 4°C. The supernatant was decanted and 1 to 2mL PBS added to each tube. Tubes were centrifuged 5 min at 300g and 4°C. The supernatant was removed and 875µL cold PBS was added to the pellet and mixed gently. Cold 2%PFA/PBS (125µL) was added to each tube, mixed, and incubated for 1hr at 4°C. Tubes were centrifuged 5 min at 300g and 4°C and the supernatant removed. Cells were resuspended in 1mL permeabilisation solution and incubated 15 min at 37°C. PBS (1mL) was added to each tube and centrifuged 5 min at 300g and 4°C. The supernatant was removed and cells resuspended to $2 \times 10^6$/mL in 25µg/mL 7AAD solution. Cells were incubated 30 min at 4°C and analysed within 2hr.

### 5.2.15 Statistical analysis

Control and LPS-stimulated values were compared for all experiments. Two-way ANOVA to compare differences at particular time points between unstimulated and stimulated populations, with Tukey's multiple comparison post test to compare differences between time points in a population, and one-way ANOVA to test differences overall between unstimulated and stimulated populations were performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All data shown are pooled means from a number of experiments. All error bars shown are the standard error of the mean.
5.3 Results

5.3.1 Cell concentration and viability

The overall response of the cultures to *S. enteritidis* LPS was examined by determining the total numbers and viability of cells in culture using trypan blue exclusion (Figure 5.1A and B). In unstimulated control cultures, the cell number and, to a lesser extent, viability decreased over the initial culture periods. Moderate to high concentrations of LPS, however, appeared to cause proliferation and increased viability. There were significantly increased cell numbers, in comparison to non-stimulated controls, in cultures containing 100ng/ml, 1ug/ml and 10ug/ml LPS. The percentage viability in the cultures stimulated with 1pg, 1ng, 10ng, and 100ng/mL LPS were also significantly different overall to that of the control culture (Figure 5.1B). The percentage viability was more stable in the cultures stimulated with the higher concentrations of LPS (100ng/mL, 1μg/mL and 10μg/mL), as there were no significant differences in viability over time.

5.3.2 Biomass increases in LPS-stimulated cultures

A further measure of the overall response to LPS stimulation was the change in biomass. Figure 5.2 shows the relative changes in biomass of control and LPS stimulated splenic lymphocyte cultures. There were no significant differences in biomass in the control culture over the course of the experiment. In the cultures stimulated with 1μg/mL and 10μg/mL LPS the biomass increased significantly up to 72hr and then decreased at 96hr, although the 96hr values were still highly significantly different to the control value for that time point (p<0.0001). This was similar to the profile seen for 50μg/mL LPS in Figure 4.4. The biomass in the culture stimulated with 100ng/mL LPS also increased significantly over the culture period (after 24hr), without the decrease seen at 72hr. All the LPS-stimulated cultures were significantly different overall to the control culture.

5.3.3 Cell number of B cell subsets

The number and concentration of B cell subsets was determined using a combination of flow cytometric analysis and cell counts using Trypan Blue exclusion staining (Figure
5.4). As shown in Figure 5.3, cells were gated first by size (forward scatter) and internal complexity (side scatter) (A) to select lymphocytes. The 7AAD fluorescence of these gated cells was then examined to select only viable cells (B), that is, those with low 7AAD fluorescence. Phycoerythrin and FITC-conjugated isotype matched controls were used to set the positive and negative levels (C) prior to analysis of anti-CD5-PE and anti-CD19-FITC staining levels (D). CD19 was used rather than B20 as the objective was to more clearly discriminate between the subsets in order to enhance the results obtained with the 3rd colour rather than to use these markers to discriminate between the subsets AND assess activation.

While the number of viable B-2 cells decreased over time in the non-stimulated cultures, the inclusion of LPS caused a dose-dependent accumulation of these cells. As shown in Figure 5.4A, there was a sharp drop in the number of B-2 cells present in control cultures from the 0 to 24 hour timepoints. This decrease continued to 48 hours and then stabilised. Addition of low concentrations of LPS (<10ng/ml) appeared to have no affect on the number of B-2 cells in culture. However, high concentrations of LPS (>10ng/ml) led to significantly increased B-2 cell numbers in the cultures.

In contrast to the response of B-2 cells, the number of B-1 cells in culture did not appear to be affected by LPS at the concentrations that were tested. For B-1 cells there was also an initial drop in cell numbers in non-stimulated control cultures at 24 hours post seeding, and there was little further change to 96 hours (Figure 5.4B). A similar pattern to the control was seen in LPS-stimulated cultures. Although an overall statistical difference was observed between the control cultures and those stimulated with 1pg/ml and 100pg/ml, if anything, the numbers of B-1 cells in these cultures was decreased in comparison to the control.

5.3.4 CD80 expression increased with LPS stimulation

The activation of B-1 and B-2 cells in response to S. enteritidis LPS was investigated by determining changes in CD80 and MHC expression. This was done was using the gating protocol outlined in Figure 5.5. At the start of the experimental period 9.40% (± s.e.m. 1.77%) of B-2 cells expressed CD80 compared to 63.23% (± s.e.m. 2.68%) of B-1 cells (Figure 5.6). CD80 expression was significantly different between the B-1 and
B-2 subsets in the control and LPS-stimulated cultures. There was no increase in the percentage of B-2 or B-1 cells expressing CD80 in the control and 100ng/mL LPS-stimulated cultures. B-2 cells in the 1µg/mL and 10µg/mL LPS-stimulated cultures express significantly more CD80 overall than cells in the control culture. There was a significant difference overall in the percentage of B-1 cells that were CD80+ in the cultures stimulated with 1µg/mL and 10µg/mL LPS compared with the unstimulated populations.

5.3.5 LPS stimulation increases division index in B-1 and B-2 cells

As shown in Figure 5.7, cells were gated to select B-1 and B-2 subsets for further analysis of CFSE staining. No CFSE analysis was performed at the 0hr time point as fluorescence was too bright immediately after staining the cells (A. Smith, Centenary Institute, personal communication). The staining profile of all B cells and the B-1 and B-2 subsets at 24hr (A) and 96hr (B) for the control and 10µg/mL LPS-stimulated cultures is shown in Figure 5.7. At 24hr no difference (apart from count) can be discerned between any of the staining profiles shown. However, at 96hr a tail of cells with lower fluorescence than the undivided peak is evidence that division has taken place in all subsets in both the control and LPS-stimulated cultures. B-2 cells in cultures stimulated with 1µg/mL and 10µg/mL LPS were significantly different to the control population (Figure 5.8A). This increase in division index mirrors the increases in viable cell concentration and percentage of B-2 cells expressing CD80 shown in Figure 5.4A and Figure 5.6A respectively. B-1 cells in cultures stimulated with 1µg/mL and 10µg/mL LPS were significantly different to the control culture. This is consistent with the data shown in Figure 5.4B and Figure 5.6B, where respective increases in viable cell concentration and percentage of cells CD80+ were found.

5.3.6 B-1 cells produce more IgM than B-2 cells

Intracytoplasmic IgM expression was determined for the B-1 and B-2 subsets 0 – 3hr after LPS stimulation. Cells were gated to select lymphocytes, isotype-matched controls were used to determine the level of non-specific staining for the monoclonal antibodies used in the experiment (Figure 5.9), and unlabelled anti-IgM was used to block surface
IgM. IgM production (as measured by internal IgM) was not significantly higher in the LPS-stimulated cultures compared to the control culture (Figure 5.10). The B-1 subset profile of IgM expression (examining control and LPS-stimulated cultures at all time points) was significantly different overall (p=0.0047) to that of the B-2 subset, and the B-1 subset was also significantly different to the B-2 subset at the 0hr time point – B-1 cells produced more IgM than B-2 cells – B-1 peak channel is 49.33 (± s.e.m. 16.292) and B-2 peak channel is 31.667 (± s.e.m. 13.715).

