THE UNIVERSITY *of* EDINBURGH

Mechanisms of microenvironmental conditioning in non-Hodgkin's lymphoma

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For the degree of Doctor of Philosophy

The University of Edinburgh

2012

Acknowledgements

I would like to express my sincere gratitude to my principle supervisor Professor Christopher Gregory for his constant guidance and encouragement. I greatly appreciate all his contributions of time and ideas which makes my PhD experience fruitful and stimulating. His joy and enthusiasm for research is inspirational for me, especially during tough times of my PhD.

My sincere thanks also go to my second supervisor, Dr. Lesley Forrester, for her guidance and constructive advice for the ES cell work. It is also difficult to overstate my gratitude to my daily supervisor, Dr. John Pound, who provided constant encouragement, reliable help, sound advice, excellent teaching and numerous great ideas throughout my PhD study. Special thanks to Dr. Catriona Ford for her teaching and encouragement, helpful discussions, and insightful comments. I particularly wish to thank Mrs Helen Taylor for enlightening me the trick for ES cell culture and useful advice to generate ESDM. I would also like to thank Jorine Willems for all the help she gave me during the time we worked together and helping me with cold-shock induced apoptotic cells for the phagocytosis assay; Lynsey Melville for performing procedures on animals to harvest mouse primary macrophages; David Dorward for supplying me with human neutrophils; our nice secretary, Ms Gillian MacLeod, for assisting me in different ways. I also thank all the present and previous members of *the Inflammation and Cancer* group for their friendship and encouragement.

Lastly but most importantly, I wish to thank my parents and my boyfriend for their constant support and unconditional love without which the thesis wouldn't have been possible. To them, I dedicate this thesis.

Declaration

The thesis and the work presented within were original work by me, unless otherwise acknowledged. All sources of information in the thesis have been acknowledged by reference. The work has not been submitted in any previous application for a degree.

Abstract

Tumours are not autonomous transformed cell populations, but rather a society composed of both malignant and normal, including immune, cells that together foster tumour growth and development. Tumour-associated macrophages have been reported to enhance tumour growth, progression and metastasis. In high-grade non-Hodgkin's lymphomas, prototypically the B-cell neoplasm, Burkitt's lymphoma (BL), infiltrating macrophages engulf large numbers of apoptotic tumour cells. Evidence suggests that apoptotic BL cells can condition the tumour microenvironment to promote lymphoma development by selectively attracting macrophages while inhibiting neutrophil infiltration and by stimulating macrophages to produce the B-cell growth and survival factor.

Tumour cells grow in a hypoxic and nutrient-deficient environment and the resultant cellular stress can induce apoptosis. It is therefore possible that hostile environmental conditions in the tumour also contribute to the generation of a pro-tumour microenvironment. This thesis describes investigations which examined this hypothesis.

BL cells were cultured at high density to mimic conditions of metabolic stress existing in the tumour environment. Cell-free supernatants from such stressed BL cells demonstrated potent chemoattractive activity for mononuclear phagocytes. Supernatants from BL cells that were protected from apoptosis by over-expression of bcl-2 had similar ability, confirming that chemoattractant release was apoptosis-independent. The observation that apyrase and suramin could inhibit the chemotactic activity of these supernatants suggested that nucleotides might be the apoptosis-independent chemoattractant. Detection of ATP in stress supernatants by bioluminescence assay was consistent with this proposal. Significantly, supernatants

from BL cells and those transfected with bcl-2 were both found to inhibit neutrophil migration, suggesting the occurrence of a neutrophil migration inhibitory factor whose release was apoptosis-independent. Furthermore, stress supernatants could promote BL cell proliferation in vitro, which was apoptosis and cell line-independent.

In order to study the role of TAM in the tumour microenvironment, a novel macrophage model was devised using mouse embryonic stem cells (ES cells). Cells derived from ES cells generated in vitro expressed macrophage-specific markers and were free of dendritic cells and undifferentiated ES cells. ES cell-derived macrophages (ESDM) could migrate towards apoptotic BL cells and engulf them. However, ESDM migrated to stress supernatants with decreasing efficiency as they matured. Preliminary data indicated that the phagocytic ability of ESDM to engulf apoptotic cells increased as they matured, consistent with distinct roles for circulating monocytes and tissue macrophages with regard to this function.

Considering the high yields and purities of ESDM described here, together with their non-malignant nature and genetic versatility these cells should provide a superior source of undifferentiated mononuclear phagocytes with which to elucidate the molecular mechanisms underlying tumour infiltration and microenvironmental conditioning by TAM.

In conclusion, this work suggests that under conditions of pre-apoptotic stress, BL cells have the capacity to regulate their micro-environment upstream of their apoptosis programme to promote net tumour growth through paracrine signals that attract supportive macrophages and inhibit destructive neutrophils and through release of autocrine/juxtacrine tumour growth factors.

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List of abbreviations

ADP	Adenosine diphosphate
AI	Apoptosis index
AIDS	Acquired immune deficiency syndrome
AIF	Apoptosis-inducing factors
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AP	Alkaline phosphatase
Apaf-1	Apoptotic protease activating factor-1
ATF6	Activation transcription factor 6
ATP	Adenosine triphosphate
AxV	Annexin V
BAFF/BLyS	B cell-activating factor of the TNF family/B lymphocyte stimulator
Bcl	B cell lymphoma/leukemia gene
BH3-only	Bcl-2-homology domain 3 ony
BL	Burkitt's lymphoma
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
C5a	Complement component 5a
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CD	Cluster designation
CM	Conditioned medium
conc.	Concentration
CSF-1	Colony-stimulating factor-1
DAB	3,3'-diaminobenzidine
DAMP	Dangre-associated molecular pattern
DAPK	Death-associated protein kinase
DC	Dendritic cells
DLBCL	Diffuse Large B-cell lymphoma
DMSO	Dimethyl sulfoxide
EBs	Embryoid bodies
EBV	Epstein-Barr virus
ECL	Enhanced chemiluninescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay

ENDOG	Endonuclease G
ENPP	Ectonucleotide pyrophosphatase/phosphodiesterase
ENTPD	Ectonucleoside triphosphate diphosphohydrolase
EPC	Endothelial progenitor cells
ER	Endoplasmic reticulum
ES cells	Embryonic stem cells
ESDM	Embryonic stem cell-derived macrophages
EU	Endotoxin units
FACs	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FCM	Flow cytometry
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FKN	Fractalkine
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
GPCRs	G protein-coupled receptors
HIF1α	Hypoxia-inducible factor 1α
HL	Hodgkin's lymphoma
HMDM	Human monocyte-derived macrophages
hr	Hour
Hsp	Heat shock proteins
HTRA2	High temperature requirement protein A2
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMS	Intermembrane space
iNOS	Inducible nitric oxide synthase
IRE1	Inositol-requiring protein-1
IRF-3	Interferon regulatory factor 3
JNK	Jun N-ternimal kinase
kDa	Kilodalton
LAL	Limulus amoebocyte lysate
LFN	Lactoferrin
LIF	Leukemia inhibitory factor
LPC	Phospholipid lysophosphatidylcholine
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex

MMP	Matrix metalloproteases
MMTV-PyMT	Mouse mammary tumour virus-polyoma middle T oncoprotein
MOMP	Mitochondrial outer membrane permeabilisation
MPs	Microparticles
MW	Molecular weight
NF-κB	Nuclear factor-κB
NGS	Normal goat serum
NHL	Non-Hodgkin's lymphoma
NK cell	Natural killer cell
NLRP3	NOD-like receptors family, pyrin domain containing 3
NMS	Normal mouse serum
PD-L1	Programmed cell death 1 ligand 1
PERK	Phycoerythrin
PERK	Protein kinase RNA-like ER kinase
PI	Propidium iodide
PI-3K	Phosphoinositide 3-kinase
PIDD	P53-induced protein with a death domain
PP2A	Protein phosphatase 2A
PS	Phosphatidylserine
PUMA	P53-upregulated modulator of apoptosis
RAIDD	Receptor-interacting protein (RIP)-associated ICH-1/CED-3 homologous death protein with a death domain
ROS	Reactive oxygen species
RT	Room temperature
S1P	Sphingosine-1-phosphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sHsp	Small heat shock proteins
SLE	Systemic lupus erythematosus
Smac	Second mitochondria-derived activator of caspases
SQSTM1	Sequestosome 1
STAT	Signal transducer and activator of transcription
TAM	Tumour-associated macrophages
TGF-β	Transforming growth factor-β
TIGAR	TP53-induced glycolysis and apoptosis regulator
TNF	Tumour necrosis factor

TP53	Tumour protein 53
TP53INPI	Tumour protein p53-inducible nuclear protein 1
Treg	Regulatory T cells
UDP	Uridine diphosphate
UPR	Unfolded protein response
UTP	Uridine triphosphate
UV	Ultraviolet
VDAC	Voltage-dependent anion channels
VEGF	Vescular endothelial growth factor

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Chapter 1 Introduction

1.1 Tumour microenvironment

The prototypical concept that the microenvironment is a crucial regulator of tumourigenesis was first proposed by Paget in his famous 'seed and soil' theory, as cited by Stephen, when Paget was trying to investigate the distribution of metastatic breast cancer, (Stephen 1889). He thought that all cancer cells possess the ability to develop further, but could only grow into a secondary cancer in certain organs which could sustain the new growth. The hypothesis 'languished in the shadows' for almost a century. However, the biology of the tumour microenvironment has been widely studied since, especially over the past decade, and tumours have increasingly been recognized as organs rather than a collection of relatively autonomous and homogeneous cancer cells. Today the tumour microenvironment is thought to comprise both malignant cells and normal cells, such as fibroblasts, epithelial cells, endothelial cells, cells of the innate and adaptive immune system as well as the subsequently generated cytokines and chemokines. It should be noted that, as genetically mutated cells, malignant cells have the inherent ability to sustain proliferation, restrain cell death, induce angiogenesis and further activate invasion and metastasis. Nevertheless, studies into the functions of each component of the tumour microenvironment in tumourigenesis suggest that they all participate in the process of, and have proved to be important, in the development of tumours (Hanahan and Weinberg 2011).

1.1.1 Tumour-associated inflammation

As cited by Balkwill and Mantovani, Rudolf Virchow made the first proposal about the role of inflammation in cancer at 1863. He hypothesized that the emergence of cancer occurred at the site of chronic inflammation (Balkwill and Mantovani 2001). However,

the hypothesis was largely neglected for a long time, and the importance of inflammation in tumourigeneis has not been well accepted until the last decade. The presence of inflammation, demonstrated by the markers of inflammatory cells and mediators, has been detected in almost all the tumours, although with different intensities (Pages, Galon et al. 2010). Different types of inflammation are present in tumours, and they are associated with different stages of tumourigenesis with various tumour promoting effects (Grivennikov, Greten et al. 2010). Although a direct causal relationship between inflammation and cancer has not yet been validated, the connection between inflammation and cancer has been summarized in two pathways: an extrinsic pathway and an intrinsic pathway (Allavena, Garlanda et al. 2008). The extrinsic pathway refers to chronic inflammation or infection at certain sites that can facilitate the initiation of neoplasia as well as tumour invasion and metastasis. The intrinsic pathway is induced by oncogene activation which subsequently causes neoplasia and produces inflammatory mediators to generate an inflammatory microenvironment within a developed solid malignancy. These two pathways are interconnected and lead to the activation of transcription factors in tumour cells, mainly nuclear factor- κB (NF- κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1 α (HIF1α) and the resulting production and release of inflammatory cytokines and chemokines. These inflammatory mediators can further recruit immune cells from blood and alter their activation status (Karin 2006; Allavena, Garlanda et al. 2008).

Both innate and adaptive immune cells can be detected in the tumour microenvironment, including tumour-associated macrophages, tumour-associated neutrophils, T cells, mast cells and B cells. The environment is further enriched by cytokines and chemokines released from them, such as IL-12, IFNγ (anti-tumour) and IL-6, IL-23 (pro-tumour) (de Visser, Eichten et al. 2006; Lin and Karin 2007). However, the pro- or anti-tumour effect of the inflammatory component within the

tumour microenvironment depends on the combination and the activation status of immune cells as well as those immune mediators (Smyth, Dunn et al. 2006).

Tumour-associated macrophages

Tumour-associated macrophages (TAM) are the major immune component of the tumour microenvironment and an important source of cytokines. In more than 80% human tumours, a high content of macrophages is associated with poor prognosis which is supported by pathological evidence and genetic profiling (Bingle, Brown et al. 2002; Tsutsui, Yasuda et al. 2005). Macrophage deficiency is also found to correlate with decreased tumour mitosis and poor vascularisation in an in vivo murine model (Nowicki, Szenajch et al. 1996). But there are exceptions. For example, depletion of liver macrophages (Kupffer cells) was found to support the growth of metastatic carcinoma, suggesting that Kupffer cells played an important role in suppressing tumour growth in the liver (Heuff, Oldenburg et al. 1993). Nevertheless, the vast majority of data from human and animal models indicate that tumour-associated macrophages promote tumour progression and metastasis (Qian and Pollard 2010).

Macrophages are cells with plasticity that can adapt their cell profiles according to the specific immunological environment (Mantovani, Sica et al. 2005). Two extremes of macrophage activation continuum have been designated: M1 and M2. The M1 phenotype can be induced by IFNγ alone or combined with microbial products, such as LPS, and macrophages activated in this way can release pro-inflammatory cytokines, such as TNF-α, IL-12 and IL-23, express high levels of major histocompatibility complex (MHC) molecules and inducible nitric oxide synthase and have cytotoxic activity against neoplastic cells. On the other hand M2, or ‘alternatively activated’ macrophages are induced by Th-2 type cytokines, such as IL-4, IL-10, and IL-13. M2 macrophages release anti-inflammatory cytokines, such as IL-10, express high levels of arginase and scavenger receptor A, have poor antigen-presenting capability but

enhanced debris clearance ability and promote wound-healing (Mantovani, Sozzani et al. 2002).

TAM can exhibit different activation statuses and functions in the tumour microenviroment. During tumour initiation, immune cells, including macrophages which exhibit a M1 phenotype and NF-κB signalling, are essential for this process (Karin and Greten 2005). Deletion of IKKβ, which phosphorylates NF-κB bound IκB and liberates NF-κB dimers to enter the nucleus to activate its transcriptional functions, in myeloid cells reduced tumour incidence by almost 50% and showed tumours with smaller sizes in a murine colitis associated cancer model (Greten, Eckmann et al. 2004). NF-κB promotes the transcription and release of inflammatory cytokines, such as IL-6, IL-8 and TNF-α, as well as inducible nitric oxide synthase (iNOS). It has been hypothesized that these immune cells generate reactive oxygen and nitrogen species to induce further mutations or genetic instability in adjacent developing tumour cells, thus forming a pro-tumour microenvironment (Pang, Zhou et al. 2007; Colotta, Allavena et al. 2009).

After initiation, when tumours gradually progress, in most, but not all tumours investigated, TAM switch from M1 to a phenotype polarized to the M2 type (Torroella-Kouri, Silvera et al. 2009; Mantovani and Sica 2010). The phenotype switch is suggested to be dependent on nuclear overexpression of p50 which is a NF-κB inhibitory homodimer (Saccani, Schioppa et al. 2006; Porta, Rimoldi et al. 2009). In cancers that arise without association with obvious inflammation, tumour-associated macrophages still exhibit an alternatively activated state and they are believed to be recruited into tumours from blood monocytes (Mantovani, Bottazzi et al. 1992). Several chemokines have already been identified as the possible chemoattractive signals. CCL2 (MCP-1) was the first discovered monocyte chemoattractant which was derived from human ovarian cancer (Negus, Stamp et al.

1995). CCL5, as well as CCL2, are found to attract tumour-associated macrophages in breast cancer (Soria and Ben-Baruch 2008). However, in glioma, CCL7 (MCP-3) instead of CCL5 was responsible for the macrophage infiltration (Okada, Saio et al. 2009). CSF-1 has been confirmed in vivo as a mononuclear phagocyte chemoattractant in a murine breast carcinoma model (Lin, Nguyen et al. 2001; Wyckoff, Wang et al. 2007). The tumour microenvironment is rich in chemokines and cytokines, other factors, like fractalkine, CXCL8, CXCL12, and VEGF, have also been suggested to be implicated in the process of recruiting monocytes (Balkwill 2004; Allavena, Garlanda et al. 2008; Truman, Ford et al. 2008; Mantovani, Savino et al. 2009).

However, after tumour initiation or after monocytes are recruited into tumour sites, the signals that are responsible for tuning the phenotype of macrophages to M2 are not sufficiently understood. Various in vitro investigations have been performed. For example, using a co-culture assay, human ovarian cancer cells were able to polarize macrophages to a M2 phenotype, indicating that the signals can derive from tumour cells (Hagemann, Wilson et al. 2006). Further in vitro assays suggested that the effect of polarization of macrophages was from soluble factors released by tumour cells (Kuang, Wu et al. 2007). CCL2 and IL-6, the most prevalent cytokines in the tumour microenvironment, can induce polarization to a M2-type during the differentiation of human blood monocytes to macrophages in vitro (Roca, Varsos et al. 2009). In vitro co-culture also suggested that metastatic melanoma derived VEGF could induce the polarization of peripheral blood mononuclear cells from Th1 to Th2 response (Nevala, Vachon et al. 2009). The production of IL-10 and CSF-1 by a variety of tumours has been accepted which thus are speculated to be involved in the transition of macrophages from M1 to M2 (Hamilton 2008; Sato, Terai et al. 2011). The only direct demonstration of the mechanism of macrophage polarization in vivo was performed in the MMTV-PyMT (the expression of polyoma middle T oncoprotein under the control

of mouse mammary tumour virus) mouse model. It has been shown that IL-4, produced by CD4-positive T cells, accumulates in the tumour environment and contributes to the polarization of macrophages into M2 (DeNardo, Barreto et al. 2009). Therefore, further investigations are still required to understand the mechanisms of the well-known M2 polarization of TAM.

M2 type TAM have been found to contribute to tumour progression by supporting various stages of tumour development, including angiogenesis, tumour invasion and metastasis as well as suppressing adaptive anti-tumour immune response (Solinas, Germano et al. 2009). Among all the functions, angiogenesis is the most studied and well-known pro-tumour function of TAM. In human cancers, the accumulation of TAM is associated with increased angiogenesis in many tumours such as glioma, breast, bladder and prostate carcinoma (Leek, Lewis et al. 1996; Nishie, Ono et al. 1999; Hanada, Nakagawa et al. 2000; Lissbrant, Stattin et al. 2000). Macrophage depletion using liposome-encapsulated clodronate has been found to inhibit angiogenesis in different tumour xenograft models including melanoma, lung carcinoma and rhabdomyosarcoma (Zeisberger, Odermatt et al. 2006; Gazzaniga, Bravo et al. 2007; Kimura, Watari et al. 2007). TAM are normally recruited into the hypoxic regions of tumours which induces the expression of HIF-1-dependent molecules in macrophages, most with pro-angiogenic effects, such as VEGF and FGF family members (Salcedo, Wasserman et al. 1999; Lewis and Hughes 2007; Murdoch, Muthana et al. 2008). TAM from breast cancer exhibited an enriched transcription of angiogenic factors as demonstrated by high-density gene expression analysis (Ojalvo, King et al. 2009).

The role of TAM in tumour invasion and metastasis has been known for a long time and macrophages have been described as an ‘obligate partner for tumour cell migration, invasion, and metastasis’ (Condeelis and Pollard 2006). The first

conclusive demonstration of TAM having a role in metastasis was performed in a macrophage-deficient mouse model of breast cancer (Lin, Nguyen et al. 2001). Absence of macrophages did not affect the growth of the primary tumour but delayed the development into invasive, metastatic malignancy. Further investigations suggested a paracrine loop between macrophages and tumour cells in mammary tumours. Tumour cells synthesize CSF-1 which recruits macrophages and induces them to produce epidermal growth factor (EGF) which, in turn, activates tumour cell migration (Wyckoff, Wang et al. 2004). This paracrine loop is also instrumental in the macrophage-assisted tumour cell intravasation (Wyckoff, Wang et al. 2007). The effect of macrophages in promoting metastasis has been demonstrated in a murine ovarian tumour model which might be mediated by stromal VEGF production (Robinson-Smith, Isaacsohn et al. 2007). On the other hand, TAM can produce a range of matrix metalloproteases (MMP), such as MMP-2, MMP-7, MMP-9 and MMP-12, modulating the extracellular matrix to facilitate the metastasis process (Mantovani, Allavena et al. 2008).

The initiation and progression of tumours indicates the lack of immunological limitation and TAM are believed to have immunosuppressive and pro-tumour functions considering their M2 phenotype, although their roles in vivo have not been clearly demonstrated. Gene transcription studies of TAM indicated an upregulation of immunosuppressive cytokines IL-10 and TGF-β and a downregulation of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α thus demonstrating the M2 phenotype. The defective activation of NF-κB but functional interferon regulatory factor (IRF-3)/signal transducer and activator of transcription 1 (STAT1) is suggested to lead to the differential gene expression of TAM (Biswas, Gangi et al. 2006). Furthermore, several mechanisms have been identified to contribute to the inhibitory effect of TAM on the cytotoxic T cell response. Factors derived from tumours (hepatocellular carcinoma) and autocrine TNF-α and IL-10 can induce the expression

of PD-L1 (programmed cell death 1 ligand 1) proteins on the surface of the monocytes and the PD-L1 positive monocytes can effectively suppress cytotoxic T cell immunity thus contributing to the tumour growth in vivo (Kuang, Zhao et al. 2009). In ovarian carcinoma, macrophages and tumour cells produced chemokine CCL22 which mediates the influx of Treg cells into tumours. Treg cells are known to mediate immune tolerance and suppress the cytotoxic T cell response (Curiel, Coukos et al. 2004).

Taken together, TAM represent an important immune cell infiltrate in the tumour microenvironment and they play important roles in tumour progression, which has been suggested to recapitulate the trophic role of macrophages during development (Pollard 2009).

1.1.2 Metabolic stress

1.1.2.1 Metabolic stress in tumours

One of the key hallmarks of cancer is the sustained proliferative ability caused by genetic mutations (Hanahan and Weinberg 2011); this de-regulated and uncontrolled tumour cell proliferation demands constant and sufficient supplies of oxygen and nutrients. However, in rapidly growing tumours, insufficient oxygen and nutrient supply and consequently metabolic stress are prevalent throughout tumour development (White 2007). As tumours develop, before reaching 1mm in diameter, there is no blood supply through vasculature and the only source of oxygen and nutrients is passive diffusion whose limit is 150-220 μm thickness of tumour cell layers (Folkman 2006). Therefore, there is always oxygen and nutrient deficiency in the centre of the tumour mass. Using hypoxia as a marker for metabolic stress, hypoxia could be readily detected in the tumour mass 2 days after injection of tumour cells (Nelson, Tan et al. 2004). As the tumour progresses, angiogenesis will be switched on and the supply through newly established vasculature can significantly mitigate the

metabolic stress in tumours. However, unlike the healthy blood vessels in normal tissues, the vasculature in tumours is often abnormal and fragile which might intermittently collapse, causing continual disruption of nutrient supply into established tumours. Besides, tumour cells more than 200 μm away from open microvessels still suffer from shortages of oxygen and nutrients which thus form the 'perivascular cuffs' composed of viable tumour cells near the vessel and necrotic tumour cells away from the vessel, evidently demonstrating the metabolic burden of the tumour cells away from the supply tunnel (Judah 2003). An emerging hallmark of cancer is the ability of tumour cells to reprogramme their energy metabolism to undergo 'aerobic glycolysis' (Warburg and Dickens 1930; Warburg 1956; Warburg, House et al. 1956; Hanahan and Weinberg 2011), an inefficient way to produce ATP from glucose, compared with the mitochondrial oxidative phosphorylation in normal cells which is 9-fold more efficient but nonetheless a way of sustaining ATP production under hypoxic conditions. The short supply of energy and low efficiency of energy production, together with the highly proliferative nature of tumour cells, exacerbates the metabolic stress.

1.1.2.2 Metabolic stress and relevant cellular stress response

Through evolution, cells develop sets of machinery to respond to the stress of the environment. There are two main responses. One is the cellular homeostasis response which serves to adapt the cellular homeostasis to a particular environmental change, and the effect is permanent until the environment changes again. The other is the cellular stress response which, by sensing damages to the intracellular macromolecules or the redox (reduction-oxidation) condition caused by different types of stress, the cell will either repair the damaged macromolecules and restore the redox potential inside the cells to promote cell survival if damage is minor, or remove seriously damaged cells by inducing programmed cell death. The two responses are interconnected and complementary in response to environmental stresses (Kultz 2005).

In the cellular response to stress, some common regulatory proteins involved in different stress responses are conserved between species, including heat shock proteins (Kultz 2005). Heat shock proteins (Hsp) are molecular chaperones which, based on their molecular weight, can be categorized into Hsp100, Hsp90, Hsp70, Hsp60 and small Hsp (sHsp) families. As molecular chaperones, Hsp proteins can be induced and activated by many types of stresses to perform their functions of 'protein holding' and 'protein folding' thereby preventing protein aggregation and providing a transient protection (Calderwood, Khaleque et al. 2006). Hsp70 and Hsp90 families are principal holding proteins and can perform their cytoprotective function by binding to unfolded sequences in polypeptide substrates (Wegele, Muller et al. 2004; Mayer and Bukau 2005). After the holding proteins release their substrates, the folding proteins like Hsp60 and other chaperone proteins will start the ATP-dependent protein-folding process (Spiess, Meyer et al. 2004; Young, Agashe et al. 2004). However, sHsp, like Hsp27, can mediate both protein-holding and protein-folding in an ATP-independent manner (Jakob, Gaestel et al. 1993).

Apart from these indirect mechanisms for cellular protection, different lines of evidence have demonstrated that Hsp27 and Hsp70 can directly inhibit apoptosis as well. In the intrinsic apoptosis pathway, Hsp27 can inhibit the formation of the apoptosome and the activation of downstream caspases by binding to both cytochrome c and procapase-3 and by inhibiting the second mitochondria-derived activator of caspases (Smac) release from mitochondria (Bruey, Ducasse et al. 2000; Pandey, Farber et al. 2000; Concannon, Orrenius et al. 2001; Chauhan, Li et al. 2003). Hsp can inhibit the extrinsic apoptosis pathway by inhibiting DAXX, an adaptor protein linking the Fas receptor and the downstream signalling components (Charette and Landry 2000). Hsp70 can perform its anti-apoptotic activity at different stages of apoptosis signalling. Hsp70 can inhibit apoptosis by preventing cytochrome c release from mitochondria (Steel, Doherty et al. 2004). Hsp70 can also perform its

anti-apoptotic function by preventing Apaf-1 oligomerization and the recruitment of procaspase-9 to the Apaf-1 apoptosome (Beere, Wolf et al. 2000; Saleh, Srinivasula et al. 2000). Furthermore, Hsp70 has been shown to be able to bind to procaspases-3, -7 therefore suppressing the caspase-dependent apoptotic signalling (Komarova, Afanasyeva et al. 2004). Taken together, heat shock proteins are molecules with pro-survival and anti-apoptotic functions.

Another factor closely involved in the cellular stress response is p53. p53, also named tumour protein 53 (or TP53) after its molecular weight of 53 kDa, is one of the best known tumour suppressor proteins (Levine, Momand et al. 1991) and is also a transcription factor which regulates genes in a variety of different responses (Laptenko and Prives 2006). p53 has a role in receiving stress signals intracellularly and assessing the damage caused by stress which consequently leads to a cell fate decision. p53 can respond to a wide range of stresses, including nutrient deprivation, telomere erosion, hypoxia, DNA damage, ribosomal stress and oncogene activation (Levine, Hu et al. 2006). In the face of low or constitutive stress, p53 can arrest cell-cycle progression to allow the self repair of the cell and to promote cell survival by regulating cell cycle regulatory proteins such as p21. For example, it has been demonstrated that a low glucose level resulting from metabolic stress can activate p53 which contributes to the short-term survival of the cell under starvation conditions (Jones, Plas et al. 2005). If the stress is acute and intense, p53 can induce apoptosis to eliminate the damaged cells (Vousden and Lane 2007). p53 induces the expression of apoptotic proteins, such as BH3-domain proteins NOXA and PUMA, which lead to caspase activation and apoptosis (Jeffers, Parganas et al. 2003; Villunger, Michalak et al. 2003). On the other hand, p53 can also function as a BH3-only protein in the apoptosis pathway independent of transcription (Vousden 2005).

In the context of tumours, rapidly proliferating tumour cells are prone to suffer from

starvation-induced metabolic stress and several lines of evidence support the role of metabolic stress in inducing apoptosis of tumour cells. For example, in non-angiogenic lung carcinoma micro-metastases, high rates of tumour cell apoptosis can be detected in the midst of proliferating tumour cells where metabolic stress persists (Holmgren, O'Reilly et al. 1995). However, after the angiogenic switch, when tumours acquired oxygen and nutrient supplies from freshly developed vasculature, a marked (3- to 4- fold) decrease in tumour cell apoptosis was observed. The mechanisms underlying the trigger of apoptotic cell death by metabolic stress have not been determined. It has been suggested that metabolic stress could signal through the BH3-domain protein Bim (Nelson, Tan et al. 2004; Tan, Degenhardt et al. 2005). However, the mechanism of Bim activation following metabolic stress has not been clearly deciphered. Another possible mechanism of metabolic stress-induced apoptosis is through the apoptosis function of p53 as mentioned above (White 2007). In tumours with defects in apoptosis, metabolic stress could trigger autophagy which is an interruptible programmed cell death providing energy for cell survival by consuming self cellular organelles. Further inhibition of autophagy in tumours with apoptosis deficiency could lead to tumour cell death by necrosis (Degenhardt, Mathew et al. 2006).

1.2 Apoptosis and its micro-environmental conditioning

1.2.1 Cell death

The field of cell death has developed rapidly over the past 20 years and the definition of cell death by the Nomenclature Committee on Cell Death has shifted from morphological observation to biochemical and functional characterisation. Based on morphology, there are three well-defined types of cell death: apoptotic cell death, ‘autophagic’ cell death and necrotic cell death. Although other types of cell death have

been described, like mitotic catastrophe, cornification, anoikis, pyroptosis, entosis, they are either a characteristic of a very specific physiological process or have overlapping phenotypes with other types of death and have insufficient characterisation to be identified as a distinct type of cell death (Kroemer, Galluzzi et al. 2009). A new molecular definition of cell death based on the understanding of the biochemical and functional mechanisms of cell death was proposed (Galluzzi, Vitale et al. 2012). Cell death described in this section is focused on the three well-understood types of cell death with brief introduction of their molecular features.

The term apoptosis was first proposed by Kerr et al.(Kerr, Wyllie et al. 1972) to describe an active molecular process and it is morphologically characterised by the shrinkage of cellular volume, condensation of chromatin, fragmentation of the nucleus and formation of apoptotic bodies by membrane blebbing. There are two defined pathways of apoptosis: intrinsic and extrinsic.

The intrinsic pathway is normally activated by cellular stresses, like DNA damage and reactive oxygen species, which subsequently cause mitochondrial membrane permeabilization - the control event of intrinsic apoptosis (Kroemer, Galluzzi et al. 2007). The permeability of the outer mitochondrial membrane is under the tight control of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL, which inhibit the release of mitochondrial factors, such as cytochrome c, from the mitochondrial intermembrane space. Whereas pro-apoptotic members of the Bcl-2 family, such as Bax and Bak, under the control of Bcl-2 homology (BH)-3-only proteins, including Bid, Bad, Bim, Bik, HRK, PUMA and NOXA, tend to translocate to mitochondria and oligomerize within the mitochondrial outer membranes, forming pores thus leading to the release of mitochondrial factors into the cytosol (Gross, McDonnell et al. 1999). Cellular stress activates BH3-only proteins and when the activation exceeds the inhibitory effect administered by anti-apoptotic Bcl-2 family

members the result is Bax/Bak pore formation on the mitochondrial outer membrane, leading to the efflux of intermembrane space proteins into the cytosol (Taylor, Cullen et al. 2008). The released cytochrome c subsequently initiates the formation of the apoptosome by interacting with oligomerised apoptotic protease activating factor-1 (Apaf-1) and caspase-9. Apoptosome formation activates caspase-9 which eventually activates the executioner caspase, caspase-3 and leads to apoptotic cell death (caspase-dependent intrinsic apoptosis) (Li, Nijhawan et al. 1997; Zou, Henzel et al. 1997). Another consequence of the Bax/Bak pore formation is the release of other mitochondrial intermembrane space (IMS) proteins, such as apoptosis-inducing factors (AIF), endonuclease G (ENDOG) and high temperature requirement protein A2 (HTRA2). These factors can mediate intrinsic apoptosis in a caspase-independent manner. AIF and ENDOG can translocate from the cytosol to the nucleus and induce chromatin condensation and nucleosomal fragmentation of DNA (Susin, Lorenzo et al. 1999; Joza, Susin et al. 2001; Li, Luo et al. 2001; Buttner, Eisenberg et al. 2007). HTRA2 can trigger apoptosis in a caspase-independent manner by performing its serine protease activity (Hegde, Srinivasula et al. 2002).

There are two types of the extrinsic apoptosis pathways. One pathway is activated by the ligation of lethal ligands, such as CD95 ligand, tumour necrosis factor superfamily and TNF-related apoptosis inducing ligand, to the corresponding death receptors on the cellular membrane surface (extrinsic apoptosis by death receptors) (Wajant 2002). The ligation can activate initiator caspases, caspase-8 or -10 which can either directly cleave executioner caspases (caspase-3, -6 and -7) (in type I cells, lymphocytes) or signal through the mitochondrial-dependent apoptosis pathway by way of cleaving and activating BH3-only protein Bid and subsequently inducing mitochondrial outer membrane permeabilisation (MOMP) before activating executioner caspases (in type II cells) (Srinivasula, Ahmad et al. 1996; Scaffidi, Fulda et al. 1998; Yin, Wang et al. 1999; Barnhart, Alappat et al. 2003). Another mechanism that activates the extrinsic

apoptosis pathway is mediated by 'dependence receptors', including netrin receptors which can lead to apoptosis when their ligands are not sufficient (Mehlen and Bredesen 2011). The activation of dependence receptors activates PP2A (protein phosphatase 2A) and DAPK (death-associated protein kinase) before activating the initiator caspase, capase-9 and then effector caspases, caspase-3 (-6, -7) (extrinsic apoptosis by dependence receptors) (Bialik and Kimchi 2006; Mille, Thibert et al. 2009; Guenebeaud, Goldschneider et al. 2010).

However, recent developments in apoptosis research suggests two other possible but controversial pathways can trigger apoptosis (Green 2011). One is triggered by pathogen- or damage-associated molecular patterns to activate the initiator caspase, caspase-1 (Fernandes-Alnemri, Wu et al. 2007; Franchi, Eigenbrod et al. 2009). Caspase-1 activation can lead to cell death independently of caspase-3 activation (Brennan and Cookson 2000). In many cases, activated caspase-1 can cleave and activate the executioner caspase, caspase-7 and lead to apoptosis (Lamkanfi, Kanneganti et al. 2008). One feature of this type of cell death is that the active caspase-1 processes and promotes the secretion of interleukin-1β (IL-1β) and IL-18 (Sansonetti, Phalipon et al. 2000). However the designation of this type of cell death is controversial and it is also referred as pyroptosis (Galluzzi, Vitale et al. 2012).

Another controversial type of apoptosis is activated by specific cellular stresses, such as metabolic stress. The stress signals initiate the assembly of an activation platform named the PIDDosome which is composed of an adaptor protein, RAIDD (receptor-interacting protein-associated ICH-1/CED-3 homologous death protein with a death domain) and another protein PIDD (p53-induced protein with a death domain). Inactive caspase-2 monomers are recruited to the PIDDosome where they dimerise and are activated. Activation of caspase-2 leads to apoptotic cell death via the mitochondrial pathway by cleaving and activating Bid (Bouchier-Hayes 2010).

However a new idea has emerged that the activation of caspase-2 has to reach a certain threshold before engaging caspase-2-dependent apoptosis. The low levels of caspase-2 activation can induce other cellular responses, such as regulation of cell cycle progression (Galluzzi, Vitale et al. 2012). The controversy over the classification of the above two types of cell death needs to be further clarified in the future.

Autophagic cell death is defined by its morphology which does not include chromatin condensation but involves a massive cytoplasmic vacuolization. However, this term has been misinterpreted as cell death executed by autophagy while its true meaning is cell death with autophagy (Kroemer and Levine 2008). Its molecular definition is based on the lipidation of microtubule-associated protein 1 light chain 3 or an increase in the degradation of autophagic substrates such as sequestosome 1 (SQSTM1).

Necrotic cell death was described as a passive process resulting from extensive damage to cells with a loss of plasma membrane integrity and organelles swelling. Recent development indicates that necrosis can occur in a regulated manner, named regulated necrosis (Degterev, Huang et al. 2005; Hitomi, Christofferson et al. 2008; Cho, Challa et al. 2009; He, Wang et al. 2009; Zhang, Shao et al. 2009). The molecular features of regulated necrosis include death receptor signalling, caspase inhibition and RIP 1 and/or RIP3 activation (Galluzzi, Vitale et al. 2012).

1.2.2 Micro-environmental conditioning by apoptotic cells

In normal tissues, apoptosis is an important regulatory mechanism to clear old or damaged cells and maintain cell homeostasis in a non-phlogistic manner. Meanwhile, apoptotic cells can trigger a series of responses in both phagocytes and non-phagocytes with which they interact, which in turn affects the micro-environment of these cells thus the effects of apoptosis are far more extensive and complex than simply a regulated cell death program.

1.2.2.1 The interactions between apoptotic cells and mononuclear phagocytes

A key event in the non-phlogistic clearance of apoptotic cells is the immediate engulfment of dying cells to prevent secondary necrosis by either professional phagocytes or neighbour cells with engulfment ability. The swift engulfment of apoptotic cells is essential to immunological tolerance whereas delay or failure of clearance will result in inflammation and autoimmune diseases (Savill, Dransfield et al. 2002; Henson and Hume 2006; Elliott and Ravichandran 2010). The clearance process has been artificially rationalized into three phases termed '3Rs": recognition, response and removal (Gregory and Pound 2010).

Recognition

Since phagocytes can be neighbouring cells as well as recruited professional phagocytes, the recognition process requires not only altered molecular patterns on the surfaces of apoptotic cells ('eat-me' signals), but also chemoattractants released from apoptotic cells to recruit professional phagocytes in situ ('find-me' signals) (Gregory and Pound 2010; Ravichandran 2011). Several chemoattractants released from apoptotic cells have been identified, ranging from lipids and proteins to nucleotides, either in the soluble or microparticle-associated fashion. Two soluble lipid mediators, phospholipid lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P) are released from apoptotic cells and can recruit mononuclear phagocytes in vitro (Lauber, Bohn et al. 2003; Gude, Alvarez et al. 2008). Another factor, fractalkine (CX3CL1), can be released from apoptotic Burkitt's Lymphoma cells in a microparticle-associated manner at early stages of apoptosis and its function as a chemoattractant has been examined both in vitro and in vivo (Truman, Ford et al. 2008). Recently, ATP and UTP have been demonstrated to be released by apoptotic cells through pannexin-1 channels and their functions as chemoattractants was examined both in vitro and in vivo (Elliott, Chekeni et al. 2009; Chekeni, Elliott et al. 2010). Other mononuclear phagocyte

chemoattractants, such as ribosomal protein S19 (Yamamoto 2007), EMAPII (Shalak, Kaminska et al. 2001), MCP-1 (Kobara, Sunagawa et al. 2008) have also been identified.

The apoptosis programme is reflected on the cell membrane as alterations in the membrane composition (appearance or loss of certain molecules) thus distinguishing apoptotic cells from viable cells. The molecules that have newly appeared on the membrane of apoptotic cells, which are required for recognition by phagocytes are called 'eat-me' signals (Grimsley and Ravichandran 2003). The best known 'eat-me' signal is phosphatidylserine (PS) which moves to the outer leaflet of the plasma membrane during apoptosis (Fadok, Voelker et al. 1992). There are also membrane molecules inhibiting the recognition process, the loss of which can lead to successful recognition, and these molecules are named 'don't-eat-me' signals. For example, CD31 and CD47 are identified to be 'don't-eat-me' signals (Brown, Heinisch et al. 2002; Gardai, McPhillips et al. 2005). In the meantime, the recognition also requires phagocytes to be equipped with certain pattern recognition receptors, such as PS receptors (BAI1, TIM-4, and stabilin-2) (Miyanishi, Tada et al. 2007; Park, Tosello-Trampont et al. 2007; Park, Jung et al. 2008), CD14 (Devitt, Moffatt et al. 1998), CD36 and integrins (Savill, Hogg et al. 1992) as well as some bridging molecules, like milk fat globule-EGF factor 8 (MFG-E8). The complex tethering interaction between apoptotic cells and phagocytes forms the basis of further responses and is termed the 'phagocytic synapse' (Fadok, Bratton et al. 2001).

Response and removal

The recognition of apoptotic cells and the formation of tight tethering leads to the activation of two partially redundant pathways upstream of the Rho family GTPase Rac1, resulting rearrangement of actin and subsequent engulfment and internalization of apoptotic cells by phagocytes (Kinchen 2010). In mammalian cells, the first

pathway involves a transmembrane scavenger receptor MEGF-10, an adaptor protein (GULP) and an ABC transporter (ABCA1 or ABCA7), and the key mediators in the second pathway include an adaptor protein CrkII and a guanine nucleotide exchange factor Dock 180-ELMO (Kinchen and Ravichandran 2007). The internalized corpses will then enter the lysosomal digestion procedure for degradation and recycling.

Non-phagocytic effects

Apart from the phagocytic response, mononuclear phagocytes have other responses to apoptotic cells which affect the surrounding microenvironment. Mononuclear phagocytes generally have anti-inflammatory responses to apoptotic cells in vitro and different mechanisms of inducing these responses have been identified. Several lines of evidence indicate that by interacting with apoptotic cells, either binding or engulfing, mononuclear phagocytes can increase the secretion of anti-inflammatory cytokines, such as TGF-β and IL-10, and decrease the secretion of pro-inflammatory cytokines, such as TNF-α and IL-1β (Voll, Herrmann et al. 1997; Fadok, Bratton et al. 1998; McDonald, Fadok et al. 1999). Further investigations confirmed that mononuclear phagocytes only require direct contact with apoptotic cells to release anti-inflammatory mediators and the process is independent of the phagocytic event and any soluble factors (Cvetanovic and Ucker 2004; Lucas, Stuart et al. 2006). The contact-dependent anti-inflammatory effect ensures the induction of a local anti-inflammatory microenvironment surrounding the apoptotic cells rather than a systematic suppression of inflammation. However, molecules required for the contact between apoptotic cells and phagocytes have not been fully delineated although one molecule identified to be required for this process is PS (Huynh, Fadok et al. 2002; Gaipl, Beyer et al. 2003).