5.3.7 B-1 cell cycle profiles are different to B-2 profiles

The cell cycle profiles of B-1 and B-2 cells were examined in order to determine whether any differences in the response of these subsets to LPS stimulation was due to cell cycle perturbations or changes. Cells were gated using forward and side scatter to select the lymphocyte population, quadrants for anti-CD5-PE and anti-CD19-FITC were set using isotype matched controls (not shown) and B-1 (CD5+/CD19+) and B-2 (CD5- /CD19+) subsets were analysed further for 7AAD fluorescence. The percentage of cells in each subset at each phase of the cell cycle was then determined using three markers on the 7AAD fluorescence histogram (Figure 5.11). Overall, the B-1 subset profiles were significantly different to the B-2 subset profiles (Figure 5.12). In particular, at the time of culture seeding (0 hours), a higher proportion of B-1, in comparison to B-2 cells were in either the S or G2/M phases of the cell cycle. Following culture with LPS, there were no statistically significant changes in the percentage of cells in either the S or G2/M phases. However, for both B-1 and B-2 populations, there was a significant LPS dose-dependent increase in the number of cells in the G0/G1 phase.
Figure 5.1 Cell concentration and viability in control and *Salmonella enteritidis* LPS-stimulated cultures

The cell concentration (cells/mL) (A) and viability (B) were determined for control and *Salmonella enteritidis* LPS-stimulated cultures of splenic lymphocytes. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of five pooled experiments.
Figure 5.2 Change in biomass over the culture period in control and *S. enteritidis* LPS-stimulated splenic lymphocyte cultures

The MTT-formazan assay was used to examine the change in biomass in control and 100ng/mL, 1μg/mL and 10μg/mL *S. enteritidis* LPS-stimulated splenic lymphocyte cultures. This was measured by determining the difference in absorbance for the yellow soluble substrate and the purple insoluble product. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 5.3 Representative FACScan plots for flow cytometric analysis (7AAD/CD5/CD19) showing gating strategy

A specific gating strategy was utilised in order to examine only lymphocytes from the splenocyte cultures. Cells were first gated by forward (FSC) and side (SSC) scatter to select cells of the correct size and internal complexity (A). Cells meeting these criteria were further gated by their 7AAD fluorescence and side scatter to determine viable and non-viable cells (B). Apoptotic and degenerate cells are also able to be identified using 7AAD. Fluorescently-labelled isotype controls were used to set the quadrant parameters for positive and negative events, using only those cells that were gated as viable lymphocytes (C). Identification of subsets of viable lymphocytes was then able to be performed using, for example, anti-CD5-PE and anti-CD19-FITC (D) to identify B-1 cells (CD5+/CD19+, upper right quadrant), B-2 cells (CD5+/CD19-, lower right quadrant), T cells (CD5+/CD19+, upper left quadrant) and non T or B cells (CD5+/CD19-, lower left quadrant). Data shown are from a single representative experiment.
Figure 5.4 Concentration (cells/mL) of viable B-2 and B-1 cells in culture

The concentration of viable B-2 (A) and B-1 (B) cells for all concentrations of *S. enteritidis* LPS was determined using a combination of 7AAD/CD5/CD19 flow cytometric analysis and Trypan Blue exclusion staining. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of ten pooled experiments.
Figure 5.5 Representative FACScan plots for flow cytometric analysis: CD5/CD19/CD80

Cells were first gated by forward (FSC) and side (SSC) scatter to select cells of the correct size and internal complexity (not shown). Fluorescently-labelled isotype controls were used to set the quadrant parameters for positive and negative events, using only those cells that were gated as viable lymphocytes (A and B). Identification of subsets of viable lymphocytes was then able to be performed using, for example, anti-CD5-PE and anti-CD19-Cy-Chrome (C) to identify B-1 cells (CD5+/CD19+, upper right quadrant), B-2 cells (CD5+/CD19-, lower right quadrant, T cells (CD5+/CD19+, upper left quadrant) and non T or B cells (CD5+/CD19-, lower left quadrant). The level of CD80 expression, determined using anti-CD80-FITC, was examined for B-2 (R2 of C) and B-1 (R3 of C) cells. Data shown are from a single representative experiment.
Figure 5.6 CD80 expression of B-2 and B-1 cells

CD80 expression was examined in viable B-2 (A) and B-1 (B) cells. The percentage of cells CD80⁺ is shown for each time point and concentration of S. enteritidis LPS used in the experiments. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 5.7 Representative FACScan plots for flow cytometric analysis: CFSE/CD5/CD19

As in Figure 5.5, cells were gated using forward and side scatter and the subdivided into B-2 and B-1 subsets using CD5-PE and CD19-CyChrome staining patterns (positive regions set using isotype matched controls). CFSE staining patterns are shown for 24hr (A) and 96hr (B) after seeding for both control and 10μg/mL LPS-stimulated populations. At each cell division the level of CFSE fluorescence is halved and the number of divisions cells have undergone can be determined by examining the level of this fluorescence. Data shown are from a single representative experiment.
Figure 5.8 CFSE division index B-1 and B-2 cells

CFSE division index at 24, 48 and 72hr is shown for B-2 (A) and B-1 (B) subsets for control and S. enteritidis LPS-stimulated cultures. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Overall significant differences (p<0.05), determined by one-way ANOVA, between the control and LPS-stimulated cultures are denoted by a † below the culture descriptor on the X axis. Data shown are the means and s.e.m. of three pooled experiments.
Figure 5.9 Representative FACSscan plots for flow cytometric analysis:
CD5/CD19/IgM

Cells were gated using forward and side scatter to select lymphocytes and positive regions for fluorescent staining were determined using fluorescently conjugated isotype matched controls. Lymphocytes were divided into B-1 and B-2 B cell subsets using CD5-PE and CD19-Cy-Chrome staining characteristics and then analysed for IgM expression using anti-IgM-FITC. Data shown are from a single representative experiment.
Figure 5.10 IgM production in B cells

Peak channel (the fluorescence channel with the greatest number of counts) was used to examine the level of IgM production in all B lymphocytes (A) and in the B-2 (B) and B-1 (C) subsets for control and S. enteritidis LPS-stimulated cells at 0, 1, 2, and 3 hours (n=4 experiments). There were no statistically significant differences as determined by two-way ANOVA with Tukey’s multiple comparison post test and one-way ANOVA. Data shown are the means and s.e.m. of four pooled experiments.
Figure 5.11 Representative FACScan plots for flow cytometric analysis: CD5/CD19/7AAD (cell cycle)

Cells were gated using forward and side scatter to select lymphocytes (and to reduce the number of doublets in the analysis) and positive regions for fluorescent staining were determined using fluorescently conjugated isotype matched controls. Lymphocytes were divided into B-1 and B-2 B cell subsets using CD5-PE and CD19-FITC staining characteristics and then analysed for cell cycle phases using 7AAD. M1 shows cells in G0/G1, M2 is cells in S, and M3 is cells in G2/M phase. Data shown are from a single representative experiment.
Figure 5.12 Cell cycle in B-2 and B-1 cells

The percentage of B-2 and B-1 cells in each phase of the cell cycle, as measured using 7AAD in conjunction with anti-CD5-PE and anti-CD19-FITC, is shown for each concentration of *S. enteritidis* LPS. Statistically significant differences (p<0.05) between the control and LPS-stimulated cultures, as determined by two-way ANOVA, are denoted by a * above the relevant time point. Statistically significant differences between time points overall and the 0hr values, as determined by one-way ANOVA, are denoted by a † below the time category. Data shown are the means and s.e.m. of three pooled experiments.
5.4 Discussion

The data presented in this chapter indicate that B-1 and B-2 cells have different basal levels of activation and do respond differently to *S. enteritidis* LPS. However, contrary to the original hypothesis, it appears that B-1 cells are less sensitive than B-2 cells to the proliferation-enhancing effects of LPS.

As was noted in Chapter 4, B-1 cells appeared to have a higher basal level of activation than B-2 cells. In non-stimulated cultures, more B-1 than B-2 cells were in the S and M phases of the cell cycle (Figure 5.12), indicating that there was a higher basal level of proliferation for these cells. Furthermore, over the course of the culture period, more viable B-1 cells remained than B-2 cells (Figure 5.4). In addition to the inherently higher proliferative capacity of B-1 cells, a higher percentage of unstimulated B-1, as compared to B-2 cells, expressed the activation marker CD80 (Figure 5.6). Furthermore, B-1 cells inherently produced more IgM (Figure 5.10). This overall picture is consistent with the known biology of B-1 cells. As described in Chapter 1, they are self-renewing and may either have a high intrinsic level of activation, or might be stimulated by self-antigens within the cultures.

When the response of B-1 and B-2 cells to varying concentrations of *S. enteritidis* LPS was examined, it was found that although moderate to high levels of LPS stimulated the proliferation of both B cell subtypes, only B-2 cells accumulated in culture. The net number of cells of each subpopulation, at any given time, should largely be determined by the combined effects of proliferation and apoptosis. When challenged with high doses of LPS, B-2 cells accumulated in number in comparison to control cultures (Figure 5.4A). B-1 cells, in contrast, did not show any accumulation in response to LPS (Figure 5.4B). As shown in Figure 5.8, both cell populations had significantly raised division indices in response to 1μg/ml or 10μg/ml LPS, in comparison to controls. Although the proliferation indices of B-2 cells were slightly higher than those of B-1 cells, which suggested that B-2 cells might be proliferating more rapidly, the magnitude of this difference was not large. This indicated that other factors, such as changes in the rate of apoptosis, might be influencing the number of each cell type. Attempts were made at assessing apoptosis in the cultures by quantifying the
hypodiploid peak for each cell type in cell-cycle analysis histograms (cells below region M1 in Figure 5.11), however technical difficulties were encountered (apoptotic cells tended to clump and stain with antibody non-specifically - data not shown). Nevertheless, it is possible that the increase in B-2 cell number in response to LPS is due to decreased apoptosis. The lack of change in B-1 cell numbers could equally be a result of increased apoptosis in response to LPS.

The finding that the CD5⁺ B cell subset was essentially unresponsive at low concentrations of LPS is in contrast to earlier reports that this subset is very responsive to mitogenic stimuli with respect to enhanced secretion of autoantibodies (Hardy et al., 1987; Hayakawa et al., 1990; Chace et al., 1993). CD5⁺ B cells in the thymic compartment, however, have been demonstrated to have low responsiveness to LPS (Miyama-Inaba et al., 1988; Inaba et al., 1990).