Other than the immuno-regulatory effect, exposure of mononuclear phagocytes to apoptotic cells can modulate the microenvironment in other ways. Hepatocyte growth

factor, which is a growth factor for bronchial epithelial cells and alveolar type II cells, can be released from macrophages that have engulfed apoptotic neutrophils in an acute lung injury model (Morimoto, Amano et al. 2001). Endothelial progenitor cells (EPC) can function as amateur phagocytes to engulf apoptotic cells and the engulfment promotes the proliferation and differentiation of the EPC, possibly facilitating the repair or construction of the endothelium although the molecular mechanism of this is unknown (Hristov, Erl et al. 2004). Another study demonstrated that the phagocytosis of apoptotic bodies leads to the secretion of VEGF from macrophages to promote the survival and growth of endothelial cells (Golpon, Fadok et al. 2004). These studies suggest that apart from clearing cell debris, conditioning of macrophages by apoptotic cells can contribute to the microenvironment in more ways than expected, including immune regulation, cell survival and growth as well as angiogenesis.

1.2.2.2 The interactions between apoptotic cells and other neighbouring cells

Apoptotic cells can also be cleared by cells other than professional phagocytes, such as neighbouring homotypic cells (Wyllie, Kerr et al. 1980; Parnaik, Raff et al. 2000), mesenchyal and epithelial cells (Saunders 1966; Wood, Turmaine et al. 2000; Monks, Rosner et al. 2005). Similar to professional phagocytes, non-professional phagocytes, such as epithelial cells and fibroblasts, and some non-phagocytic cells, such as neuronal and lymphoid lineage cells, also exhibit contact-dependent anti-inflammatory effects upon their exposure to apoptotic cells. However, unlike the interaction with professional phagocytes, the effect is independent of phosphatidylserine (PS), although the molecules essential to the contact process are still undefined. A model involving MAPK signalling has been speculated to be the mechanism underlying the ubiquitous apoptotic cell recognition by both phagocytes and nonphagocytes. Recognition of apoptotic cells activates p38 and JNK signalling pathways and inhibits ERK1/2, leading to the formation of inactive AP1 complexes

which sequester limiting transcriptional co-activators and exert transcriptional trans-repression (Cvetanovic, Mitchell et al. 2006).

1.2.2.3 Micro-environmental conditioning signals derived directly from apoptotic cells

Macrophages are not the only source of anti-inflammatory cytokines in the apoptotic environment; apoptotic cells can themselves release anti-inflammatory cytokines, such as IL-10, TGF-β, contributing to the immunosuppressive micro-environment (Gao, Herndon et al. 1998; Chen, Frank et al. 2001). On the other hand, each of these anti-inflammatory mediators can enhance the ability of macrophages to clear apoptotic cells, ensuring the immediate and successful clearance (Szondy, Sarang et al. 2003; Ogden, Pound et al. 2005). Furthermore, it has been shown that TFG-β can exert an inhibitory effect on innate immunity and tune the phenotypes of macrophages and neutrophils from M1, N1 to M2, N2, respectively (Flavell, Sanjabi et al. 2010). Other factors released from apoptotic cells are found to influence microenvironment in multiple ways. Lactoferrin which can be released from apoptotic cells, can suppress inflammation by inhibiting the migration of inflammatory neutrophils and eosinophils towards apoptotic cells (Bournazou, Mackenzie et al. 2009; Bournazou, Pound et al. 2009) and it also has multiple immunomodulatory and anti-inflammatory properties (Legrand, Elass et al. 2005). Fractalkine, a mononuclear phagocyte chemoattractant released from apoptotic cells, has been shown to have anti-inflammatory activities on monocytes as well as microglia, macrophages in the central nervous system (Zujovic, Benavides et al. 2000; Ollivier, Faure et al. 2003; Cardona, Pioro et al. 2006). Fractalkine has also been shown to have anti-apoptotic, pro-survival and pro-proliferation effects on human vascular smooth muscle cells (White, Tan et al. 2010).

A compensatory proliferation in surrounding cells induced by apoptotic cells has been

observed in Drosophila. However, this compensatory proliferation was only observed in response to apoptosis induced by stress or damage instead of developmental apoptosis. This phenomenon was observed more than three decades ago, in which X-ray irradiated cells underwent pyknosis while stimulating remaining survival cells to undergo additional proliferation (Haynie and Bryant 1977). The underlying mechanism was revealed in recent years. In apoptotic cells, two mechanisms have been found to be responsible for triggering compensatory proliferation depending on the developmental state of the apoptotic cells. The initiator caspase, Dronc, mediates the compensatory proliferation induced by apoptotic proliferating tissues whereas, effector caspases, DrICE and Dcp-1 mediate the compensatory proliferation induced by differentiating tissues (Kondo, Senoo-Matsuda et al. 2006; Wells, Yoshida et al. 2006; Fan and Bergmann 2008). Therefore, it has been proposed that apoptosis in proliferating and differentiating tissues leads to the activation of two different signalling pathways. In differentiating tissues, where effector caspases, DrICE and Dcp-1, are involved, Hedgehog is upregulated and released to mediate the neighbouring cell proliferation. In proliferating tissues, where the initiator caspase is in charge, Wingless (Wg; Drosophila Wnt homolog) and Decapentaplegic (Dpp; Drosophila homolog of TGF-β) are the possible compensatory proliferation mediators, and p53 and Jun N-terminal kinase (JNK) signalling are suggested to mediate their release (Fan and Bergmann 2008).

1.3 The tumour microenvironment in non-Hodgkin's lymphoma

1.3.1 Non-Hodgkin’s lymphoma

Non-Hodgkin’s lymphomas (NHL) are a collection of more than 60 heterogeneous malignant neoplasms composed of mainly aberrantly proliferating lymphocytes, exhibiting distinct morphological, immunophenotypic and molecular genetic features.

Some lymphomas can develop both within lymphoid organs, such as lymph nodes and spleen, and at extranodal sites. The standard system for the classification of NHL is known as the 'Revised European-American Classification of Lymphoid Neoplasms' (REAL) classification, which takes into account not only the immunologic features, but also genetic mutations together with the clinical manifestations. There are 13 most frequent NHL (Armitage and Weisenburger 1998). Despite all the discrepancy between them, they share the common property of being lymphocyte malignancies, either B cells or T cells, arising from cells at various stages of differentiation with disruption of different phases of normal development.

1.3.2 The tumour microenvironment in non-Hodgkin's lymphoma

In contrast to Hodgkin's lymphoma (HL) which is characterized by the classical Reed-Sternberg cells and the presence of only a few malignant cells (often less than 1% of cells in the tumour tissue) with abundant benign infiltrated immune cells (neutrophils, macrophages, eosinophils, mast cells, T and B cells), most of the cells in the NHL are malignant. Therefore, unlike the well recognized and studied roles of the tumour microenvironment in HL (Aldinucci, Gloghini et al. 2010), the role of the tumour microenvironment in NHL has largely been overlooked. For many years, the research and treatment for NHL has been focused on the genetically mutated neoplastic cells, since almost every NHL has been found associated with specific genetic mutations that disrupt the balance between normal cell birth and cell death.

With the gradually recognized role of the tumour microenvironment in favouring tumour progression, especially epithelial-related tumours, the microenvironment of NHL has gained increasingly more attention with time. The landmark discovery was made by gene profiling of follicular lymphoma and diffuse large B-cell lymphoma (DLBCL) which identified the correlation between the tumour-associated

non-malignant immune cells at diagnosis and the length of survival after various standard treatments. In DLBCL, Lenz et al. demonstrated that the 'stomal-1' gene cluster, which is rich in genes of extracellular matrix proteins and cells of monocytic lineage, is associated with a favourable prognosis whereas the 'stromal-2' cluster which is rich in genes involved in endothelial cell biology and adipocyte function indicates inferior survival (Lenz, Wright et al. 2008). Gene profiling of follicular lymphoma suggested that the tumour heterogeneity might originate from the infiltrated non-malignant cells: two signatures, both belonging to the microenvironment of the tumour, were related to prognosis; the 'immune response-1' signature, composed mainly of T cell genes, was linked to a favourable outcome and the 'immune response-2' signature, enriched with myeloid cell genes, indicated poor prognosis (Dave, Wright et al. 2004). All the above findings emphasized an important interplay between the tumour microenvironment and malignant cells and suggested that different components of the tumour microenvironment, including lymphoid stromal cells, macrophages, T cells, dendritic cells as well as chemokines and their receptors might all contribute to the development and progression of malignancy (Coupland 2011).

One of the hallmarks of cancers is their inherent ability to suppress cell death, including apoptosis (Hanahan and Weinberg 2011). However, oncogene activation, such as c-myc and e2f, does not only result in sustained proliferative signalling, it is also coupled with apoptosis, especially when growth factor is limited (Evan, Wyllie et al. 1992; Lowe, Cepero et al. 2004). The presence of apoptosis within tumours is broadly accepted (Wyllie 1985) and it is evidently demonstrated in several classes of NHL, prototypically Burkitt's lymphoma (Hori, Xiang et al. 2001; Zenger, Abbey et al. 2002; Park, Kim et al. 2005; Wang and Boxer 2005). In malignant NHL, apoptosis indices correlate significantly with lethality (Leoncini, Del Vecchio et al. 1993). Apoptotic index (AI) has been found associated with the grade of NHL, and high AI

predicts high grade of NHL (Symmans, Cangiarella et al. 2000). Nevertheless, the role of apoptosis in tumour microenvironmental conditioning has not been well studied. The abundance of apoptotic cells in NHL is always associated with a high rate of infiltrated macrophages. The histological stained tumour sections always contain a darkly stained, almost homogeneous background of tumour cells scattered with lightly stained macrophages with abundant cytoplasm, forming a characteristic 'starry-sky' pattern (Berard 1969; Hori, Xiang et al. 2001). Nevertheless, the functions of the massively infiltrated macrophages are still mostly unknown, aside from the clearance of apoptotic cells demonstrated by some tumour cell debris inside the cytoplasm of macrophages. In order to better understand the tumour microenvironment of NHL, Burkitt's lymphoma was adopted as a prototypical model (Figure 1.1).

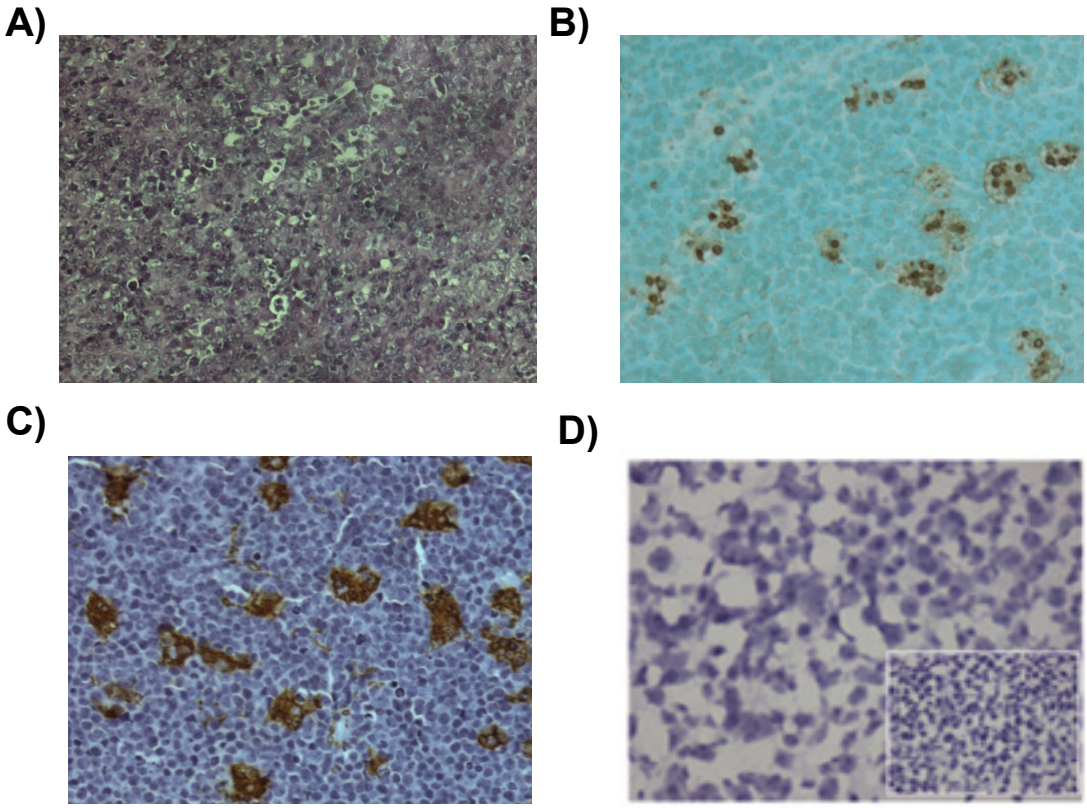


Figure 1.1 Microenvironment in Burkitt's lymphoma.
A) 'Starry-sky' morphology in BL (Kindly provided by Dr. John Pound). B) Apoptotic cells in BL revealed by TUNEL staining. C) Macrophages in BL revealed by CD68 staining. (B and C were kindly provided by Dr. Catriona Ford. D) The absence of neutrophils in BL revealed by Gr1 staining (Taken from (Bournazou, Pound et al. 2009)).

1.3.3 Burkitt's lymphoma (BL) as a prototypical model of NHL microenvironment

1.3.3.1 Burkitt's lymphoma

Burkitt's lymphoma (BL) is a highly aggressive, B-cell NHL named after Dennis Burkitt who first described the disease in Africa (Burkitt 1958). This type of BL later designated as endemic BL according to WHO, often arises in 4-7 year old African children and is normally associated with Epstein-Barr virus (EBV) which was the first virus to be described in association with human tumour pathogenesis. BL is also found at a low prevalence in sporadic areas around the world (sporadic BL); the exception being HIV positive patients (immunodeficiency-associated BL) which have a high incidence, around 40% (Ferry 2006). There are three variants based on their morphology and immunohistochemistry, classical BL, BL with plasmacytoid differentiation and atypical/Burkitt's-like lymphoma. Despite their difference in the nuclear pleomorphism and the cytoplasmic immunoglobulin, all three variants share some common morphological features: uniform and medium-sized neoplastic cells with abundant, RNA rich cytoplasm which is deep blue on Giemsa stain. The tumour cells are admixed with numerous pale stained infiltrated macrophages on H&E stain with many phagocytosed apoptotic tumour cells. The interspersed light-stained macrophages among the deep blue tumour cells compose the renowned 'starry-sky' morphology (Berard 1969).

The 'starry-sky' histological pattern is a prominent feature of BL but it is not specific to BL; other neoplasms, for example poorly differentiated malignant lymphomas and certain cases of acute leukaemia, share a similar histological appearance (Berard 1969). The defining feature of BL is the translocation of the c-myc gene, either juxtaposed with the IgH gene (80%) or with the gene for the kappa or lambda light chain (20%). The translocation results in the c-myc expression under the regulation of the

transcriptionally active Ig enhancer, which leads to de-regulated over-expression of the c-myc gene (George 1983). Myc protein is a DNA-binding transcription factor and its target genes constitute 15% of known genes therefore the over-expression of Myc protein can increase cell growth and proliferation as well as apoptosis and cell senescence, which is consistent with the high rates of proliferation as well as apoptosis in BL both in vivo and in vitro (Dang, O'Donnell et al. 2006; Allday 2009).

Evidence accumulated over time suggests that BL was derived from peripheral germinal centre B-cells. Biopsies from non-endemic BL were selectively involved with germinal centres, indicating there was some relationship between neoplastic cells and normal germinal centre B lymphocytes (Mann, Jaffe et al. 1976). Further evidence was provided by comparing immunofluorescence staining of a spectrum of cell surface antigens in endemic BL and a population of tonsillar germinal centre B cells which carried similar surface markers, positive for CD10, CD20 and CD77 while negative for CD23 and CD39 (Gregory, Tursz et al. 1987). In contrast to mature, circulating B cells, BL cells share other similar properties to germinal centre B cells, including prominent cell loss and lack of expression of the anti-apoptotic protein bcl-2 (Pezzella, Tse et al. 1990; Henderson, Rowe et al. 1991; Liu, Johnson et al. 1992). Further finding of the presence of hypermutated regions in translocations in sporadic BL proved further that germinal centre B cells were the origin of the neoplasm (Cario, Stadt et al. 2000).

1.3.3.2 Burkitt's lymphoma microenvironment

Despite the c-myc translocation and its perennial activation, rather than being a tumour of autonomous growth, cross-talk between neoplastic B cells and their microenvironment is an integral part in the tumour pathogenesis. In the BL microenvironment, there is a delicate balance between pro- and anti-apoptotic signals. Histological analysis of BL sections has revealed a high rate of innate apoptosis

(Ogden, Pound et al. 2005). Since apoptotic cells are engulfed instantly and degraded rapidly by professional and amateur phagocytes in vivo (Savill 1997), the frequent detection of apoptotic tumour cells in the presence of abundant infiltrated macrophages suggests there is a high rate of apoptotic cell death occurring in BL. When cultured in vitro, cells derived from BL show a similar tendency to apoptosis (Gordon, Knox et al. 1993). However, their inherent tendency to undergo apoptosis has to be balanced by anti-apoptotic signals from the BL micro-environment and therefore net tumour growth can be achieved. In vitro investigations using a group I BL cell line to model the BL micro-environment suggested that survival signals can be generated from different components of the BL micro-environment including fibroblasts, follicular dendritic cells, macrophages and activated T cells and even from BL cells themselves (Levens, Gordon et al. 2000). It was also discovered that macrophages conditioned by IL-10, expressed by both tumour cells and infiltrated macrophages, can release B cell-activating factor of the TNF family/B lymphocyte stimulator (BAFF/BLyS) which is a B cell survival factor to promote BL cell growth and proliferation (Ogden, Pound et al. 2005).

Previous work has identified that the persistence of apoptotic BL cells in the BL micro-environment, instead of being a tumour-suppressive mechanism, can actually promote tumour growth. On the one hand, apoptotic BL cells can regulate the immune cell infiltration by releasing a combination of chemokines to selectively recruit immune cells: 1) fractalkine is a 'find-me' signal to attract mononuclear phagocytes (Truman, Ford et al. 2008); 2) lactoferrin is a 'keep-out' signal to inhibit neutrophil and eosinophil infiltration (Bournazou, Mackenzie et al. 2009; Bournazou, Pound et al. 2009). On the other hand, macrophages, conditioned by IL-10, exhibit an enhanced ability to clear apoptotic cells (Ogden, Pound et al. 2005). Since clearance of apoptotic cells was found to polarize macrophages, recruited macrophages in BL can provide an important mechanism in tumour microenvironmental conditioning. As mentioned

earlier, lactoferrin has multiple immunomodulatory and anti-inflammatory properties (Legrand, Elass et al. 2005), whereas fractalkine has anti-inflammatory activities as well as anti-apoptotic, pro-survival and pro-proliferation effect (Zujovic, Benavides et al. 2000; Ollivier, Faure et al. 2003; Cardona, Pioro et al. 2006; White, Tan et al. 2010), they might also contribute to tumourigenesis in currently unidentified ways.

Although the presence of apoptosis in BL and its role in tumour microenvironmental conditioning is recognized, the mechanisms driving apoptosis in situ are not completely understood, apart from the identified role played by c-myc. BL often presents as a rapidly growing solid tumour and is among the fastest growing tumours observed, with a short in vitro doubling time of 19-36 hours (Iversen, Iversen et al. 1974) and more than 95% of cells have been observed to be actively engaged in cell cycle progression, as revealed by positivity for Ki-67 staining of BL sections (Cooper, Frank et al. 1966). Unlike normal cells, which can check and regulate the rate of proliferation and metabolism in response to hypoxia and nutrient limitation (Lum, Bauer et al. 2005; Brahimi-Horn, Chiche et al. 2007; Semenza 2007), BL cells have de-regulated over-expression of myc oncogene which continually drives cellular proliferation despite limited supplies of oxygen and nutrients, exacerbating the metabolic stress in the tumour mass. However, the role of metabolic stress in triggering the induction of apoptosis in BL has not been addressed.

Although apoptotic cells can release ‘find-me’ signals to attract mononuclear phagocytes, the stage of apoptosis at which the cells start to release those ‘find-me’ signals has yet to be identified. However, some studies have demonstrated that the release of chemoattractants (eg. LPC and ATP) can be dependent on the apoptotic effector protease, caspase-3 (Lauber, Bohn et al. 2003; Chekeni, Elliott et al. 2010), suggesting that there is cross-talk between the apoptosis program and the mechanisms controlling chemoattractants release. It is appreciable that the early release of

'find-me' signals would facilitate the mobilization and infiltration of the responsive phagocytes to locate and clear apoptotic cells quickly. In vivo analysis of surface membrane PS positive BL cells also reveals the presence of very early apoptotic cells with condensed chromatin and loss of electron density of the cytoplasm as well as cells with characteristics of other stages of apoptosis, including late apoptotic cells with advanced chromatin condensation, nuclear pyknosis and cellular fragmentation (Van den Eijnde, Boshart et al. 1997). Thus, another question raised is whether early stage apoptotic cells or even pre-apoptotic cells can release 'find-me' and 'keep-out' signals to regulate the infiltration of phagocytes, and therefore participate in the tumour microenvironmental conditioning.

1.3.3.3 Burkitt's lymphoma cell lines

Various BL cell lines have been established in vitro from both EBV positive and negative Burkitt's lymphoma in an attempt to study BL properties in vitro and they have been categorized into three different groups according to their cell surface phenotypes (Rowe, Rooney et al. 1985). Group I BL lines grow as a single cell suspension and express the BL marker CD10 and CD77 without any B cell activation markers, which reflects the biopsy features. Group II cultures contain small cellular aggregates and express some of the activation markers, like CD23, CD30, CD39 and CD70, apart from the BL marker CD10 and CD77. Group III lines are normally present as large cellular clumps and only expressed high levels of activation markers without any of the BL markers. Despite the variations between the three groups of cell lines, they all display a c-myc chromosome translocation, t(8:14) or t(8:22), demonstrating their malignant identity as Burkitt's lymphoma.

1.4 Generation of macrophages from murine embryonic stem cells

For the study of macrophages in vitro, especially their interaction with apoptotic cells, an appropriate model is required. Currently commonly used in vitro macrophage models are derived from two main sources: tumour-derived cell lines and primary cells isolated from animals both of which have their own advantages and disadvantages. Tumour-derived cell lines, such as RAW267.4 and THP-1 cells, are at different stages of maturation so differ in phenotypes and functions. Although they have the advantage of genetic tractability and a limitless supply of homogenous population of cells, their oncogenic nature makes them somewhat different from the normal state. For example, some cancer cell lines cannot be activated by IFN-γ which is an important factor regulating macrophage functions (Stojdl, Lichty et al. 2000). However, they still preserve some functions of their normal counterparts which make them still widely used as macrophage or macrophage precursor models. On the other hand, macrophages from primary sources, either peritoneal macrophages or macrophages derived from primary precursor cells in human blood or murine bone marrow, reflect normal macrophages with normal physiology. However, the cells derived from living organisms are always limited in numbers, thus restraining the availability for experiments. Furthermore, primary cells have limited replicative ability and are resistant to genetic manipulation which confines them from being applied in the studies of the molecular mechanisms in macrophage research (Oliveira, Lima et al. 2003).

Advances in the culture and directed differentiation of embryonic stem cells (ES cells) provides a better in vitro macrophage model. ES cells are pluripotent cells derived from the inner cell mass of blastocysts which can differentiate into any type of cells except those of the placenta. Since the derivation process is different from conventional transformation methods, ES cells preserve their normal physiology and

can therefore contribute to embryo development if returned to the embryo environment (Smith 2001). Cell lineages, belonging to the mesoderm, endoderm and ectoderm have already been generated from murine ES cells (Keller 2005). Furthermore, another advantage of ES cells is their amenability to genetic manipulation in both gain-of-function and loss-of-function studies. Gain-of-function can be achieved by over-expression of specific genes (Chambers, Colby et al. 2003) and conventional homologous recombination and RNAi can result in loss-of-function (Hamaguchi, Woods et al. 2000; Ma, Ramezani et al. 2003). The expression can be controlled both temporally and spatially by using site-specific recombinases which can avoid the potential effect of genetic manipulation on ES cell differentiation (Kyba, Perlingeiro et al. 2002). Therefore, macrophages derived from ES cells have the advantage of being normal cells with high purity as well as the limitless feature of in vitro cell lines. Furthermore, they can be applied to molecular mechanism studies that require genetic manipulation.

1.5 Aims of the project

As discussed above, macrophages conditioned by apoptotic cells can acquire some vital functions similar to those of tumour-associated macrophages, such as anti-inflammatory and immunosuppressive effects, release of survival and growth factors and participation in angiogenesis, together with the direct microenvironmental conditioning effects by apoptosis, it is reasonable to speculate that in NHL, apoptotic cells can condition the tumour microenvironment to promote tumour growth (Gregory and Pound 2010; Gregory and Pound 2011). Since the delay or failure to clear apoptotic cells in vivo can lead to the activation of the immune system and may cause anti-tumour immune responses (Nagata, Hanayama et al. 2010), it is also rational to hypothesize that apoptotic cells might release microenvironmental conditioning signals at a very early stage, such as stressed, pre-apoptotic stage, to ensure the prompt clearance of apoptotic cells as well as the timely conditioning of the microenvironment.

The main aim of the project was to test the hypothesis that stressed, pre-apoptotic cells can contribute to tumour microenvironmental conditioning in Burkitt's lymphoma. Considering the microenvironment of BL which is characterised by a high rate of spontaneous apoptosis, abundant infiltration of macrophages but no neutrophil infiltration (Truman, Ogden et al. 2004; Bournazou, Pound et al. 2009), the microenvironmental conditioning effects assessed were focused on the recruitment of macrophages and neutrophils and the generation of the anti-apoptotic/mitogenic signals which are essential for net tumour growth under the circumstances of a high rate of spontaneous apoptosis.

In order to generate an in vitro model of pre-apoptotic tumour cells and mimic the physiological trigger (metabolic stress) that induces apoptosis in the tumour microenvironment, a high density stress model was devised which employed a group I BL cell line. The mechanisms underlying the apoptosis-inducing effect of high density stress was also examined. Secondly, cell-free supernatants generated from stressed BL cells were assessed for their ability to recruit macrophages and neutrophils and the identity of the molecule responsible for the recruitment and its release mechanism were examined. Finally, the effect of the cell-free supernatants generated from stressed BL cells on the survival/proliferation of viable cells was assessed to examine the presence of any anti-apoptotic/mitogenic signals as well as their identities.

Although interactions between apoptotic cells and phagocytes have been widely studied, the underlying molecular interactions have not been fully elucidated. Further understanding of these may be facilitated by using ESDM, especially because of their amenability to genetic manipulations. Therefore, the second aim of the project was to develop an optimal protocol for in vitro differentiation of murine ESC into ESDM. ESDM were characterised phenotypically and their functions, including chemotaxis towards and phagocytosis of apoptotic cells, were investigated.

Chapter 2 Materials and Methods

2.1 Cells

2.1.1 Isolation and culture of primary cells

2.1.1.1 Mouse bone marrow-derived macrophages (BMDM) preparation

Mouse femurs (Balb/c) were removed by dissection, and cleaned in ethanol in a sterile hood. The bones were then placed into $DMEM_{supp}$ (see Appendix 1) and the ends were cut off the bones. Bone arrow was flushed with $DMEM_{supp}$ using a 10ml syringe (BD Plastipak) and a 26G needle (BD Microlance) into a 50ml conical tube (BD Falcon). Cells were then passed through a 23G needle (BD Microlance) using a syringe and plated onto 95 mm Petri dishes in 20 ml $DMEM_{supp}$. The medium was changed after 2 hours and then after 4 days. On day 7, differentiated macrophages were used for experiments.

2.1.1.2 Peritoneal cell preparation

Peritoneal lavage was performed in Balb/c mice using sterile PBS and the cells were centrifuged and re-suspended in 20 ml $DMEM_{supp}$. Then they were plated onto 95 mm Petri dishes for 2 hours before use.

2.1.1.3 Human blood leukocyte separation

Human leukocytes were obtained from peripheral blood of healthy donors. Peripheral venus blood was drawn using 19-gauge needle from an antecubital vein and transferred to polypropylene centrifuge tubes containing 3.8% (w/v) sodium citrate for anticoagulation. Anticoagulated blood was centrifuged at 350g for 20 min with brake-off to fractionate plasma.

The supernatant which was platelet-rich plasma was collected into sterile glass universal bottles supplemented with $CaCl_2$ (final concentration 40 mM) and incubated at 37°C for 1 h to clot and generate autologous serum. Autologous serum was heat-inactivated at 56°C for 45 min and centrifuged at 2000g for 10 min to remove any precipitate.

Plasma-free cell pellet was uniformly resuspended in dextran (final concentration 0.6%, w/v) (Pharmacia, Buckinghamshire, UK) and left to stand undisturbed for 15-30 min to allow erythrocytes to sediment under gravity. The supernatant which was rich in leukocytes was collected and centrifuged at 220g for 6 min. Three different concentrations of Percoll (55%, 68% and 79%) were prepared from the stock (Pharmacia) using phosphate buffered saline (PBS) (Ca^{2+}/Mg^{2+}-free). For each 30 ml of blood, 68% Percoll was carefully layered over 5 ml 79% in a conical polypropylene tube. The leukocytes pellet was resuspended in 55% Percoll (Cells from 30 ml of blood should be resuspended in 3 ml of 55% Percoll) and 3 ml of the cell suspension was layered on top of the gradient. The gradient was centrifuged at 700g for 20 min with brake-off. The mononuclear phagocytes were at the 55%/68% Percoll interface and polymorphonuclear cells (neutrophils and eosinophils) at the 68%/79% Percoll interface.

Mononuclear phagocytes were harvested after removing most of 55% Percoll layer. Harvested mononuclear cells were washed and centrifuged at 220g for 5 min. The pellet was resuspended in serum-free Iscove's Modified Dulbecco's Medium containing penicillin, streptomycin and glutamine (IMDM+P/S+Glu) at 4×10^6 cells/ml. Cells were pated onto 25 cm^2 tissue culture flasks (10 ml per flask) and incubated for one hour at 37°C/5%CO_2. The medium was then aspirated and IMDM+P/S+Glu containing 10% heat-inactivated autologous serum was added. The cells were cultured for 2 days to generate day 2 human blood monocyte-derived

macrophages (HMDM).

Polymorphonuclear cells were harvested after removing most of 68% Percoll layer. Harvested polymorphonuclear cells were analysed by cytospin and neutrophils represented >95% of the population (Figure 4.7 A). The cells were used directly for chemotaxis assays.

2.1.2 Maintenance of cell lines

2.1.2.1 Culture of human Burkitt's lymphoma (BL) cells

Two human BL cell lines were used, BL2 (EBV-negative) (Lenoir, Vuillaume et al. 1985) and BL2-bcl2. BL2-bcl2 are BL2 cells stably transfected with bcl2 to suppress apoptosis (Wang, Grand et al. 1996). The BL2-bcl2 line used in the present study was a stable bcl-2 transfectant established by C D Gregory and S. Douglas. The cells were cultured in suspension in BL media (Appendix 1). These cell lines were cultured at 37°C, 5% CO2 and maintained in the exponential phase (10^5-10^6/ml).

2.1.2.2 Culture of human monocytic cell lines, THP-1 and MonoMac 6

THP-1 cells were established from the blood of a boy with acute monocytic leukemia (Tsuchiya, Yamabe et al. 1980). The cells were cultured in THP-1 culture medium (see Appendix 1) at 37°C, 5% CO_2 and maintained in the exponential phase (10^5-10^6/ml).

MonoMac6 cells were established from peripheral blood of a patient with monoblastic leukemia with characteristics of mature monocytes (Ziegler-Heitbrock, Thiel et al. 1988). The cells were cultured in MonoMac6 culture medium (See Appendix 1) at 37°C, 5% CO_2 and maintained in the exponential phase (0.3-1.0 × 10^6/ ml).

2.1.2.3 Culture of murine embryonic stem cells (ES cells)

Murine embryonic stem cell line E14, derived from the 129/Sv mouse strain, was used. It was originally derived on a feeder layer and has now been adapted to grow on gelatine-coated tissue culture dishes (Handyside, O'Neill et al. 1989). Fetal calf serum (FCS) was batch tested using a colony forming assay and was designated normal (growth-supporting) FCS and Diff (differentiation-promoting) according to the variance in its ability to promote ES cell self-renewal. Normal FCS was used for routine ES cell culture and Diff was used for ES cell differentiation. ES cells were cultured in ES cell culture medium ($GMEM_{FCS}$, see Appendix 1). Its sterility was tested by tryptose phosphate broth (Gibco) before use. ES cells were cultured in $GMEM_{FCS}$ supplemented with 100 units /ml leukemia inhibitory factor (LIF) at 37 °C, 5% CO_2. ES cells were seeded at the density of 1×10^6 cells in 10 ml media in gelatinized T25 cm^2 flask and were usually ready to be passaged 2 days later when the cells were confluent. The LIF–containing supernatant was from COS7 cells transfected with a LIF-expressing plasmid and its efficacy was tested according to published methods (Jackson, Taylor et al. 2010).

2.1.2.4 Culture of L929 cells and in-house production of L929 cell-conditioned medium (L929 CM)

Colony stimulating factor-1 (CSF-1) is highly expressed in the mouse lung fibroblast line L929 (Burgess, Metcalf et al. 1985) and L929 cell-conditioned media is widely used as a source of CSF-1. L929 cells were thawed and expanded in L929 cell culture medium (Appendix 1). The adherent cells were passaged 1 in 30 every 3 to 4 days at confluence using trypsin/EDTA to detach the cells. To harvest the conditioned medium, cells were cultured 3 days after they became confluent. The conditioned medium was harvested directly from the adherent monolayer of cells and filtered through a 0.22μm membrane to remove cell debris. The L929 CM was stored at - 20°C.

2.2 Induction and evaluation of apoptosis

2.2.1 Induction of apoptosis by UV-irradiation

In order to induce apoptosis, 100mJ/cm^2 UV-B was used as a trigger (Truman 2005). A UV meter (SLS) was used to determine the intensity of UV exposure and the meter was placed under the cover of the flask used for the cells to mimic the exact intensity of UV irradiation that the cells would receive. Time required for UV treatment was calculated as follows (in seconds): 100,000 μJ/meter reading = time required. UV-treated BL2 cells were returned to culture at 37°C (in a humidified 5% CO2 atmosphere) for 3 hours to induce high rate of apoptosis.

2.2.2 Induction of apoptosis by staurosporine

BL2 cells from exponentially growing cultures were centrifuged for 5 min at 400g and washed twice in Dulbecco's PBS (without Ca^{2+} /Mg^{2+}/ phenol red, PAA) and once in serum-free RPMI 1640 (with L-glutamine and penicillin/streptomycin). The cells were resuspended in serum-free RPMI 1640 at 1×10^6 cells/ml and apoptosis was induced by addition of 1 μM staurosporine (Calbiochem) followed by 3 hour incubation at 37 °C, 5% CO_2.

2.2.3 Induction of apoptosis by cold-shock

BL2 cells were washed in serum-free RPMI 1640, resuspended at 5x10^6 cells/ml in HypoThermosol (BioLife Solutions, Bothell, WA, USA) in a 50 ml conical tube (BD Falcon) and incubated at 4˚C for 18hrs. Cells were then washed twice in serum-free RPMI, resuspended at 2x10^6 cells/ml in $ESDM_{Cult}$ and cultured for 90 min at 37˚C, 5% CO_2 to induce apoptosis.

2.2.4 Assessment of apoptosis by Annexin V-FITC/Propidium iodide staining

The level of apoptosis was evaluated by binding of Annexin V (AxV)-FITC (Invitrogen) and uptake of propidium iodide (PI) (Sigma-Aldrich), by FCM on a Beckman XL flow cytometer. In short, 0.5×10^6 cells were put in a polystyrene 75×12 mm tube, washed with 1ml ice-cold Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ in dH_2O, pH 7.4), centrifuged and re-suspended in 100 µl binding buffer. 2.5 µl of Annexin V-FITC was added per tube and incubated for 15 min in the fridge. 400 µl of binding buffer was further added into the tube after incubation. 10 µl of propidium iodide (20µg/ml stock) was added to each tube 45 seconds prior to flow cytometric analysis.

2.2.5 Assessment of apoptosis by measuring mitochondrial membrane potential

Measurement of mitochondrial membrane potential was performed by flow cytometry using a Mito-ID® Membrane Potential Cytotoxicity Kit, Enzo Life Sciences. In brief, 0.8×10^6 cells/ml BL2 cells treated with 4 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 30 mins were used as a positive control for the membrane potential loss. CCCP was equilibrated to room temperature before use. 0.8×10^6 cells/ml staurosporine treated BL2 cells were used as a positive control for apoptosis. Untreated BL2 cells (0.8×10^6 cells/ml) were used as a negative control. One hour stressed cells were assessed. All the samples were centrifuged and washed once with 4ml 1× Assay Solution. 10 ml of 1 × Assay Solution was composed of 1ml 10 × Assay Buffer, and 0.2 ml 50× Assay Buffer into 8.8 ml deionized water. The supernatant was carefully removed and the pellet was gently resuspended in 50 µl Mito-ID™ MP Detection Reagent. Samples were incubated in dark and protected from light for 15 min at room temperature before being analyzed via flow cytometry using a

488nm laser with the FL1 channel for the green fluorescent signal of the dye, the FL2 channel for the orange fluorescent signal from the dye.

2.3 Detachment of macrophages from Petri dishes

Cell culture media was removed and macrophages were washed twice with Hank's BSS (without Ca^{2+} /Mg^{2+}/ phenol red, PAA) and incubated in the detachment buffer (Appendix 1) at 4°C for 20 mins before being detached with a cell lifter (Corning). Cells were washed twice with Hank's BSS (without Ca^{2+} /Mg^{2+}/ phenol red) before use.

2.4 Cytocentrifugation

ESDM were detached, washed and blocked in Dulbecco's PBS (without Ca^{2+} /Mg^{2+}/ phenol red, PAA) with 10% normal mouse serum (v/v, NMS) (Harlan) at 4°C for 30 mins. After cytocentrifuging on a cytospin (300rpm, 5 mins) (Thermo Shandon), the slides were dried and fixed in ice-cold acetone at 4°C for 10 mins. The slides were air-dried again before further staining.

2.5 Diff-Quick staining

Slides were dipped in Diff-Quick solution I 10 times and then in solution II 10 times (Reagena, Finland). Then they were rinsed in distilled water and air-dried overnight before mounting.

2.6 Immunostaining

2.6.1 Immunoperoxidase staining of macrophages

The primary antibodies and isotype controls used were from Serotec and included rat-anti-mouse CD68 (MCA1957, purified, working concentration 5μg/ml),

F4/80(MCA497GA, purified, 2µg/ml) and CD11b (MCA711, purified, 2µg/ml). The negative control for CD68 was Rat IgG2a (MCA1212, purified, 5µg/ml), for CD11b and F4/80 was rat IgG2b (MCA1125, purified, 2µg/ml). The secondary antibody was biotinylated anti-rat IgG (H+L) (mouse absorbed) (Vector, BA-4001). The concentration used was 2.5µg/ml.

After being washed twice in PBS, slides were blocked in Dakoprotein (Dako) for 10 minutes at room temperature. Slides were then incubated with primary antibodies overnight at 4°C, followed by two washes in PBS and incubation with secondary antibodies for 30 minutes at room temperature. After two more washes, Vector ABC reagent, which is conjugated to peroxidase, was added to the slides for 30 minutes at room temperature, and Dako DAB chromagen was added for 5 minutes at room temperature. Finally, the slides were washed with distilled water and counterstained in haematoxlin for 2.5 minutes, washed in distilled water again and air-dried before mounting with Histomount (Friel, Fisher et al. 2006).

To quantify the percentage of positively stained cells, 10 non-overlapping fields from one slide at 200× magnification were chosen and the numbers of both positively and negatively stained cells were quantified per field. Data were expressed as the percentage of positive cells ±SD per field.

2.6.2 Immunofluoresence staining of macrophages analysed by flow cytometry

Macrophages were detached and blocked with normal mouse serum (NMS) (10% v/v) in staining buffer (normal goat serum (NGS, 5% v/v, Biosera) in Dulbecco's PBS (without Ca^{2+} /Mg^{2+}, PAA)) on ice for 10 minutes. 3 x 10^5 cells were stained in 100µl final volume per sample at 4°C for 30 mins. Antibodies against F4/80 (0.5µg /ml, PE-conjugated, Caltag), CD11b (1µg /ml, PE-conjugated, BD Pharmingen) and

CD11c (2.5µg /ml, FITC-conjugated, eBioscience) were used to analyse the phenotype of macrophages. The unfixed samples of BMDM were analysed with FACS Calibur (Becton-Dickinson), and the unfixed samples of ESDM and peritoneal cells were analysed with Epics XL (Beckman Coulter). The forward and side light scatter data was also recorded. Flow cytometry list mode data was analyzed with Flowjo (Treestar).

2.7 ELISA

2.7.1 TNF-alpha sandwich ELISA assay

The level of TNF-alpha in ESDM culture supernatant was examined by sandwich ELISA (DuoSet ELISA Development kit, R&D systems). Fresh $ESDM_{cult}$ (see Appendix 1) was used as a negative control and ESDM supernatant from cells stimulated with recombinant mouse Interferon-gamma (5ng/ml, R&D systems) and E.coli 0111 B4 LPS (1ng/ml, Sigma) for 24 hours was a positive control. All the samples and standards were set up in triplicate. The assay was performed following the manufacturer's instructions. In brief, 96-well ELISA plates (Mantovani, Allavena et al.) were coated with capture antibody (goat anti-mouse TNF-α, 0.8µg/ml, 100µl per well), sealed, and incubated overnight at room temperature (RT). Plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) and blocked with reagent diluent (1%BSA in PBS, pH 7.2-7.4, 300µl per well) at RT for 1 hour before being thoroughly washed 3 times. 100µl of supernatant or standard was added per well and incubated for 2 hours at RT. Following 3 washes, 100µl of the Detection Antibody (biotinylated goat anti-mouse TNF-α, 0.2µg/ml, 100µl per well) was added per well and incubated for 2 hours at RT. The plates were washed 3 times and were incubated for 20 minutes with 100µl of Streptavidin-horseradish-peroxidase (1:200 dilution) per well at RT. After another 3 washes, 100µl of substrate solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) was added per well for 20 minutes at RT. The reaction was stopped by adding 50µl of stop solution (2N H_2SO_4) to each well. Absorbances were

measured on an Anthos 96-well plate reader at a wavelength of 450nm.

2.7.2 S1P competitive ELISA

S1P ELISA was performed using a Sphingosine-1-Phosphate Assay Kit purchased from Echelon and following the manufacturer's instruction. The assay was based on the principle of a competitive ELISA. In brief, a 96-well plate was pre-coated with S1P and blocked at room temperature for 1 hour to reduce non-specific binding. Diluted S1P standards and samples were mixed with the provided biotin-labelled anti-S1P antibody before the mixture was added to the S1P-coated plate. S1P on the plate and in the samples competed for binding to the antibodies. Following 1 hour incubation at room temperature and four washes with PBS, streptavidin-HRP was added to the plate to bind to the biotin-labelled anti-S1P antibodies that were bound to the plate. After an additional 1 hr incubation at room temperature and washes, TMB substrate was added to the plate for 30 min incubation before the reaction was stopped by addition of 1 N sulphuric acid. Absorbances were measured on an Anthos 96-well plate reader at a wavelength of 450nm.