It was considered that the increased B-2 cell number in response to LPS might be a result of the action of rheumatoid-like IgM produced by B-1 cells acting on B-2 cells. A preferential role for CD5⁺ B cells in the secretion of rheumatoid factor (RF)-like "regulatory" autoantibody has been demonstrated (Hardy et al., 1987; Hayakawa and Hardy, 1988; Hayakawa et al., 1990). It has been postulated that these cells help in the activation and development of conventional CD5⁻ B cells (Andersson et al., 1977; Hardy et al., 1987; Hardin et al., 1990; Hayakawa et al., 1990). Also in support of this hypothesis are findings that human B-1 cell-derived IgM can bind to membrane-bound Ig on murine B-2 cells (Weston and Raison, 1991), and that 60% of murine B cells respond to anti-μ by entering S phase (DeFranco et al., 1985). If B-1 cell derived IgM was responsible for B-2 cell proliferation in this system, CD5⁺ B cells would have to respond to low levels of LPS by increasing production and secretion of IgM (Chace et al., 1993). However, the data shown in Figure 5.10 do not support this hypothesis, as there is no increase in IgM staining in cultures challenged with low concentrations of LPS.

The studies presented in this chapter did not show a difference between B-1 and B-2 cell responses to low concentrations of LPS. However, the responses of both subsets
were able to be examined and further studies could be undertaken within this system to analyse other aspects of the B-1 and B-2 cell response to LPS.
Chapter 6 General Discussion

The aim of the research presented in this thesis was to investigate, using in vitro systems, the response of B-1 and B-2 cells to enterobacterial and non-enterobacterial LPS to shed light on the pathogenesis of chronic periodontitis and other diseases involving B cell accumulation and autoantibody production.

The hypotheses tested were:

1. B cells respond differently to enterobacterial and non-enterobacterial LPS.
2. B-1 cells are activated by a lower concentration of LPS than B-2 cells.
3. LPS stimulation results in preferential accumulation of B-1 cells.

Findings consistent with these hypotheses would provide new evidence for different roles for B-1 and B-2 cells in immune responses and that LPS stimulation could lead to B-1 cell accumulation in diseases thus characterised.

Initial experiments investigated the responses of representative B-1 and B-2 cell lines to P. gingivalis and S. enteritidis LPS. In both cell lines, there were obvious differences in the responses to the different LPS preparations. Since S. enteritidis LPS appeared to have more pronounced effects on both B cell populations, this LPS was used to further investigate B cell subset responses in a mixed splenocyte culture system. The results of the splenocyte culture experiments clearly demonstrated that there are differences in the responses of B-1 and B-2 cells to LPS. However, overall, the results are inconsistent with LPS driving the accumulation of B-1 cells in disease states.

6.1 B cell responses to enteric and non-enteric LPS

It has long been clear that the immune system is capable of activating a variety of effector mechanisms in response to different pathogens. However, despite the huge array of effector cells and pathways, only a subset of the available mechanisms is ever used. There has been considerable effort spent over many years to understand the
pathways underlying this phenomenon. It has become apparent that the eventual effector modality may be 'decided' by the innate immune system and this decision may occur very early in a developing immune response. The phylogenetically conserved cells and receptors of the innate immune system recognise invariant pathogen-derived molecules (such as LPS) and are capable of setting very distinct effector pathways in train. Indeed, since vertebrate immune systems have co-evolved with pathogens over millions of years, it is perhaps not surprising that subtle distinctions in the structures of pathogen-derived molecules may be sensed, and that this may ultimately lead to differing profiles of effector activation. An example of this specificity is the putative ability of immune cells to respond differently to LPS of enterobacterial and non-enterobacterial origin (see section 1.5.2.1.6).

Given the reported accumulation of B cells in periodontitis (see section 1.7.3), it was hypothesised that LPS from this organism might be capable of activating B cells to cause proliferation and differentiation into antibody secreting cells. This was initially examined using the CH12 and WEHI 279 cell lines. The effects of *P. gingivalis* LPS were compared with those of LPS derived from a typical enteric organism, *S. enteritidis*. Results from both cell lines suggest that B cells can respond differently to LPS of different origins, and that *P. gingivalis* LPS can drive B-1 accumulation.

The effects of either LPS preparation on WEHI-279 cells were not marked, but there were indications of diverging responses. *S. enteritidis* LPS failed to affect growth at any concentration (Figure 3.2), although viability was increased at the highest dose tested (Figure 3.1). In contrast, at high concentrations, *P. gingivalis* LPS appeared to actively inhibit proliferation (Figure 3.10). Measurement of an array of cellular parameters, including investigation of the expression of cytokines, co-stimulatory molecules, TLR and Bcl2/Bax messages was used in attempt to dissect the mechanisms involved in the response. These were largely uninformative, although results did suggest that IL-6 and IL-10 may be slightly increased in response to high concentrations of *S. enteritidis* LPS (Figure 3.8). These cytokines may drive both the proliferation and differentiation of B cells (see section 1.5.2.2). No further differences were found in the response of this cell line to the two LPS preparations. Taken at face value, this suggests that B-2 cells may not be strongly affected by either LPS preparation. Although the WEHI-279 line is a
useful tool for studying B-2 biology (see section 1.6.2), the effects of inter-subset interactions are unable to be examined. A possible method for examining such interactions could include a mixed culture of WEHI-279 and CH12 cells, however as these lines were cultured in different media (DMEM and RPMI-1640 respectively), this was not feasible in the time available.

The response of the CH12 cell line contrasted with that of the WEHI-279 line. CH12 cells did respond to high concentrations of *P. gingivalis* LPS by proliferating (Figure 2.20B and Figure 2.21). However, low concentrations actually appeared to inhibit proliferation. In response to *S. enteritidis* LPS, there was protection from cell death at high concentrations of LPS (Figure 2.6A), and a marked proliferation (Figure 2.6B and Figure 2.8).

Investigation of the cellular processes triggered in CH12 cells in response to different LPS preparations showed diverging patterns. Although the concentrations of the different LPS preparations at which CH12 cells proliferated were approximately the same, in a range of assays investigating further parameters of the cellular response, CH12 cells did not appear to be affected by *P. gingivalis* LPS. In contrast, high concentrations of *S. enteritidis* LPS did affect antibody and cytokine production, as well as the regulation of Toll-like receptor expression. In response to high concentrations of *S. enteritidis* LPS, there was an increase in the expression of secretory IgM message, which indicates that this LPS induced differentiation into plasma cells. This is consistent with previous reports that showed increased production of IgM by CH12 cells in response to enteric LPS (Stockdale *et al.*, 1987; Arnold *et al.*, 1988; Bost *et al.*, 1990). There was also a small increase in IL-10 mRNA in response to *S. enteritidis*, but not *P. gingivalis*, LPS (Figure 2.19B and Figure 2.27B). This increase in cytokine message was similar to that seen in the WEHI-279 cells and suggests that there may be similar pathways involved in both cell lines in response to *S. enteritidis* LPS. It is possible that autocrine stimulation by either or both of these cytokines may be involved in driving the proliferation of both cell lines in response to *S. enteritidis*, but not *P. gingivalis*, LPS. Unfortunately, sufficient time and resources were not available to conduct antibody blocking experiments, which would have answered this question conclusively.
A distinct change in the expression of RP105 mRNA in CH12 cells in response to *S. enteritidis* LPS may shed some light on the processes involved in LPS-stimulation of these cells. While there were no changes in TLR expression in CH12 cells in response to *P. gingivalis* LPS, culture with *S. enteritidis* LPS led to a clear decrease in the message of RP105 (Figure 2.18). Modulation of TLR2 has been observed in response to bacterial lipoprotein (Wang *et al.*, 2002), while down-regulation of TLR4 has been implicated in the phenomenon of endotoxin tolerance in macrophages (Medvedev *et al.*, 2000; Nomura *et al.*, 2000; Wang *et al.*, 2002). The present study is the first report of regulation of the B cell specific RP105 by non-enteric LPS. Given the apparent specificity observed in this regulation process (bacterial lipoprotein binds to TLR2 and enteric LPS binds to TLR4 — see section 1.5.2.1 and Appendix 4) it appears that decreases in TLR expression may be part of a negative feedback loop. The exact initial processes involved in B cell activation of by LPS are unclear. RP105 has been shown to be highly expressed on B cells (Akashi *et al.*, 2000; Zarembek and Godowsdi, 2002), and LPS from the enteric bacterium *E. coli* has previously been shown to stimulate B cells via RP105. However, activation via RP105 also requires the presence of some level of TLR4 expression (Ogata *et al.*, 2000). In an alternate murine CD5⁺ B cell line, TH2.52, it was reported that the response to LPS requires the presence of CD14 (Koide *et al.*, 2001). The role of CD14 in LPS signalling is controversial (see section 1.5.2.1), but it appears that it may co-localise with TLR4 and together they may bind LPS. It is possible that CD14 could also act in concert with RP105 to bind LPS, although this has not been demonstrated. It does seem likely that *S. enteritidis* LPS acts via RP105. Down-regulation of RP105 following prolonged LPS stimulation may represent a mechanism to limit B cell proliferation and antibody production. The receptors involved in CH12 activation by *P. gingivalis* LPS are, however, less clear. Although *P. gingivalis* LPS has been postulated to act via TLR2 (see section 1.5.2.1.3), there was no modulation of this receptor. The absence of regulation of TLR2 message, in itself, does not mean that TLR2 is not involved in the response to *P. gingivalis* LPS. It could also reflect the absence of a pathway linking TLR2 signalling to regulation of TLR4 expression.