2.8 Western blotting

2.8.1 Whole cell lysate preparation

Cells were collected from experimental cultures, centrifuged at 400 g for 5 min and supernatant was removed as much as possible. Cell pellets were resuspended in lysis buffer (1 mM EDTA, 10 mM HEPES, 1% Triton-X-100) (12.5 µl of the buffer per 1×10^6 cells) and transferred to eppendorf tubes. 1 in 12.5 diluted protease inhibitor cocktail (Sigma P8340) was added per sample before samples were vortexed intensively for 20 seconds. Samples were then incubated on ice for 30 min, followed by centrifugation at 14,000 g, 4°C for 10 min. Cellular debris-free supernatant was transferred to fresh 1.5 ml eppendorf tubes and stored at - 80°C.

2.8.2 Bradford protein assay

Protein concentrations in whole cell lysate samples were assessed using Bradford assay (Bio-Rad). Samples were thawed and diluted 1/ 20 or 1/50 in PBS. Human IgG diluted in PBS (ranging from 1mg/ml to 15.63 μg/ml), were used as standards. The standards and samples were set up in triplicate, 10 μl per well into 96-well plate. 190 μl filtered 1 in 5 dilution of Bradford's assay solution was supplemented to each well. The assay colour developed instantly and was read on plate reader at 595 nm. Protein concentrations were calculated according to standard curves.

2.8.3 Sample preparation

Each sample contained 30 μg cell lysate proteins, 2.5 μl 4 ×NuPAGE Sample Buffer (Invitrogen) and 1 μl 10 ×NuPAGE Reducing Agent, and the total volume was made up to 10 μl by adding dH_2O. Samples were denatured at 70°C for 10 min before loading into gels. 15-well 1.0 mm 10% bis-tris gel was run using the NuPAGE kit (Invitrogen).

2.8.4 Electrophoresis

Electrophoresis of samples was performed using the Invitrogen NuPAGE system and reagents, according to the manufacturer's protocol. SeeBlue Plus2 pre-stained standard (Invitrogen) was used as the molecular weight standard. Prepared samples were loaded into NuPAGE pre-cast 15-well 1.0 mm 10% Bis-Tris gels and gels were run at a constant voltage of 200 V for 1 hr in NuPAGE MOPS SDS buffer.

2.8.5 Western Blotting

Afterwards, proteins on gels were transferred on to PVDF membranes using 10% methanol in the NuPAGE transfer buffer, at 30 V for 75 min. The membranes were blocked in 5% milk/PBS-0.1%Tween-20 for 1 hr at room temperature. Next, the

membranes were probed with different primary antibodies overnight at 4 °C and antibodies used were in Table 2.1.

Table 2.1

Primary antibodies	Producers	Catalog no.	Dilution used	Band size
Mouse anti-hu/mo Hsp27	Abcam	Ab2790	1/1000	24kDa
Mouse anti-hu/mo Hsp70	Abcam	Ab5439	1/5000	70-78 kDa
Mouse anti-hu/mo Hsp90	Abcam	Ab1429	2 μg/ml	90kDa
Moue anti-hu/mo HIF-1α	Novus Biologicals	NB100-131	1/1000	100-120kDa
Rabbit anti-hu/mo caspase 3	Cell Signalling Technology	9662	1/1000	35kDa, 19+17kDa
Mouse anti-α actin	Sigma	A1978	1/10000	40kDa
Rabbit anti-phospho-Akt Ser473	Cell Signaling Technology	4060	1/2000	60kDa
Rabbit anti-phospho-Akt Thr308	Cell Signaling Technology	2965	1/2000	60kDa
Rabbit anti-pan-Akt (C67E7)	Cell Signaling Technology	4691	1/2000	60kDa

Primary antibodies were diluted in 5% milk/PBS-0.1%Tween-20 (TBS-Tween system was used for blotting phosphor-Akt). Subsequently, the probed membranes were washed 6 ×5 min in PBS with 0.1%Tween-20 and incubated in secondary antibodies diluted in 5% milk/PBS-0.1%Tween-20 for 1 hr at room temperature. List of Secondary antibodies is in Table 2.2.

Table 2.2

Secondary antibodies	Producers	Catalog no.	Dilution used
anti-mouse-HRP	Amersham NXA931	351908	1/10,000
Anti-rabbit-HRP	Sigma	A6154	1/5000

The blots were then washed 6 × 10 min in PBS with 0.1% Tween-20. Specific protein bands were visualised using Amersham ECL reagents (GE Healthcare) and developed onto X-Ray film using a film developer (Konica SR×101A Developer, Med Imaging Ltd).

2.9 High density stress model

BL2 or BL2/bcl-2 cells were harvested from exponentially growing cultures and washed with Dulbecco's PBS (without Ca^{2+} /Mg^{2+}/ phenol red, PAA) twice, and with serum-free RPMI 1640 once. The cells were then resuspended in serum-free RPMI at 100 million cells/ml and put into either T25 or T75 cm^2 tissue culture flask. The cells were cultured at 37 °C (in a humidified 5% CO_2 atmosphere) for 1 hour or for longer.

2.10 Glucose level measurement

Glucose level in the culture medium was measured using Accu Check Aviva Nano (Roche) following the manufacturer's instructions. Briefly, for one measurement, the meter was switched on and one test strip was inserted into the testing slot. A small amount of liquid was pipetted and loaded onto the test strip. Displayed reading was recorded.

2.11 Generation of stress supernatant

After one hour of high density stress, cells were harvested and centrifuged at 400g, 4°C for 5 min to remove cells. The supernatant was then centrifuged at 870g, 4°C for 10 min to remove cell debris and stored at -80°C.

2.12 ATP concentration measurement

ATP concentration was measured using bioluminescent assay kit (Sigma) following the manufacturer's instructions. The assay was based on the principle that the light

emitted during the oxidation of D-luciferin, catalysed by firefly luciferase, is proportional to the amount of ATP present when ATP is the limiting reagent. White plates (non-treated, Nunc) were soaked in 1N hydrochloric acid (Acors Organics, 1N standard solution) for at least 4 hr to reduce the background luminescence and rinsed with deionized water several times before dried completely. Assay mix, which contained luciferase and luciferin, was diluted 1 in 100 with dilution buffer and 100 µl was loaded into each well of pre-treated white plate (assay mix and dilution buffer were provided with the kit). ATP standards were serially diluted with sterile water (Baxter) from 1×10^{-6} M to 1×10^{-8} M and sterile water was a standard blank control. Stressed BL2 and BL2/bcl-2 supernatants were examined and serum-free RPMI was a blank control. 100 µl of either ATP standards or samples was added to the pre-loaded assay mix solution using multichannel pipette, immediately before the plate was read on the luminescence reader (BioTek, Synergy HT).

2.13 ATP channel antagonists

In the experiment examining channels responsible for ATP release, after washing, BL2 cells were treated with several ATP channel antagonists (Table 2.3) before stress. After high density stress, the same procedure was applied to generate stress BL2 supernatant.

Table 2.3

	Supplier	Catalog no.	Working concentration used	Target
Gadolinium Chloride	Sigma	439770-5G	50 µM	Maxi-anion channel
Aranchidonic Acid	Sigma	A3555-10mg	20 µM	Maxi-anion channel
Carbenoxolone disodium salt	Sigma	C4790-1g	100 µM	Connexin and pannexin channels
Probenecid	Sigma	P8761-25G	1 mM	Pannexin Channel

Gadolinium chloride and carbenoxolone disodium salt were dissolved in water to make up stock solutions, 50 mM and 100 mg/ml respectively. Arachidonic acid and probenecid were dissolved in DMSO to make up stock solutions, 30 mM and 1 M respectively (Liu, Sabirov et al. 2008; Chekeni, Elliott et al. 2010). They were added directly to BL2 cells to make the working concentrations immediately before experiment.

Since Gd^{3+} is known to interfere with the luciferin-luciferase reaction (Boudreault and Grygorczyk 2002), the luciferin-luciferase assay mix was supplemented with 600 μM EDTA when the samples were treated with $Gd^{3+.}$

2.14 Isolation and incubation of microparticles from stress supernatant

Stress supernatant was transferred to ultracentrifuge tubes and centrifuged at 100,000g, 4ºC, for 30 min. The supernatant was removed, and the cell pellet, which was a greasy, almost transparent dot sticking on the side of the ultracentrifuge tube, was resuspended in serum-free RPMI 1640 of the same volume as the original stress supernatant and vortexed until the pellet was dissolved. Since the whole ultracentrifuge process was non-sterile, in order to culture isolated microparticles for ATP measurement, resuspended microparticle solution was passed through a 0.22 μm syringe filter before incubation. Microparticles were incubated at 37°C, 5% CO_2 for 1 hr, 5 hr, 8 hr, and 24 hr, and ATP level at each time point was measured using ATP bioluminescent assay. The separated microparticle-free stress supernatant was used as a control. Fresh serum-free RPMI 1640 was the 0 hr control.

2.15 Cell proliferation assay

Cells were seeded at $0.2x10^6$/ml in medium appropriate to the cells being used in the

assay and were cultured at 37 °C (in a humidified 5% CO_2 atmosphere) for up to 96 hr. Viable and total cell concentrations were determined using Trypan Blue exclusion every 24 hr.

In the serum-free culture system, the cell proliferation assay was performed in different concentrations of X-VIVO 20 (Lonza) which was diluted in serum-free RPMI 1640.

2.16 Cell cycle analysis using propidium iodide

Cell cycle was monitored by measuring DNA content using the DNA-binding fluorescent dye propidium iodide (PI). Each sample contained $1.5x10^6$ cells. Samples were washed once in Dulbecco's PBS (without Ca^{2+} /Mg^{2+}/ phenol red, PAA) and centrifuged at 870g for 5 min. Cell pellets were resuspended in 300 µl PBS to a single cell suspension and drop-wise 700 µl 'fridge-cold' absolute ethanol (VWR) to each sample while vortexing. Samples were then kept at 4°C in the fridge for at least 30 min for fixation. After washing three times with 1 ml PBS, samples were resuspended in 380 µl PBS and stained by adding 20 µl 1mg/ml PI. They were incubated at room temperature for 30 min in dark before binding analysis on an Epics XL flow cytometer (Beckman Coulter).

2.17 Preparation of S1P

Sphingosine-1-phosphate was purchased from Avanti Polar Lipids. 2.64 ml of methanol (Fisher Scientific) was added into 1 mg of S1P and was heated in a 50°C water bath, vortexed and sonicated in a water-bath sonicator (Decon) until S1P was dissolved. The solution was aliquoted at 100 µl per glass vial and dried to powder under a stream of N_2 gas. Glass vials were then sealed with stoppers and foil caps with a crimper before storing frozen at – 80°C. Each dried aliquot contained 100 nmol of S1P.

To dissolve S1P aliquots for experiments, no more than 1 hr before use, 1ml serum-free RPMI containing 4 mg/ml low-endotoxin bovine serum albumin (BSA) (PAA) (carrier protein) was added to each vial of S1P and warmed in a 37°C water bath for 20 min. The vial was vortexed vigorously until S1P was dissolved. The solution was 0.22 μm filtered before being added to cells for use in the cell proliferation assay.

2.18 Limulus amoebocyte lysate (LAL) assay

In-house-produced L929 conditioned media was endotoxin screened by chromogenic LAL assay (QCL-1000, Lonza), following the manufacturer's instruction. The principle of the assay is as follows: Gram-negative bacterial endotoxin can catalyze the activation of a proenzyme in the LAL which can further catalyze the colourless substrate into a yellow product. The absorbance of the final product is in proportion to the amount of endotoxin present which can be calculated from a standard curve. Disposable endotoxin-free glass assay tubes (10×75 mm, Cambrex #25-415) were used. Four concentrations of LAL were prepared to make the standard curve: 0.1, 0.25, 0.5 and 1.0 EU/ml, and LAL reagent water was used as the blank control. All the standards and samples were analysed in duplicate. Samples or standards were dispensed into assay tubes (50 μl per tube) in a 37°C waterbath. At time 0 (T=0), 50μl of LAL was added to each assay tube and the solutions were mixed thoroughly. Reagents were pipetted in the same order from tube to tube and at the same rate to ensure the exact timing for each reaction tube. At T=10 minutes, 100μl of substrate solution (pre-warmed to 37°C) was added and mixed thoroughly. At T=16 minutes, 100μl of stop reagent was added and mixed before the absorbance was read at 405 nm on an Anthos 96-well plate reader.

2.19 Self-renewal assay

In order to determine the presence of undifferentiated ES cells present in a cell suspension, a self-renewal assay was performed. ESDM and E14 cells were seeded, at

different densities, 10^3, 10^4, 10^5 and 10^6 cells per well, in duplicate, onto gelatin-coated 6-well plates in $GMEM_{FCS}$ containing 100 units /ml LIF. The cells were cultured for 1 day at 37 °C (in a humidified 5% CO_2 atmosphere) before being transferred to $GMEM_{FCS}$ with or without LIF. After a further culture of 5 days at 37 °C (in a humidified 5% CO_2 atmosphere), culture media was removed and colonies were fixed and stained for alkaline phosphatase (AP leukocyte kit, Sigma) which is highly expressed by undifferentiated ES cells (Pease, Braghetta et al. 1990). The procedures carried out were as follows: colonies were fixed with 1 ml fixative per well (25ml citrate solution, 65ml acetone, and 8ml 37% v/v formaldehyde) which was warmed to room temperature before use. Citrate solution was supplied with the kit. The fixative was applied for 30 seconds before being carefully washed away with deionized water for 45 seconds. The substrate (Appendix 2) was mixed thoroughly and 1ml was applied into each well, followed by 15 minutes incubation in the dark. After staining, the colonies were rinsed carefully with water, drained and air dried before they were examined microscopically for stem cell, mixed, or differentiated colonies.

The colonies were categorised and scored based on the status of alkaline phosphotase staining. E14 cells were used as a positive control for different colonies. ESDM growing in $GMEM_{FCS}$ with LIF for one day and then in $ESDM_{cult}$ for five days were used as a control for ESDM viability during the assay process.

2.20 Flow cytometry-based phagocytosis assay

2.20.1 Labelling of BL2 cells with far-red fluorescent dye

BL2 cells from culture were centrifuged at 400g for 5 minutes at RT and washed twice in Dulbecco's PBS (without Ca^{2+}, Mg^{2+}, without Phenol Red, PAA). Cell pellet was resuspended in serum free RPMI at 20×10^6 cells/ml. Resuspended cells were mixed with far-red fluorescent dye DDAO-SE (Invitrogen) at the ratio of 1µl of dye per 10×10^6 cells and incubated at 37°C (in a humidified 5% CO2 atmosphere) for 15

minutes. The cells were centrifuged, resuspended at 4×10^6 cells/ml in serum free RPMI and placed into tissue culture flasks.

2.20.2 Induction of labelled BL2 cell apoptosis

As described in sections 2.2.1 and 2.2.3. Afterwards, the cells were centrifuged and resuspended in $ESDM_{cult}$ at 2×10^6 cells/ml.

2.20.3 Assessment of apoptosis by Annexin V-FITC/Propidium iodide staining

As described in section 2.2.4.

2.20.4 ESDM preparation

A new ES cell line, GFP#7a, which expresses eGFP constitutively (Gilchrist, Ure et al. 2003), was used and it was established from the ES cell line E14tg2a subclone IV. ESDM expressing endogenous eGFP were derived from GFP#7a following the same differentiation protocol and used as phagocytes in the phagocytosis assay. The assay was adapted from the published flow cytometry-based phagocytosis assay (Jersmann, Ross et al. 2003)

D7 ESDM were detached, washed and resuspended in fresh $ESDM_{cult}$ at 4×10^5 cells/ml and seeded into 48-well plates (0.5ml per well). Plates were incubated at 37°C (in a humidified 5% CO2 atmosphere) for 24 hr.

2.20.5 Phagocytosis incubation and sample preparation for flow cytometry

Before the assay, medium was removed from ESDM by aspiration and ESDM were washed gently with PBS before 0.5ml of prepared BL2 cell suspension (DDAO-SE-labelled and incubated for 3 hours after UV-treatment) was loaded. The phagocytosis incubation was either 0.5 or 1 hr at 37°C (in a humidified 5% CO2 atmosphere). ESDM only and BL2 cells only were used as controls. All the samples were set up in triplicate.

After incubation, media was removed and wells were washed gently with PBS once. 0.5 ml of Trypsin/EDTA (PAA) was added to each well, followed by 15 minutes of incubation at 37°C (in a humidified 5% CO2 atmosphere) and then 15 minutes of incubation on ice to detach ESDM. Finally, the cells were harvested from each well by vigorous pipetting for analysis by flow cytometry.

2.21 Chemotaxis assay

Chemotaxis was assayed using an adapted from Boyden chamber assay (Figure 2.1). A transwell, which is a cylindrical cell culture insert (6.5 mm diameter), was placed inside a well of a 24-well plate; the insert contained a porous, polycarbonate membrane which divided the well into upper and lower chambers. Polycarbonate membrane transwells with either 5μm or 8 μm pore size (Costar Corning), depending on the migratory cells, were used in the assay. RPMI 1640 (Gibco) supplemented with L-glutamine (2mM, PAA), penicillin (100 IU/ml, PAA), and streptomycin (100μg/ml, PAA) was used as chemotaxis assay medium. 100 μl input migratory cells were seeded in the upper chamber and 600 μl chemoattractants were loaded in the lower chamber. Time for incubation at 37 °C (in a humidified 5% CO_2 atmosphere) varied depending on cell type. Samples are set up in duplicate.

The detailed protocols for the assay are given below.

2.21.1 Initial ESDM chemotaxis protocol based on which optimization was performed:

The chemotaxis assay was performed with transwells of 8μm pore size membrane. Recombinant human C5a (R&D systems) (10 ng/ml) was used as a positive control and assay medium, a negative control. C5a and negative control were loaded into lower chambers and incubated with transwells at 37ºC for 2 hr to allow equilibration before loading ESDM. ESDM were detached from Petri dishes, washed twice with Hank's BSS and once in assay medium before resuspending in assay medium at 2×10^6/ml. ESDM were added to the upper chambers and were incubated at 37 °C (in a humidified 5% CO2 atmosphere) for 4 hr. At the end of the assay, non-migrated cells were removed from the inside of transwells with cotton buds and the transwells were fixed with 100% methanol for 10 min, air-dried and subsequently stained with Diff-Quick Blue (Reagena, Finland) for 20 min.

Since migrated ESDM adhered to the underside of the transwell (no cells were observed in lower chambers), the migration of ESDM was presented as the mean of the number of migrated cells per high power field from ten random areas (×400 magnification) on the underside of the transwell using an inverted microscope (Axiovert 25, Zeiss, UK).

2.21.2 ESDM chemotaxis assay protocol after optimization

Only two steps were altered from the initial protocol. 1) ESDM were detached from Petri dishes on day 6 and cultured in 20 ml fresh $ESDM_{cult}$ medium in suspension culture in Teflon pots for 24 hr before ESDM were harvested and prepared for experiments. 2) No equilibrium was performed and ESDM and chemoattractants were

loaded at the same time.

2.21.3 THP-1 chemotaxis assay

For THP-1 chemotaxis assay, 5 μm pore size membrane was used and THP-1 cells were prepared at 2×10^6/ml. Most of the migrated THP-1 cells were in lower chambers (very few were attached to the underside of transwells), therefore, at the end of the assay, 450 μl of media from one lower chamber was harvested for cell counting using flow cytometry. The data were presented as the mean fold increase compared to the control.

Suramin sodium salt (Sigma) was dissolved in water and 1mM freshly-made suramin solution was used to antagonize P2 receptors on THP-1 cells (working concentration 100 μM) at room temperature 30 mins before chemotaxis assay.
Apyrase (New England Biolabs) was 1 in 2000 diluted with working concentration of 25 mU/ml.

2.21.4 Neutrophil chemotaxis assay

For neutrophil chemotaxis assay, 5 μm pore size membrane was used and neutrophils were prepared at 1×10^6/ml. 100 nM fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine, Sigma) was used as a positive control, and assay medium was a negative control. The assay incubation time was 60 min. At the end of the assay, migrated neutrophils on the membrane were stained and quantified under microscope.

2.21.5 Preparation of other chemoattractants

Recombinant human CCL5 (Prospec) was reconstituted at 100 μg/ml in sterile deionized water and the aliquots were stored at -20°C. Recombinant human VEGF

(R& D systems) was reconstituted at 10µg/ml in sterile PBS containing 0.1% low endotoxin BSA and aliquots were stored frozen at – 20°C. They were diluted in assay medium to working concentration for experiments. Recombinant CCL2 (R&D) was reconstituted at 100 µg/ml in sterile PBS and the aliquots were stored at -20°C. fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine, Sigma) was reconstituted in DMSO to a 10 mM stock solution and aliquots were stored at -20°C.

2.22 Statistical analysis

Results from multiple experiments are presented as means + standard errors of the means (SEM). Mann-Whitney test was performed to compare the difference between two groups of quantitative data and calculate the P value. P values of 0.05 or less were considered statistically significant. Data analysis was performed using GraphPad Prism 5 (GraphPad, La Jolla, USA).

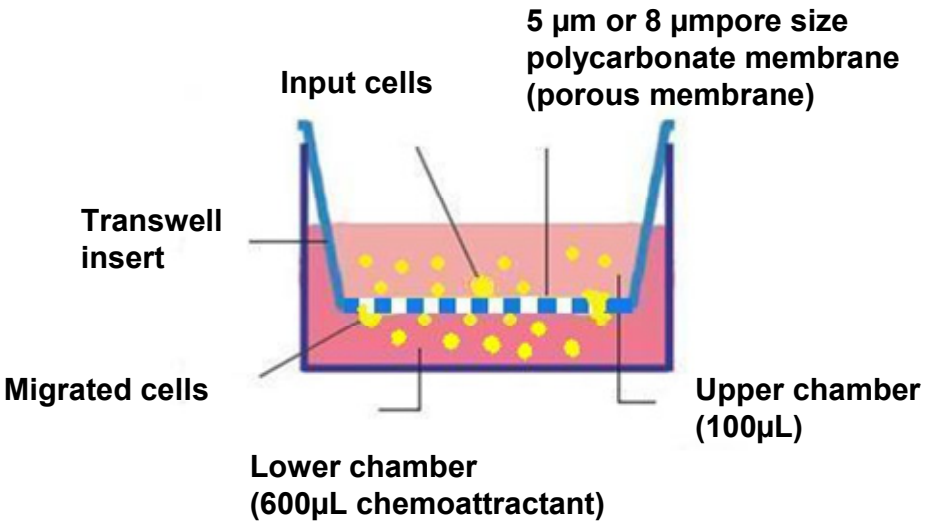


Figure 2.1 Schematic diagram of chemotaxis assay.

Chapter 3 Results: High density stress-modelling the pre-apoptosis induced by metabolic stress

3.1 High density stress model

In an attempt to address the question whether stressed (pre-apoptotic) cells can promote tumour growth via microenvironmental conditioning, a stress model that induced pre-apoptotic cells was required. In solid tumours, metabolic stress is a common feature due to nutrient, growth factor, and oxygen deprivation (Judah 2003) and it has been shown that such stress is a potent trigger of apoptotic cell death (Degenhardt, Mathew et al. 2006), therefore, to mimic the in vivo tumour microenvironment and model the pre-apoptosis caused by metabolic stress, a stress model was established in which cells were incubated at high density for 1 hr.

3.2 One hour high density stress did not induce apoptosis of BL2 cells

Before and after BL2 cells were subjected to one hour high density stress, the exposure of phosphatidylserine on the outer plasma membrane leaflet and the loss of mitochondrial membrane potential, which are events happening in the early stage of apoptosis (Castedo, Hirsch et al. 1996), were assessed.

Staining with AxV and PI was performed before and after treatment of the cells (Vermes, Haanen et al. 1995). Untreated cells were used as controls. The early apoptotic cell population, which was AxV positive and PI negative, did not increase after the one hour stress, indicating that one hour high density stress did not induce the apoptosis of BL2 cells (Figure 3.1).

A cationic mitochondrial membrane potential dye, which exists as fluorescent green aggregates in the cytosol and fluorescent orange aggregates in the energized mitochondria, was used. The dye is released from mitochondria when the mitochondrial membrane potential is compromised and diffuses into the cytosol, becoming exclusively fluorescent green (Figure 3.2). Unstained BL2 cells were negative in both orange (FL2) and green (FL1) fluorescence channels, excluding the possible influence by the autofluorescence of BL2 cells. Untreated cells were used as controls. Control cells were positive for both green and orange fluorescence, suggesting that they had energized mitochondria. When BL2 cells were treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which is a mitochondrial membrane potential perturbation agent, they lost the orange fluorescence and became only positive for green fluorescence, suggesting that the mitochondria lost their membrane potential. BL2 cells, which were treated with staurosporine for three hours, became exclusively fluorescent green as well, indicating that apoptotic cells lost their mitochondrial membrane potential. However, for one hour stressed BL2 cells, they were still double positive for green and orange fluorescence, implying that these cells still had intact mitochondrial membrane potential and they did not undergo apoptosis.

Results from the above two independent assessments were consistent in their conclusion that one hour high density stress did not induce the apoptosis of BL2 cells.

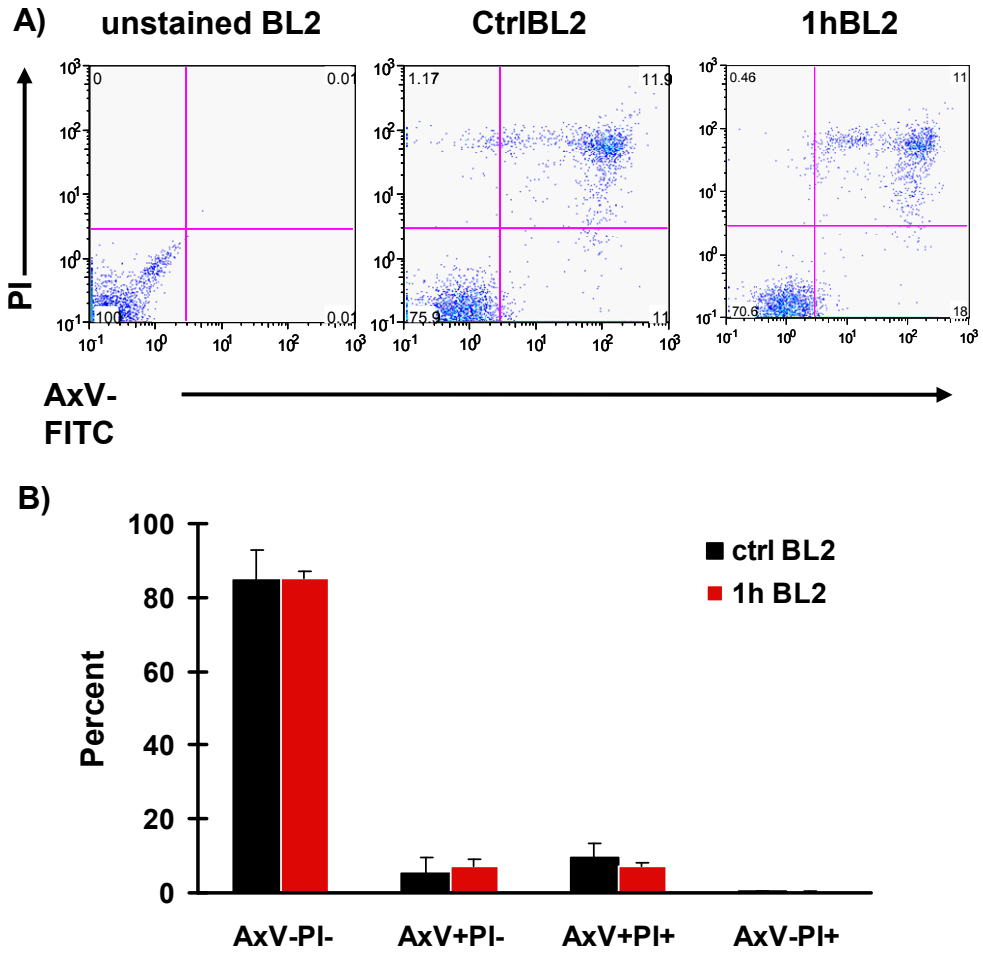


Figure 3.1 1 hour high density stress did not induce the exposure of phosphatidylserine on BL2 cells.

BL2 cells were subjected to one hour high density stress (1h BL2). PS exposure on cell surface was assessed by AxV staining and cell membrane permeability was assessed using trypan blue exclusion. Cells before high density stress were controls (ctrl BL2). A) Light scatter plot of AxV/PI staining. One representative result from over twenty similar is shown. B) Summary graph shows the percentage of viable (AxV-PI-), early apoptotic (AxV+PI-) and late apoptotic (AxV+PI+) cells in ctrl BL2 (black bars) and 1h BL2 (red bars). Error bars indicate SEM for three experiments.

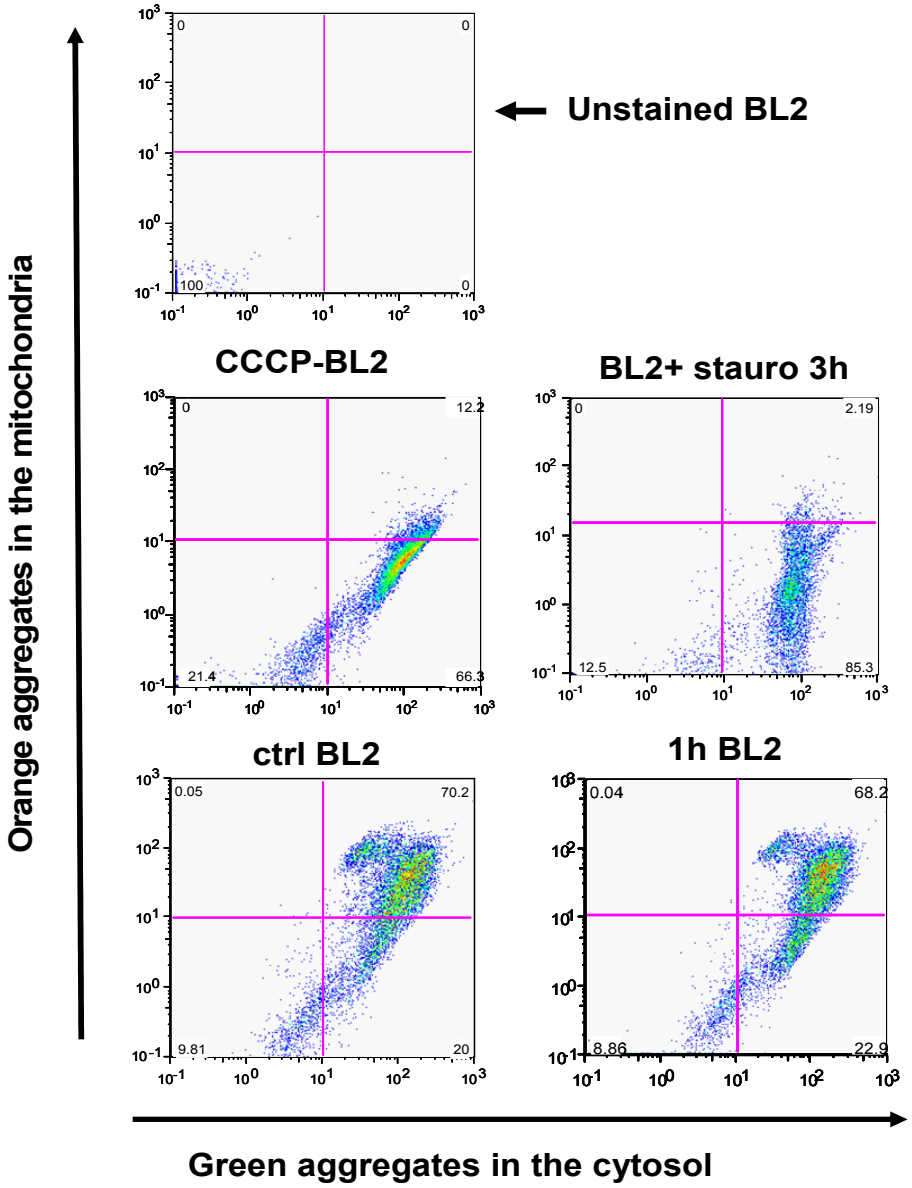


Figure 3.2 1 hour high density stress did not affect mitochondrial membrane potential of BL2 cells.

The mitochondrial membrane potential of BL2 cells after 1 hour high density stress was assessed by a dual-emission mitochondrial membrane potential-sensitive dye whose fluorescence emission shifts from orange to green upon the collapse of the membrane potential. CCCP-BL2 : BL2 cells treated with CCCP (a membrane potential perturbation reagent); BL2+stauro 3h: BL2 cells treated with staurosporine and incubated for 3 hours; ctrl BL2: BL2 cells before high density stress; 1h BL2: BL2 cells from 1 hour high density stress. One representative experiment of three is shown.

3.3 One hour high density stress generated pre-apoptotic cells which were committed to apoptosis

Since one hour stress did not induce apoptosis in BL2 cells, further experiments were carried out to identify whether cells had been committed to apoptosis under these conditions. BL2 cells harvested from exponential phases were put into one hour high density stress and afterwards, the cells were cultured at lower density: 1×10^6 cells/ml, for up to eight hours. Apoptosis and cell death were assessed by AxV/PI staining every 2 hours (Figure 3.3). The results show that immediately after one hour's stress, BL2 cells were not induced to undergo apoptosis. However, there was a significant increase in early apoptosis after two hours in lower density culture, both in serum-free RPMI and RPMI with serum, and the apoptotic cells gradually underwent secondary necrosis with time. The viable cells in the population started proliferating when back in low density culture. This result indicated that during the one hour high density stress, some BL2 cells were pre-apoptotic and committed to undergo apoptosis and this commitment could not be reversed either by low density culture or by factors in serum.

3.4 Commitment to apoptosis during stress can be inhibited by overexpression of bcl-2

To confirm that commitment to apoptosis had occurred during one hour exposure to high density stress, BL2 cells stably transfected to express bcl-2 protein (BL2/bcl-2) and therefore resistant to apoptosis, were employed. Immediately after one hour stress, there was no increase in early apoptotic BL2/bcl-2 cells shown by AxV/PI staining (Figure 3.4). When stressed BL2/bcl-2 cells were brought back to low density culture, there was no increase in early apoptotic cells, no matter in serum-free RPMI or in RPMI with serum, indicating that bcl-2 over-expression inhibited the commitment to apoptosis during stress.

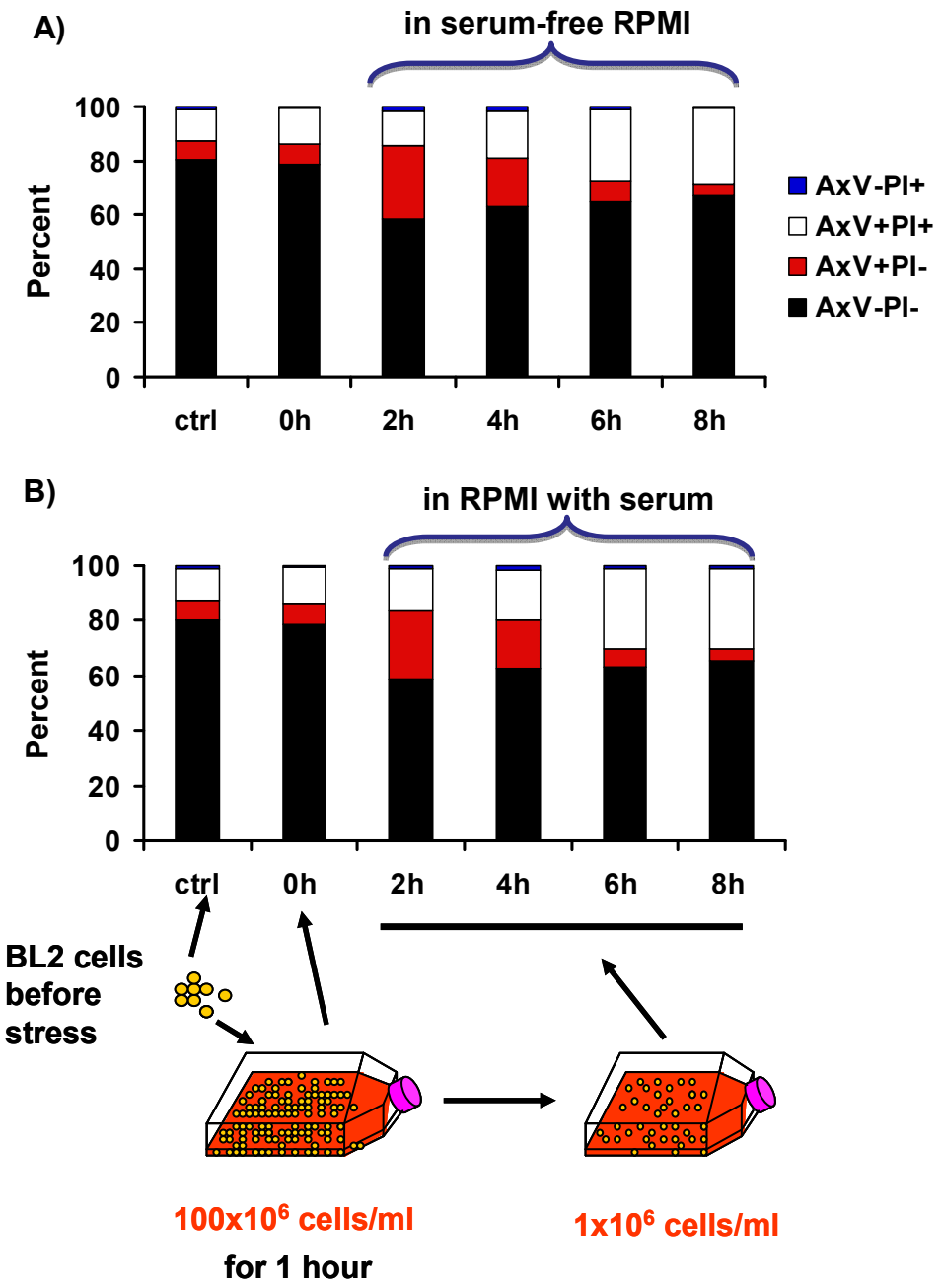


Figure 3.3 One hour stress generated pre-apoptotic cell which were committed to apoptosis.

1 hour stressed BL2 cells were seeded at $1x10^6$ cells/ml in either serum-free RPMI (A) or RPMI with serum (B) for different length of time and cell death was assessed by AxV/PI staining. Ctrl: BL2 cells before stress; 0h: BL2 cells after 1 hour stress; 2h: 1hr stressed cell were seed at $1x10^6$ cells/ml in serum-free RPMI or RPMI with serum for 2 hours. Red bars represent early apoptotic cells. Data presented are representative of three independent experiments.

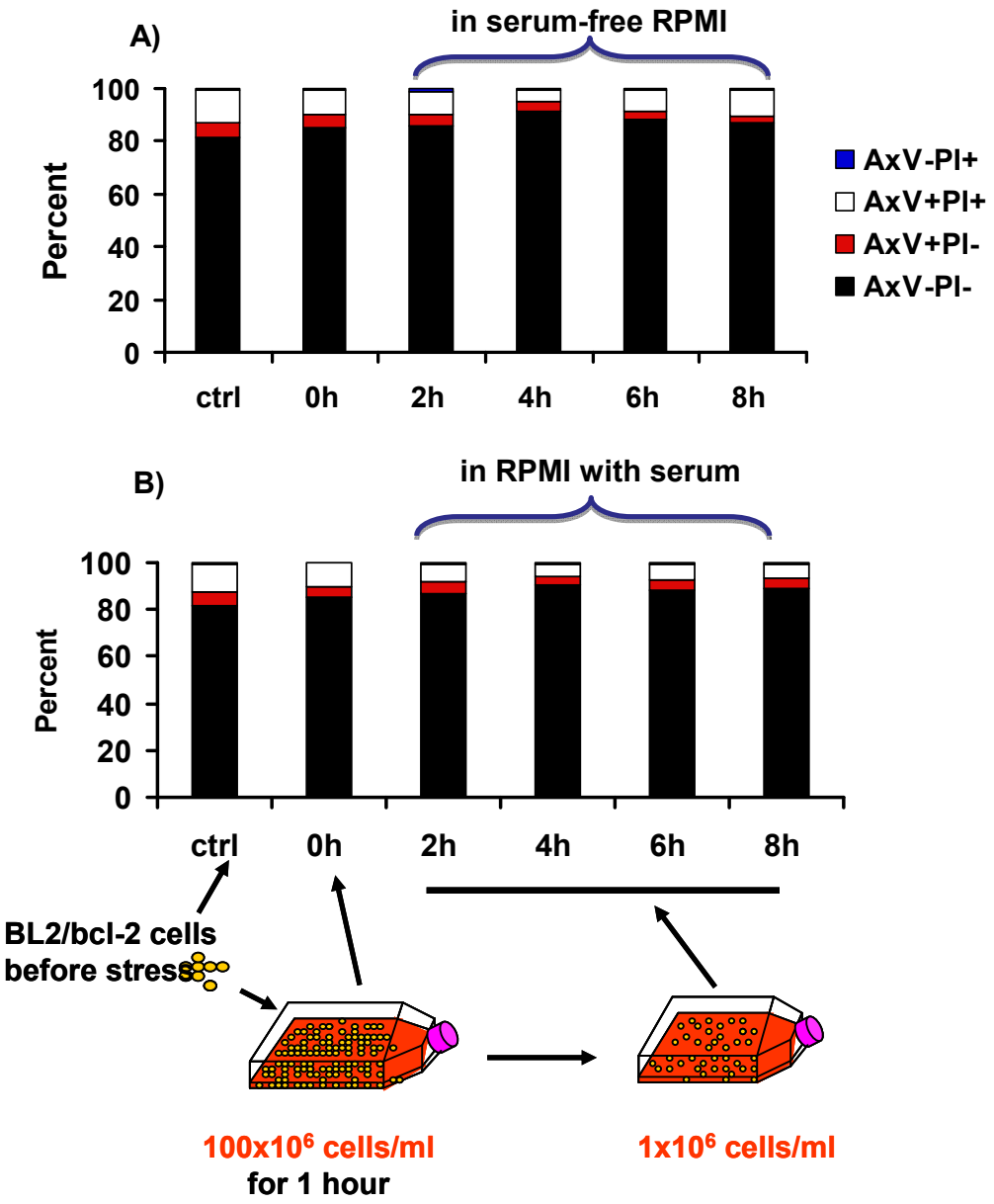


Figure 3.4 Commitment to apoptosis during stress can be inhibited by bcl-2 overexpression.

1hour high density stressed BL2/bcl-2 cells were seeded at $1x10^6$ cells/ml in either serum-free RPMI (A) or RPMI with serum (B) for different length of time and cell death was assessed by AxV/PI staining. ctrl: BL2/bcl-2 cells before stress; 0h: BL2/bcl-2 cells after 1 hour stress; 2h: 1hr stressed cells were seed at $1x10^6$ cells/ml in serum-free RPMI or RPMI with serum for 2 hours. Red bars represent early apoptotic cells. Data presented are the representative of three independent experiments.