Taken as a whole, the responses of the two cells lines to *P. gingivalis* LPS are consistent with patterns seen in periodontitis. In both the peripheral blood of periodontitis patients
(Afar et al., 1992), and within the lesions (Sugawara et al., 1992), CD5+ cells are over-represented. It is possible that patients with an underlying disorder that increases the responsiveness of CD5+ B cells may be predisposed to periodontitis, however, the data presented here suggest that *P. gingivalis* LPS may directly drive the expansion of B-1 cells while inhibiting the proliferation of B-2 cells.

### 6.2 Differences in the response of B-1 and B-2 cells to LPS

Differential responsiveness of B-1 and B-2 cells to LPS, if demonstrated, could represent a mechanism that enables an organism to mount a co-ordinated and effective immune response to pathogens. In some circumstances, however, it could also contribute to the development of immunopathology. As described in sections 1.4.2 and 1.4.1, B-1 and B-2 cells have very different functions in the immune system. One view is that B-1 cells are ‘first-responders’ in an immune response. They produce antibodies that are polyreactive, and stimulation of B-1 cells is likely to lead to the production of antibodies that may not only bind directly to foreign antigen, leading to its clearance (see section 1.3.2.2), but may stimulate the development of specific B-2 responses (section 1.4.1). From first principles it seems that it would be advantageous to an organism if B-1 cells were more sensitive to LPS than B-2 cells. Rapid production of natural antibodies would help protect the organism in the early stages of infection, and would also assist the development of a more specific adaptive response. A higher activation threshold in B-2 cells would mean that these cells, most of which would be unlikely to bind to LPS, would not be activated. As with many aspects of immune effector functions, however, production of natural antibodies may be a double-edged sword. Natural antibodies, by their nature, are polyreactive and may recognise many self-antigens (see section 1.3.2.2). Prolonged, uncontrolled B-1 cell activation could lead to tissue damage mediated by autoantibodies.

Data from previous studies has suggested that B-1 cells may indeed be very sensitive to LPS (Chace et al., 1993; Koide et al., 2001). The data presented in this thesis demonstrates that B-1 and B-2 cells do respond differently to LPS, however these
differences were not as marked as the literature has previously suggested. Further, while experiments using cell lines suggested that B-1 were indeed more sensitive to LPS, the results of more physiological mixed splenocyte cultures suggested that, in fact, B-2 were more responsive than B-1 cells. It may be that B-1 cells are relatively refractive to LPS stimulation in order to prevent "overpopulation" in a subset that, "due to their long life span and self-renewal capabilities, capabilities...have an increased ability to clonally expand and escape negative selection to become malignant" (Zhang et al., 2001). Transient increases in number may ensure proportional relativity in comparison to increased numbers of B-2 cells in infection, thus retaining proportionate amounts of "natural" IgM.

6.2.1 Effects of LPS on viability and proliferation of B cell subsets

6.2.1.1 Cell lines

Initial experiments used cell lines to investigate the response of B-1 and B-2 cells to LPS. Cultures that were stimulated with P. gingivalis LPS did not show a clear-cut response, but overall the data was consistent with increased sensitivity of CH12 cells when compared with the WEHI-279 line. P. gingivalis LPS did not affect viability in CH12 cells, but did cause increased cell numbers in culture when present at 100ng/ml and higher concentrations (Figure 2.20). In contrast, when WEHI-279 cells were cultured with P. gingivalis LPS, low concentrations appeared to enhance proliferation of WEHI-279 cells to a slight degree, but high concentrations (similar to those that caused proliferation in CH12 cells) actually caused inhibition of growth (Figure 3.9 and Figure 3.10).

The CH12 studies presented in Chapter 3, using the CD5⁺ CH12 cell line, showed that LPS did affect the survival of these cells. Addition of 100ng/mL and above of S. enteritidis LPS to cultures led to increased viability and cell concentration when compared to the unstimulated cells (Figure 2.6). The division index of cells stimulated with 10ng/mL and upwards of S. enteritidis LPS was significantly different to that of the unstimulated control population (Figure 2.8). Essentially, addition of S. enteritidis LPS "rescued" these cells from death due to serum starvation and changed the dynamics
of the population to resemble that of the unstimulated population cultured in medium supplemented with 10% FCS (Figure 2.6). In contrast to its effects on CH12, *S. enteritidis* LPS caused only minimal changes in WEHI-279 proliferation and viability (Figure 3.2 and Figure 3.1). Only the highest dose of LPS tested, 10 μg/ml, led to increased viability (Figure 3.1A) and, possibly, to proliferation (Figure 3.2).

When viewed as a whole, the cell line experiments supported the hypothesis that B-1 cells were more sensitive to the effects of LPS. Although both cell lines have been used extensively as models to investigate B cell function (see sections 1.6.1 and 1.6.2), it is uncertain if the responses of these isolated populations of immortalised cells accurately represent *in vivo* responses. The individual cells lines could, for example, display idiosyncratic responses to LPS. Alternatively, the use of isolated populations ignores interactions between cell types. It was therefore decided to examine the response of B-1 and B-2 cells in primary culture. A mixed splenocyte culture system of was developed that allowed interaction between a range of immune cell types, and simultaneously enabled the investigation of the responses of both B-1 and B-2 subsets (Chapter 4). This culture system was subsequently used to determine the relative sensitivities of the two B cell subpopulations to *S. enteritidis* LPS (Chapter 5).

6.2.1.2 Splenocyte cultures

Surprisingly, given both the published literature (see section 1.5.2.1.7) and the evidence gained using cell lines in Chapters 3 and 4, the results of the mixed culture system supported neither the hypothesises of increased B-1 cell sensitivity to LPS nor the that of LPS driving B-1 accumulation.

When the response of CD5+ cells to *S. enteritidis* LPS was measured by the number of cells in culture (Figure 5.4), there was no effect seen in any of the LPS concentrations tested. Interestingly, however, high concentrations of LPS did appear to cause increased *proliferation* of B-1 cells (Figure 5.8). Such a discrepancy may be explained by a change in the balance of proliferation and cell death. An increase in proliferation that was balanced by a simultaneous increase in cell death would lead to a neutral net change in cell numbers in culture. As described in section 5.4, there were technical difficulties in assessing apoptosis in the different B cell subpopulations, however,
considerable apoptosis was seen in cultures stimulated with high dose LPS (Figure 4.9), indicating that it was definitely occurring within these cultures. It is possible that LPS not only increases the proliferation of B-1 cells, but also sensitises them to apoptosis. Such a mechanism could help to limit the expansion of B-1 cells in response to LPS and could conceivably protect the organism from the deleterious effects of excessive expansion and activation of this population.

In direct contradiction to the original hypotheses, B-2 cells were responsive to S. enteritidis LPS at similar concentrations to B-1 cells (Figures 4.8 and Figure 5.4). Furthermore, and in contrast with B-1 cells, it was B-2 cells that accumulated in number in LPS-challenged splenocyte cultures (Figure 5.4). As with B-1 cells, these changes appeared to result from changes in the balance of proliferation and death. The accumulation of B-2 cells probably resulted from increased proliferation (Figure 5.8) and, possibly, decreased cell death. These observations, at first, appear difficult to reconcile with the cell line studies (Chapters 3 and 4). However, there are a number of possible explanations. Although both of these cell lines have been used in a range of studies investigating many aspects of B cell biology (see sections 1.6.1 and 1.6.2), it is possible that one or both of the cell lines might have non-characteristic responses to LPS. Alternatively, it may be that there are as yet undefined interactions occurring within the more complex mixed culture system. Purification of primary B cell subsets and investigation of their separate responses to LPS per the cell line experiments presented in this thesis would allow the determination of characteristic subset responses, if indeed this is different to that of the cell lines, but would not elucidate any cross-subset interactions, as is possible in the mixed culture system.

The central thrust of the studies presented here was to investigate potential pathogenic mechanisms underlying periodontitis. Consequently, P. gingivalis LPS, as well as S. enteritidis LPS, was used in initial studies investigating the effects on cell lines. As the response to S. enteritidis LPS was more marked, this was chosen for further studies in the mixed splenocyte culture system. These experiments provided useful evidence that supported the idea that B-1 and B-2 cells respond differently to LPS. However, these studies were unable to directly address the role of P. gingivalis LPS in periodontitis. It of may be that P. gingivalis LPS has different effects to S. enteritidis LPS on primary B
cells. It is still possible that B-1 cells may be more sensitive to P. gingivalis, as opposed to S. enteritidis LPS. Unfortunately, time constraints, and a developing severe reaction of the candidate to mice, precluded further experiments in this system. Ultimately, however, the evidence provided by the mixed system is not consistent with either of the two final hypotheses behind these studies; firstly, that B-1 cells do not respond to lower concentrations of LPS than do B-2 cells, and secondly, that B-1 cells do not preferentially accumulate in response to LPS stimulation.

6.3 Further observations on B-1 cell biology

6.3.1 Activation

In addition to the direct investigation of the hypotheses of differential B cell responsiveness to LPS and responsiveness to enteric and non-enteric LPS, a further salient observation was made over the course of the studies that contributes to the understanding of B cell biology. Evidence was found that B-1 cells have a high basal level of activation.

When CH12 cells were grown under standard cell culture conditions, the majority of these cells were CD80+. This suggests that this line is constitutively activated. Stimulation with P. gingivalis or S. enteritidis LPS did not affect the level of CD80 mRNA expression. It is possible that the CH12 line constitutively expresses a maximal level of CD80 (and possibly CD86) and further stimulation will not cause any increase. LPS stimulation did not have any effect on CD80 or CD86 mRNA expression in WEHI-279 cells. This may be due to either a lack of any effect on CD80 and CD86 expression or that this line is also maximally expressing these activation markers.

In the splenic lymphocyte cultures, activation status as measured by CD80 expression, was different for B-1 and B-2 cells. A majority (63%) of B-1 cells expressed CD80 at the start of the experimental period compared to 9% of B-2 cells (Figure 5.6). The percentage of B-2 cells expressing CD80 increased when the cells were stimulated with concentrations of LPS of 1μg/mL and above. A similar pattern is seen for B-1 cells, although the increase in CD80+ cells is significantly less than that for B-2 cells (Figure
4.11). An observed decrease at 72hr in the 1μg/mL and 10μg/mL LPS-stimulated cultures was most likely due to overgrowth and acidification of medium, followed by cell death. Increased activation in both B cell subsets is reflected in the MTT assay data (Figures 4.4 and 5.2) and the morphological data shown in Figures 4.1, 4.2 and 4.3. Within these studies it was not possible to determine whether the cells shown were B-1 or B-2 cells, but it was evident that activation did occur.

It is relevant to note that binding of ligands to CD80 and CD86 may regulate apoptosis in B cells. In murine splenic B cells, ligation of CD80 or CD86 may modulate antibody production, while in a B cell lymphoma, cross-linking of CD80 enhanced the expression of the pro-apoptotic molecules caspase-3, caspase-8, Fas, FasL, Bak, and Bax (Suvas et al., 2002). In contrast, signalling through CD86 increased the expression of anti-apoptotic molecules Bcl-w and Bcl-x(L) (Suvas et al., 2002). Thus, it is possible that ligation of CD80 and or CD86, by other cells in mixed splenocyte cultures, may affect B cell survival. The ratio of Bcl-2 to Bax, anti-apoptosis and pro-apoptosis proteins respectively, in CH12 cells in LPS-stimulated cultures was not significantly different to the control culture. The ratio was significantly higher at 48hr than at 24hr and this might reflect the survival of the cells in the cultures, rather than any LPS-specific effect as the ratio in the unstimulated populations was also increased. A trended increase of the Bcl-2/Bax ratio across the LPS-stimulated and 10% FCS control populations was noted, but the differences were not significant. Stimulation with *P. gingivalis* LPS did not affect the Bax/Bcl-2 ratio. Despite increases in proliferation and viability in WEHI-279 cells when stimulated with *S. enteritidis* and *P. gingivalis* LPS, no change in the Bax/Bcl-2 ratio was seen for this cell line.

Taken together, this evidence suggests that B-1 cells may have a high basal level of activation. Such basal activation is compatible with involvement of these cells in the early response to pathogens. A low but continuous level of activation could be one of the mechanisms by which CD5+ B cells are able to maintain antibody production without stimulation from the foreign antigens for which the antibody is (poly)specific. It is of note that signal transducer and activator of transcription (STAT) protein-3 is constitutively phosphorylated in B-1 cells, reflecting or causing the activated state of B-
1 cells and possibly contributing to the self-renewing characteristics of this B cell subset (Karras et al., 1997).

6.4 Implications for pathogenesis of periodontitis

Advanced/progressive human periodontitis is considered by many to be a B lymphocyte and plasma cell lesion (Okada et al., 1983; Tew et al., 1989; Gemmell and Seymour, 1991). Gemmell and Seymour examined the phenotype of B lymphocytes in periodontitis and found that a majority of these cells were activated (Gemmell and Seymour, 1991). Up to 38% of B cells from inflamed gingival tissues were determined to be CD5+ and many of these cells produced anti-collagen (auto-) antibodies (Sugawara et al., 1992). An increase of CD5+ B cells was also found in the peripheral blood of periodontitis patients (Afar et al., 1992).

Chronic periodontitis is believed to be exacerbated by the colonisation of the subgingival region by organisms such as Porphyromonas gingivalis, Prevotella intermedia, and Fusobacterium nucleatum (Wilson, 1995). It has been suggested by Tew et al., and other investigators, that polyclonal activation of these B cells by bacterial mitogens derived from such organisms is important in the pathogenesis of the periodontal lesion (Tew et al., 1989; Ranney, 1991). LPS (Ringden et al., 1977; Hammarstrom et al., 1980; Matsushima et al., 1985; Gaidano et al., 1989; Settimacher et al., 1990), together with pertussis toxin (Kolb et al., 1990), the B subunit of cholera toxin (Dugas et al., 1991), Mycoplasma arthritidis MAM (Cole et al., 1996), synthetic oligodeoxynucleotides (Liang et al., 1996), Dermatophagoides farinae antigens (Fujii et al., 1996), and Nocardia opaca water soluble mitogen (Brochier et al., 1976) among others, have been shown to be polyclonal human B cell activators. The amount of LPS associated with the root surface of periodontally diseased teeth was shown to range from 19 to 394ng per tooth (Wilson, 1995), and the local concentration available to the conventional B cells proximal to a diseased tooth could be high enough to result in polyclonal activation.

6.4.1 Polyclonal B cell activation in periodontitis

Progressive disease could be considered to represent a state of failure to prevent the ingress of microbial products into the tissues; accordingly, high levels of components
such as LPS would be expected. CD5⁺ B cells are known to be self-renewing in the periphery (Hardy and Hayakawa, 1986). The maintenance of stable B-1 cell numbers in control unstimulated cultures is most likely due to this mechanism of self-renewal and homeostasis (see Figures 4.8 and 5.4). It can be postulated that during the self-renewal process only a certain number of these CD5⁺ B cells survive, perhaps only one of the two daughter cells produced after mitosis. The perturbation of this state could lead to an increase in the number of these cells, as seen in Figure 5.8, resulting in an accumulation of this subset in pathologies such as chronic periodontitis, as reported by Sugawara et al. (Sugawara et al., 1992).

The data suggest that CD5⁺ B cells are able to respond to LPS, and that LPS perturbs the normal population dynamics of this subset. This suggests an extension of the above mechanism for the accumulation of CD5⁺ B cells in chronic periodontitis and other pathologies involving such accumulations; trafficking B-1 cells are perhaps kept at a site of pathogen infection by chemotactic means where the high local levels of LPS result in a perturbation of the normal steady-state/self-renewal of B-1 cells. Fewer B-1 cells die resulting in more cells available for proliferation, and B-1 cells accumulate through decreased death and increased proliferation. Perhaps more trafficking B-1 cells are recruited and this removal of B-1 cells from peripheral blood (PB) results in proliferation of remaining PB B-1 cells to replenish cell numbers to their usual levels. The removal of many of the controls of B-1 cell activation and proliferation in this model could lead to the accumulation of this subset.

### 6.4.2 $T_H1/T_H2$ profile

The underlying drive behind the present study has been that B cell accumulation in periodontitis could be explained by direct stimulation of B cells by pathogenic bacteria and bacterial products, including *P. gingivalis* LPS. It is possible, however, that other less direct pathways are responsible. The most likely candidate pathway is that a strong $T_H2$ response is occurring. It has been postulated by a number of investigators that the $T_H1/T_H2$ profile is skewed towards $T_H2$ in chronic periodontitis (Seymour et al., 1996; Gemmell and Seymour, 1998; Yun et al., 2001; Fokkema et al., 2002).

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*NB: There is a discrepancy in the absolute numbers of B-1 cells between these two Figures, however for each experimental set present, the B-1 data was internally consistent with the B-2 data.*
*P. gingivalis* LPS may be capable of skewing immune responses towards a T\(_{H2}\) phenotype by its ability to signal via TLR2. In mice, *P. gingivalis* LPS, when injected with soluble protein antigen, leads to the expression of T\(_{H2}\)-associated cytokines (IL-5, IL-10 and IL-13) in a non-TLR4-dependent manner. This is in contrast to *E. coli* LPS, which stimulates IFN-\(\gamma\) production *in vivo*, and IL-12p70 production from cultured dendritic cells (Pulendran *et al.*, 2001). Indeed, not only does *P. gingivalis* LPS appear to signal solely through the TLR2, it is able to antagonize signalling through TLR4 (Darveau *et al.*, 2002; Hajishengallis *et al.*, 2002; Yoshimura *et al.*, 2002).