Therefore, although one hour high density stress did not increase the early apoptotic cell number, it generated some pre-apoptotic cells, which, when cultured at low density, could execute the apoptosis program and this effect could be inhibited by overexpression of bcl-2.

3.5 Characterisation of high density stress

3.5.1 Longer stress induced non-apoptotic BL2 cell death

To further characterise the stress model, BL2 cells were subjected to high density stress for longer, up to seven hours and cell death was assessed by AxV/PI staining (Figure 3.5A). After one hour there was slight increase in early apoptosis. However, data presented here was only a representative of three independent experiments and as demonstrated in Figure 3.1B, the increase of early apoptosis after one hour stress was not statistically significant. This might be caused by the variations in the percentages of viable BL2 cells applied in each experiment. Further analysis of apoptosis induction after stress was performed using Western blot to examine caspase 3 expression and activation (Figure 3.5 B). Constant levels of pro-caspase 3 (35 kDa) were detected in all the samples, however, similar to 0 hr, there was no active caspase 3 (17-19 kDa) detected at 1hr and 4hr, confirming the previous observation that apoptosis was not induced directly after high density stress. The slight increase in the level of PS exposure in Figure 3.5A was therefore not a valid indication for apoptotic cells, since it has been shown previously that AxV binding in BL cell line was not a reliable indicator of apoptosis (Holder, Barnes et al. 2006). However, there is a defect in the experimental design. Despite the fact that the same antibodies against pro- and active caspase-3 have been used successfully in Western blot to detect active caspase-3 in apoptotic cells (C.Ford, personal communication), a positive control, such as staurosporine induced apoptotic cells, is still needed in this study to demonstrate that the absence of active caspase-3 was not caused by lack of binding of the antibodies.

With time, the apoptotic cells gradually underwent secondary necrosis and there was no further increase in early apoptosis (Figure 3.5 A). When BL2/bcl-2 cells were subjected to longer high density culture, there was no increase in early apoptosis after one hour. Around three hours, these cells started to become permeable to PI (Figure 3.5C). The above results suggested that long term culture at high density induced non-apoptotic cell death.

3.5.2 Characterisation of stressors in high density stress

In order to clarify the nature of high density stress, the model was further analysed by incubating BL2 cells at high density for 1 hour and 4 hours. The changes of BL2 cells at these two time points were assessed to identify the key stressors.

First, stressed BL2 cell lysates were examined using Western blot for the expression of heat shock proteins (Hsp), given that the induced expression of Hsp is a common feature in response to extensive stressors. Hsp70 and Hsp 90 were expressed at constant levels in all samples (Figure 3.6A). Hsp27 was found in all 3 samples, however, the protein level gradually decreased during stress with time.

Furthermore, the existence of hypoxia was analysed by detecting HIF-1α expression levels by western blot, however, the expected product band, which is around 100-120 kDa, was not detected at any of the samples (Figure 3.6B). A 65 kDa band and a doublet at 90-95 kDa was found at a constant level in all 3 samples, however, their identities were still unknown. Thus, it is likely that hypoxia was not a stressor in the model. However, considering that a positive control, such as cells treated with hypoxia, was missing here, it could not exclude the possibility of the ineffectiveness of the antibodies. Therefore, further detection of HIF-1α in the presence of a positive control should be performed.

In the end, measurement of glucose level in longer stress indicates that glucose level in the media dropped significantly from 12mM to around 5mM within one hour, and almost depleted around four hour, in both BL2 and BL2/bcl-2 culture (Figure 3.6C). The rapid glucose depletion during the stress was consistent with the lack of apoptosis during stress, given that apoptosis is an ATP-dependent programme (Leist, Single et al. 1997). Therefore, glucose limitation might be the key stressor in the high density stress model.

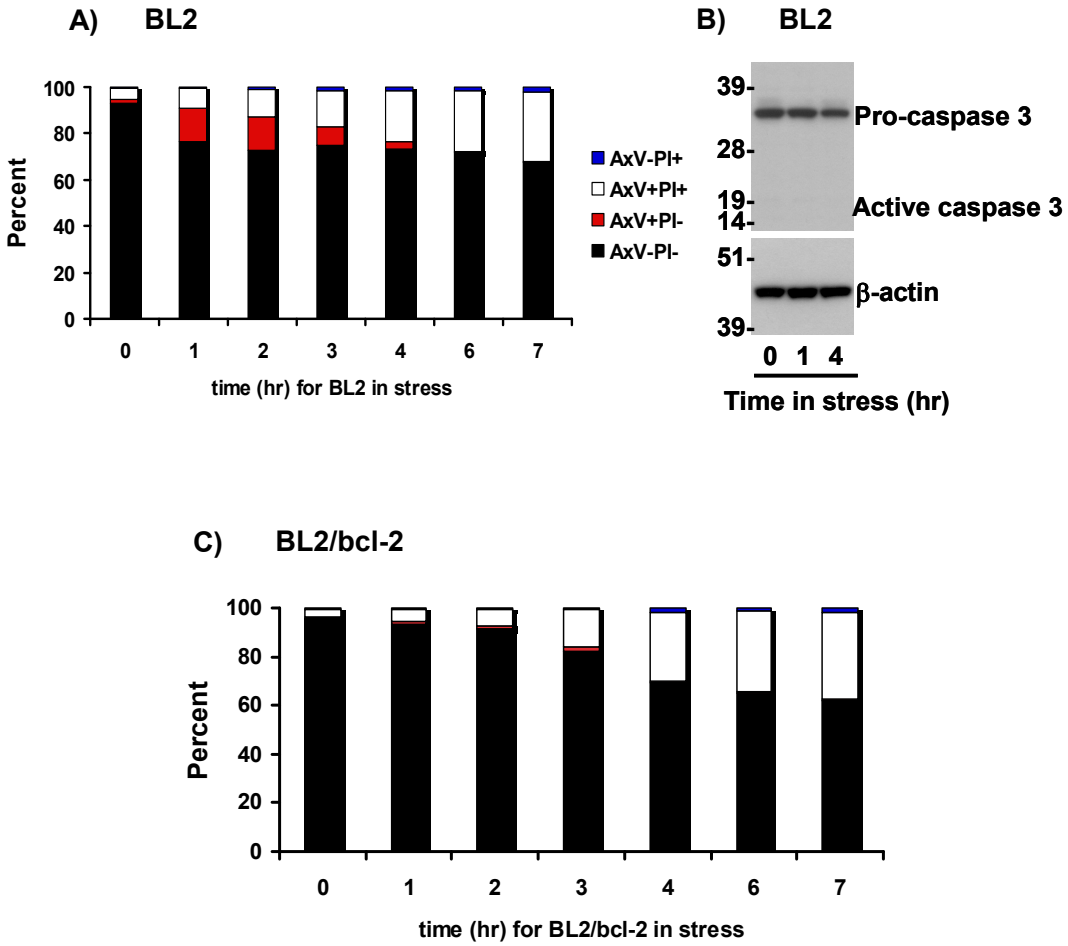


Figure 3.5 Longer stress induced non-apoptotic cell death.

A), C). BL2 and BL2/bcl-2 cells were subjected to high density stress for up to 7 hours and cell death was assessed by AxV/PI staining every hour. B) 1 hour and 4 hour stressed BL2 cells whole cell lysates were prepared and immunoblotted with rabbit anti-human caspase 3. β-actin was detected as a loading control. 0 hr was BL2 cells before stress. Data presented are the representative of three independent experiments.

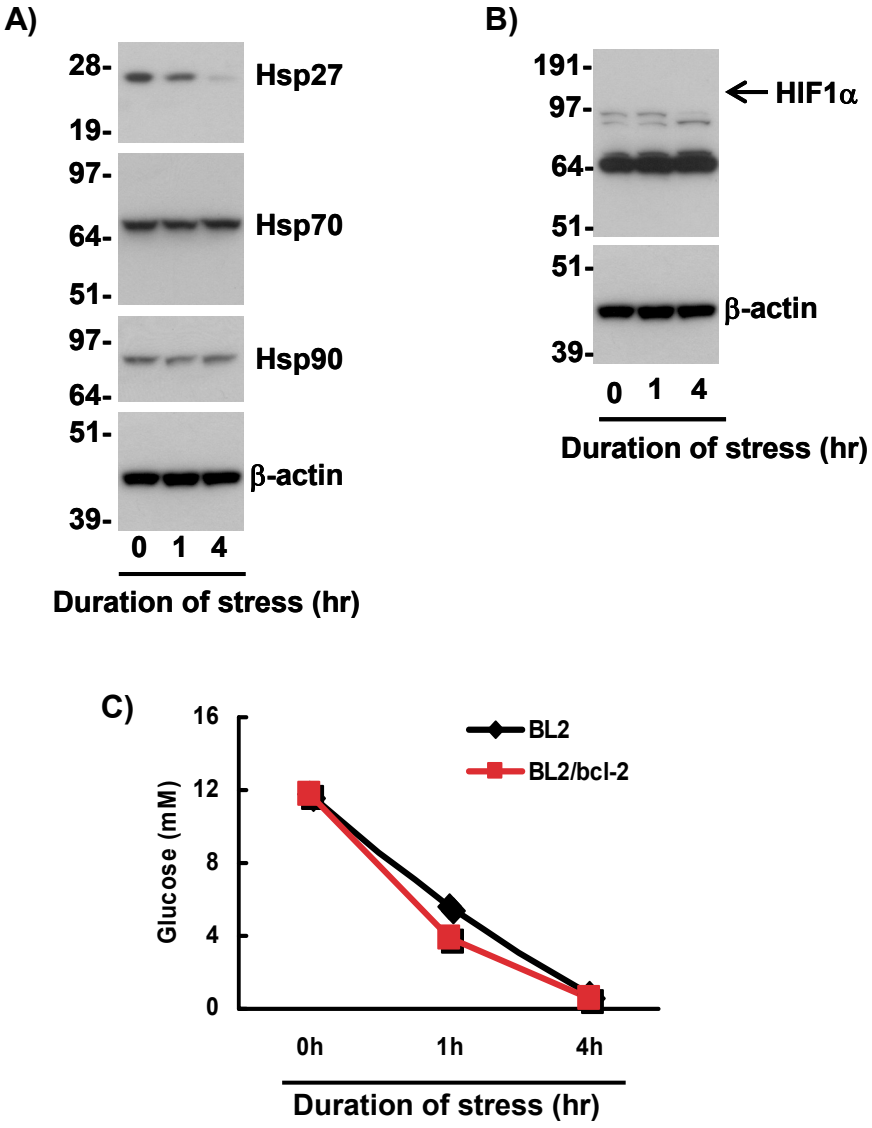


Figure 3.6 Longer stress characterisation.

BL2 cells were subjected to high density stress for 1 hour and 4 hours. A), B) : 1 hour and 4 hour stressed whole cell lysates were prepared and immunoblotted using mouse anti-human Hsp27, mouse anti-human Hsp70, mouse anti-human Hsp90 (A), mouse anti-human HIF-1α (B). β-actin was detected as a loading control. 0hr was BL2 cells before stress. C) Glucose levels were measured in stress cell cultures after 1 hour and 4 hours during stress. 0 hr was the glucose level in fresh serum-free RPMI 1640 medium. Representative data from one of two independent experiments is shown.

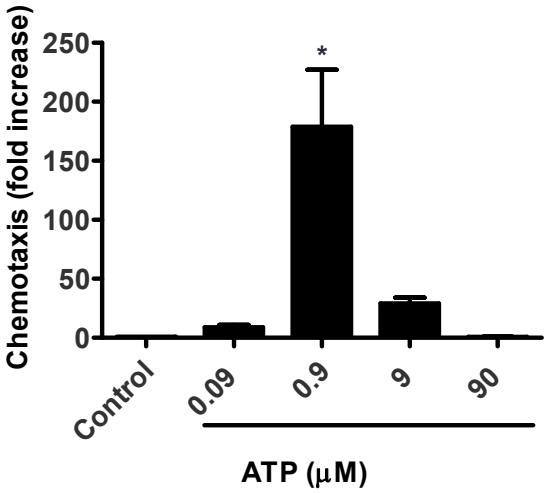
Chapter 4 Results: Stressed tumour cells released ATP to selectively recruit mononuclear phagocytes and inhibit neutrophil infiltration

4.1 ATP is a chemoattractant for THP-1 cells

Although it has been identified that ATP is a novel mononuclear phagocyte chemoattractant (Elliott et al., 2009), recent studies on both macrophage (Kronlage, Song et al. 2010) and neutrophil chemotaxis (Chen, Corriden et al. 2006; McDonald, Pittman et al. 2010) raised doubts that instead of being a chemoattractant, ATP functions only as an autocrine signal to help cells navigate in chemotactic grandients (Junger 2011). Therefore, chemotaxis assays were performed to examine whether ATP was a mononuclear phagocyte chemoattractant or a navigation signal which promotes chemokinesis.

As introduced before (Chapter 1, 1.1.1 Tumour-associated macrophages), some TAM are derived from monocytes recruited from blood (Mantovani, Bottazzi et al. 1992), thus THP-1 cells, whose phenotype are mainly monocytic, were used in the assay. First, the migratory ability of THP-1 cells to ATP was assessed and four different concentrations of ATP were used, ranging from 0.09 μM to 90 μM (Figure 4.1A). The migratory response of THP-1 cells towards ATP produced a bell-shaped dose-dependent response and the optimal concentration was 0.9 μM. To confirm the response was indeed chemotaxis instead of chemokinesis, 0.9 μM ATP was loaded into upper wells (Figure 4.1B). Comparing with the potent response towards 0.9 μM ATP in lower wells, there was no migratory response to ATP in upper wells, suggesting that ATP did not induce random migration of THP-1 cells and the response to ATP was chemotaxis, not chemokinesis.

A)



B)

ATP		Chemotaxis (fold increase)	
Lower well	Upper well	Experiment 1	Experiment 2
-	+	1	1.5
+	-	10	200
-	-	1	1

Figure 4.1 ATP was a chemoattractant for THP-1 cells.

A) Chemotaxis assays were performed using THP-1 cells and different concentrations of ATP. Assay medium was the control. The data presented are the mean fold increase compared to the control+SEM, n=3. *, P<0.01, compared with the control (Mann-Whitney test). B) THP-1 cells were added to the upper wells of the chambers and migration assessed when ATP (0.9 μm) was added to the upper or lower wells. Migration in the absence of ATP was control. The data are the mean fold increase compared to the control from duplicate analyses.

4.2 Effect of apyrase on mononuclear phagocyte chemotaxis

Since it has been found that macrophage chemotaxis in response to a gradient of chemoattractant required autocrine purinergic signalling which functioned as a navigation system, recombinant apyrase, an enzyme which can remove the gamma and beta phosphate from nucleoside triphosphates and diphosphate, was used to investigate the effect of purinergic signalling disruption on THP-1 chemotaxis. Two most commonly used THP-1 cell chemoattractants CCL2 and CCL5, whose chemoattractive ability are resistant to the hydrolase function of apyrase, were adopted as chemoattractants. Various concentrations of CCL2 and CCL5 were assessed (Figure 4.2). ATP stimulated a potent migratory response by THP-1 cells whereas responses to CCL2 and CCL5 were very weak or absent. This might be caused either by the inactivity of recombinant CCL2 and CCL5 proteins or by lack of receptor expression on THP-1 cells. Therefore, the chemoattractive activity of CCL2 was further examined using another human monocytic cell line, MonoMac6.

MonoMac6 cells could migrate to CCL2, and the response also produced a bell-shaped dose-dependent response (Figure 4.3A). The data excluded the possibility of CCL2 activity loss, and suggested that THP-1 cells used in the assay might be a clone of cells with defects in CCL2 receptor expression. Thus, MonoMac6 cells were used to examine the effect of apyrase treatment on chemotaxis. Before the assay, CCL2 was treated with apyrase or its control buffer (Figure 4.3B). According to the data, the control buffer could interact with CCL2 to induce higher chemotaxis response compared with pure CCL2; however the mechanism for this is unknown. Apyrase diminished this elevated chemotactic response suggesting apyrase might disrupt the autocrine/paracrine purinergic signalling of MonoMac6 cells to partially impair their migratory ability. However, the effect of ATP on MonoMac6 migration as a chemoattractant has not been assessed.

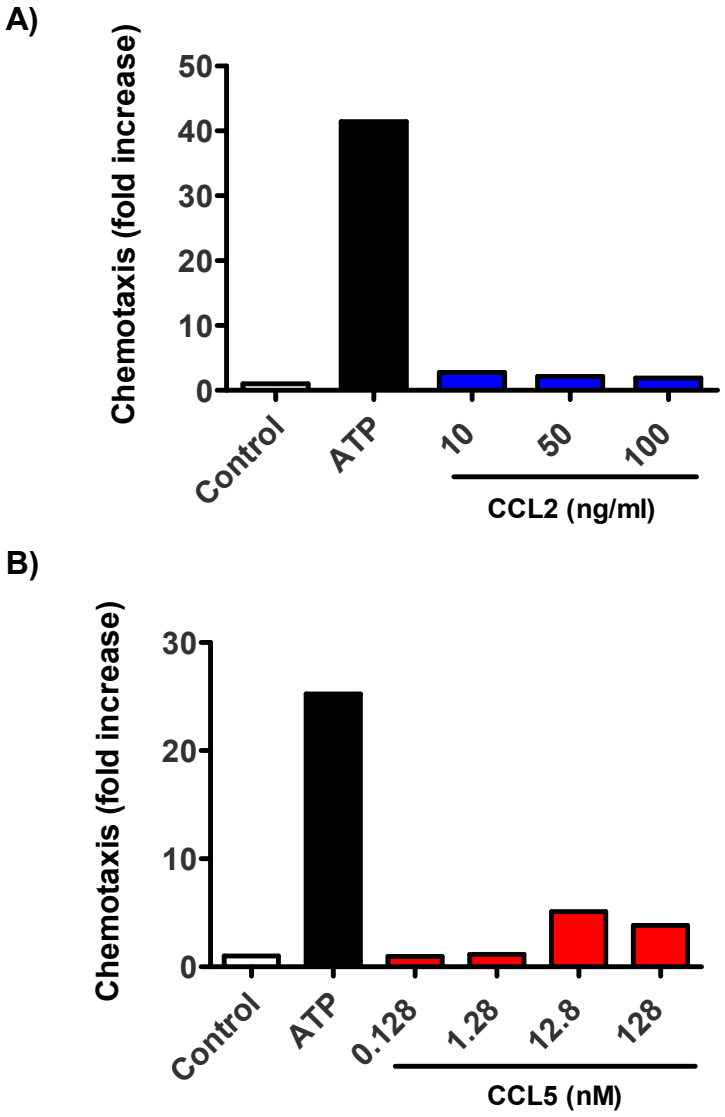


Figure 4.2 THP-1 cells did not migrate to CCL2 and CCL5.

Chemotaxis assays were performed to identify whether CCL2 (A) and CCL5 (B) were chemoattractants for THP-1 cells. Assay medium was used as a negative control and 0.9μM ATP was used as a positive control. The data presented is the mean fold increase of duplicate samples compared to the control. Data from one representative experiment of two is shown.

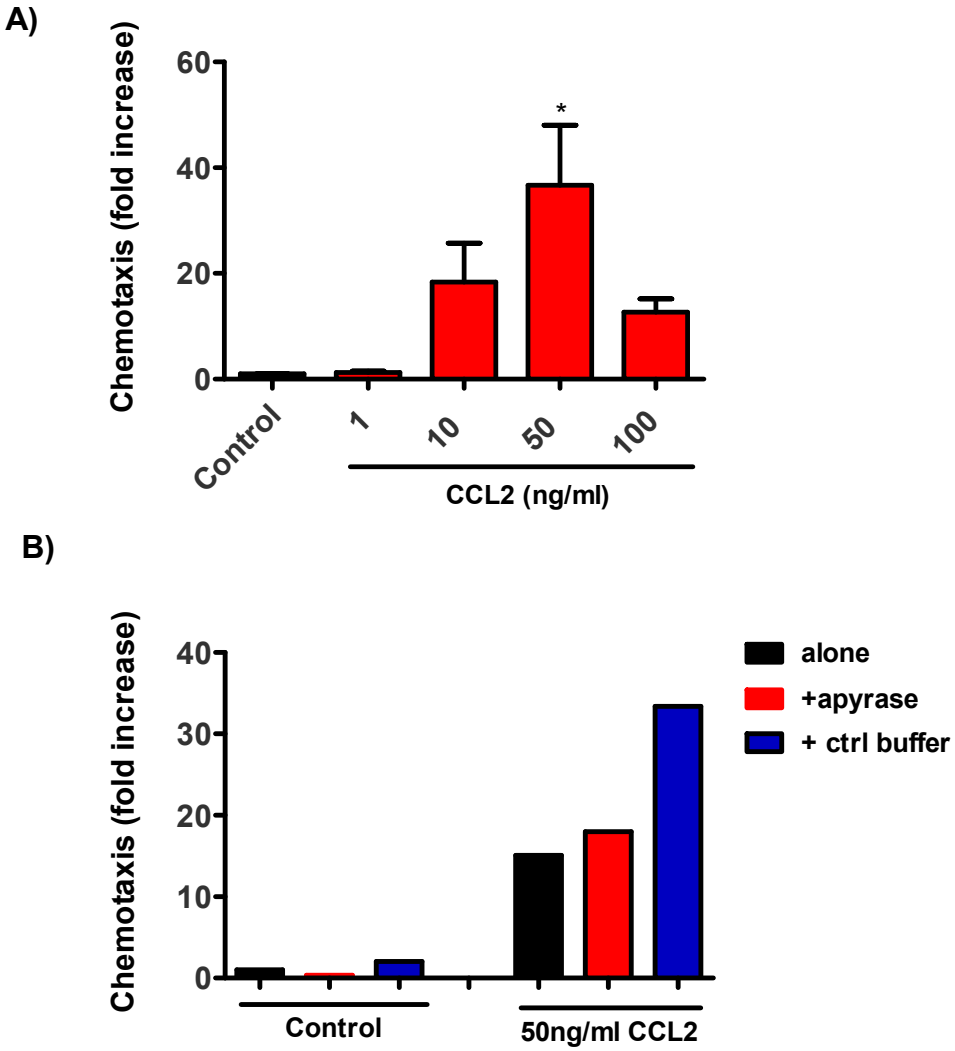


Figure 4.3 Apyrase partially decreased the migration of MonoMac6 cells to CCL2.

A) The migration of MonoMac6 cells to different concentrations of CCL2 was examined. Assay medium was used as a negative control. The data presented are the mean fold increase compared to the control±SEM, n=3. *, P<0.05, compared with the control (Mann-Whitney test). B) Assay medium and 50ng/ml CCL2 were treated either with apyrase or with apyrase control buffer. Assay medium and 50ng/ml CCL2 alone were used as controls. The data presented are the mean fold of increase compared to the control. Data shown is from one experiment.

4.3 THP-1 cells migrated to stressed BL2 supernatant

Having validated that ATP was a chemoattractant for THP-1 cells and disruption of purinergic signalling could impair macrophage chemotaxis, chemotaxis assays were carried out to examine whether stressed BL2 supernatant could help to condition tumour microenvironment by attracting mononuclear phagocytes and whether ATP was a chemoattractant responsible for the attraction (Figure 4.4). The results demonstrated that stressed BL2 supernatant could induce high level of migratory response from THP-1 cells. When the supernatant was treated with apyrase, the chemoattractive effect was abolished. This could be explained, in part, by the disruption of the autocrine purinergic signalling in THP-1 cells, as the data presented above in which apyrase was only able to partially reduce the response to CCL2 (Figure 4.3 B). On the other hand, the abrogation of response indicated that aside from interrupting autocrine purinergic signalling, apyrase also affected chemoattractants in stressed BL2 supernatant, which possibly were nucleoside triphosphates (ATP, UTP) (Figure 4.4A).

Suramin is an antagonist of the P2 family (P2X and P2Y) of nucleotide receptors. When THP-1 cells were treated with suramin, the chemoattractive ability of the stress supernatant was significantly decreased (Figure 4.4B). The inability of suramin to completely prevent THP-1 chemoattraction to stress supernatants could be due to its relatively low potency and its inability to block all P2Y receptors: $P2Y_4$ and $P2Y_6$ receptors, which are receptors for ATP, uridine triphosphate (UTP) ($P2Y_4$) and uridine diphosphate (UDP) ($P2Y_6$) (Ralevic and Burnstock 1998) are highly resistant to suramin-dependent inhibition. Taken together, these observations indicate that stressed BL2 supernatant can attract THP-1 cells and nucleoside triphosphates are a likely candidate for the chemoattractive response.

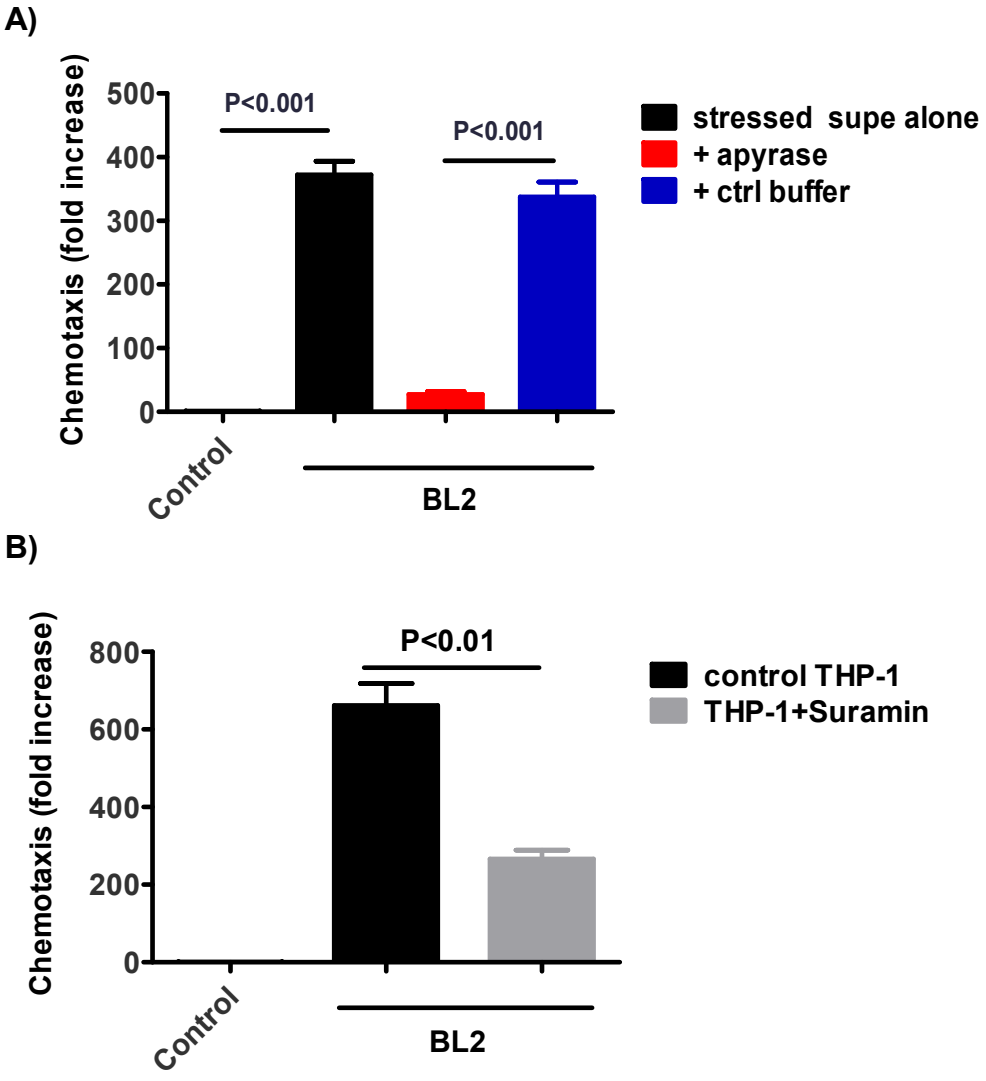


Figure 4.4 THP-1 cells migrated to supernatants from stressed BL2 cells.

Chemotaxis assays were performed to assess the chemoattractive capability of supernatants from stressed BL2 cells. Supernatant was treated with apyrase (A) or THP-1 cells were treated with suramin (B) to examine the contribution of nucleotides to the chemoattractive response. Controls in (A) and (B) were the migration of THP-1 cells to assay medium. Data presented are the means of the fold increase compared to the control ±SEM, n=3 (Mann-Whitney test).

4.4 THP-1 cells migrated to stressed BL2/bcl-2 supernatant

It has been previously demonstrated that the release of nucleoside triphosphates from apoptotic cells to chemoattract mononuclear phagocytes is a caspase-dependent process (Elliott, Chekeni et al. 2009). Since the one hour stress model has been shown not to induce apoptosis, in order to elucidate whether chemoattractant release in stressed BL2 supernatant was due to spontaneous apoptosis in BL2 cells, BL2/bcl-2 cells, which had inherent resistance to apoptosis due to the over-expression of bcl-2, were stressed and their supernatant was assessed. Comparable to stressed BL2 cells, stressed BL2/bcl-2 supernatant exhibited a similarly potent ability to attract THP-1 cells, which could also be abolished by apyrase treatment and significantly decreased by suramin exposure (Figure 4.5). The data suggested that the release of the chemoattractants in stress supernatants was not attributed to spontaneous apoptosis.

4.5 Apoptosis-independent ATP release from stressed BL cells

In order to examine whether ATP contributed to the chemoattractive ability of the stress supernatants, the presence of ATP was measured using an ATP bioluminescent assay (Figure 4.6A). Around 200 nM and 300 nM ATP were present in stressed BL2 and BL2/bcl-2 supernatants respectively, but the difference between these was not statistically significant. The presence of ATP indicated that ATP might be one of the chemoattractants in stress supernatants.

Since there was no increase in the dead cell number after stress (Figure 3.2B), ATP should not come from a gross leakage of the pool of cytosolic ATP. Although it has been demonstrated that the release of chemoattractants in stress supernatants was not attributed to apoptosis, there was still the possibility that ATP could be released from

low level of spontaneous apoptotic cells in the population, considering the high cell density used in the stress model. Therefore, stressed BL2 and BL2/bcl-2 cells were analysed for their apoptotic cell number after stress relative to the amount of ATP released from 1 ml of $100x10^6$ cells (Figure 4.6B). Although the difference in apoptotic cell number between BL2 and BL2/bcl-2 was not significant (P=0.1420, calculated by paired Mann-Whitney test), the data still demonstrated clearly that there were more apoptotic cells in stressed BL2 cells than in BL2/bcl-2 cells and despite this, stressed BL2 supernatant had much lower levels of ATP than stressed BL2/bcl-2 supernatant, suggesting that ATP release in the stress model was not apoptosis-dependent.

4.6 Stress supernatants inhibited neutrophil migration

Neutrophil infiltration cannot be seen in Burkitt's lymphoma and lactoferrin released from apoptotic BL cells has been found to be a neutrophil infiltration inhibitory factor in this system (Bournazou, Pound et al. 2009). Since stressed cells can release chemoattractants to recruit mononuclear phagocytes, it was therefore interesting to determine whether stressed cells could further condition the tumour microenvironment by regulating neutrophil infiltration. Given that there was ATP in the stress supernatants and it has been shown that the autocrine signalling of ATP and adenosine helps neutrophils to navigate towards a chemoattractant gradient and accelerate the migration velocity (Chen, Corriden et al. 2006), the migratory responses of neutrophils to stress supernatants along with pure ATP were assessed. fMLP was used as a positive control (Figure 4.7). Within the concentrations examined, especially high concentrations of ATP (9 μM and 90 μM) induced a migratory response; however, the possibility of chemokinesis had not been excluded. Compared with the negative control, stress supernatants, including BL2 and BL2/bcl-2, despite containing ATP, could inhibit neutrophil migration suggesting that stressed BL could release neutrophil infiltration inhibitory factor(s) independent of apoptosis. However, the identification

of the factor(s) requires further investigation.

However, a drawback of these experiments is that the controls used in the assays (assay medium, serum-free RPMI) for stress supernatant is not the optimal control. An additional and more suitable control would be the conditioned medium from healthy, unstressed BL2 cells which could help clarify whether the chemoattractant was specifically derived from stressed cells or was simply a concentrated effect from tumour cells.

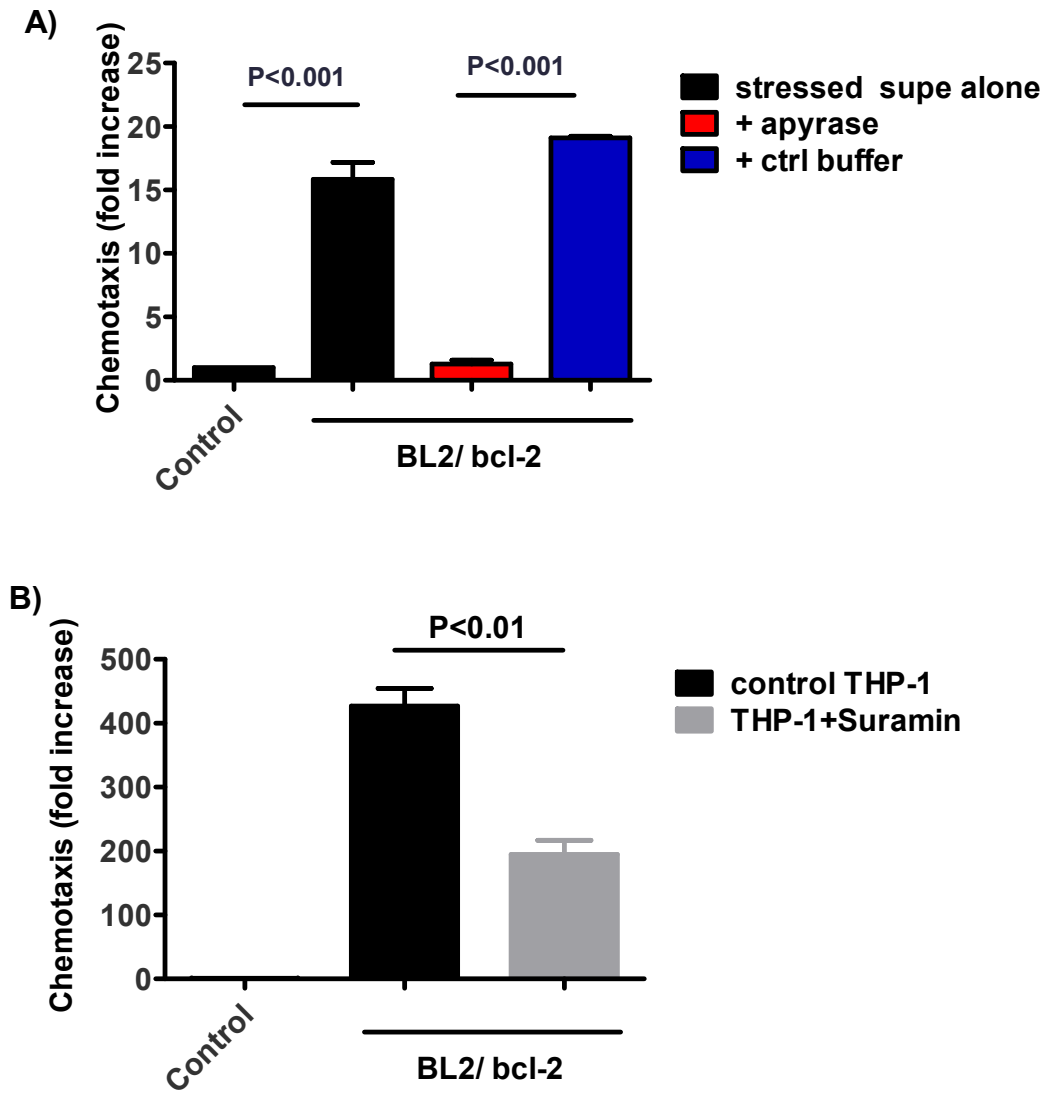


Figure 4.5 THP-1 cells migrated to supernatants from stressed BL2/bcl-2 cells.

Chemotaxis assays were performed to assess the chemoattractive ability of supernatants from stressed BL2/bcl-2 cells. To examine the contribution of nucleotides to the chemoattractive responses of THP-1 cells, stress supernatant was treated with apyrase (A) or the THP-1 cells were treated with suramin (B). Controls in (A) and (B) were the migration ofTHP-1 cells to assay medium. Data presented are the means of the fold increase compared to the control and error bars are SEM, n=3 (Mann-Whitney test).

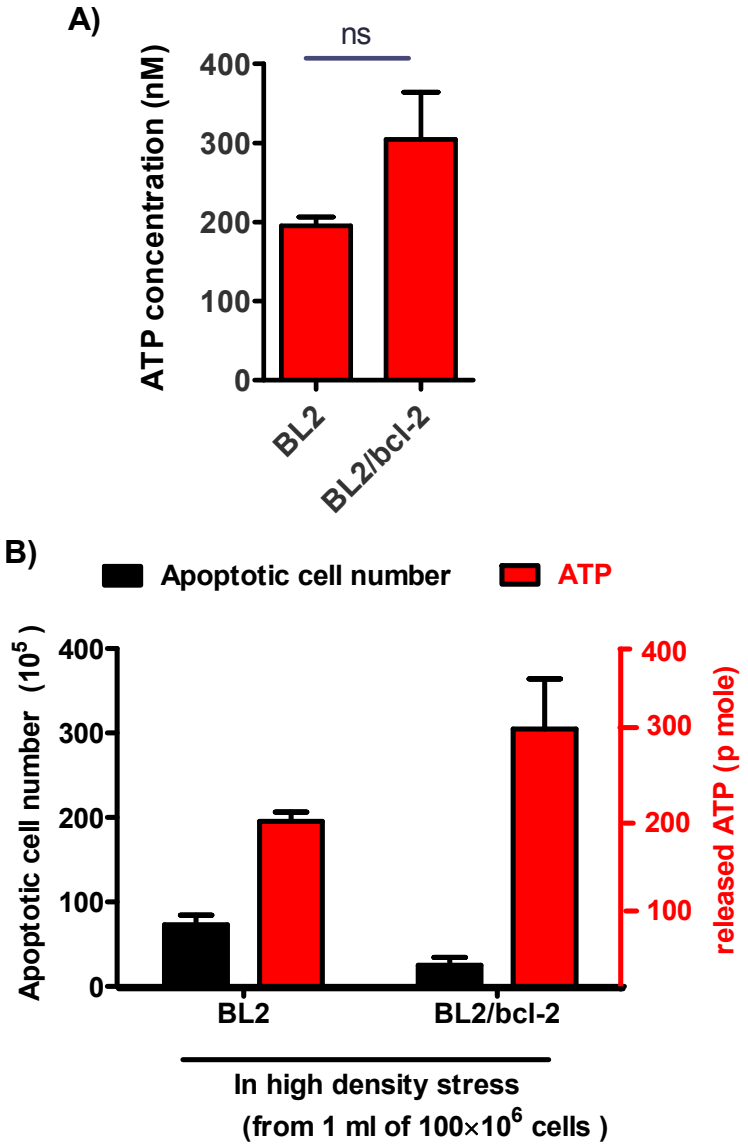


Figure 4.6 Apoptosis-independent ATP release in stress supernatants.

A) ATP concentrations in stressed BL2 and BL2/bcl-2 cell supernatants were assessed using ATP bioluminescent assay. The difference between the concentrations of ATP in BL2 and BL2/bcl-2 was not significant by the Mann-Whitney test. B) Apoptotic cell number (AxV+PI- cells) and ATP released from 1 ml of 100×10^6 cells were compared between stressed BL2 and BL2/bcl-2 cells. Error bars are SEM, n=3. The difference between the apoptotic cell numbers in BL2 and BL2/bcl-2 was not significant by the Mann-Whitney test.

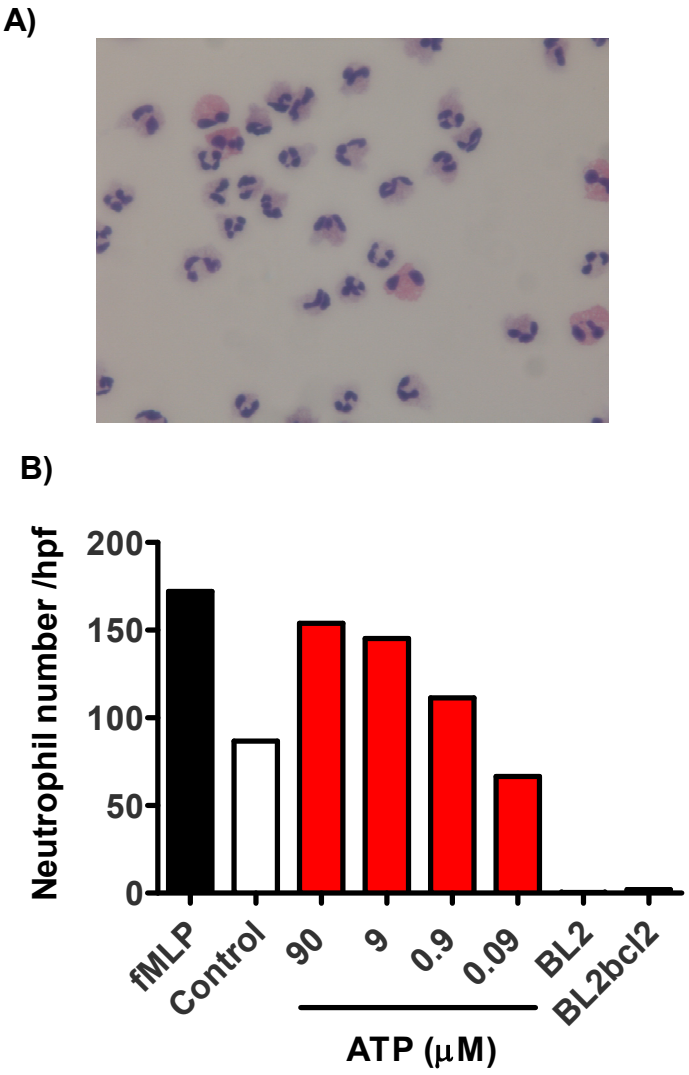


Figure 4.7 Stress supernatants inhibited neutrophil migration.

A) Analysis of the purity of separated human blood polymorphonuclear cells by cytospin and H&E staining. B) Neutrophil chemotaxis assays were performed and the chemoattractive ability of ATP and stressed BL2, BL2/bcl-2 supernatants were assessed. fMLP (100nM), positive control; control, assay medium. The data presented are the mean of migrated neutrophil number/hpf of duplicate samples. One representative of three experiments is shown.

4.7 ATP was not released from microparticles (MPs) of stress supernatant

In recent years, the occurrence of controlled ATP release from intact mammalian cells under different conditions has been gradually accepted, however, the mechanisms of ATP release from these cells is not well understood. The two most studied and widely accepted modes of ATP release are exocytosis of secretory vesicles that contain ATP and ATP release through specialized channels (Junger 2008; Praetorius and Leipziger 2009). Besides, hypoxia could stimulate vesicular ATP release from rat osteoblasts (Orriss, Knight et al. 2009). Thus, pilot experiments were performed to identify the possible ATP releasing mechanism in the stress model, including secretory vesicles and ion channels. Given that ATP functioned as a chemoattractant for mononuclear phagocytes, and the release of fractalkine, a chemokine known to attract mononuclear phagocytes in BL, was microparticle-associated (Truman et al., 2008), it was first speculated that the release of ATP is microparticle-associated. Hence, microparticles from stressed BL2 supernatant were separated and cultured for up to 24 hours to observe ATP release. ATP levels were measured at different time points (Figure 4.8). The data demonstrated that around 200nM ATP was present in soluble form in microparticle-free stress supernatant, and there was no ATP release from cultured microparticles at 37ºC within 24 hours.