Both TLR2 and TLR4 are expressed in human periodontal tissues, and the ratio of TLR2-positive cells was highest overall in connective tissue subjacent to pocket epithelium of the severe group and that of TLR4-positive cells was higher in the severe group than in the other groups. PBMC from 8 out of 31 patients with insulin-dependent diabetes mellitus showed a significant increase in IL-10 production following challenge with 0.01, 0.1 and 1\(\mu\)g/mL *P. gingivalis* LPS, and only these patients had elevated levels of CD5\(^+\) B cells (Stein *et al.*, 1997). Aramaki *et al.* found that the proportion of CD5\(^+\) B cells and the amount of IL-6 and IL-10 were significantly higher in inflamed gingival tissues from patients with adult periodontitis than in peripheral blood from healthy subjects (Aramaki *et al.*, 1998). Stein *et al.* postulate that in “susceptible” IDDM patients, exposure to *P. gingivalis* LPS could induce a hyperactive IL-10 response in both monocytes and B cells leading to expansion of CD5\(^+\) B cells and increased levels of autoantibody directed against gingival tissue components (Stein *et al.*, 1997). Mahanonda also found that IL-10 significantly enhanced the B cell proliferative response, when it is present together with *P. gingivalis* (Mahanonda *et al.*, 2002). Periodontopathic bacterial-activated B cells could therefore serve as possible antigen presenting cells in periodontitis lesions (Mahanonda *et al.*, 2002). These results suggest that TLR2 and TLR4 participate in the innate immune response to stimulation by bacterial products in periodontal tissues (Mori *et al.*, 2003). The dominance of B cells/plasma cells in the advanced/progressive lesion would suggest a role for T\(_{H2}\) cells. If the innate response is poor, low levels of IL-12 would be produced and a poor T\(_{H1}\) response may occur which may not then contain the infection. Polyclonal B cell activation and the subsequent production of IL-4 would encourage a T\(_{H2}\) response,
further B cell activation, and antibody production. If these antibodies are protective and clear the infection, the disease will not progress, but if on the other hand they are non-protective, the lesion will persist and continued B cell activation may result in the unregulated production of large amounts of IL-1 and subsequent tissue destruction (Seymour and Gemmell, 2001).

In addition to differential LPS signalling via TLR2, a further pathway that could lead to T\(_{H2}\) deviation has been recently described. Cysteine proteinases (gingipains) of \(P.\ gingivalis\) have been found to cleave IFN\(_\gamma\) (Yun et al., 1999), hydrolyse IL-12 and reduce the IL-12-induced IFN\(_\gamma\) production from CD4\(^+\) T cells (Yun et al., 2001). LPS upregulates expression of IFN\(_\gamma\)R on LPS-activated B cells and induces production of IFN\(_\gamma\) (Abed et al., 1994). IFN\(_\gamma\) produced by T\(_{H1}\) and NK cells activates monocytes and macrophages and inhibits IgM secretion and B cell activation. Interferon-gamma may therefore be part of a negative feedback loop that regulates B cell response to polyclonal activators (Abed et al., 1994). Decrease in IFN\(_\gamma\) may permit increased sensitivity to B cell PBA (Chace et al., 1995). Macrophages produce IFN\(_\gamma\) (reviewed in Gessani and BeIardelli, 1998), and the unresponsiveness of macrophages within the periodontal tissue (Chapple et al., 1998), coupled with the cleavage of IFN\(_\gamma\) by gingipains could result in unregulated B cell activation by periodontal pathogens. The lack of IFN\(_\gamma\), together with B cell-produced IL-10, could contribute to the skewing of the T\(_{H}\) profile to that of T\(_{H2}\), further promoting polyclonal B cell activation. Interestingly, recent work in our laboratory has found that \(P.\ gingivalis\) gingipains are able to polyclonally activate human B cells and induce IL-4R expression (Yun et al., 2003), thus contributing further B cell accumulation stimuli.

The periodontitis lesion is characterised by tissue destruction and high levels of auto-antibodies, suggesting a high availability of self-antigen (see section 1.7.3). B-1 cells are known producers of auto-reactive antibodies (section 1.3.2.2), and stimulation by self-antigens, in conjunction with foreign mediators of activation may further drive the polyclonal response and accumulation of B-1 cells with periodontitis lesions.

It can be postulated that unregulated polyclonal B cell activation of the B-1 cell subset contributes to the pathogenesis of chronic periodontitis through skewing of the T\(_{H}\)
response to the $T_H 2$ profile via production of IL-10 and destruction of tissues via autoantibodies. Accumulation of B-1 cells, constitutively or inducibly, producing IL-10, even if the amount produced per cell is relatively low, could lead to locally high concentrations of the cytokine and increased opportunity to skew the $T_H$ response.

6.5 Future work

Further studies to elucidate the roles and mechanisms of B-1 and B-2 cell involvement in the pathogenesis of periodontitis should include an extension of the mixed population studies using $P. gingivalis$ LPS in order to better understand the interactions of the B cell subsets with each other and with other cells (for example, T cells and macrophages). Specifically, gene expression studies and proteomics would provide data concerning cytokine expression and mechanisms of cell death agonists and antagonists. An extension of the model would require the sorting of murine B-1 and B-2 cells and pulsing each subset with a different tracking dye using for example, PKH26 and PKH67. The sorted and labelled cells would then be placed into the same culture and the kinetics of each subset examined, together with gene and protein expression. Similar studies could be undertaken with human B cells.

Studies presenting modulation of TLR expression (Wang et al., 2001) and function by $P. gingivalis$ LPS (Hajishengallis et al., 2002), together with the differential effects of LPS-stimulation on TLR expression presented in this thesis suggest interesting avenues of study regarding the differential effects of TLR signalling in the immune response and the mechanisms of differential responses from the same TLR.

In order to draw more detailed parallels between the mixed cell culture system and human disease, an examination of biopsies from healthy controls and patients with periodontitis is suggested. This should include the study of the same mediators and markers examined in the cell culture work.

6.6 Conclusions

The studies presented in this thesis have shown that there are differences between B-1 and B-2 cell responses to enteric and non-enteric LPS, which are consistent with
differential roles of these cells in immune responses. LPS was shown to perturb the normal homeostatic mechanisms of B-1 cells, a subset which was found to have high basal levels of activation and this, together with the “rescue” of serum-starved CH12 cells, suggests a mechanism by which B-1 cell accumulation in diseases such as periodontitis could occur. This has implications both for the treatment of diseases characterised by B-1 cell accumulations and postulated roles for this subset in the immune response.
Appendix 1
Formulations

Ammonium chloride erythrocyte lysing solution

10x

NH₄Cl (1.5M) 80.2g
NaHCO₃ (100mM) 8.4g
disodium EDTA (1mM) 3.7g
H₂O to 900mL
Adjust pH to 7.4 with 1M HCL or NaOH
Add H₂O to 1000mL
Store up to 6 months at 4°C

1x

Dilute 1:10 with H₂O fresh before use
Store cold and discard unused portion

10% BSA

BSA (Sigma A4503) 1g
dH₂O 9mL
Place in 37°C waterbath and allow to dissolve
Filter (Sartorius minisart-plus)
Freeze

CFSE stock

10mM in DMSO
Stored desiccated at −70°C as 20μL aliquots
CFSE from Molecular Probes, Eugene, Oregon, USA. Cat: C-1157
mw 557.47
5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester *mixed isomers*

Fixation solution for FACS analysis

PFA 2g
PBS 100mL
Heat to not more than 70°C to dissolve
Adjust pH to 7.2 with 0.1M HCl or NaOH
Store protected from light up to 1 month at 4°C

**L-Glutamine (200mM solution)**

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<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>L-Glutamine</td>
<td>2.922g</td>
</tr>
<tr>
<td>Medium of choice</td>
<td>100mL</td>
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<td>Store frozen</td>
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**Karnovsky’s fixative for Electron Microscopy**

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<tr>
<td>4% PFA</td>
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<tr>
<td>PBS</td>
<td>17.8mL</td>
</tr>
<tr>
<td>Glutaraldehyde (50%)</td>
<td>2.5mL</td>
</tr>
<tr>
<td>Mix</td>
<td></td>
</tr>
<tr>
<td>Store 4°C up to 1 week</td>
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**Karnovsky’s fixative (EM Unit)**

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<th>Amount</th>
</tr>
</thead>
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<tr>
<td>(2.5% glutaraldehyde, 2.4% formaldehyde in 0.1M MOPS</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>30mL</td>
</tr>
<tr>
<td>(Polysciences 16% 10mL phials)</td>
<td></td>
</tr>
<tr>
<td>25% Glutaraldehyde</td>
<td>20mL</td>
</tr>
<tr>
<td>1.0M MOPS</td>
<td>20mL</td>
</tr>
<tr>
<td>dH2O to 150mL</td>
<td></td>
</tr>
<tr>
<td>Adjust pH to 7.4 with 1.0M NaOH</td>
<td></td>
</tr>
<tr>
<td>dH2O to 200mL</td>
<td></td>
</tr>
</tbody>
</table>

**LPS extraction for P. gingivalis**

Method modified from Westphal, O. & Jann, K. (1965) *Bacterial Lipopolysaccharides. Extraction with phenol-water and further applications of the procedure*. Methods Carbohydrate Chemistry 5:83-91

1. For 20g dry weight of bacteria (or equivalent), resuspend in 350mL of dH2O at 65-68°C (in a water bath).
2. Add 350mL of 90% phenol solution, preheated to 65-68°C, with vigorous stirring for 10-15mins at 65-68°C.
3. Cool mixture to 10°C on ice.
4. Centrifuge to separate phases at 8000rpm for 10min (~10000 x g). Remove and keep the water phase (normally the upper phase, unless it contains > 0.5M salt). Do not disturb the interface.
5. Repeat the extraction procedure with the phenol/interface phase using another 350mL of water at 65-68°C. Keep the water phase after centrifugation.
6. Dialyse the water phase with dH₂O with frequent changes to remove phenol.
7. May elect to reduce volume using Aquacide etc. before lyophilisation.
8. Resuspend the pellet in 30mL of buffer (20mM Tris, 2mM MgCl₂, 20mM NaCl, 0.1mM DTT, pH 8). Add pancreatic DNase I and pancreatic RNase A at 100 µg/mL and 25 µg/mL, respectively.
9. Incubate for 2hr. at 37°C.
10. Add Proteinase K at 100 µg/mL and CaCl₂ to 1mM concentration. Incubate for a further 2hr at 37°C. Add another 50 µg/mL Proteinase K and incubate for 1 hour. [Alternatively, add 1mg/mL of Protease Type XIV (from Streptomyces griseus) and 500 µg/mL, respectively.]
11. Remove proteins by adding 10mL of neutral water-saturated phenol, emulsify and separate the phases by centrifugation. Keep the aqueous phase. Repeat.
12. Dialyse against dH₂O to remove the phenol and small molecular weight molecules post DNase/RNase/protease treatment.
13. Lyophilise

**MOPS 3 (MOPS) Stock 1.0M**

-(N-morpholino) propane sulfonic acid 209.27g
dH₂O 1L
(add gradually until dissolved)
NaN₃ 0.8g

**MTT (5mg/mL)**

(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

MTT 25mg
PBS 5mL
Vortex
Stand 30min room temperature
Filter sterile
Store 4°C in foil wrapped bottle
Absorbance measured at 570nm, background subtraction at 630nm - 690nm

**Osmium tetroxide 5% stock**

2 ampoules Osmium (1g each) into PTFE bottle
Add 40mL dH₂O and mix gently
Leave in fume cupboard for 1 day to dissolve then refrigerate
**Osmium tetroxide in 2% 0.1M Cacodylate buffer**

5% Osmium tetroxide 4mL  
Sodium cacodylate 1.0M 1.0mL  
Adjust to pH 7.4 with 0.1N HCl 0.62mL  
Add dH2O up to 10mL

**4% Paraformaldehyde**

PBS (Sterile) 100mL  
Paraformaldehyde (PFA) 4g  
Heat to 70-80°C shaking gently  
Allow to cool down

**Permeabilisation solution for FACS analysis**

Tween 20 200μL (0.22g)  
PBS 100mL  
Store up to 1 month in amber container at 4°C  
Warm to RT before use

**Phosphate Buffered Saline (PBS)**

NaCl 8g/L  
KCl 0.2g/L  
KH₂PO₄ 0.2g/L  
Na₂HPO₄·2H₂O 1.15g/L  
Milli Q Water to required volume

**PBS/2% FCS/0.1% NaN₃**

Foetal Calf Serum 2mL  
PBS/NaN₃ 100mL  
Store 2 - 8°C for 1 month

**PBS/0.1% NaN₃**

NaN₃ 100mg  
PBS 100mL  
Store 2 - 8°C for up to 2 months
**PBS/2% PFA/0.1% NaN₃**

Paraformaldehyde 2g  
PBS 100mL  
Heat to 56°C in a water bath until the PFA goes into solution  
allow to cool to room temperature and adjust pH to 7.4 (0.1M NaOH, 0.1M HCl)  
Add 100mg NaN₃ and dissolve  
Store 2 - 8°C for 1 month

**RPMI 1640**

<table>
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<tr>
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<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>1 pack</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>3.43μL</td>
</tr>
<tr>
<td>Sodium Bicarbonate (7.5% solution)</td>
<td>27mL</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (5000IU/mL solution)</td>
<td>10mL</td>
</tr>
<tr>
<td>Milli Q water to 1000mL</td>
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</tr>
<tr>
<td>Supplement with 10% FCS and L-Glutamine if required (2mL/100mL 200mM stock)</td>
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**Sodium cacodylate Stock 1M**

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<tr>
<td>Sodium cacodylate</td>
<td>214.03g</td>
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<tr>
<td>dH₂O</td>
<td>1L</td>
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**Spurr low-viscosity epoxy resin**

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<tr>
<td>Vinylcyclohexene dioxide ERL 4206 (VCD)</td>
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</tr>
<tr>
<td>Diglycidyl ether of polyethylene glycol (DER736)</td>
<td>32.5g</td>
</tr>
<tr>
<td>Nonenyl succinic anhydride (NSA)</td>
<td>130g</td>
</tr>
<tr>
<td>Diethyl amino ethanol (DMAE)</td>
<td>2g</td>
</tr>
<tr>
<td>(cure time 8hr)</td>
<td></td>
</tr>
<tr>
<td>Mix thoroughly using mechanical stirrer in fume cupboard</td>
<td></td>
</tr>
<tr>
<td>Dispense into bottles, store in freezer</td>
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**Variations of Spurr**

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<th>Reagent</th>
<th>Std</th>
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<th>Fast cure</th>
<th>Slow cure</th>
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<td>10</td>
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</tr>
<tr>
<td>DER736</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>6</td>
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<tr>
<td>NSA</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>DMAE0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>1.0</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Cure at 70°C</td>
<td>8 hr</td>
<td>8 hr</td>
<td>8hr</td>
<td>3 hr</td>
<td>16 hr</td>
</tr>
</tbody>
</table>
Trypan Blue Dye

Trypan Blue (40% Dye Content) 400mg
PBS 100mL
Filter sterilise
Store 2 - 8°C protected from light

4% aqueous Uranyl acetate

Uranyl acetate (Fluka) 2g
dH2O 50mL
Cover flask with foil and mix until dissolved
Store 4°C

Washing buffer for FACS analysis

Tween 20 100μL (0.11g)
PBS 100mL
Store up to 1 month at 4°C

Working solution - 0.1M MOPS (tissue wash)

1M MOPS stock 100mL
dH2O 800mL
pH to 7.4 with NaOH
Make up to 1L

Working solution - 0.1M (tissue wash)

Sodium cacodylate 1M 100mL
dH2O 800mL
pH to 7.4 with HCl
Make up to 1L
Appendix 2

Spleens

The method of cell suspension preparation required some preliminary testing. Initially, the protocol was as follows;

1. Mice sacrificed using Halothane, spleens removed and placed into RPMI 1640 medium, on ice;
2. Spleens were cut into smaller pieces using a sterile scissors and then squashed through a metal mesh, using the butt of a 20mL syringe, into a petri dish;
3. The cell suspension was then layered over 10mL FCS and left to stand for 10 min at room temperature;
4. The red upper layer was then removed and layered over 10mL FCS in a new tube;
5. The layered suspension was then centrifuged for 10 min (300g, 4ºC);
6. The supernatant was decanted and 4mL RPMI 1640 was added to the resuspended pellet;
7. The suspension was then layered over 4mL Ficoll (Pharmacia) in a 15mL centrifuge tube;
8. The layered suspension was then centrifuged for 30 min (400g, 18ºC);
9. The lymphocyte layer was removed and added to 40mL RPMI in a 50mL centrifuge tube;
10. The cell suspension was centrifuged was then centrifuged for 10 min (300g, 4ºC), and;
11. The supernatant was decanted and 10mL RPMI/10% FCS was added to the resuspended pellet.

However, the lymphocyte yields from harvested spleens prepared in this manner were highly variable and generally lower than expected (see Figure B.1A and B).