However, there were limitations for this experiment. First, the identity of microparticles after ultracentrifugation should be examined. Although the same ultracentrifugation protocol has been used to generate microparticles from apoptotic BL cells whose identity has been confirmed by Annexin V staining using flow cytometry (Truman, Ford et al. 2008), the identity of microparticles in this study (generated from stressed BL cells) should be either by similar Annexin V staining or by Western blotting for microparticle markers, such as lipid raft protein flotillin (Lee, D'Asti et al. 2011). Second, given that the size of microparticles was unknown and the

requirement for sterility, the resuspended microparticles were passed through a 0.22 μm filter. Since there might be larger microparticles filtered out, the conclusion can only be applied to microparticles with diameters smaller than 0.22μm and smaller particles such as exosomes.

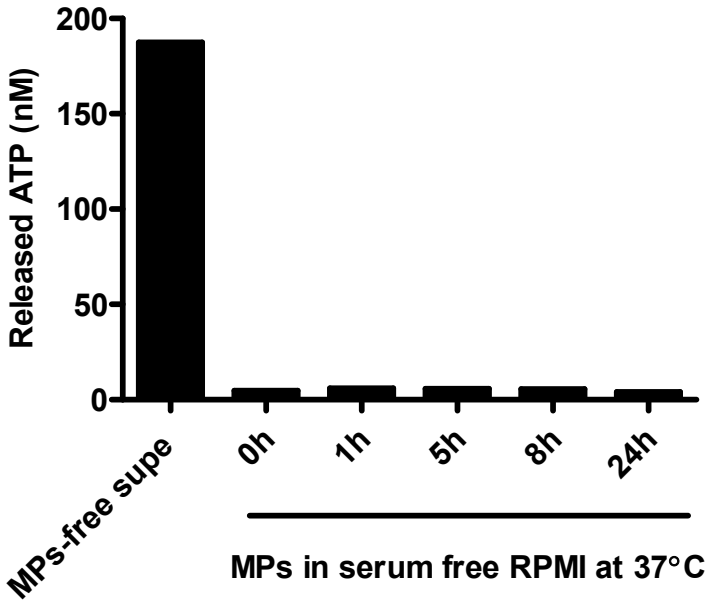


Figure 4.8 ATP was not released from microparticles (MPs) of stress supernatant.

MPs were isolated from stressed BL2 supernatant and incubated for different periods of time. ATP levels were measured at each time point. MPs-free supe: MPs-free stress supernatant from which MPs were isolated. 0h: fresh serum-free RPMI 1640. Data presented are the mean of ATP concentration of duplicate samples. Data from one experiment is presented.

4.8 Effect of ATP channel antagonists on ATP release during stress

Since the released ATP in stress supernatant was mostly in soluble form, further experiments were carried out to identify whether anion channels were responsible for ATP release. Various anion channels are responsible for ATP release in response to different stimuli. These channels include connexin and pannexin hemichannels, maxi-anion channels, voltage-dependent anion channels (VDAC), and $P2X_7$ receptors (Praetorius and Leipziger 2009). Some channels have already been identified to be responsible for the ATP release in different stress conditions. Pannexin 1 is a large pore plasma membrane channel and has been found to be involved in ATP release in various systems (MacVicar and Thompson 2010). Oxygen and glucose-deprivation can induce ATP release from pannexin 1 (Thompson, Zhou et al. 2006). Connexins have several similar biophysical and biochemical properties to pannexins and they can also conduct ATP across the plasma membrane (Harris 2007; Scemes, Spray et al. 2009). There are also reports on the induction of ATP release via a maxi-anion channel upon oxygen and glucose- deprivation (Liu, Sabirov et al. 2008). These channel proteins were also expressed by BL2 cells according to the gene profiling database previously established in our group. Considering the glucose level dropped rapidly in the stress model (Figure 3.7), the effect of all three channels on ATP release was examined using channel antagonists (Figure 4.9). $Gd^{3+}$and arachidonic acid are maxi-anion channel antagonists; carbenoxolone is a connexin and pannexin channel antagonist; and probenecid is a pannexin channel antagonist. By comparison with cells that had not been treated with inhibitor, Gd^{3+} did not affect ATP release, but arachidonic acid partially inhibited ATP release and carbenoxolone and probenecid were able to reduce the amount of released ATP. These observations implicate these channels as mediators of ATP release; however, more experiments have to be performed to confirm the result and analyze the significance.

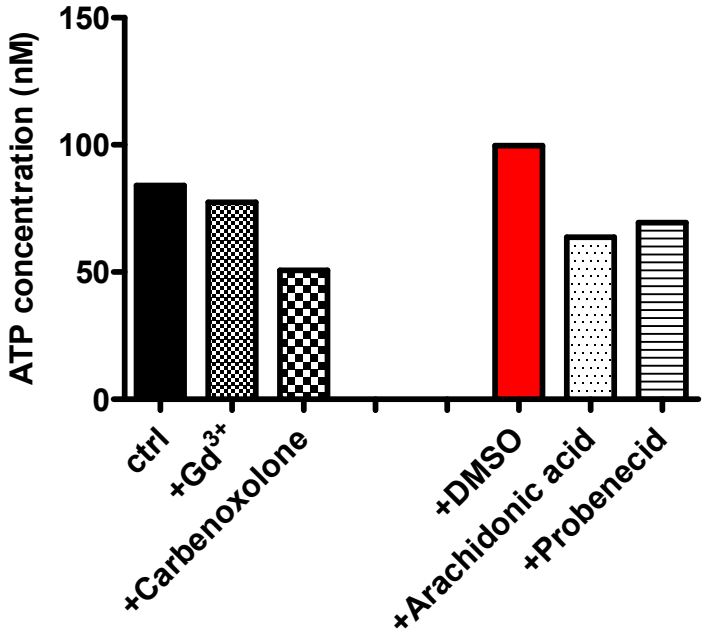


Figure 4.9 Effect of possible ATP channel antagonists and ATP release.

Before stress, BL2 cells were treated with different ATP channel antagonists and stress supernatants were generated to compare the amount of released ATP. 1) Gd3+and Aranchidonic acid: maxi-anion channel antagonists; 2) Carbenoxolone: connexin and pannexin channel antagonist; 3)Probenecid: pannexin channel Antagonist. Ctrl and DMSO were cells treated with solvents before stress. Data presented are the means of ATP concentration of duplicate samples. Data from one experiment is presented.

Chapter 5 Results: Stressed BL cells can release factors which can promote tumour cell proliferation in vitro

5.1 BL2 cell proliferation kinetics in BL culture medium and cell cycle properties

In an attempt to identify whether stressed tumour cells had any direct trophic effect on other tumour cells, the effect of stress supernatant on BL2 cell proliferation was assessed. To begin with, the kinetics of BL2 cell proliferation in their normal culture medium was established to form the basis and a positive control. The optimal concentration for seeding density of cells to reflect the exponential phase of proliferation was confirmed as being $0.2x10^6$/ml. Cells were then cultured for up to 96 hours, and their concentration was monitored every 24 hours (Figure 5.1 A). Meanwhile, cell cycle analysis was performed at each time point as cells proliferated (Figure 5.1B-F). As cells proliferated, they moved from G1 phase into S phase and G2/M phase. However by 96 hour the G1 phase was enriched and the S and G2/M phases were depleted suggesting the growth of the population of cells was reaching a plateau.

5.2 Identification of optimal serum-free culture medium with minimal proliferation effect

Since serum contains immunoglobulins and many unidentified growth factors which might mask the effect of exogenous bioactive factors on BL2 cell proliferation, use of a serum-free culture medium was adopted. The commercial product X-VIVO was examined since its serum-free formulation only contains defined exogenous growth factors, including transferrin and insulin. A range of concentrations of X-VIVO

medium in RPMI were assessed, from 1% to 50% (v/v), in the BL2 cell proliferation assays (Figure 5.2). BL2 cells grown in 1% X-VIVO exhibited minimal proliferation while maintaining cell viability. Therefore, this concentration was used as the serum-free culture medium in subsequent assays to identify exogenous bioactive factors.

5.3 Supernatants from stressed BL2 cells promoted BL2 cell proliferation

Having established the assay system, stressed BL2 supernatant was applied to the cell proliferation assay to determine its effect on the proliferation of BL2 cells thus exploring the ability of stressed tumour cells to have a direct trophic effect on other tumour cells. Considering the limited availability of stress supernatant, the effect of different dilutions was assessed in the assay, including 1 in 4, 1 in 8 and 1 in 16 (Figure 5.3). Compared with the control which was 1%X-VIVO in RPMI, stress supernatant was able to stimulate BL2 cell proliferation in a dose-related fashion. Among the dilutions examined, 1 in 4 dilution of stressed BL2 supernatant had the strongest effect, therefore, stress supernatants were all diluted 1 in 4 in future experiments. Further experiments using stressed BL2 supernatant confirmed that it could significantly promote BL2 cell proliferation (Figure 5.4).

The mitogenic effect of stress supernatants was further confirmed by examining Akt phosphorylation using Western blotting (Figure 5.5). Akt is constitutively phosphorylated on Ser473, but not on Thr308, without which Akt cannot perform its kinase function (Alessi, James et al. 1997; Stokoe, Stephens et al. 1997). When treated with stressed BL2 supernatant, Akt was phosphorylated on Thr308 and active within 30 minutes, which is consistent with the proliferation assay where stressed BL2 supernatants could support proliferation of BL2 cells. However, two batches of samples exhibited distinct patterns of phosphorylation. In Figure 5.5, samples in left

panels showed around an equal amount of pT^{308}-Akt at 30 and 60 min, and the right ones showed peaked expression at 30 min which was partially lost at 60 min. The difference might be owing to subtle difference between experiments. Despite the fact that Akt was phosphorylated upon stress supernatant treatment, it was nonetheless difficult to determine the nature of initial activation: survival or proliferation stimulation, since phosphorylated Akt has been found to both promote cell survival and stimulate cell proliferation (Vivanco and Sawyers 2002). The resulting cell proliferation might either be induced by a mitogen, or be induced by a survival factor which inhibited apoptosis leading to further cell growth and proliferation. Nevertheless, the presence of activated Akt confirmed the observation from cell proliferation assay that the stressed BL2 supernatant promoted cell proliferation despite the fact that the nature of the bioactive factors in the stress supernatant was unknown.

5.4 Mitogenic effect of stress supernatants is not apoptosis-dependent

The 1 hour high density stress did not induce apoptosis in BL2 cells (Figure 3.2), therefore the mitogenic effect of stressed supernatant was unlikely to be due to bioactive factors released from apoptotic cells. However, in order to confirm this, supernatant generated from stressed BL2/bcl-2 cells was assessed in the proliferation assay (Figure 5.6). Supernatant from stressed BL2/bcl-2 cells promoted proliferation of BL2 cells indicating the mitogenic effects of stressed supernatant were not dependent on the spontaneous apoptosis.

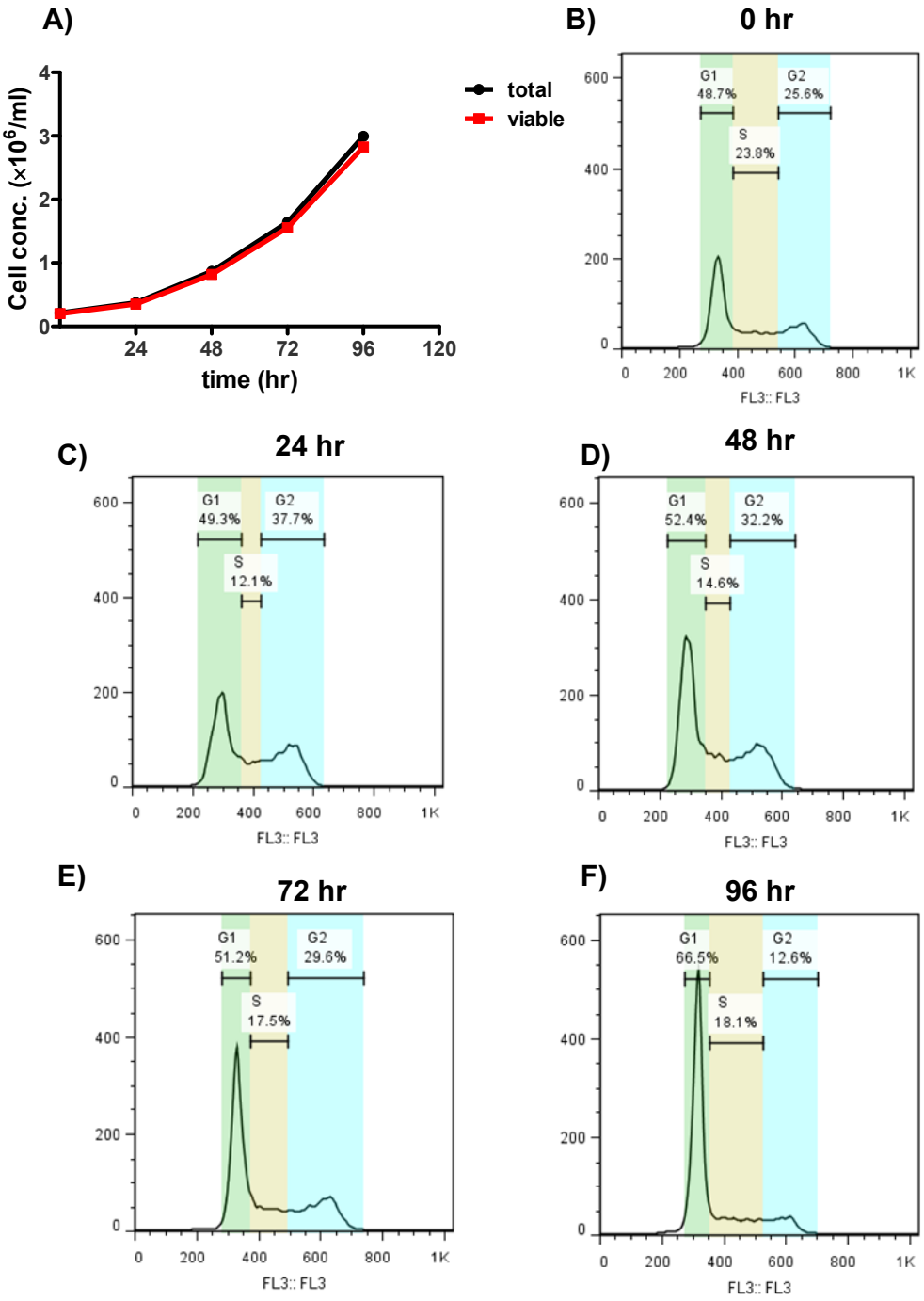


Figure 5.1 BL2 cell proliferation kinetics and cell cycle properties.

A) BL2 cell proliferation kinetics in BL culture medium. Seeding density: $0.2x10^6$/ml. Viable and total cell concentrations every 24 hours were determined using Trypan Blue exclusion. Data presented are means of triplicate samples and are representative of three independent experiments. B)-F) Cell cycle was analyzed every 24 hours using PI.

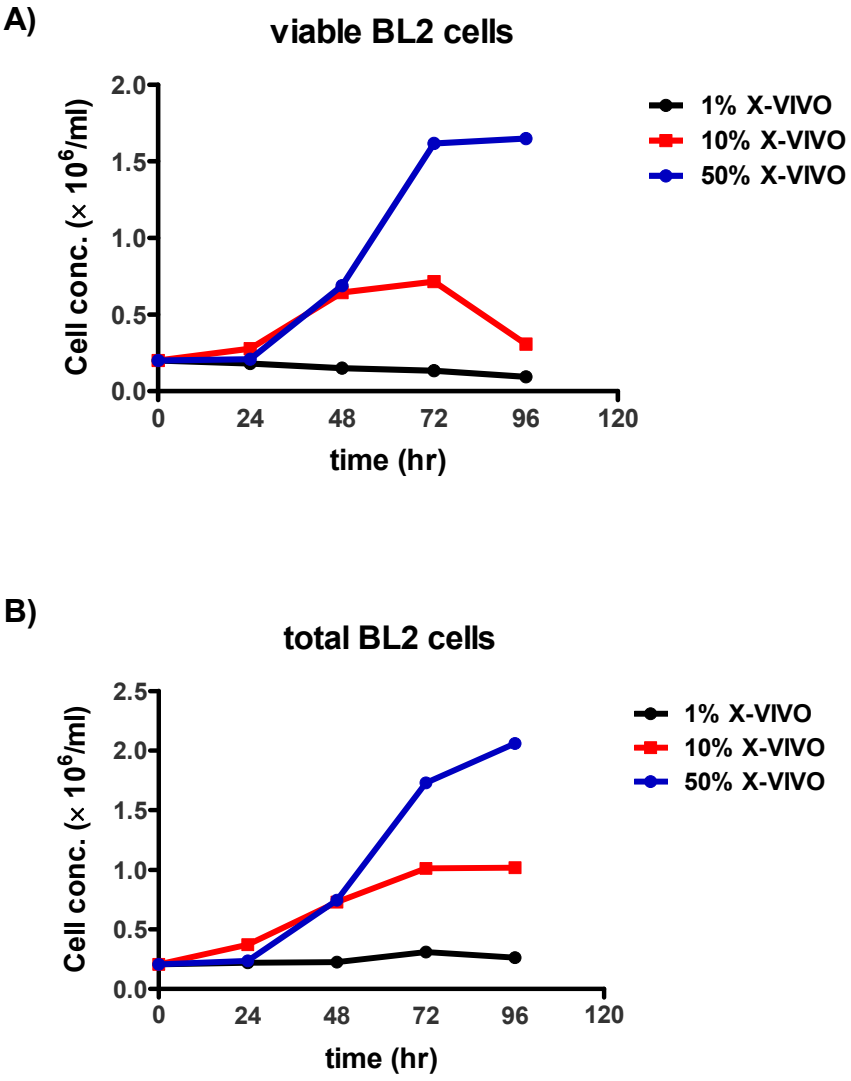


Figure 5.2 Identification of the optimal concentration of X-VIVO as a supplement for serum-free culture medium.

Various concentrations of X-VIVO were added to BL2 cells cultured in serum-free RPMI to establish the minimum concentration that could support cell viability without promoting proliferation. Viable (A) and total (B) cell concentrations were determined using Trypan Blue exclusion every 24 hours. Data are the means of triplicate samples and are representative of those from three independent experiments.

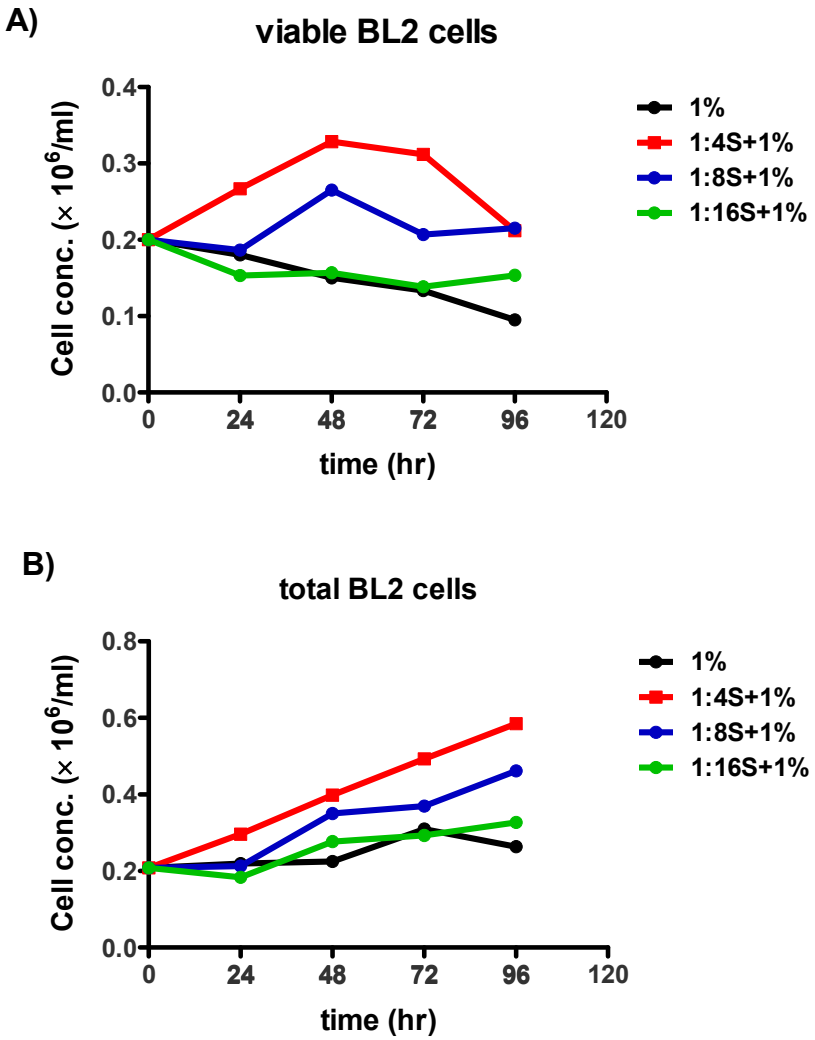


Figure 5.3 Effect of stressed BL2 cell supernatant on BL2 cell proliferation.

Various dilutions of supernatant from stressed BL2 cells were added to BL2 cells cultured in serum-free RPMI containing 1% X-VIVO (1%). Viable (A) and total (B) cell concentrations were determined using Trypan Blue exclusion every 24 hours. 1:4S+1%: stress supernatant was diluted1 in 4 in 1%X-VIVO; 1:8S+1%: stress supernatant was diluted1 in 8 in 1%X-VIVO; 1:16S+1%: stress supernatant was diluted1 in 16 in 1%X-VIVO. Data presented are means of triplicate samples

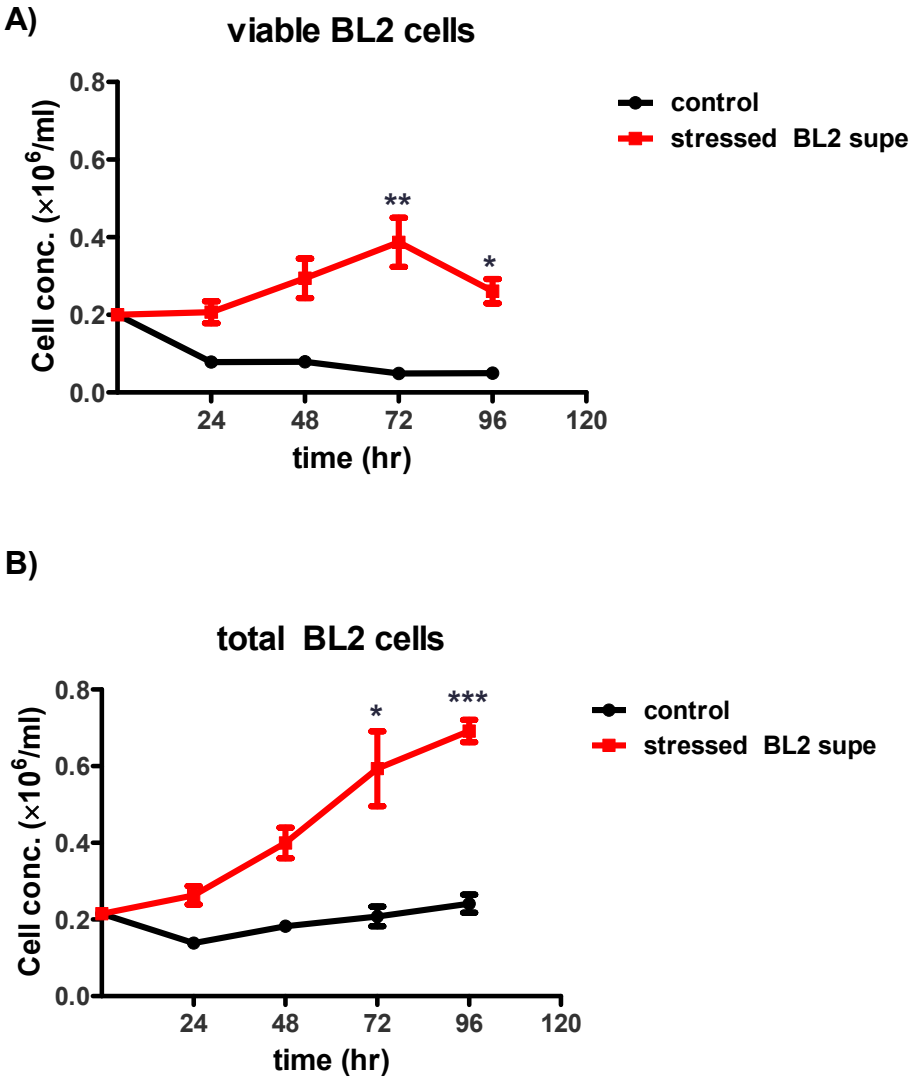


Figure 5.4 Stressed BL2 supernatant promoted BL2 cell proliferation.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) supernatant from stressed BL2 cells that was diluted 1 in 4. Viable (A) and total (B) cell concentrations were determined using Trypan Blue exclusion every 24 hours. Data are means±SEM (n=3) *, P<0.05; **, P<0.01; ***, P<0.001, compared with control at the corresponding time point (Mann-Whitney test).

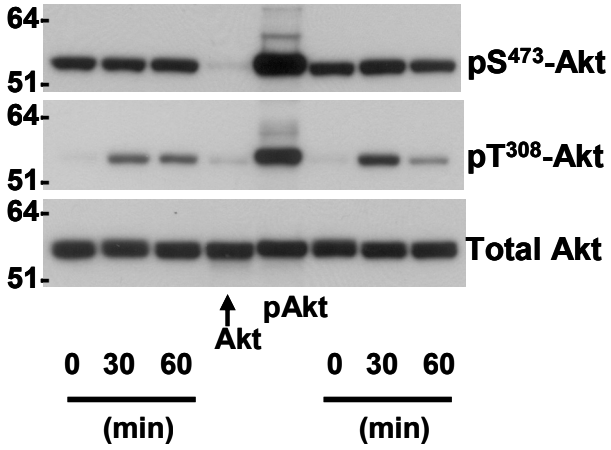


Figure 5.5 Akt is phosphorylated after stress supernatant treatment.

BL2 cells were incubated in serum-free RPMI containing 1% X-VIVO and supernatant from stressed BL2 cells that was diluted 1 in 4. After 30 and 60 min. respectively, a cell lysate was prepared. Time 0 was lysate from BL2 cells incubated without stress supernatant. Samples from two independent preparations were immunoblotted with rabbit anti-human phospho-Akt (Ser473), phospho-Akt (Thr308) and total-Akt. Akt and phospho-Akt were controls from cell extracts purchased commercially (Cell signaling technology).

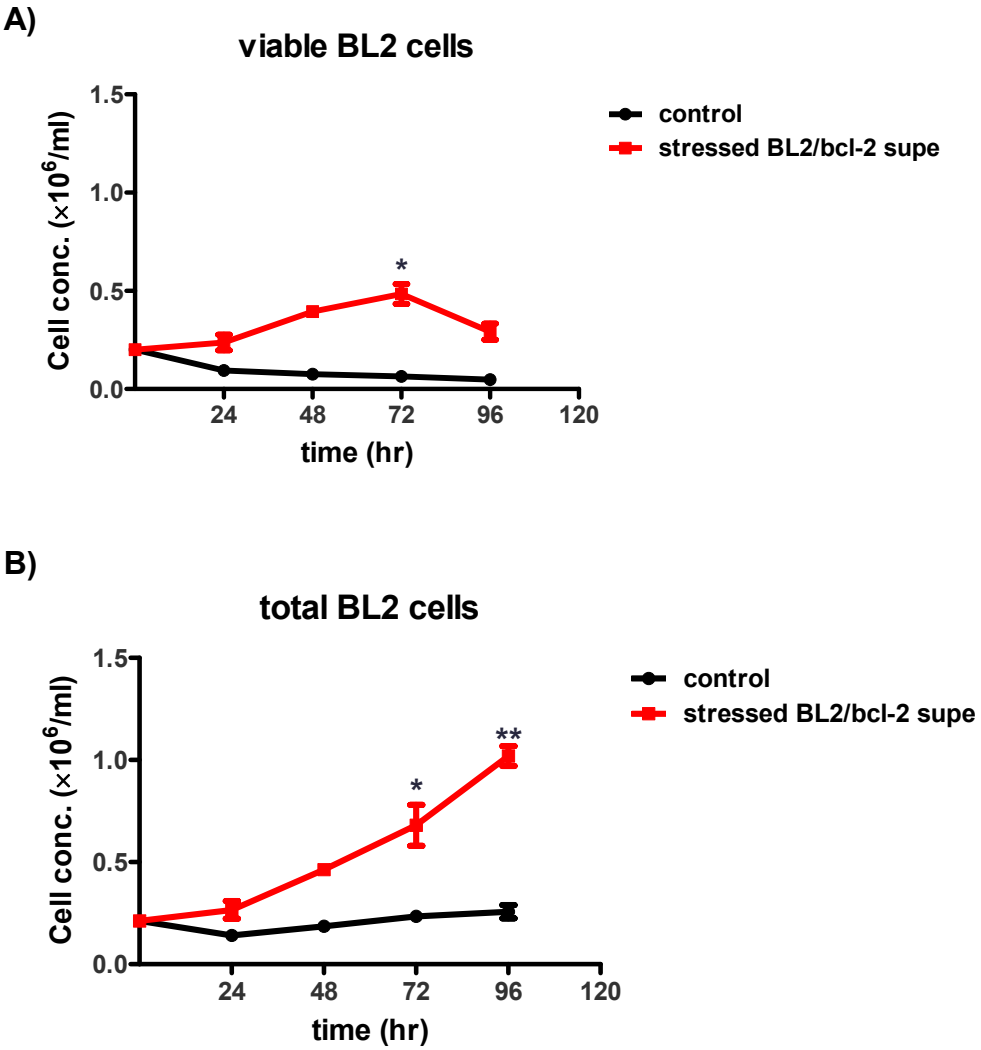


Figure 5.6 Supernatant from stressed BL2/bcl-2 cells promoted BL2 cell proliferation.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) supernatant from stressed BL2/ bcl-2 cells that was diluted 1 in 4. Viable (A) and total (B) cell concentrations were determined using Trypan Blue exclusion every 24 hours. Data are means±SEM (n=3). *, $P<0.05$; **, $P<0.01$, compared with control at the corresponding time point (Mann-Whitney test).

5.5 Mitogenic effect was not cell line specific

In order to determine whether the stress supernatants were able to induce proliferation in cell types other than the BL2 line, their effects on THP-1 cells were studied (Figure 5.7). Compared with the control (1%X-VIVO), stressed BL2 supernatant significantly promoted THP-1 cell proliferation. Thus, the mitogenic effect was not cell line specific.

5.6 Identification of bioactive factors responsible for stress-induced proliferation

5.6.1 ATP and lactoferrin were not the bioactive factors in stress supernatants

As demonstrated in chapter 4, there was sufficient ATP in stress supernatants to contribute to conditioning of the tumour microenvironment by recruiting mononuclear phagocytes. To determine whether ATP could also condition the tumour microenviroment by performing direct trophic effects on the tumour cells, exogenous ATP was added to BL2 cells and the growth of the cells was observed over 96 hours (Figure 5.8). The addition of ATP at various concentrations to BL2 cells in 1% X-VIVO in RPMI did not have any effect on the proliferation of the lymphoma cells, suggesting that ATP was not the growth-promoting bioactive factor of stress supernatant.

Another candidate for the mitogenic effect of stress supernatant was lactoferrin (LFN) which is a member of the transferrin family and iron-saturated transferrin has been found to promote cell growth (Kovar and Franek 1989). Moreover, it can be released from apoptotic BL cells and has been found to promote BL cell proliferation (Bournazou 2010). It was thus speculated that stressed BL cells could also release LFN

and that this was responsible for the mitogenic effect of stress supernatant. Based on the concentration of LFN used by Bournazou, two concentrations were examined in the cell proliferation assay (Figure 5.9). However, neither concentration of LFN could promote BL2 cell proliferation, suggesting that lactoferrin was not the bioactive factor.

5.6.2 Heat-stable bioactive factor(s)

In an attempt to further characterise the stress-derived factor that was responsible for promoting proliferation of BL2 cells, stressed BL2 supernatant was boiled for 10 min before being applied to the proliferation assay thus determining the heat stability of the bioactive factor (Figure 5.10). Heat-inactivation did not affect the proliferation stimulating effect compared with the untreated stress supernatant, indicating that the bioactive factor(s) was heat stable which might be either lipid or small peptides.

Sphingosine-1-phosphate (S1P) has been found to promote macrophage survival (Weigert, Johann et al. 2006) and stimulate glioma cell proliferation (Van Brocklyn, Letterle et al. 2002). S1P is also a key factor skewing macrophages towards an M2 phenotype (Weigert, Weis et al. 2009). Therefore, it might be a vital factor regulating the tumour microenvironment. Given that it was also a small lipid, S1P was considered to be another candidate for the bioactive factor in stress supernatant. Thus, different concentrations of S1P were applied to the proliferation assay and S1P was found to significantly promote BL2 cell proliferation, compared with its carrier protein control (bovine serum albumin, BSA) (Figure 5.11). It seemed that S1P might be the factor causing the proliferation stimulating effect.

To confirm the role of S1P, S1P level in stress supernatants was measured using a S1P ELISA. It was a competitive ELISA therefore the less S1P in the sample, the higher the final absorbance, as demonstrated by its standard curve (See Appendix 3). Since S1P has been found at high concentrations in the circulation and is capable of binding to

serum proteins (Scherer, Schmitz et al. 2009) and low-endotoxin BSA has been observed to stimulate proliferation of BL2 cells grown in minimal culture media, the level of S1P in 4 mg/ml BSA (low-endotoxin, and lipid-free BSA as a control) was also examined. 1 μM reconstituted S1P (commercially purchased) was assessed to validate the concentration of S1P used in the proliferation assay (Figure 5.12). The result for 1 μM reconstituted S1P was consistent with its concentration which also confirmed the validity and accuracy of the ELISA assay. There was no S1P in low-endotoxin BSA, and there seemed to be a tiny amount of S1P in the lipid-free BSA. However, the absorbance of stress supernatants (BL2 and BL2/bcl2) was as high as 0 μM S1P, suggesting that there was no S1P in the stress supernatants. The result indicated that S1P was not the proliferation stimulating factor of stress supernatant.

However, another possible explanation is that S1P was enclosed in microparticles released by BL2 cells, since substantial numbers of microparticles are known to be released from dying BL cells (Segundo, Medina et al. 1999; Truman, Ford et al. 2008) and microparticles released by tumour cells (human hepatocarcinoma and human breast carcinoma) are known to be a site for S1P production (Rigogliuso, Donati et al. 2010). In order to examine whether the proliferation stimulating effect was microparticle-related, microparticles were separated from stressed BL2 supernatant by ultracentrifugation and applied to the cell proliferation assay (Figure 5.13). Microparticle-free and the original stress supernatant were used as controls. The majority of the proliferation-stimulating effect of the stress supernatant was found to be in the microparticle-free stress supernatant suggesting microparticles from the stress supernatant did not have any proliferation-stimulating effect. However, since it was necessary to sterilise the microparticles by filtration therefore it was not possible to assess the effect of microparticles larger than 0.2 μm. Larger microparticles (larger than 0.2 μm) thus may contain the growth-promoting factors of stress supernatant, potentially including S1P.

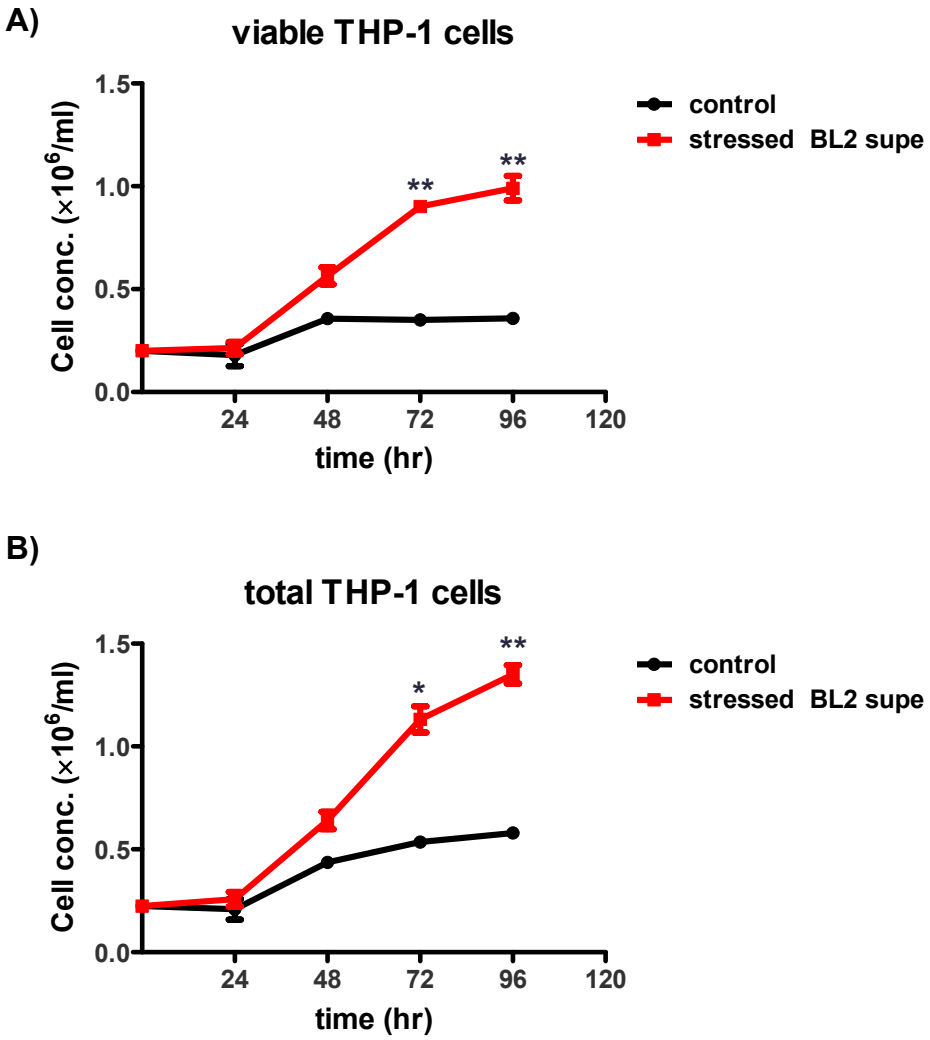


Figure 5.7 Mitogenic effect of supernatant from stressed BL2 cells was not cell line-specific.

THP-1 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) supernatant from stressed BL2 cells that was diluted 1 in 4. Viable (A) and total (B) cell concentrations were determined using Trypan Blue exclusion every 24 hours.
Data presented are means±SEM (n=3) *, $P<0.05$; **, $P<0.01$, compared with control at the corresponding time point (Mann-Whitney test).

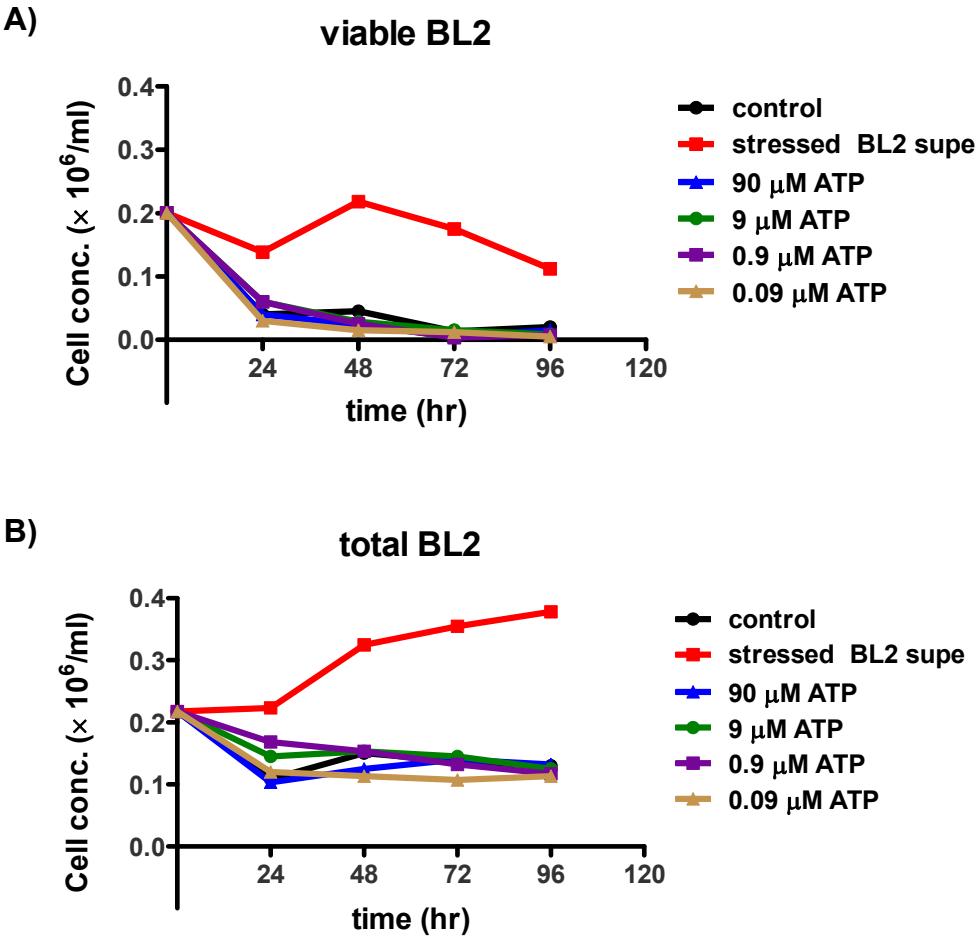


Figure 5.8 ATP could not promote BL2 cell proliferation.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) the shown concentrations of ATP. The mitogenic effect of stressed BL2 supernatant diluted ¼ is also shown. Data are means of triplicate samples

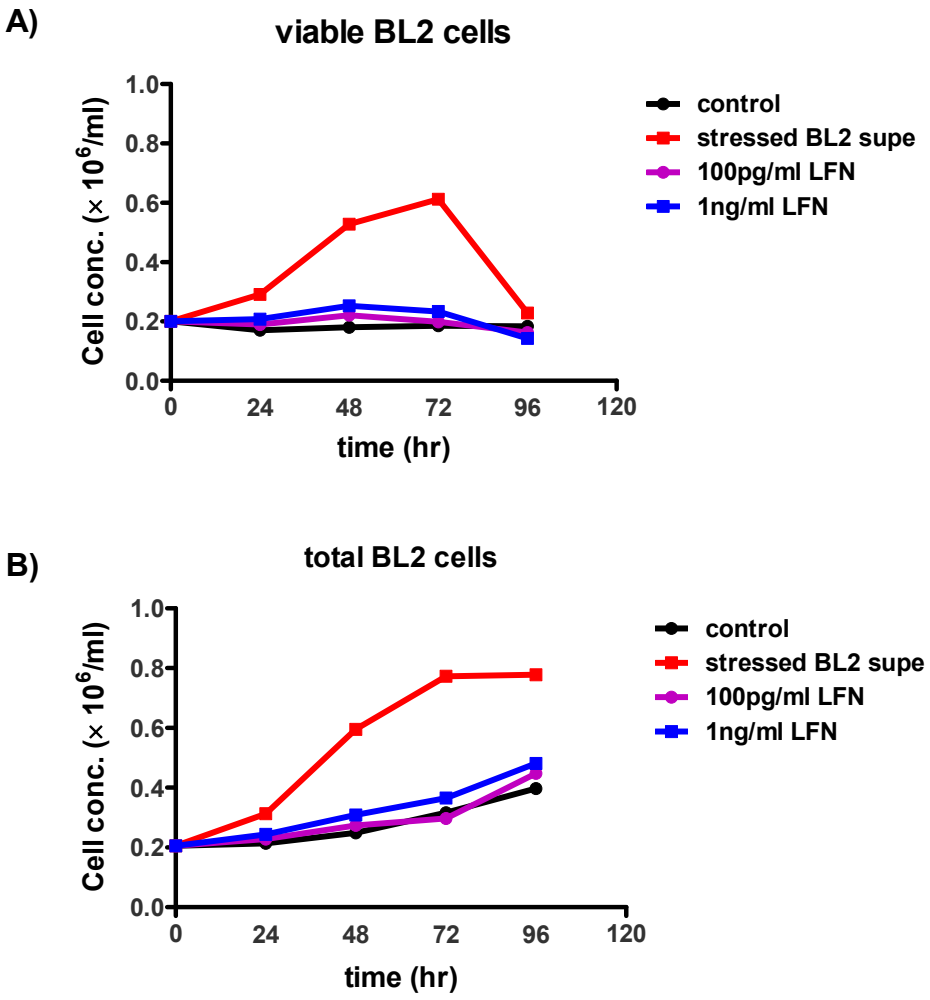


Figure 5.9 Lactoferrin could not promote BL2 cell proliferation.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) the shown concentrations of lactoferrin (LFN). The mitogenic effect of stressed BL2 supernatant diluted ¼ is also shown. Data are means of triplicate samples.