A new preparation method, suggested by Dr WLK Massey (personal communication), and the one subsequently used for the experiments shown, with an addition of an erythrocyte lysis step, resulted in significantly higher lymphocyte yields from harvested spleens (Figure B.1B, C and D – new method used from 14/7/97).
The new method was as follows;

1. Mice were sacrificed using Halothane, spleens removed and placed into RPMI 1640 medium, on ice;
2. Spleens were placed in petri dish and mashed using the butt of a 20mL syringe
3. The cell suspension was suctioned up with a syringe and 18g needle and jetted over the petri dish surface a series of times
4. An autoclaved piece of nylon mesh (200μm pore size) was “tented” into the top of a 50mL centrifuge tube. The cell suspension and debris were then poured through the mesh.
5. The suspension was washed twice with RPMI 1640/10% FCS
6. After the final wash the pellet was resuspended in erythrocyte lysing buffer (approx 5mL/spleen)
7. The suspension was incubated 5 minutes at room temperature with occasional shaking
8. RPMI was added to fill the tube and the suspension was centrifuged for 10 minutes (200g, 4°C)
9. The pellet was washed again and resuspended in RPMI 1640/10% FCS
10. The cell suspension was transferred into a centrifuge tube precoated with FCS and underlaid with an equal volume of Histopaque 1083
11. The layered suspension was centrifuged for 30 minutes (400g, 18°C)
12. The buffy layer containing the lymphocytes was removed and added to 40mL RPMI 1640/10% FCS
13. The cell suspension was centrifuged for 10 minutes (300g, 4°C) and the remaining pellet resuspended in RPMI 1640/10% FCS prior to seeding
Figure A.1 Average lymphocyte yield/spleen
The average lymphocyte yield per spleen is shown for experiments conducted in 1996 (A), 1997 (B), 1998 (C) and 1999 (D). (No error bars are shown as the averages were calculated by dividing the final total viable lymphocyte count (after preparation) by the number of spleens harvested for that experiment.)
**Trial Enrichment of B cells**

It has been reported that the high level of expression of CD23 and IgD on conventional B cells, and the absence of these markers on B-1 cells, allows B-1 cells to be selectively enriched by magnetic bead separation (Metzger et al., 1992). Accordingly, biotin-conjugated CD23 and IgD were bound to Strep-Magnaspheres and used to selectively enrich B-1 cells from splenic lymphocyte populations (through negative selection to reduce any activation of this subset during the enrichment process). However, this enrichment process proved problematic (data not shown), with unacceptably low yields of cells.

Therefore, the unenriched, mixed population of splenic lymphocytes (T cells, conventional and B-1 B cells) was used as the combination of fluorescently labelled monoclonal antibodies used for the flow cytometric analysis allowed the discrimination of T cells, conventional B cells (B-2 cells) and B-1 cells in the splenic lymphocyte populations without the requirement for prior enrichment.
Appendix 3

Sample plate map

<table>
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<th>1</th>
<th>2</th>
<th>3</th>
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The plate mat above is for the B sample set for CD80 (no fill) and CD86 (dark grey shading) analysis. Wells A8 and A9 contain the CD80 positive and negative control samples respectively whilst wells E8 and E9 contain the positive and negative controls for CD86.

All four cell sample types were included on each plate (one for each of the three experimental runs; A, B, and C);

- WEHI-279 with *S. enteritidis* LPS (WEHI SE)
- WEHI-279 with *P. gingivalis* LPS (WEHI PG)
- CH12 with *S. enteritidis* LPS (CH12 SE)
- CH12 with *P. gingivalis* LPS (CH12 PG)
Appendix 4

The importance of innate immunity in instructing adaptive immune responses

All multicellular organisms have defence mechanisms that can be triggered by infection to protect the host organism. These defence mechanisms can be divided into the innate and adaptive systems. Vertebrates are the only organisms that have an adaptive immune system, but the contribution of an innate immune system, combined with the resistance to reinfection inherent in an adaptive immune system, provides a selective advantage (Parish and O'Neill, 1997). Chondrichthyes (sharks, rays, and other cartilaginous fish) are the most phylogenetically primitive vertebrates with an adaptive immune system; including immunoglobulins (Igs), T cell receptors (TCRs), and MHC class I and II receptors (Bartl et al., 1997). The Ig genes in cartilaginous fish are restricted in terms of diversity relative to mammalian prototypes (Haire et al., 1997).

The initiation of adaptive immune responses and the induction of particular effector function mechanisms appear to depend on the signals provided by the innate system (Parish and O'Neill, 1997; Medzhitov and Janeway, 1998). Medzhitov and Janeway suggested that the evolution of innate immunity is directed toward recognition of invariant molecular constituents of infectious agents. The relatively slow rate of evolution and adaptation of the innate immune system receptors would not be a major selective disadvantage in this case as the targets of these receptors are also stable. The targets of this recognition represent molecular patterns rather than particular structures (Medzhitov and Janeway, 1997). The innate immune system uses germ line-encoded receptors for the recognition of microbial pathogens, so changes in receptor specificity reflecting changes in pathogen structure occur only during meiosis. Many of the components of the innate immune system are conserved across classes and even phyla. In the fruit fly, Drosophila melanogaster, the protein encoded by the toll gene induces the innate anti-fungal immune response. The plant tobacco-mosaic-virus resistance gene encodes a protein very similar to Toll and murine and human homologues have also
been identified and characterised (Medzhitov et al., 1997; Rock et al., 1998; Yang et al., 1998; Takeuchi et al., 1999; Takeuchi et al., 1999; Miyake et al., 2000; Ogata et al., 2000; Rhee and Hwang, 2000; Hayashi et al., 2001; Rassa et al., 2002). It therefore appears that the immune-response system mediated by Toll represents an ancient host defence mechanism (Medzhitov et al., 1997).

A number of individual Toll-like receptors (TLR) have been identified, and each has a distinct specificity for pathogen-derived products. These include flagellin (TLR5) (Hayashi et al., 2001), dsRNA (TLR3) (Alexopoulou et al., 2001), lipopeptides (TLR2 homodimers) (Ozinsky et al., 2000), peptidoglycan (TLR2/6 heterodimers) (Ozinsky et al., 2000), unmethylated CpG motifs (TLR9) (Hemmi et al., 2000) and LPS (TLR4) (Poltorak et al., 1998; Poltorak et al., 1998).

TLR2 and TLR4 recognise different bacterial cell wall components and TLR2 plays a major role in Gram-positive bacterial recognition (Takeuchi et al., 1999). Enterobacterial LPS binds to TLR4 and P. gingivalis LPS binds to TLR2 but not TLR4 (Bainbridge and Darveau, 2001; Hirschfeld et al., 2001) and TLR2 signalling pathways appear to be different to those activated by TLR4 (Arbibe et al., 2000). Different TLRs as well as different combinations of TLR heterodimers can be used by cells of the immune system to differentiate among the large number of pathogen associated molecular patterns (PAMP) found on pathogens (Akira et al., 2001).

The activation of TLRs can also lead to tissue injury, including the manifestations of septic shock (Poltorak et al., 1998) and the induction of apoptosis (Hoshino et al., 1999). Bacterial lipoproteins (BLP) induce apoptosis in THP-1 monocytic cells through human Toll-like receptor–2(hTLR2). BLPs also initiated apoptosis in an epithelial cell line transfected with hTLR2. In addition, BLPs stimulated nuclear factor–kB, a transcriptional activator of multiple host defence genes, and activated the respiratory burst through hTLR2. Thus, hTLR2 is a molecular link between microbial products, apoptosis, and host defence mechanisms and signals for both cell activation and apoptosis (Aliprantis et al., 1999).
Mammalian Toll-like receptors (TLRs) signal through the adapter protein MyD88 (Medzhitov et al., 1998; Muzio et al., 1998; Kawai et al., 1999). Human MyD88 contains an N-terminal death domain, followed by an intermediate domain and a C-terminal TIR domain. MyD88 is recruited to activated Toll and IL-1Rs through homophilic interactions between the TIR domains of the receptor and MyD88 (Medzhitov et al., 1998; Muzio et al., 1998). The death domain of MyD88, in turn, associates with the death domain of the serine threonine protein kinase IL-1R-associated kinase (IRAK) (Cao et al., 1996; Muzio et al., 1997; Wesche et al., 1997). Recruitment of IRAK leads to its activation and association with another adapter, tumour necrosis factor receptor-associated factor (TRAF6) (Cao et al., 1996). Oligomerisation of TRAF6 results in activation of IkB kinases, followed by phosphorylation and degradation of IkB. On IkB degradation, NF-kB factors translocate into the nucleus, where they induce transcription of target genes (Ghosh et al., 1998; Horng and Medzhitov, 2001).

Mycobacteria contain well-defined ligands for TLR-2 (Means et al., 1999) and prolonged signalling through TLR-2 by the 19-kDa lipoprotein of *M. tuberculosis* blocked IFN-γ activation of human macrophages (Gehring et al., 2003). Signalling through TLR-2 has been described to cause apoptosis of human macrophages (Aliprantis et al., 1999; Aliprantis et al., 2000). Apoptosis of macrophages in response to *M. tuberculosis* p19 was found to be caspase-8 dependent and caspase-9 independent consistent with a transmembrane pathway signalling cell death through TLR-2 (Lopez et al., 2003).

TLR2 was expressed by Schwann cells and the activation of Schwann cells with a lipopeptide comprising the functional N-terminal portion of the putative *M. leprae* 19-kDa lipoprotein triggered nuclear apoptosis. Schwann cells in leprosy lesions were also found to have undergone nuclear apoptosis. These data suggest that the activation of TLR2 on Schwann cells contributes to nerve damage in leprosy (Oliveira et al., 2003). TLR2-induced apoptosis has been shown to involve a novel pathway requiring myeloid differentiation factor 88 and nuclear factor B and involving Fas-associated death domain protein and caspase 8 (Aliprantis et al., 2000).
Aliprantis has suggested that apoptotic signalling through TLR4 is likely to be mechanistically similar to apoptotic signalling mediated by TLR2 (Aliprantis et al., 2000). TLR4 activation by LPS has been shown to lead to extensive neuronal death in vitro that depends on the presence of microglia (Lehnardt et al., 2003).
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