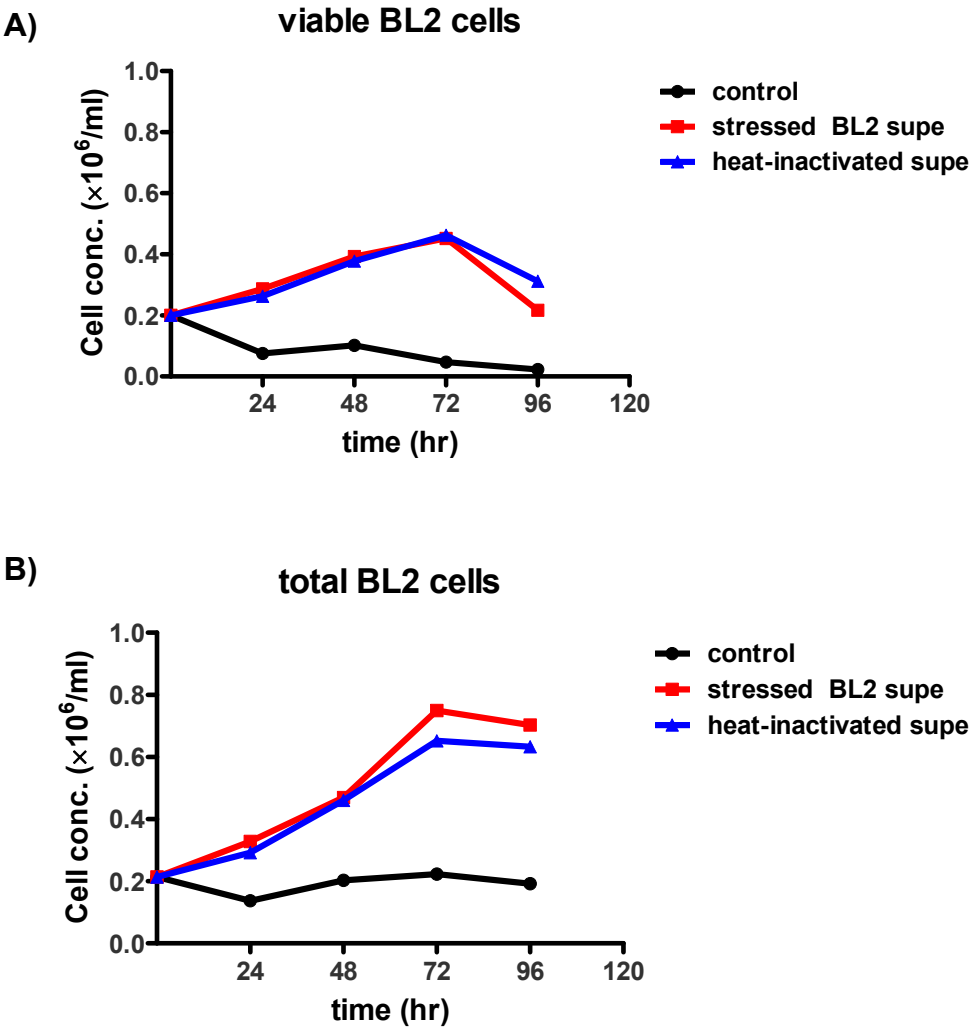


Figure 5.10 Heat stability of mitogenic factor(s) in supernatants from stressed BL2 cells.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) supernatant from stressed BL2 cells that was boiled for 10 min. The mitogenic effect of stressed BL2 supernatant diluted ¼ is also shown. Data are means of triplicate samples and are representative of those from three independent experiments

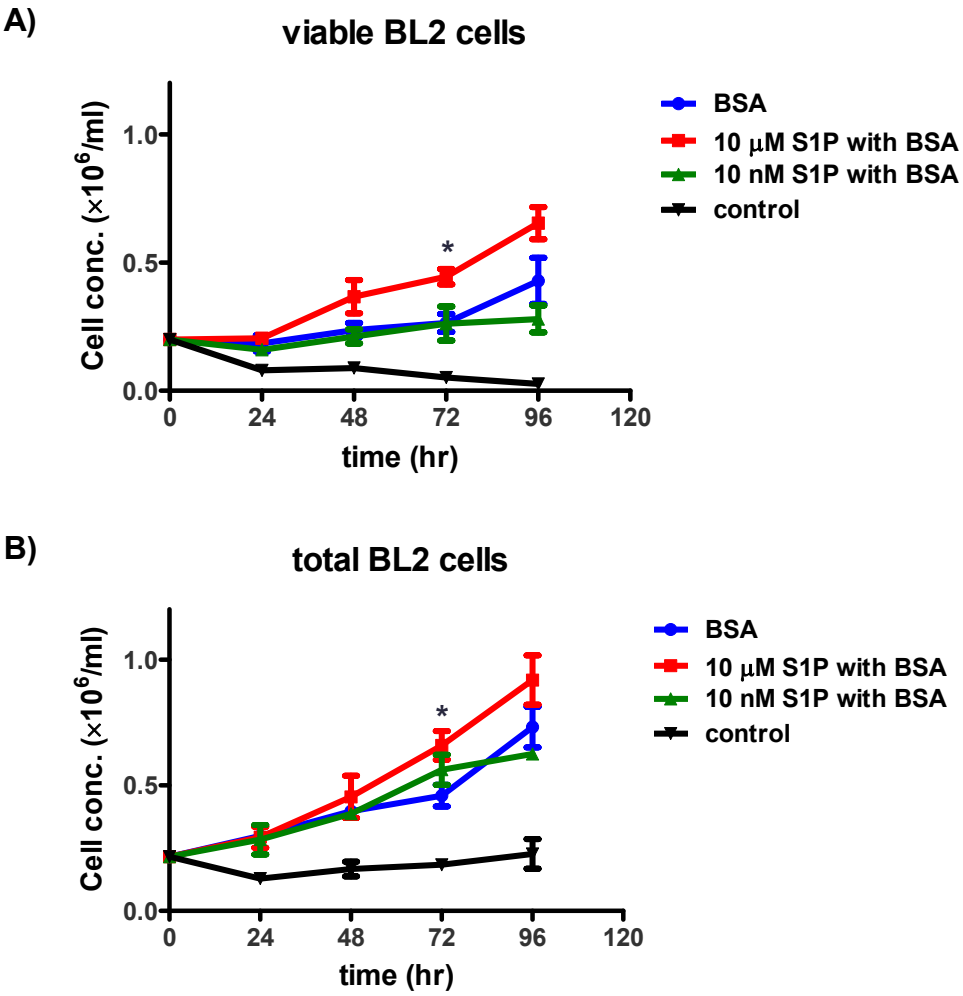


Figure 5.11 S1P could promote BL2 cell proliferation.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) the shown concentrations of S1P. BSA was the carrier protein for S1P. Data presented are mean±SEM (n=3). *, $p<0.05$, compared with BSA control at the corresponding time point (Mann-Whitney test).

Sample	Calculated concentration of S1P (μ M)
1 μ M S1P	1.295
0 μ M S1P	0.031
Stressed BL2 supernatant	0.0352
Stressed BL2/bcl-2 supernatant	0.0251
4 mg/ml low-endotoxin BSA	0.0362
4 mg/ml lipid-free BSA	0.0632

Figure 5.12 S1P was not released from stressed cells.

S1P level in stress supernatant was measured using S1P ELISA. Calculated S1P concentrations according to the standard curve are shown. Purified S1P was used as a control. 4 mg/ml low-endotoxin and lipid-free BSA were examined for the existence of S1P. The standard curve is in Appendix 3.

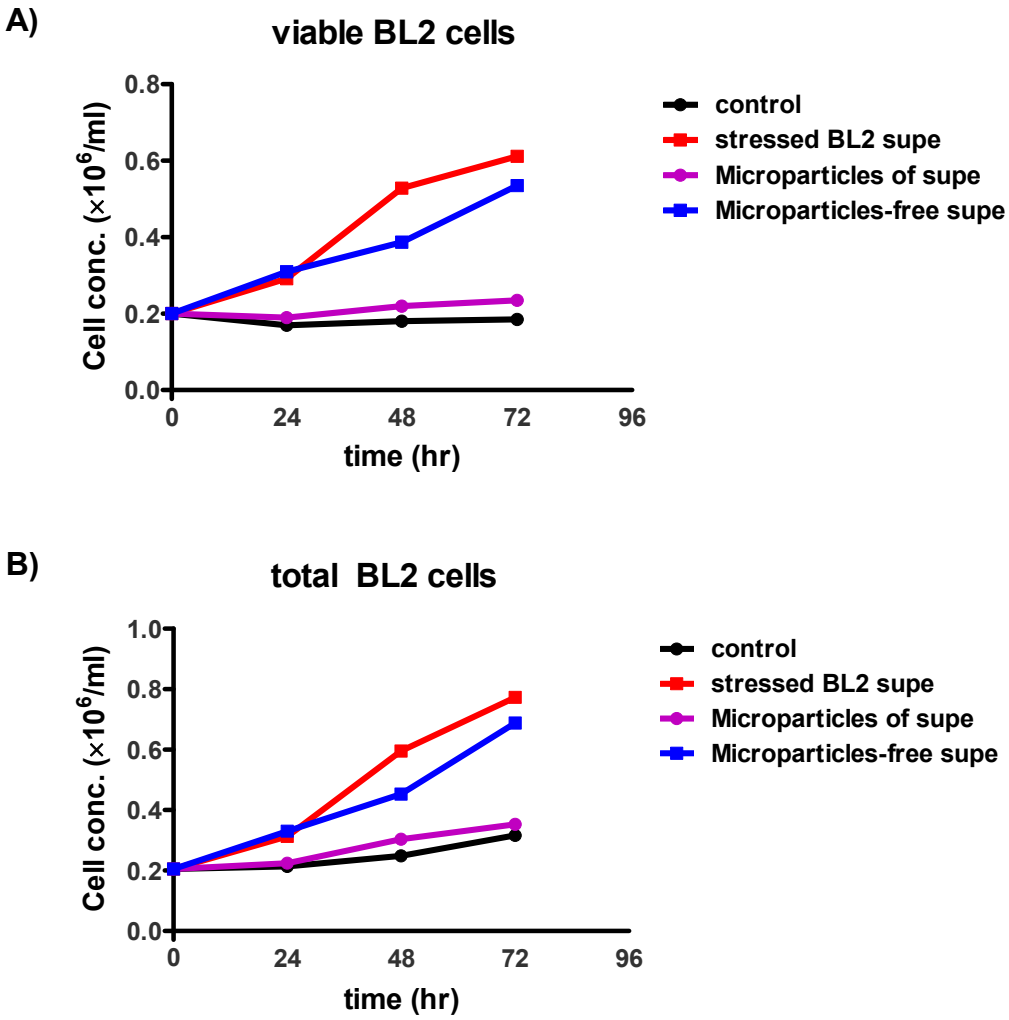


Figure 5.13 The mitogenic effect was not microparticle-associated.

BL2 cells were cultured in serum-free RPMI containing 1% X-VIVO with or without (control) microparticles isolated from supernatant from stressed BL2 cells or supernatant depleted of microparticles. The mitogenic effect of stressed BL2 supernatant diluted ¼ is also shown. Data are means of triplicate samples.

Chapter 6 Results: Generation and phenotypical characterisation of ESDM

6.1 Directed differentiation of murine embryonic stem cells into macrophages

The most common way of generating differentiated cell types from ES cells is to induce them to form three-dimensional multicellular aggregates, which are named embryoid bodies (EBs). There are three basic methods for EBs formation, including suspension culture in bacterial-grade dishes, hanging drops culture, and methylcellulose culture.

When this project was initiated, all published protocols for differentiating murine embryonic stem cells into macrophages adopted the differentiation systems in methylcellulose semisolid media (Moore, Fabunmi et al. 1998; Chawla, Barak et al. 2001; Odegaard, Vats et al. 2007). Although methylcellulose allows seeded ES cells to generate clonal and reproducible cell aggregates (EBs), it disturbs the unrestricted transfer of growth factors added and the handling of the medium by pipettes is difficult (Wiles 1993). The cost of the media is another limiting factor for the scale of experiments. Efficiency of hematopoietic differentiation has been compared between three basic EBs formation methods (Dang, Kyba et al. 2002). Therefore, based on the findings of my previous work (Zhuang 2007), all three methods were assessed to identify the most effective way to generate macrophages from ES cells. The results consistently indicated that compared with hanging drops and methylcellulose culture, suspension culture could yield the highest number of macrophage-like cells with the most straightforward cell culture procedures, although there was a disadvantage that EBs generated in suspension culture were heterogeneous. Thus, work was carried out to further define the optimal protocol using the suspension culture system.

The media for ES cell differentiation ($GMEM_{Diff,}$ see Appendix 1) was similar to ES cell culture media $GMEM_{FCS}$ (see Appendix 1) except that FCS was batch-tested for differentiation. Interleukin 3 (IL-3) plays a key role in the lineage commitment from pluripotent stem cell to myeloid-restricted progenitor and can act on myeloid progenitors to promote their proliferation (Dorshkind 1990). As IL-3 responsive progenitor cells differentiate, they become responsive to colony stimulating factor-1 (CSF-1), which is required for the maintenance of the monocyte phenotype and capable of supporting the differentiation and the long-term survival of the macrophage-like cells (Young, Lowe et al. 1990). Therefore, both CSF-1 (15%) and IL-3 (1ng/ml) were supplemented in ES cell differentiating and EBs culture media (the medium was named $ESDM_{Diff}$, see Appendix 1) and only CSF-1 (15%) was added to supernatant cell-derived progenitor cell culture media (the medium was named $ESDM_{cult}$, see Appendix 1). In-house generated L929 cell conditioned media (L929 CM) was used as the source of CSF-1 and recombinant mouse IL-3 was purchased commercially. The main ES cell line used for differentiation was E14.

Firstly, 6×10^5 trypsin-dissociated E14 cells were seeded in 20 ml $ESDM_{Diff}$, and plated onto one 95 mm diameter bacteriological Petri dish. This time point was defined as day 0. Under the culture condition without leukaemia inhibitory factor (LIF), ES cells started to differentiate and formed three dimensional structures of various sizes which were named embryoid bodies (EBs). As EBs grew bigger, they tended to stick to the Petri dish. To avoid their adherence, EBs were dislodged and transferred to a new Petri dish on day 4 and day 6, and supplemented with $ESDM_{Diff}$. After 8 days of culture, the EBs were transferred to one gelatine-coated tissue culture dish (95 mm), supplemented with 20 ml fresh $ESDM_{Diff}$. EBs became adherent on the dish, and they started to release progenitor cells into the supernatant. Two days later (day 10) their supernatant was harvested and progenitor cells in the supernatant were isolated by centrifugation and re-suspended in 20 ml $ESDM_{cult}$ ($GMEM_{Diff}$ with L929, penicillin

and streptomycin, see Appendix 1) and cultured on one 95 mm diameter bacteriological Petri dish. Adherent EBs were replenished with 20 ml fresh $ESDM_{Diff}$ for prolonged culture of up to 20 days, and supernatants were harvested every 2 days (Figure 6.1A). Adherent cells started to appear on Petri dishes after around 3 to 5 days, and the cell number gradually increased with time and formed a confluent monolayer. On day 7, adherent cells were harvested by using cold detachment buffer and a cell lifter. Around 2 to 4 million macrophages could be harvested from one Petri dish and the viability after detachment was >90% according to trypan blue exclusion.

From one dish of ES cells over long term differentiation, 12 to 24 million macrophages could be produced in total. More macrophages could be generated when required, simply by multiplying the number of dishes of ES cells for differentiation (Figure 6.1B).

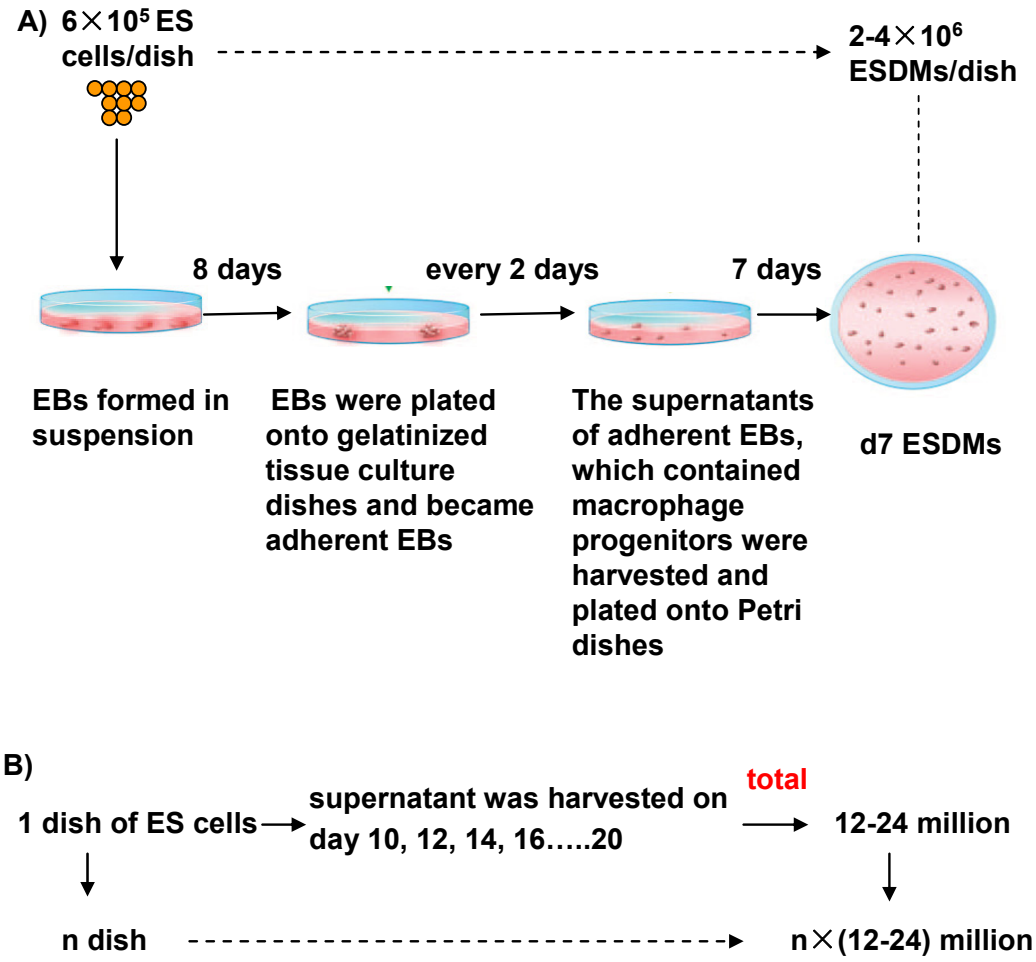


Figure 6.1 Differentiation of murine embryonic stem cells into macrophages.

A) 6x105 ES cells were cultured in suspension for 8 days with CSF-1 and IL-3 in one Petri dish to form embryoid bodies (EBs) and were then plated onto gelatinized tissue culture dishes, also with CSF-1 and IL-3. EBs became adherent after 2 days. From day 10, supernatant of adherent EBs which contained macrophage progenitor cells was collected every 2 days and put onto 95mm Petri dishes, with CSF-1. The supernatant was cultured for 7 days before ESDMs were harvested. B) Since supernatant can be collected every two days from day 10 EBs to day 20 EBs, and 1 dish of ES cells can yield up to 2-4 x10^6 ESDMs, after full term of culture, up to 12-24 million ESDMs can be harvested from 1 dish of 6x10^5 ES cells. Higher numbers of ESDMs can be obtained by multiplying the starting number of ES cell dishes.

6.2 Characterisation of putative embryonic stem cell derived macrophages (ESDM) by morphology

To confirm that the cells which adhered to bacteriological Petri dishes were macrophages, cells were first characterised by morphology both in culture and in stained cytocentrifuged preparations. In culture, the cells were strongly adhesive to plastic and extended long plasma membrane projections with varied morphology, including bipolar and multipolar forms (Figure 6.2C). The morphology indicated that these cells might be macrophages or dendritic cells. However, the cells were so strongly adherent that detachment directly with a cell lifter yielded a high ratio of dead cells, around 50% according to trypan blue exclusion. Pre-treating the cells with cold detachment buffer before scraping with a cell lifter can increase the viability to ~ 90%. Previous work has shown that mouse spleen dendritic cells are semi-adherent, and despite initially adhering to plastic surfaces, they become nonadherent after overnight culture (Steinman and Cohn 1973). Therefore, given the strong adhesive nature of the ES-derived cells, these cells are unlikely to be dendritic cells.

After detachment, the cells were prepared as single cell suspension for cytocentrifugation, followed by Diff Quick staining. Although the cells were heterogeneous in morphology, all of them were mononuclear cells which excluded the possibility of granulocyte contamination.

Most of them showed mature macrophage morphology, including large cells with low nucleocytoplasmic ratio; eccentrically placed, round or kidney-shaped nucleus and a cytoplasm containing numerous vacuoles, reflecting active pinocytosis (Figure 6.2 B, D). There were also cells which had immature macrophage morphology, including high nucleocytoplasmic ratio and dense cytoplasm (Figure 6.2B). Among the mature cells, some either bound to cell debris or had an apoptotic cell engulfed inside the cytoplasm (Figure 6.2D) which indicated active phagocytosis. The morphology of

adherent cells in cytocentrifuged preparations was distinct from that of embryonic stem cells (Figure 6.2A).

Together, the adhesive nature, morphology, pinocytosis and phagocytosis activity suggested that these adherent cells on Petri dishes were macrophages.

6.3 Characterisation of putative ESDM by macrophage-specific markers

6.3.1 Immunoperoxidase staining

6.3.1.1 Cytocentrifuge preparations

In order to confirm the identity of these adherent cells, antibodies against macrophage-specific markers were used for immunostaining. Purified F4/80, CD11b and CD68 antibodies were used to stain cytocentrifuged preparations of cells. CD68, also named macrosialin, is a heavily glycosylated transmembrane protein, expressed by tissue macrophages, Langerhans cells and at low levels by dendritic cells. It is the murine homologue of the human macrophage glycoprotein CD68, and a member of the lysosomal-associated membrane protein family which are located predominantly within the cells. Minute but significant amounts of CD68 have also been detected on macrophage surfaces (Ramprasad, Terpstra et al. 1996). Cytocentrifuge preparation and fixation with acetone made the intracellular antigen accessible to the antibody. CD11b, known as the integrin alpha M chain, is implicated in different adhesive interactions of monocytes, macrophages and granulocytes. F4/80 is a cell surface glycoprotein which is a member of the EGF-TM7 protein family and it has been found that the expression of F4/80 is heterogenous and can vary during macrophage maturation and activation. Unlike other macrophage markers, F4/80 is not expressed on other types of blood cells (Nussenzweig, Steinman et al. 1981).

Most cells showed very strong staining of CD68, CD11b and F4/80 (Figure 6.3A), suggesting high level expression of these antigens, while a minority were weakly stained, which suggested low level or negative expression of antigens. More than 80% of cells were positively stained by F4/80 and CD11b antibodies, and more than 90% of cells were CD68 positive. The staining profile strongly suggested that the adherent cells were macrophages (Figure 6.3B). The stem cell line, E14, was also stained as control and it was negative for all the three markers (Figure 6.4).

6.3.1.2 Staining of cells in chamber slides

In order to determine the expression of the above antigens without the interference of the detachment procedure, non-adherent cells from EBs cultures were transferred to chamber slides and the cells were stained directly on these. The seeding density was adjusted in order to achieve sub-confluence after 7 days culture. Almost all the cells were positively stained by CD68, F4/80 and CD11b antibodies, although some cells were only weakly stained (Figure 6.5). Furthermore, the staining on chamber slides more clearly revealed the bipolar and multipolar stellate morphology of these cells in culture.

6.3.2 Analysis by flow cytometry

Staining of ES cells-derived cells with fluorescence-conjugated antibodies against F4/80, CD11b and CD11c was analyzed by flow cytometry. Staining with CD11c antibody was used to detect dendritic cells, since the latter are also slightly adherent and have some morphological similarity to macrophages. Mouse bone marrow-derived macrophages (BMDM) and resident peritoneal cells were analyzed by the same criteria for comparison with bona fide macrophages.

Macrophages from each of the three sources had a wide range of forward and side light scatter, therefore, a log scale was used for analysis. For both BMDM and peritoneal

macrophages, three subpopulations could be identified by light scatter, named gate 1, 2, and 3 (Figure 6.6A and B). For BMDM, gate 1 cells were positive for F4/80, CD11b and CD11c and gate 2 cells had a lower expression level of F4/80, CD11b and CD11c. For peritoneal macrophages, gate 1 and gate 2 cells were positive for F4/80 and CD11b, but negative for CD11c. Notably, there was a subpopulation in gate 2 that was negative for F4/80 and negative for CD11b. For both BMDM and peritoneal macrophages, gate 3 cells were negative for all three markers. These data indicated that 1) in addition to macrophages, both BMDM and peritoneal cells also contained F4/80 and CD11b negative cells which were presumably not macrophages; 2) BMDM gate 1 and 2 cells were contaminated by classical dendritic cells as defined by CD11c.

For ESDM, three subpopulations, two major and one minor, could be defined by light scatter, named gate 1, 2 and 3 (Figure 6.6 C and D). Unlike BMDM and peritoneal cells, ESDM in all three gates were positive for F4/80 and CD11b, but negative for CD11c. Similar to resident peritoneal macrophages, there was also a small population in gate 2 that was negative for F4/80 and CD11b.

Every time after supernatant was collected, EB cultures were supplemented with fresh media, so the culture of embryoid bodies could be maintained to continually generate macrophage progenitor cells. ESDM from day 10 embryoid bodies and ESDM from day 20 embryoid bodies were analysed to see whether they had the same phenotype. Both of them were cultured for 7 days. ESDM from these two sources showed similar pattern, positive for F4/80 and CD11b, but negative for CD11c. But ESDM day 20 had slightly weaker fluorescence intensity, compared with ESDM day 10 and the unspecific binding of F4/80 increased, which might be caused by increased expression of Fc receptors (Jungi and Hafner 1986; Clarkson and Ory 1988) (Figure 6.6 D). There was also a small population in gate 2 negative for F4/80 and CD11b.

In Figure 6.6, gate 2 cells of ESDM were always less strongly stained than gate 1 cells, and there was always a small population in gate 2 that was negative for F4/80 and CD11b. Since the above fluorescence analysis was performed on the whole cell population, bearing in mind that dead cells might lose some cell surface molecules, the viability of gate 2 cells was assessed using propidium iodide (PI) to see whether gate 2 cells were simply dead macrophages. Backgating showed that PI-positive cells were not exclusively in gate 2 but were present in all the three gates (Figure 6.7), although the majority of dead cells were in that gate. The presence of around 20% dead cells within ESDM population offered one explanation of the identify of the 10%-20% unstained cells in immunoperoxidase staining and the small negatively stained population of gate 2 cells in immunofluorescence staining. However, in order to confirm this directly, fluorescence-conjugated antibody staining combined with PI binding should be performed. Nevertheless, it did not exclude other possibilities. Because only single staining was performed, it did not distinguish whether it was the same contaminating population that was negative for all three markers or they were indeed macrophages at various stages of maturation which temporarily lack of certain antigen expression. Multicolour staining with all the markers could help to resolve this.

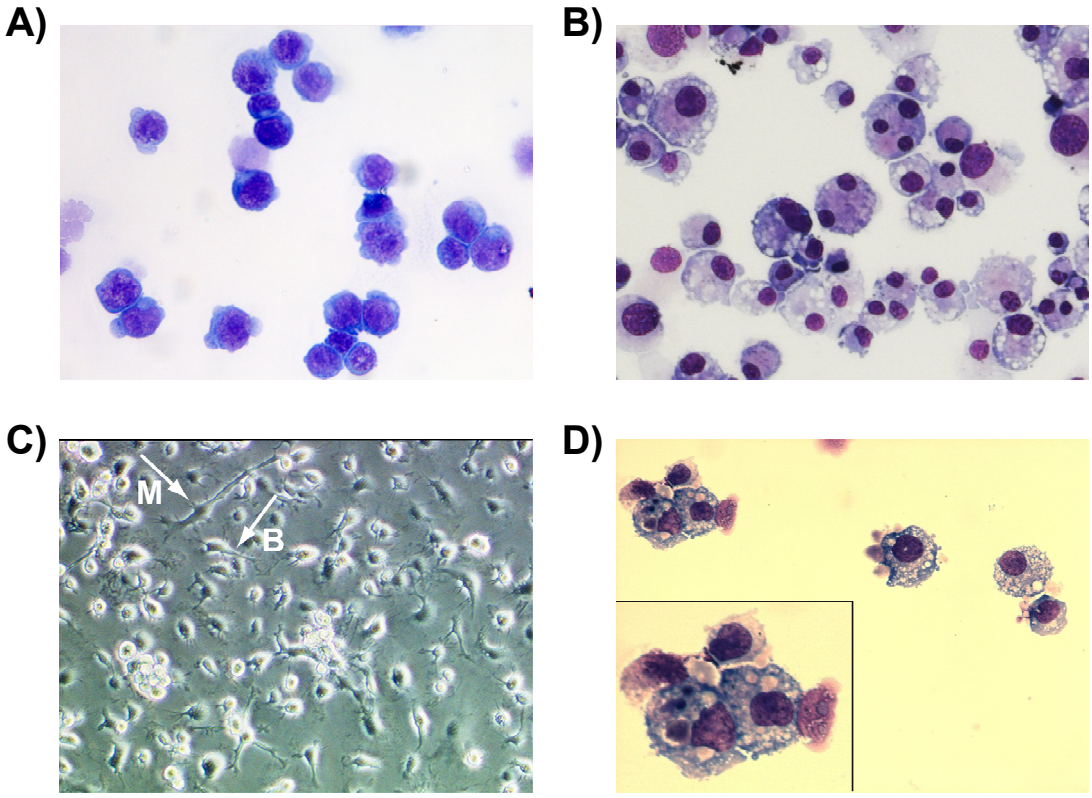


Figure 6.2 Morphological comparison of ES cells and derived macrophages.

A: Morphology of ES cells revealed by Quick-Diff staining of cytocentrifuge preparation. B: ESDM differentiated from macrophage progenitor cells by adherent culture for 7 days. Cytocentrifuge preparation of detached ESDM revealed by Quick-Diff staining. C: Phase contrast micrograph of ESDM in culture showing bipolar (arrow, B) and multipolar (arrow, M) morphologies. D: ESDM bind and engulf apoptotic cells and cell debris. Cytocentrifuge preparation of detached ESDM revealed by Quick-Diff staining. Inset: higher magnification showing internalized material.

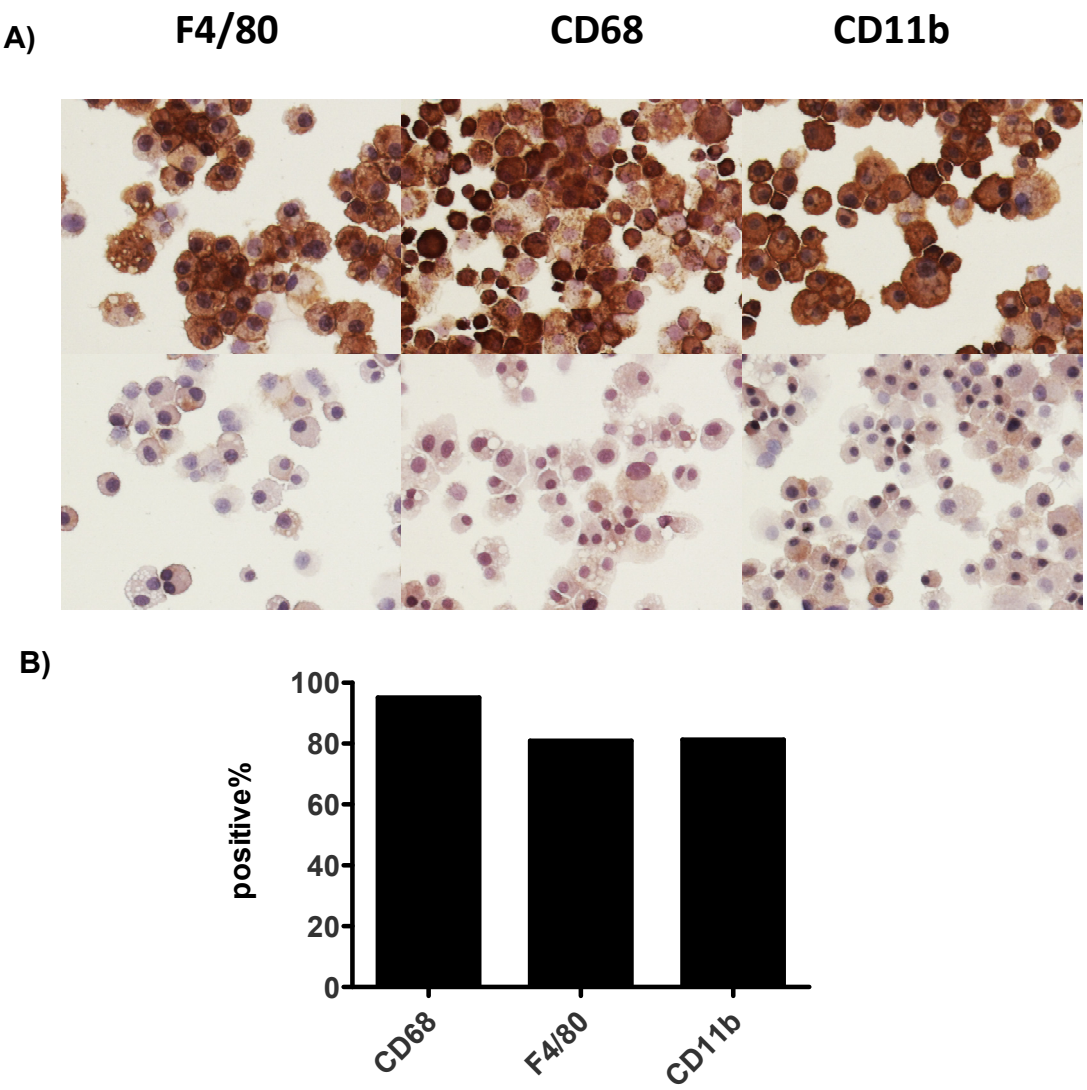


Figure 6.3 Immunocytochemical demonstration of macrophage markers expressed by ESDM

Cytocentrifuge preparations of ESDM stained with peroxidase-conjugated rat anti-mouse macrophage specific antibodies against F4/80, CD68 and CD11b (upper panel) or isotype control reagents (lower panel). Binding was detected using DAB substrate (brown) and haematoxylin was used as counterstain.

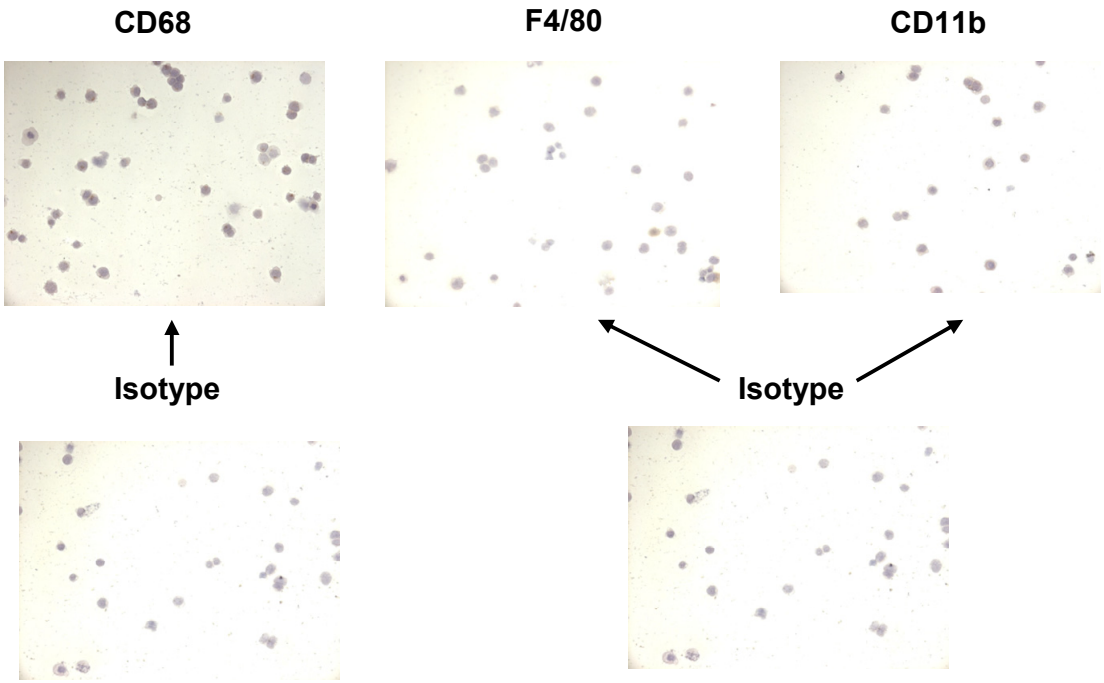


Figure 6.4 Absence of macrophage-specific markers from E14 cells.

Cytocentrifuged preparations of E14 cells were stained with rat anti-mouse macrophage markers, CD68, CD11b and F4/80 or isotype control antibody. Other details of immunoperoxidase staining were as given in legend to Fig. 6.3.

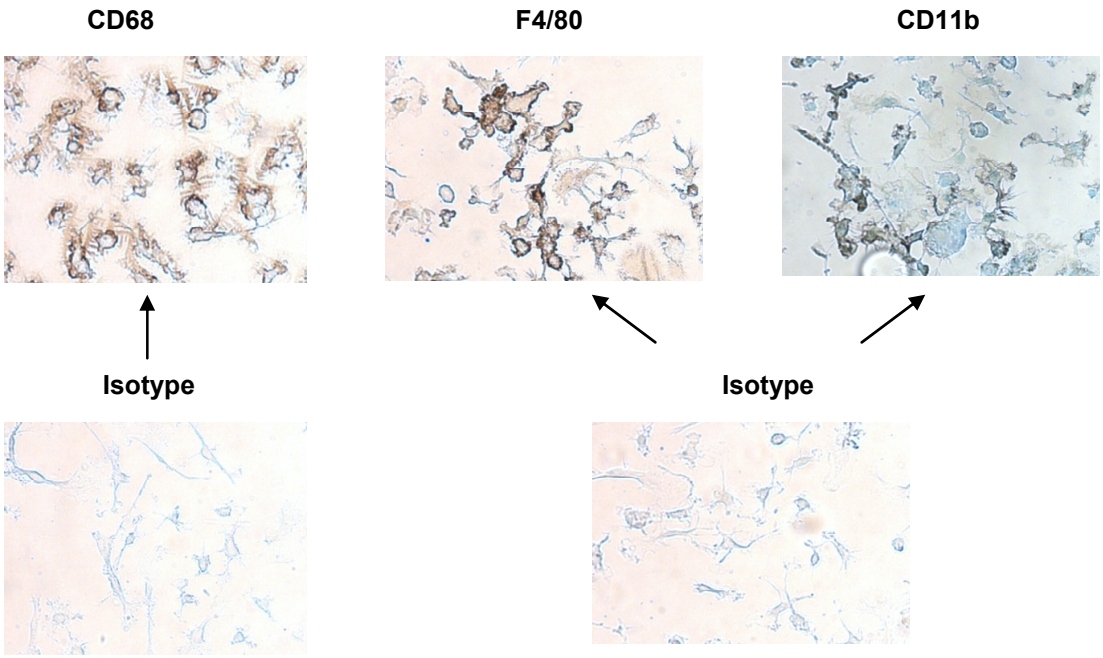
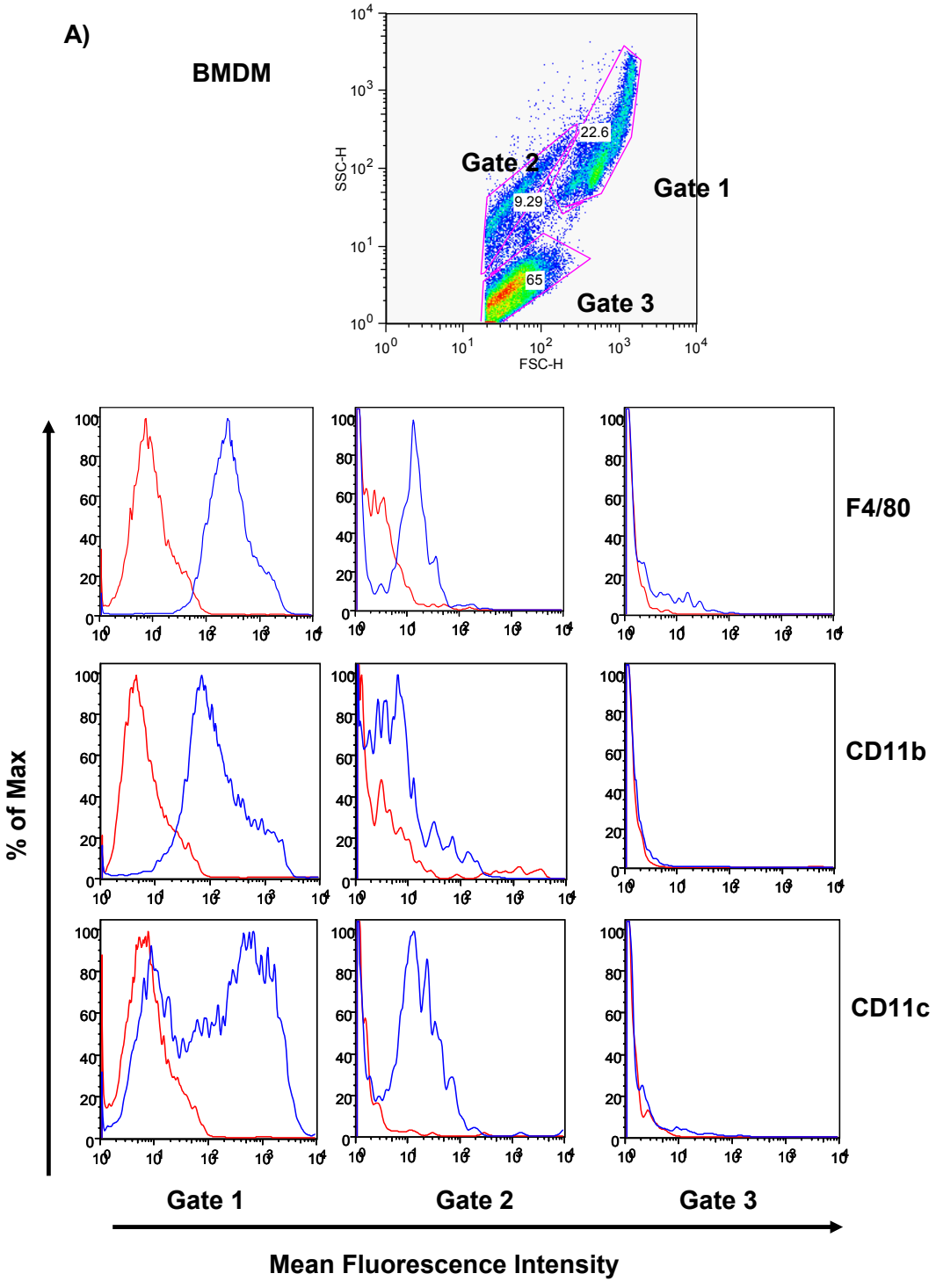
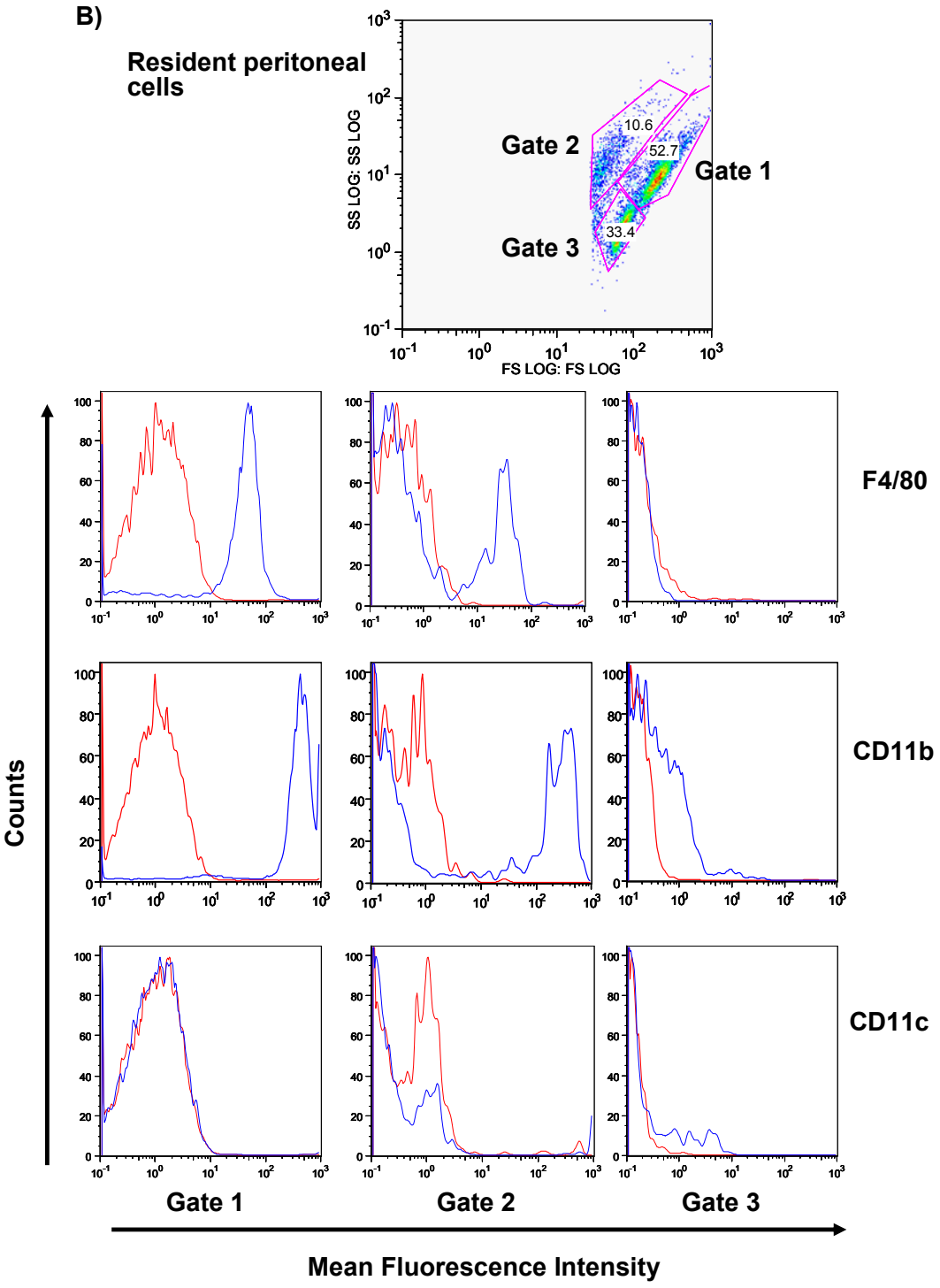


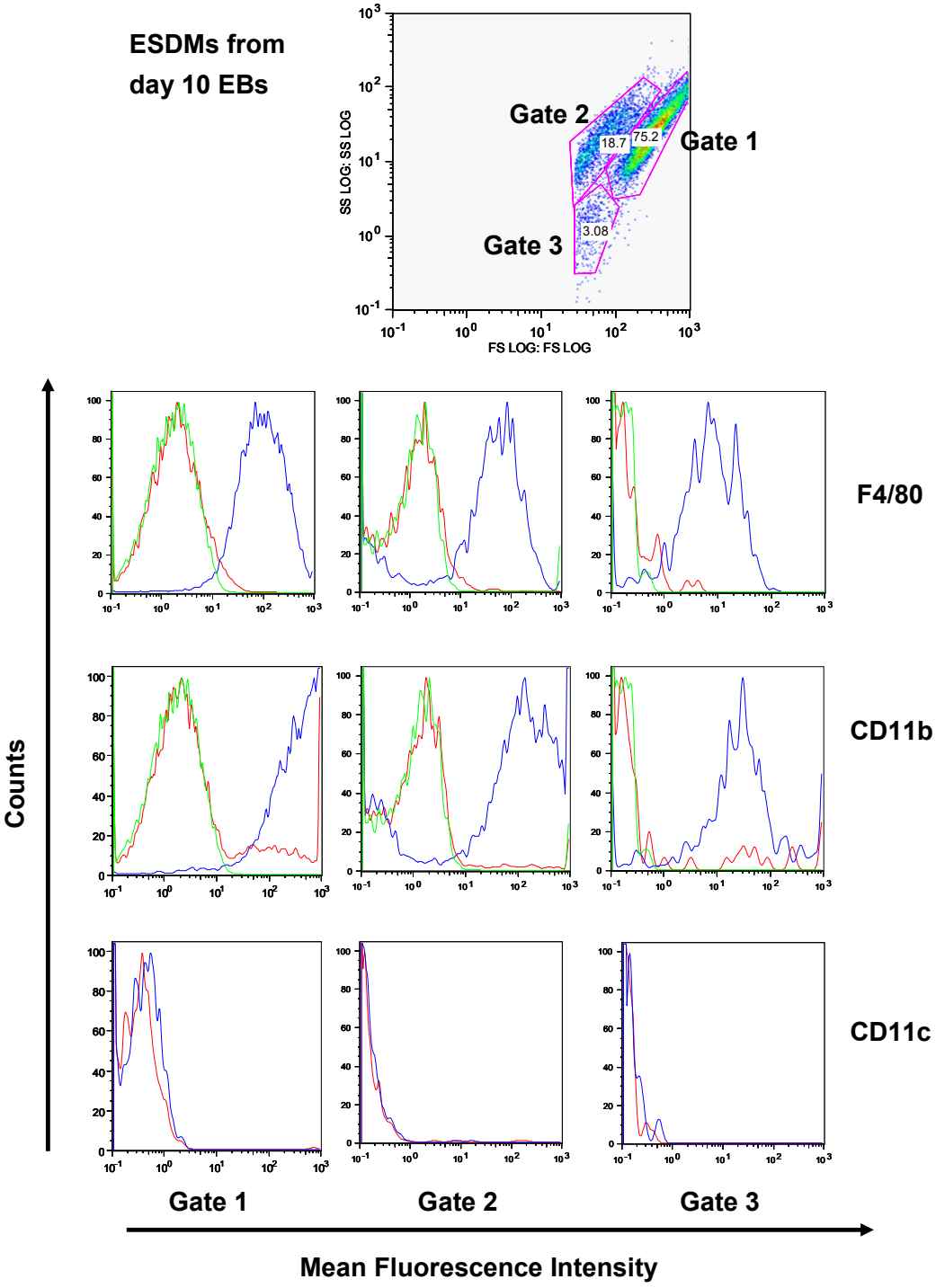
Figure 6.5 Cells derived from adherent EBs express macrophage-specific markers.

Non-adherent cells from EB cultures were transferred to chamber slides and stained with rat anti-mouse macrophage-specific markers, CD68, CD11b and F4/80 or isotype controls. Other details of immunoperoxidase staining were as given in legend to Fig. 6.3.


A)
BMDM
SSC-H
FSC-H
Gate 1
Gate 2
Gate 3
22.6
9.29
65
F4/80
CD11b
CD11c
% of Max
Gate 1
Gate 2
Gate 3
Mean Fluorescence Intensity


B)
Resident peritoneal cells
SS LOG: SS LOG
FS LOG: FS LOG
Gate 2
Gate 1
Gate 3
10.6
52.7
33.4
F4/80
CD11b
CD11c
Counts
Gate 1
Gate 2
Gate 3
Mean Fluorescence Intensity

C)


ESDMs from
day 10 EBs
Gate 2
Gate 1
Gate 3
18.7
75.2
3.08
SS LOG: SS LOG
FS LOG: FS LOG
F4/80
CD11b
CD11c
Counts
Gate 1
Gate 2
Gate 3
Mean Fluorescence Intensity

D)

ESDMs from day 20 EBs

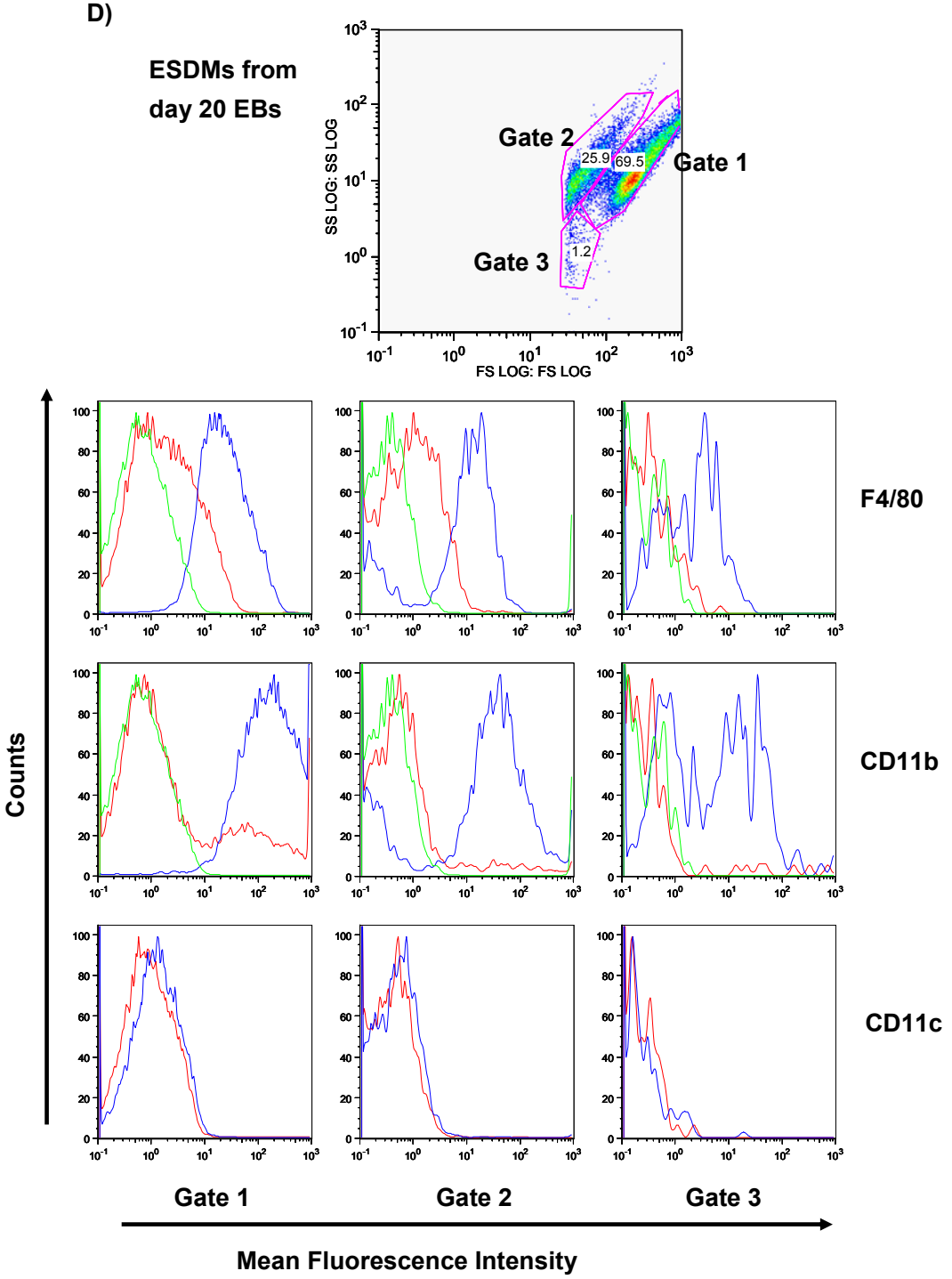


Figure 6.6 Flow cytometric analyses of macrophage and dendritic cell markers on ESDM, BMDM and resident peritoneal macrophages.

7 day-matured ESDM derived from supernatants of day 10 EBs and day 20 EBs, BMDM and peritoneal macrophages were stained with antibodies to F4/80, CD11b and CD11c or isotype controls. Cell sub-populations were defined by forward and side light scatter and staining of these was analysed by electronic gating on a flow cytometer. The histograms for fluorescent staining are shown for different gates. A). BMDM, B) Resident peritoneal macrophages, C) ESDM from day 10 adherent EBs, D) ESDM from day 20 adherent EBs. Green: unstained cells, red: isotype control; blue, antibody as indicated. BMDM and resident peritoneal macrophages were from Balb/c mice. The data are representative of those from three independent experiments.

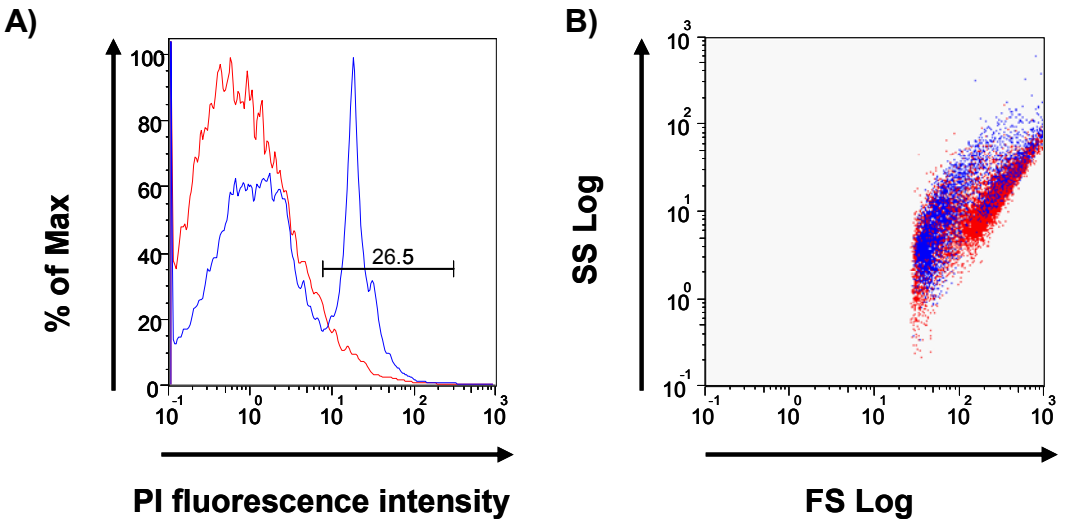


Figure 6.7 The second major ESDM subpopulation defined by light scatter contained viable cells.

ESDM were stained with PI to identify dead cells. 26% of cells were stained, shown by blue line in histogram, A); red indicates fluorescence of unstained cells. B) Backgating of PI positive staining on ungated scatter plots: blue, PI positive; red, ungated cells. Data representative of those from three independent experiments are shown.

6.4 Self-renewal assay to exclude the possibility of undifferentiated embryonic stem cell contamination

Since undifferentiated embryonic stem (ES) cells can form teratomas in vivo (Nussbaum, Minami et al. 2007), the possibility that ESDM might be contaminated by undifferentiated ES cells could hinder the future in vivo application of ESDM. Given the FACs data showed a small subpopulation in gate 2 of ESDM to be negative for both F4/80 and CD11b and the staining of cytocentrifuge preparation indicated approximately 10% of cells were negative for F4/80 and CD11b, it was necessary to exclude the possibility of ES cell contamination in ESDM. A self-renewal assay, which is based on the principle that the undifferentiated state of ES cells can be characterised by high level expression of alkaline phosphatase (Pease, Braghetta et al. 1990), was therefore performed. ESDM were seeded at different densities, 10^3, 10^4, 10^5, and 10^6 per well, with the ES cell line E14 included as a positive control. Two different culture conditions were used: $GMEM_{FCS}$ with and without LIF. LIF is the key factor for ES cell renewal. For E14 cells, three kinds of colonies were formed at 10^3 per well. At higher densities, E14 cell colonies merged (10^4, 10^5, and 10^6 per well), and it was impossible to identify and quantify individual colonies. Therefore only cultures seeded at 10^3 per well were analysed.

The colonies formed by ES cells were categorised based on their morphology and the intensity of alkaline phosphatase staining (Figure 6.8 A-C). Stem cell colonies are small, tightly packed and strongly-stained colonies and consist of only pink-stained, alkaline phosphatase positive cells. Mixed colonies have a central core of undifferentiated strongly stained ES cells surrounded by differentiated, unstained cells. Differentiated colonies are translucent, loosely packed colonies with little or no stain. The number of each type of colonies at 10^3 was quantified (Figure 6.8 G). In $GMEM_{FCS}$ with LIF, the majority were stem cell colonies, while without LIF, the majority were differentiated colonies with no stem cell colonies at all.

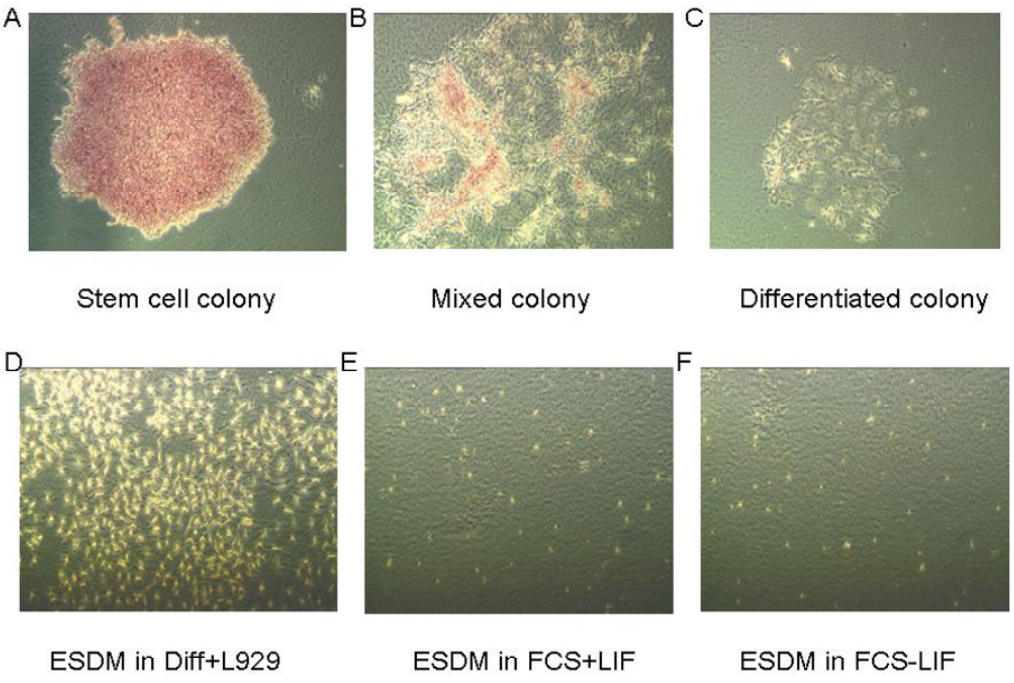
ESDM were growing well in $ESDM_{cult}$ but not in $GMEM_{FCS}$ either with or without LIF (Figure 6.8 D-F). However, there were no colonies formed in ESDM at all seeding densities and there was absolutely no pink staining with each condition. The results indicated that in up to 1 million ESDM there was no contamination by undifferentiated ES cells.

6.5 Endotoxin control of ESDM culture condition

LPS can modulate macrophage functions and profoundly affect macrophage physiology. Before performing further functional studies, it was therefore critical to test whether ESDM were grown in an endotoxin-free environment. All the cell culture media, serum and other additive reagents employed were designated to be nonpyrogenic by their commercial suppliers and were not assessed. However, the L929 CM which was produced in-house was tested for endotoxin using an endpoint chromogenic limulus amebocyte lysate (LAL) assay. Four batches of L929 CM were generated and stored at -20°C for future use. They were analyzed, and their endotoxin level were all less than 0.1 endotoxin units (EU)/ml, which is below the endotoxin limit for sterile water for injection set by the FDA, which was 0.25 EU/ml (Figure 6.9). It was also much lower than the lowest endotoxin level that has been found to activate macrophages (5 EU/ml) (Morris, Crowe et al. 1992).

Furthermore, to confirm ESDM were not activated by endotoxin over long term culture, ESDM matured for 7 days were tested for TNF-alpha in their conditioned media by TNF-alpha ELISA, since macrophages activated by endotoxin can release TNF-alpha. $ESDM_{Diff}$ was used as a negative control and the supernatant of LPS and recombinant mouse IFN-gamma treated ESDM was used as a positive control. 16 samples of ESDM conditioned media were examined (Figure 6.10). Compared with the negative control, TNF-alpha levels of all the samples were similar to background (dot pointed by red arrow) and much lower than that of classically activated ESDM

(dot pointed by blue arrow). These data suggested that during long term culture, ESDM were grown in an endotoxin-free environment or in other words, the endotoxin level in ESDM culture was not sufficient to classically activate these macrophages. Furthermore, the data demonstrated that ESDM had the machinery to be classically activated and can release high levels of TNF alpha (around 1900 pg/ml).



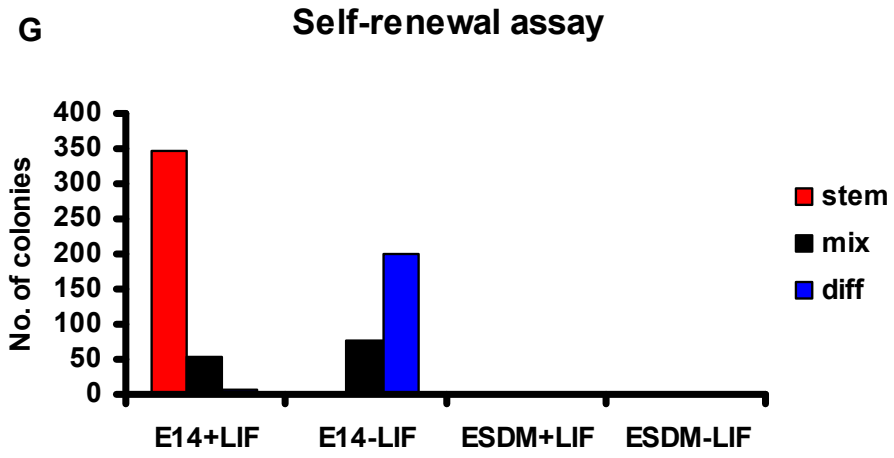


Figure 6.8 Self-renewal assays to exclude the possibility of undifferentiated embryonic stem cell contamination.

Alkaline phosphatase was used as the marker for undifferentiated ES cells under early conditions of LIF removal. Cells expressing alkaline phosphatase stained pink. In this assay, ES cells were cultured in the presence of LIF for one day to keep cell renewal before being changed to fresh media with or without LIF. After 5 days, medium was removed and colonies were fixed and stained for alkaline phosphatase. ES cells formed 3 kinds of colonies, including stem cell colony (A: tight, strongly stained, pink colony), mixed colony (B: pink stem cells and differentiated unstained cells), and differentiated colony (C: translucent dispersed colony with little or no stain). E, F: The same procedure was applied to ESDM and no colonies were derived from ESDM, with or without LIF, and there was no pink stain at all. D: ESDM were cultured in their growth medium as a control. G. Quantification of colonies formed by E14 cells and ESDM. Results are representative of two independent experiments.

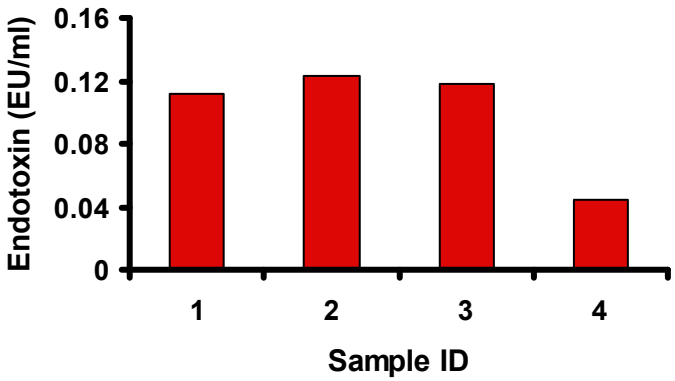


Figure 6.9 Endotoxin level of the in-house L929 conditioned medium (CM) was examined by LAL Assay.

LAL assay was performed according to the manufacturer's instruction. LAL assay standard curve is in Appendix 3. Four batches of L929 CM were analyzed.

Sample ID	1	2	3	4	5	6
TNFα (pg/ml)	<1	<1	<1	<1	<1	<1
Sample ID	7	8	9	10	11	12
TNFα (pg/ml)	<1	<1	<1	<1	<1	<1
Sample ID	13	14	15	16	17	18
TNFα (pg/ml)	<1	<1	<1	<1	<1	1964

Figure 6.10 Confirmation of long term endotoxin free environment in ESDM culture by TNF alpha ELISA.

Standard curve of TNF alpha ELISA is in Appendix 3. Samples of ESDM culture supernatant (Sample ID 1-16) were tested for the presence of TNF alpha. Sample 17: fresh ESDM culture medium alone as the negative control. Sample 18: The supernatant from ESDM treated with IFN-gamma and LPS for 24 hours as the positive control. The data shown are one representative of three independent experiments.

Chapter 7 Results: Functions of ESDM: phagocytosis and chemotaxis

7.1 Phagocytic ability of ESDM to engulf apoptotic tumour cells

An important functional feature of macrophages is their phagocytic ability and it was intended that ESDM generated in this study would be primarily applied in the future to examine the interaction between apoptotic tumour cells and macrophages in Burkitt's lymphoma (BL), therefore it was essential to evaluate the ability of ESDM to phagocytose apoptotic BL cells.

Flow cytometry-based phagocytosis was performed using the published method (Jersmann, Ross et al. 2003) due to its advantage of 1) distinguishing bound from internalized apoptotic cells 2) eliminating vigorous washings which might skew the magnitude of the overall phagocytic response.

BL2 cells were used as phagocytic targets. BL2 cells were labelled with far-red fluorescent dye DDAO-SE and induced to undergo apoptosis following UV-irradiation. Apoptosis was assessed by Annexin V/ PI staining (Figure 7.1A). Meanwhile, their red fluorescent labelling (detected using the FL4 channel) was confirmed in Figure 6.11 C (UV-BL2 cells).

ESDM constitutively expressing eGFP (GFP7a-ESDM) were used as phagocytic cells. They were derived from an ES cell line, GFP#7a, which constitutively expresses eGFP (Gilchrist, Ure et al. 2003). The macrophage identity of these cells was confirmed by F4/80 and CD11b staining (Figure 7.1 B), and their green fluorescence (FL1 channel) was demonstrated in Figure 7.1 C.

After apoptotic BL cells and ESDM were co-cultured, aside from the two single fluorescence-labelled populations, a third population that was positive for both FL1 and FL4 was observed. According to the published method, trypsin/EDTA can successfully release bound cells from the surface of phagocytes, and therefore the double positive cells are predicted to be the macrophages that have engulfed apoptotic cells.

7.2 Optimization of ESDM chemotaxis assay

7.2.1 Inconsistency of ESDM migratory response to C5a

The results presented in chapter 4 demonstrated that stressed BL cell supernatants could release nucleotide to attract THP-1 cells in vitro. However, in order to validate the finding, in vitro chemotaxis assay with primary macrophages and in vivo experiments have to be performed. ESDM stood out as a primary candidate considering their similarity to primary macrophages and feasibility for genetic manipulation. After the identification and phagocytic ability of ESDM had been appraised, it was important to evaluate their migratory ability using a chemotaxis assay. The classical macrophage chemoattractant C5a (Snyderman, Pike et al. 1975) was used as a positive control, and chemotaxis assay medium (see Appendix 1) was used as a negative control. The same experiment was repeated seven times. However, the results were inconsistent, and the ratio of migrated cell number towards positive control and towards negative control varied from <1 to 7 (Figure 7.2), indicating that ESDM have migratory machinery to respond to C5a. Nevertheless, at least under these experimental conditions, the response was very variable. Therefore, measures were taken to optimize the chemotaxis assay protocol in an attempt to achieve consistent ESDM chemotaxis responses.

7.2.2 Effect of different pore size on ESDM migration responses to C5a

Published macrophage chemotaxis assays vary widely for the same type of macrophages in the choice of membrane pore size between 5μm and 8μm. Thus, in order to identify whether pore size was the reason for the above inconsistency, the effect of pore size on ESDM migration to C5a was examined. Both 5μm and 8μm pore size were assessed (Figure 7.3). However, regardless of the pore size used, the migration of ESDM to C5a was not significantly different from the background control. The data demonstrated that migration of ESDM to C5a cannot be optimized by adjusting membrane pore size.

7.2.3 Effect of CSF-1 starvation on ESDM migration to C5a

CSF-1 has been found to be able to mobilize the macrophage cytoskeleton and induce rapid behavioural responses (Boocock, Jones et al. 1989). Macrophages stimulated with CSF-1 demonstrated migration without directional preferences (which was named chemokinesis) in response to linear CSF-1 gradients, however, CSF-1-deprived cells can orientate towards the CSF-1 gradient (Webb, Pollard et al. 1996). Given that ESDM were derived in an environment rich in CSF-1 (serum and supplemented L929 CM) for long term, in response to the stimulus of C5a, they might have lost their chemotactic ability. Hence, in an attempt to starve them of CSF-1 and enhance their sensitivity towards migration signals, ESDM were prepared in assay medium and cultured in upper chambers of the transwells for 24 hours. 5μm and 8μm pore sizes were assessed. Freshly detached, non-starved ESDM were used for comparison (Figure 7.4). The data showed that starved ESDM did not possess better migratory ability towards C5a, suggesting CSF-1 was not a significant factor in modulating ESDM in this chemotaxis model.

7.2.4 Effect of different assay media on ESDM migration

In most experiments, compared with the response to C5a, ESDM displayed high levels of migration to assay medium (control). This can be viewed as lack of a response to C5a, or from another perspective, as certain components of the assay medium may induce high levels of migration. Considering that the above optimization process did not strengthen the response to C5a, assay media were assessed for their ability to induce nonspecific chemotaxis responses which potentially masked the response to C5a. In order to address this question, another commercial protein-free medium, chemically defined hybridoma medium (CD Hybridoma Medium, Invitrogen), was assessed in the assay. Meanwhile, given that no protein was present in the assay medium and macrophages might be inclined to bind to the membrane nonspecifically, causing false high background migration, low endotoxin BSA (0.1% w/v) was added to assay medium to see whether BSA can reduce the background migration. Both 5μm and 8μm pore size were assessed (Figure 7.5). Compared with assay medium, CD Hybridoma Medium induced a higher chemotaxis response, and BSA supplementation did not reduce the background, suggesting that of the media tested, the protein-free assay medium (serum-free RPMI) was to be preferred for future experiments.

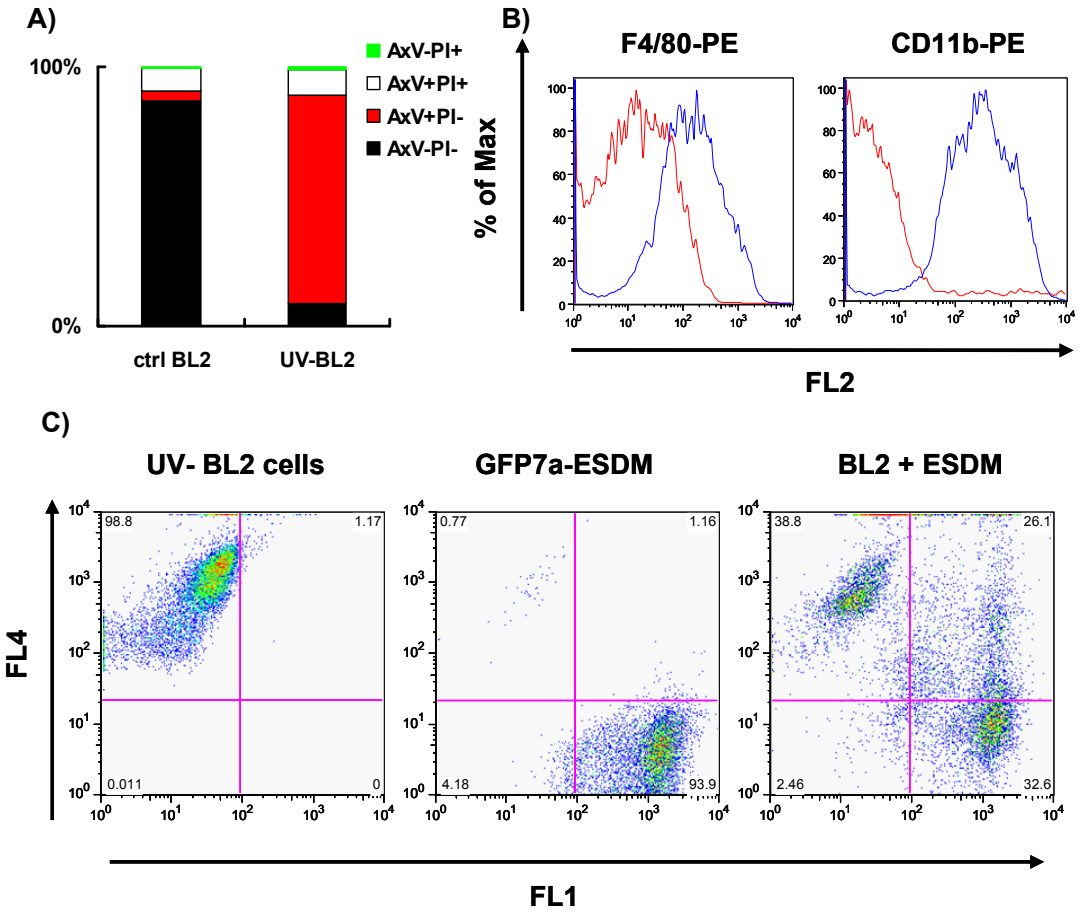


Figure 7.1 Phagocytosis of apoptotic BL cells by ESDM.

Flow cytometry- based phagocytosis assay was performed to examine the phagocytic ability of ESDM. A) BL2 cells were labeled with far-red fluorescent dye DDAO-SE before being treated with UV and incubated at 37°C for three hours to induce apoptosis. The apoptosis was assessed by AxV/PI staining. The red bar represents the early apoptotic population (Annexin V positive and PI negative). B) ESDM used in this phagocytosis assay were derived from the GFP-expressing mouse embryonic stem cell line, GFP#7a, using the same differentiation protocol and their expression of F4/80 and CD11b was assessed. C) DDAO-SE labeled apoptotic BL2 cells (UV-BL2) were co-cultured with ESDM derived from GFP#7a (GFP 7a-ESDM) for 30 mins before being washed and trypsinized for analysis by flow cytometry (BL2+ESDM).

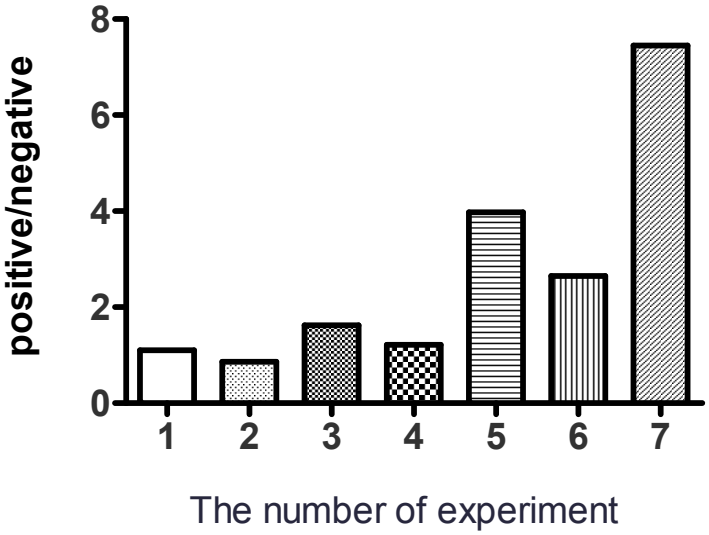


Figure 7.2 Inconsistency of ESDM migratory responses to C5a.

Chemotaxis assay was performed with 8μm pore size membrane transwells. C5a was loaded into lower chambers and incubated at 37ºC for two hour equilibrium before loading ESDM. C5a (10 ng/ml) was positive control; assay media was negative control. Samples were analysed in duplicate. The data presented are the mean ratios of migrated cell number towards positive control to that of negative control. Data from seven independent experiments are presented.

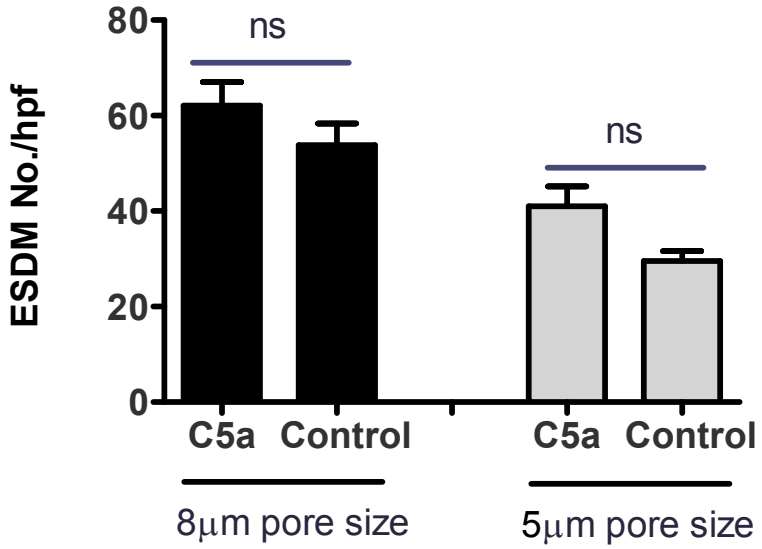


Figure 7.3 Effect of different pore size on ESDM migration in response to C5a.

Both 5 μm and 8 μm pore size membranes were assessed in chemotaxis assays. The data presented are the mean numbers of migrated ESDM per high power field. Error bars indicate SEM. 8 μm pore size: n=4; 5μm pore size: n=3; ns: P>0.05 (Mann-Whitney test).

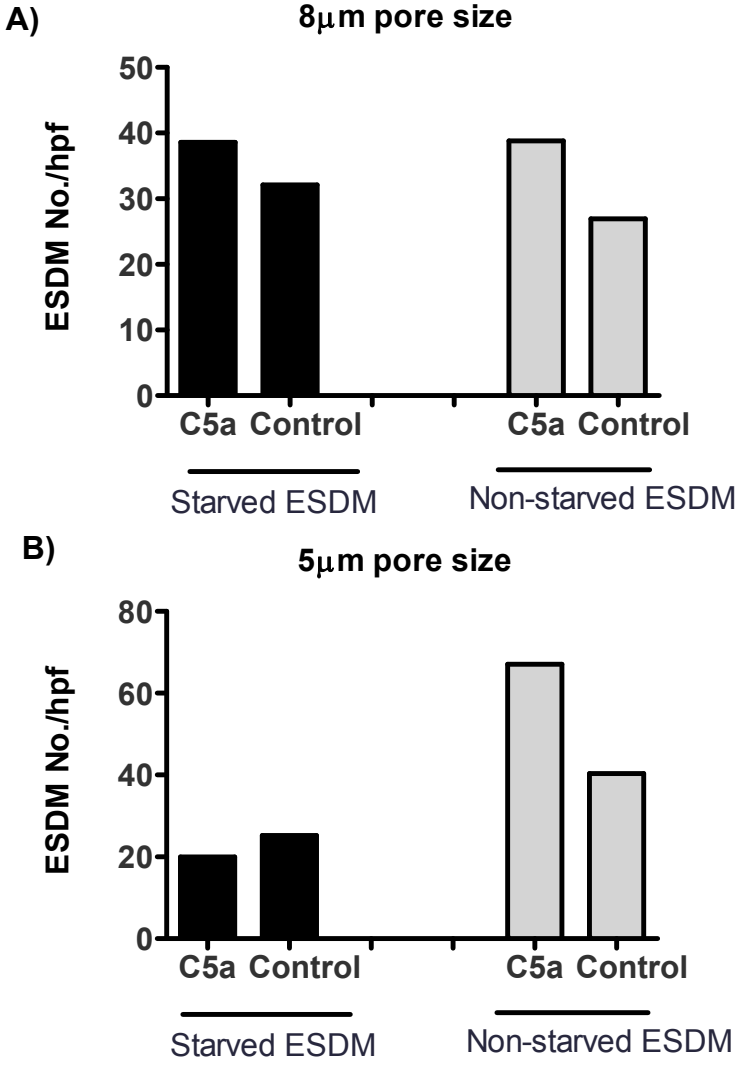


Figure 7.4 Effect of CSF-1 starvation on ESDM migration to C5a.

7 day-matured ESDM were detached and seeded into upper chambers in assay medium for 24 hour (37ºC, 5%CO2) to starve the cells from serum factors before loading C5a in the lower chamber. Both 5μm (B) and 8 μm (A) pore size membranes were assessed in the chemotaxis assay. Non-starved ESDM were used as a comparison. The data presented are the mean numbers of migrated cells per high power field.

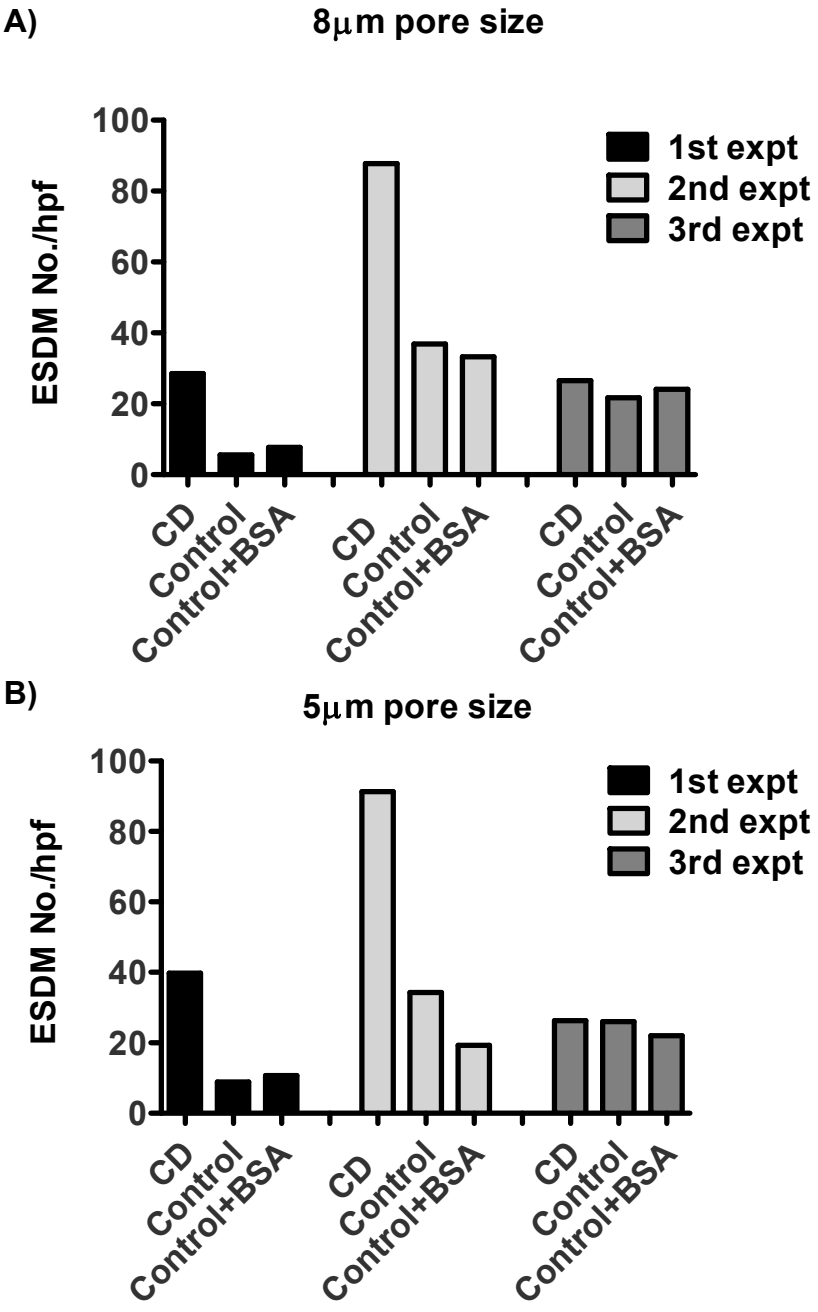


Figure 7.5 Identification of the optimal assay medium for ESDM chemotaxis.

Both 5μm (B) and 8 μm (A) pore size membranes were assessed in the chemotaxis assay. CD: Chemically Defined Hybridoma Medium (Invitrogen); Control: assay medium; Control +BSA: assay medium supplemented with 0.1% low endotoxin BSA. Data are the mean numbers of migrated ESDM per high power field and from three independent experiments

7.2.5 Effect of chemoattractant equilibrium on ESDM migration

Conventionally in chemotaxis assays, C5a is added to the lower chambers and cultured at 37 °C (in a humidified 5% CO_2 atmosphere) for an extended period (eg. 2 hours see 2.21.1) before macrophages are loaded in upper chambers to allow equilibrium conditions to be established. However, this allows the possibility that C5a lost some activity during the incubation, leading to reduced chemotaxis responses. In order to see whether ESDM can migrate to C5a without equilibrium, ESDM were loaded in upper chambers immediately after C5a was added. Both 5μm and 8μm pore size were assessed (Figure 7.6). In 8μm membrane pore size group, the response to C5a was around three times more than the background control, and the trend was reproducible in three independent experiments. However, the responses showed wide variation between experiments which might be caused by ESDM differences. The results in 5μm membrane pore size were still inconsistent between three experiments. The data suggest that under non-equilibrium conditions and with an 8μm membrane pore size, ESDM can display greater migratory responses to C5a.

The optimal conditions for ESDM to migrate towards C5a were selected as follows: 1) 8μm membrane pore size; 2) serum-free RPMI as assay medium; 3) no starvation before the assay; 4) no chemoattractant equilibrium. Therefore, another chemotaxis assay was performed using these conditions to validate the protocol and also to identify the most potent concentration of C5a to attract ESDM (Figure 7.7). Surprisingly, the response to C5a was only slightly higher than the background control, and the four different concentrations of C5a showed similar chemoattractant ability. This indicated that under certain circumstances, the optimized protocol still generated inconsistent results.

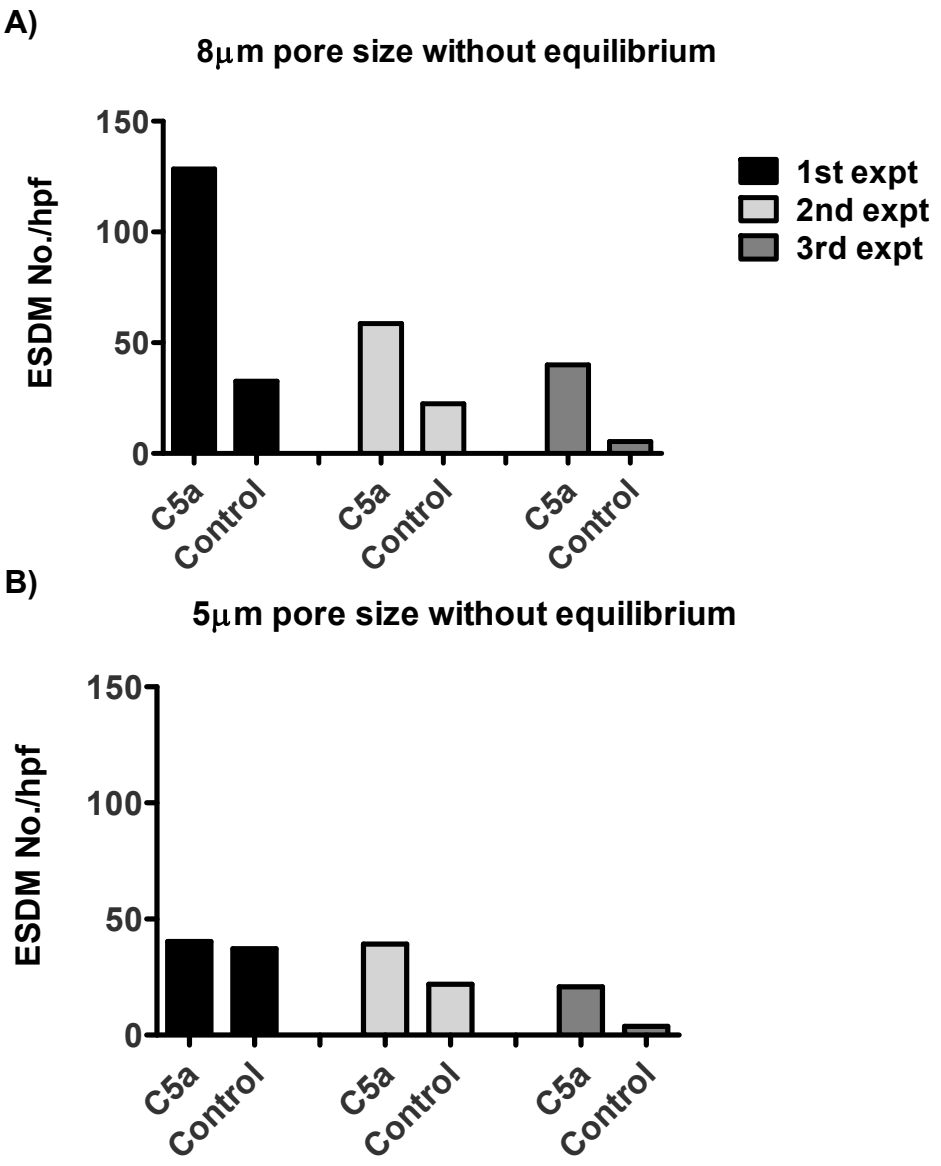


Figure 7.6 Chemotaxis of ESDM under non-equilibrium conditions.

ESDM were loaded in upper chambers immediately after C5a was added in lower chambers. Both 5μm (B) and 8 μm (A) pore size membranes were assessed in the chemotaxis assay. The data are presented as the mean numbers of migrated ESDM/hpf. The data presented are from three independent experiments.

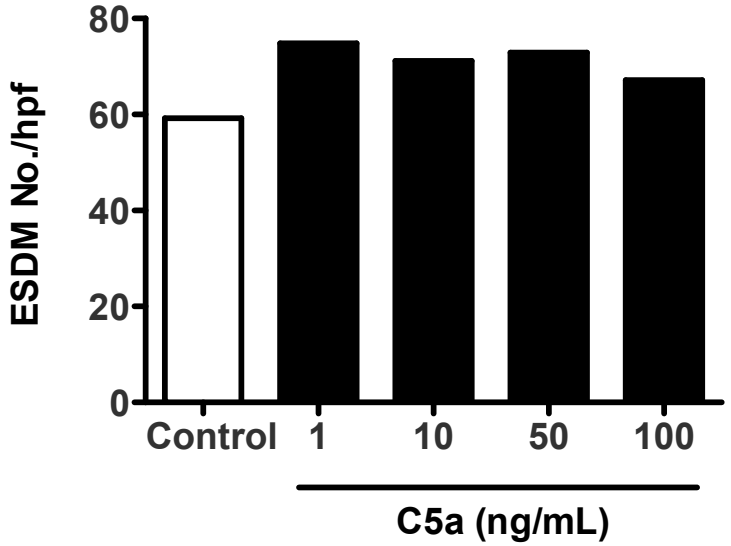


Figure 7.7 Chemotaxis of ESDM under the optimized conditions.

The following conditions were used: 1) 8μm pore size membrane 2) serum free RPMI as assay medium; 3) ESDM were not starved before the assay; 4) no equilibrium was performed before the assay. Four different concentrations of C5a were examined. The data presented are means of migrated ESDM/hpf. The data are from one experiment.

7.2.6 Culture detached ESDM in suspension in Teflon pots for 24 hours

The fact that the optimized protocol generated inconsistent results raised doubts about its validity. However, although all the above adjustments were made to the protocol, the status of the ESDM activation was not assessed. Given that only one out of four experiments showed aberrant results, and, on top of that, within the three experiments the magnitude of the response was quite different, hence, the variation in ESDM activation status might be responsible for the variable chemotaxis results, especially the effect of the harsh detachment procedure on the cells. Thus, 24 hours before the chemotaxis assay, ESDM were detached from Petri dishes and cultured in suspension in Teflon-coated pots with $ESDM_{cult}$ medium. Afterwards, ESDM were harvested from suspension culture and used in chemotaxis assays. All the other procedures and settings were the same as the optimized protocol described above. A range of classical macrophage chemoattractants, including CCL5, VEGF and C5a, were assessed with the new method (Figure 7.8). The data demonstrated that ESDM had strong migratory responses to C5a and CCL5 in a dose dependent manner and only weak response to high concentrations of VEGF. The results were reproducible, suggesting that factors associated with detachment of the ESDM had led to the irregular chemotaxis response and this effect can be prevented or reversed by 24 hours suspension culture. Furthermore, the data confirmed that ESDM had migratory machinery and both C5a and CCL5 were their potent chemoattractants.

In order to confirm the response of ESDM to C5a was indeed chemotaxis instead of chemokinesis, C5a was placed in the upper chambers (Figure 7.9). The migration induced by C5a in upper wells was similar to the background control, demonstrating that the response of ESDM to C5a was chemotaxis rather than chemokinesis.

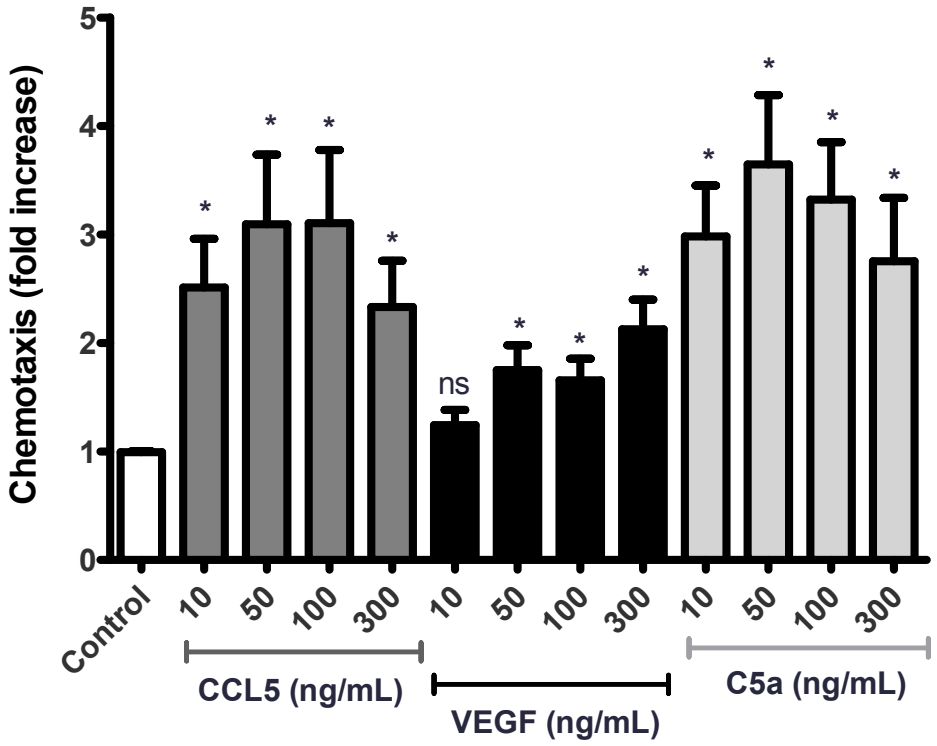


Figure 7.8 Chemotaxis assay with ESDM recovered in Teflon pots for 24 hours.

One day before the assay, ESDM were detached from Petri dishes on day 6 and cultured in fresh $ESDM_{cult}$ in Teflon pots. ESDM harvested from suspension culture were used in the chemotaxis assay. A range of macrophage chemoattractants (CCL5, VEGF, and C5a) were assessed. The data are means of fold increase compared to negative control±SEM. n=3;*, $P<0.05$, compared with control (Mann-Whitney test).

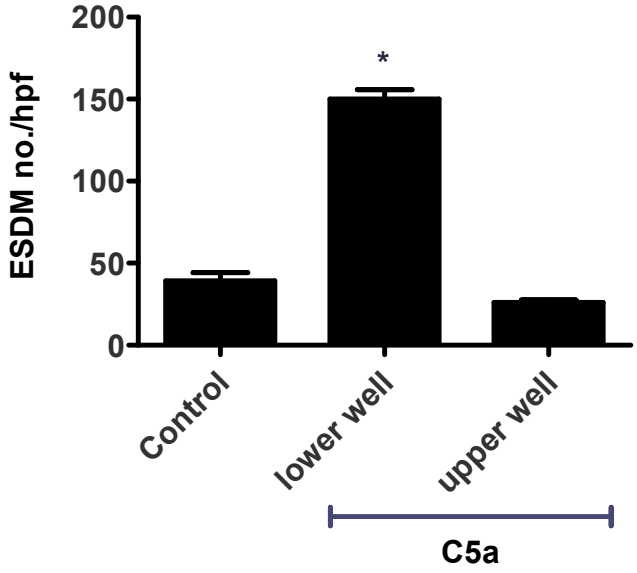


Figure 7.9 Migration of ESDM to C5a was not chemokinesis.

C5a (50ng/ml) was loaded into upper wells to see the chemokinesis response. Control: assay medium; C5a in lower wells: positive control. Data are the mean numbers of migrated ESDM/hpf±SEM. n=3; $P<0.05$, compared with control (Mann-Whitney test).

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7.3 Migration of ESDM to stressed BL2 supernatant

Since stressed BL supernatant has been identified to be chemoattractive for THP-1 cells, and the chemoattractant was found to be ATP, in order to be able to apply ESDM in future macrophage infiltration studies in BL both *in vitro* and *in vivo*, it was important to assess in the chemotaxis assay the migratory ability of ESDM towards stressed BL supernatant. Four different batches of stressed BL2 supernatant were examined with C5a as a positive control and assay medium as a negative control (Figure 7.10). However ESDM migrated to C5a, but not to any of the four samples.

7.4 Cells from earlier stages of ESDM maturation

Although the origin of tumour-associated macrophages has not been clearly identified, it has been proposed that they were derived from both monocyte extravasation and proliferation of in situ macrophages (Gordon and Taylor 2005). Based on the fact that ESDM did not migrate to supernatants from stressed BL2 cells, it was hypothesized that the ESDM used were matured to a state that they had lost the ability to respond to the chemoattractants present in the stress supernatants. During the course of these studies, a paper was published describing a similar differentiation system from human ES cells, and they identified the progenitor cells in supernatant from adherent embryonic bodies as monocytes (Karlsson, Cowley et al. 2008). Therefore, cells were harvested and examined from earlier stages of ESDM maturation (Figure 7.11 A). ESDM used for previous chemotaxis assay were matured on Petri dishes for 6 days and in Teflon pots for one day, hence they were designated ESDM d7. Earlier ESDM, which were matured on Petri dishes for 2 days and in Teflon Pots for one day, were designated ESDM d3. Non-adherent cells in the supernatant from adherent EBs were nominated ESDM d0.

To identify these cells, ESDM d0 and d3 were labeled with the same fluorescence-conjugated antibodies used for d7 ESDM (Figure 7.11 B, C). ESDM d0

and d3 displayed similar light scatter and antibody staining pattern to that of ESDM d7, although with minor variations in fluorescence intensity. The data in one way confirmed the myeloid lineage identity of ESDM d0 and d3, considering that both of them express F4/80 and CD11b. The similarity in F4/80 antibody staining pattern suggested that ESDM d0 are unlikely to be monocytes, since it has been demonstrated before that the level of F4/80 expression on monocytes is much lower than that of macrophages (Austyn and Gordon 1981; Geissmann, Jung et al. 2003; Gordon and Taylor 2005). Thus, the difference in the phenotypes and maturation stages of these cells should be characterized further.

7.5 Progressively matured ESDM showed increasing phagocytic ability to eat apoptotic BL cells

It has been shown that freshly isolated human blood monocytes did not phagocytose apoptotic cells (Newman, Henson et al. 1982), however, cells gradually acquired this capacity as they differentiated and matured into macrophages (Callahan, Halleck et al. 2003). Thus, phagocytosis assays were performed in order to identify whether ESDM, as they gradually matured on Petri dishes, can acquire the capacity to phagocytose apoptotic cells. ESDM d0, d3 and d7 were assessed, and cold-shock induced apoptotic BL2 cells were used (Figure 7.12). Only one pilot experiment was carried out due to time limitations. However, the results demonstrated evidently that ESDM d0 had weak phagocytic response, but as ESDM matured, they gradually acquired enhanced phagocytic ability to phagocytose apoptotic BL cells, suggesting that ESDM d0, d3 and d7 represented progressively matured macrophages.

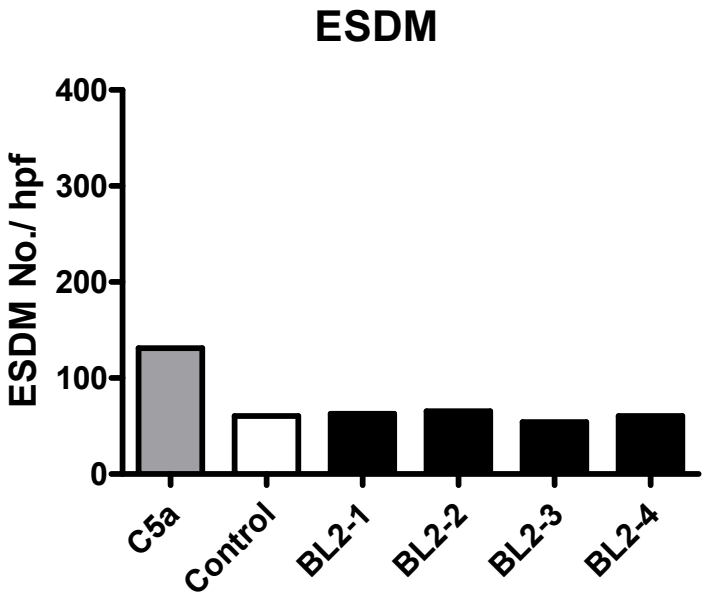


Figure 7.10 Migration of ESDM to supernatants from stressed BL2 cells.

C5a (50ng/ml) was used as a positive control. Four different batches of supernatants from stressed BL2 cells (BL2-1 to BL2-4) were assessed for their ability to attract ESDM. C5a: positive control; Control: assay medium. Data are the mean numbers of migrated ESDM/hpf.

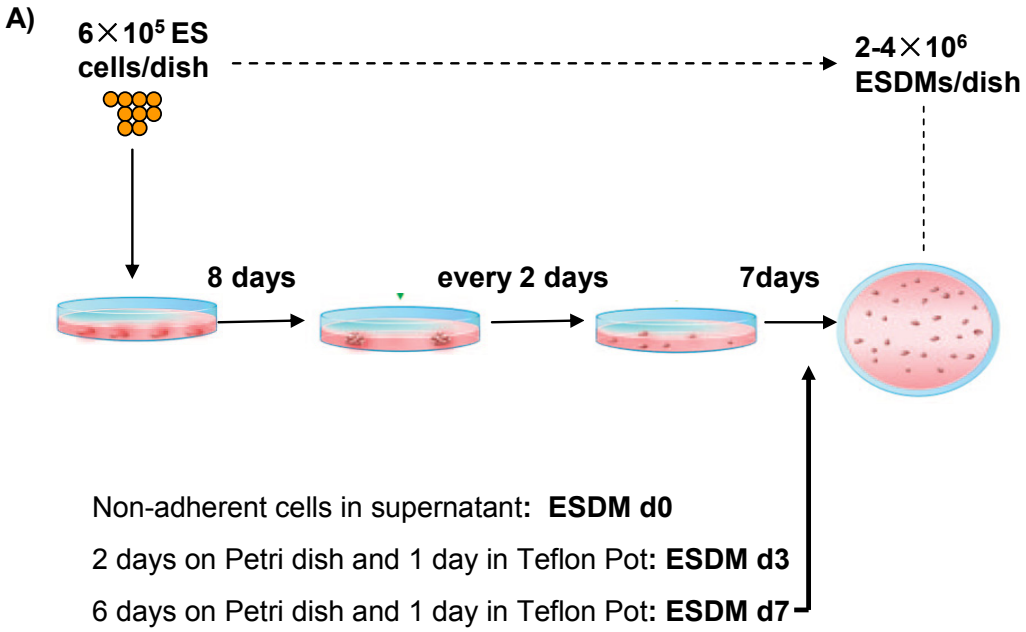
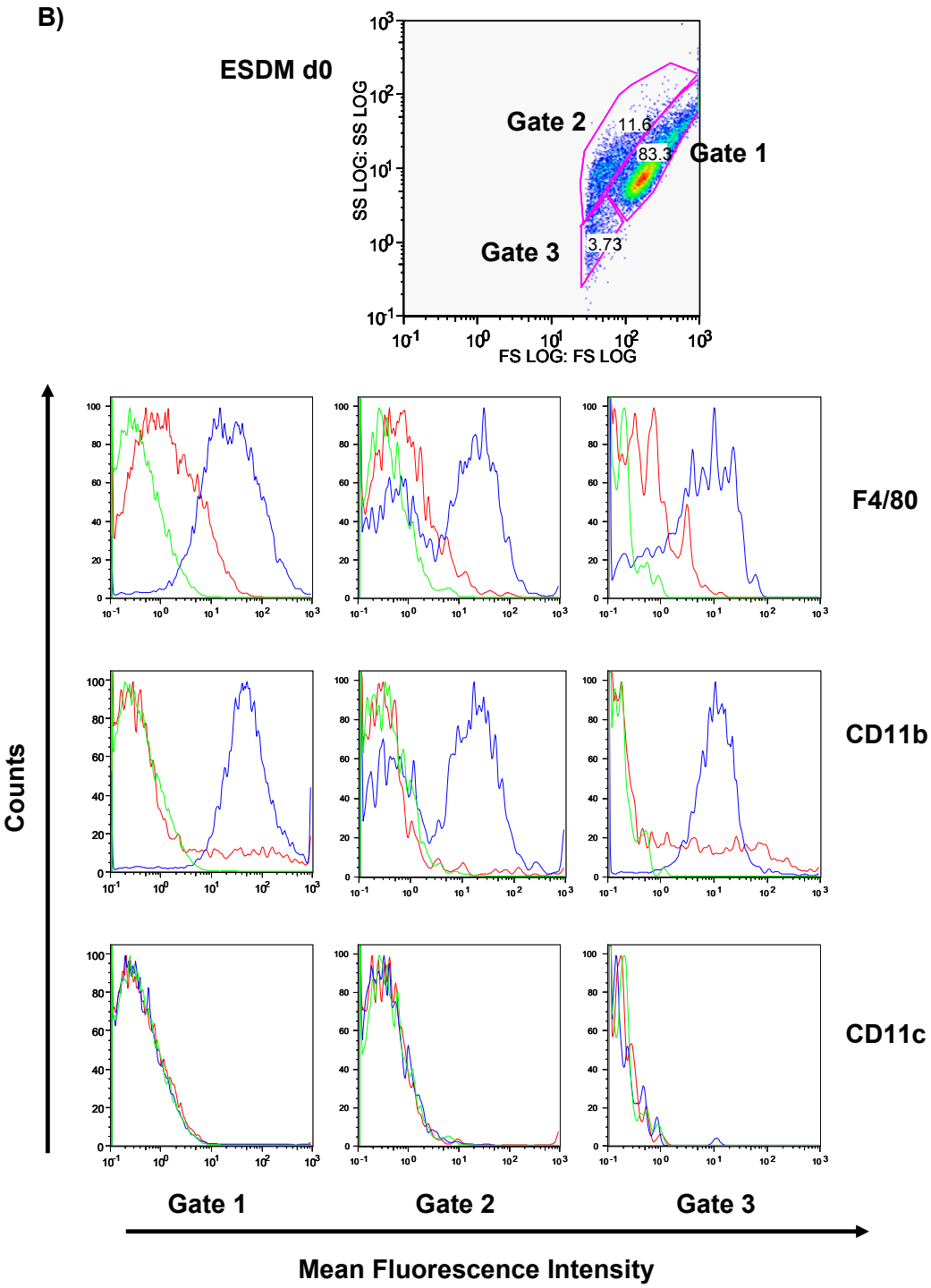
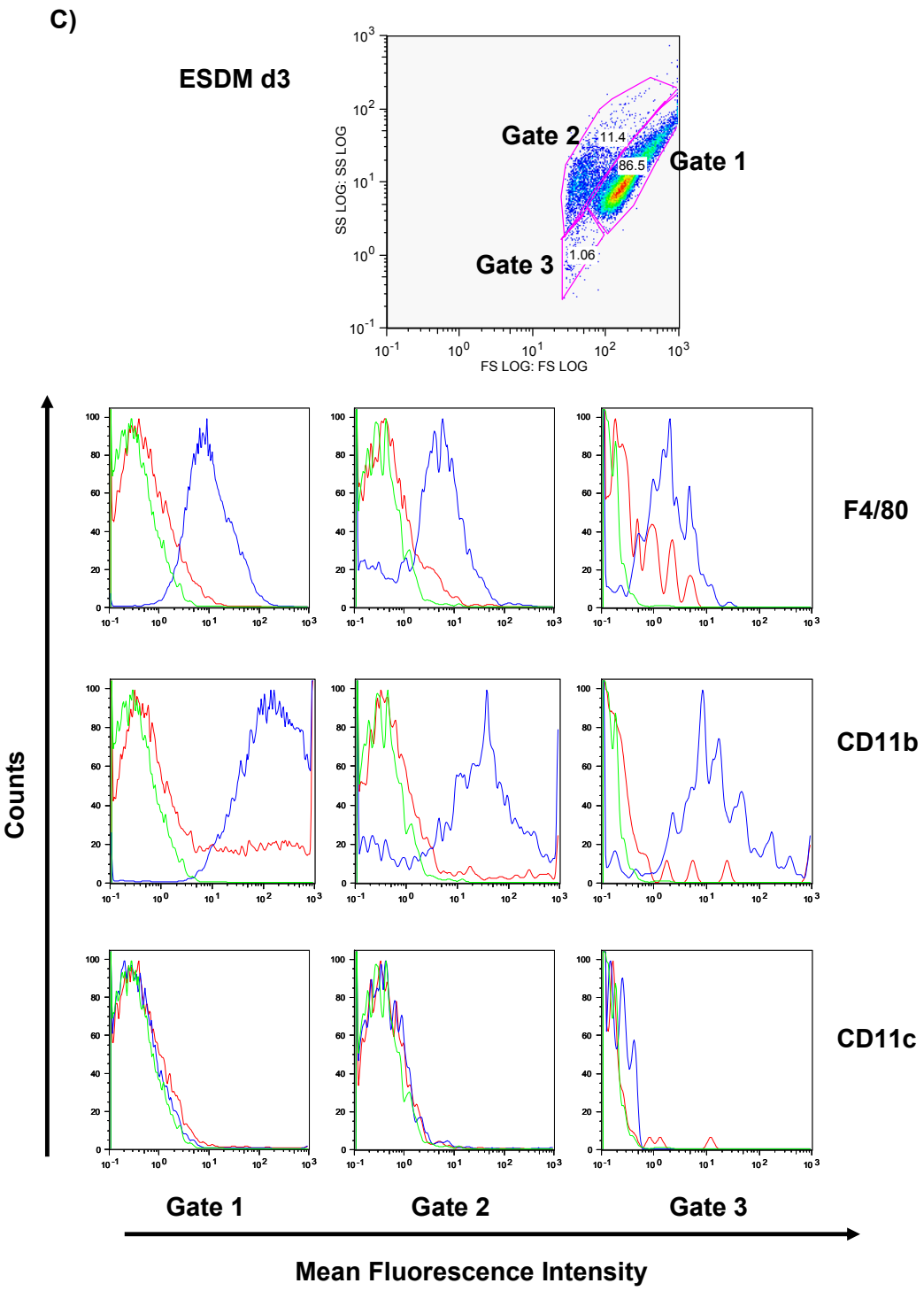


Figure 7.11 Cells from earlier stages of ESDM maturation.

A) Schematic demonstrating ESDM d0, ESDM d3 and ESDM d7. B)-C) (See the following two pages) Cell scatter and phenotype of ESDM d0 and d3 by flow cytometry. Staining of sub-populations defined by light scatter was compared. F4/80-PE, CD11b-PE and CD11c-FITC antibodies were used to label the cells. The histograms of fluorescent staining were displayed by different gates. Green line: unstained cells; red line: Isotype control; blue line: stained with Abs. The data are a representative of those from three independent experiments.


B)
ESDM d0
Gate 2
Gate 1
Gate 3
11.6
83.3
3.73
SS LOG: SS LOG
FS LOG: FS LOG
F4/80
CD11b
CD11c
Counts
Gate 1
Gate 2
Gate 3
Mean Fluorescence Intensity

C)


ESDM d3
Gate 2
Gate 1
Gate 3
11.4
86.5
1.06
SS LOG: SS LOG
FS LOG: FS LOG
F4/80
CD11b
CD11c
Counts
Gate 1
Gate 2
Gate 3
Mean Fluorescence Intensity

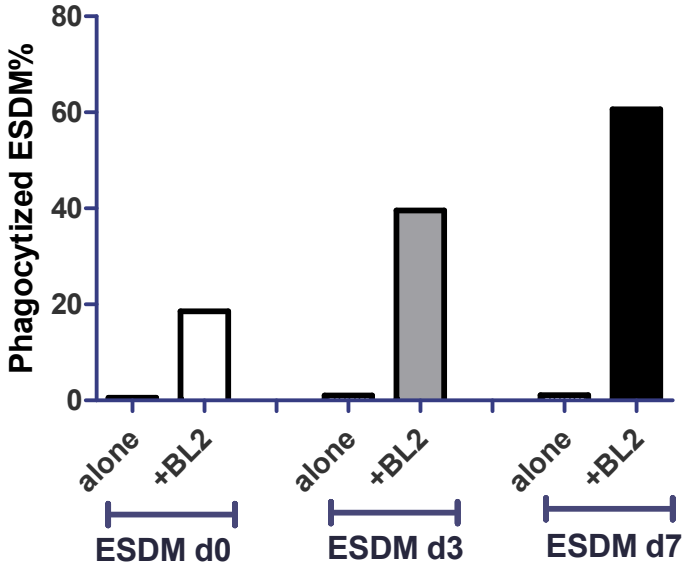


Figure 7.12 Progressively matured ESDM showed increasing ability to phagocytose apoptotic BL cells.

Flow cytometry was used to determine the uptake of apoptotic BL2 cells by ESDM d0, d3 and d7. The phagocytic co-culture with apoptotic BL2 cells was for one hour. The data presented are the mean percentages of phagocytosed ESDM in the total ESDM. Alone: ESDM alone. Details of phagocytic index are given in the text.

7.6 Migration of ESDM d0 and d3 to stressed BL2 supernatants

Having obtained evidence that ESDM d0 and d3 were from earlier stages of ESDM maturation, their migratory ability towards stressed BL2 supernatant was assessed by using the same supernatant in the previous ESDM d7 chemotaxis assay (Figure 7.10). Both ESDM d0 and d3 migrated consistently to four different batches of stress supernatant, and ESDM d0 showed much stronger responses to both C5a and supernatants (Figure 7.13). Considering the lack of responsiveness of ESDM d7, the results together indicated that ESDM d0 and d3 had greater migratory ability towards chemoattractants, especially the ones in stress supernatant, and as they matured, they gradually lost this ability. This observation accorded well with previous findings that stress supernatants could attract THP-1 cells, which is a human monocytic cell line and suggested that the chemoattractants in stress supernatants had cross-species reactivity.

7.7 Migration of ESDM to stressed BL2/bcl-2 supernatant

Since stressed BL2/bcl-2 supernatant could attract THP-1 cells, its activity on ESDM was also assessed. Stressed BL2 supernatant was used as a comparison (Figure 7.14). Similar to the result shown in Figure 7.10 and 7.13, ESDM d0 had the strongest ability migrating to C5a and stressed BL2/bcl-2 supernatant, and as cells became more mature, they gradually lost the ability. The response to BL2/bcl-2 was similar to BL2, further supporting the conclusion that the chemoattractive ability in stress supernatants was not apoptosis-dependent.

7.8 Migration of day 2 human monocyte-derived macrophages (HMDM) to ATP

In order to confirm in primary cells from a different species that stress supernatant can differentially attract macrophages of various maturation stages, responses of human monocyte-derived macrophages were studied. Only one pilot experiment was carried out using HMDM matured on Petri dishes for two days with various concentrations of ATP as chemoattractants (Figure 7.15). Day 2 HMDM exhibited dose dependent migratory response to ATP, even though the response was weak. Further experiments have to be performed to repeat and confirm this result.

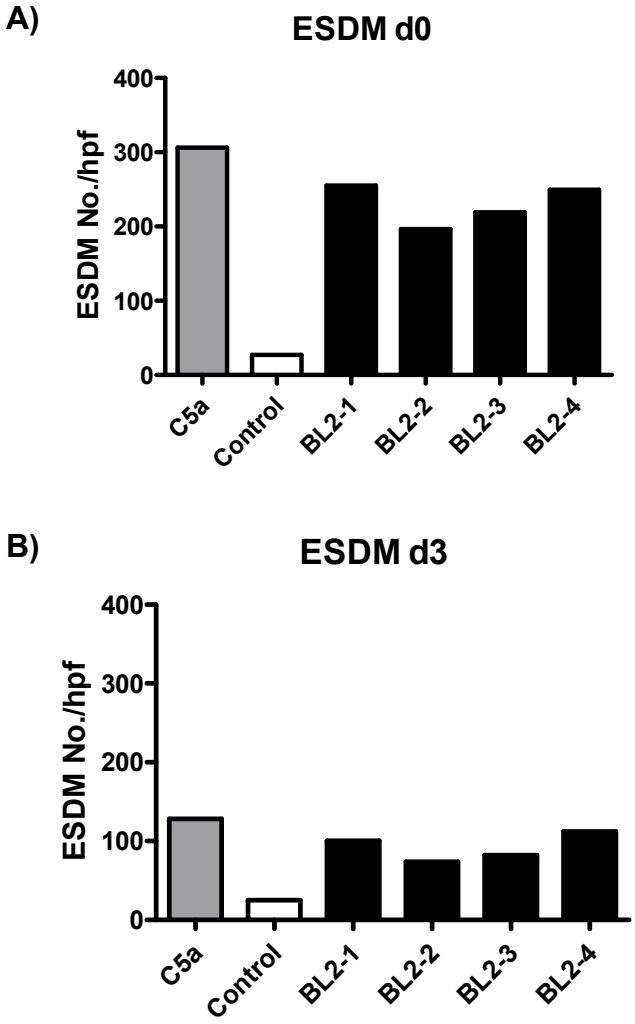


Figure 7.13 Migration of ESDM d0 and d3 to supernatants from stressed BL2 cells.

The same batches of stress supernatants as used in ESDM d7 chemotaxis assay (Figure 7.10) were applied in this experiment and ESDM d0 (A) and d3 (B) were assessed. C5a (50ng/ml): positive control; Control: assay medium. The data presented are the mean numbers of migrated ESDM/hpf.

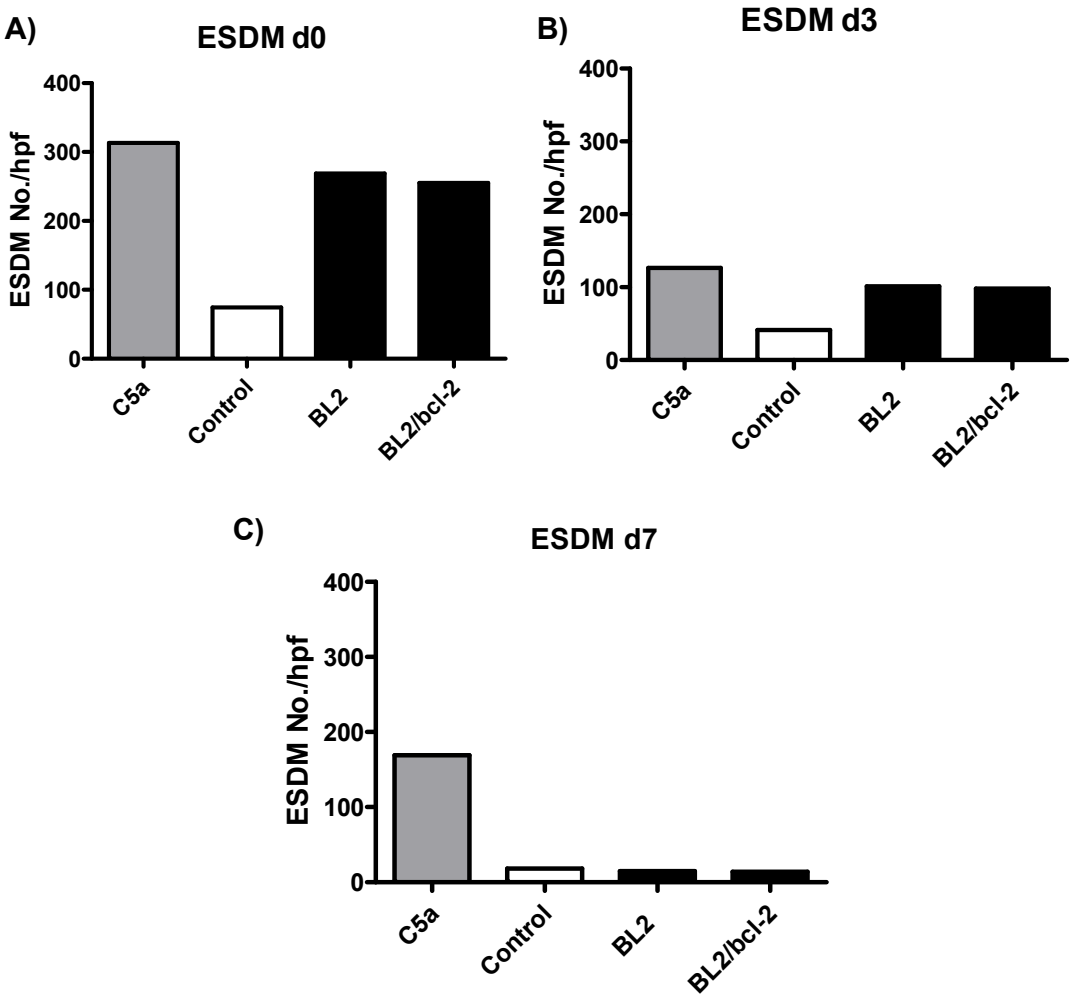


Figure 7.14 Migration of ESDM to supernatants from stressed BL2/bcl-2 cells.

Supernatants from stressed BL2/bcl-2 cells were used as chemoattractants, and supernatans from stressed BL2 cells were used as its comparison. ESDM d0 (A), d3 (B) and d7 (C) were assessed. C5a (50 ng/ml): positive control; Control: assay medium. The data are the mean numbers of migrated ESDM/hpf.

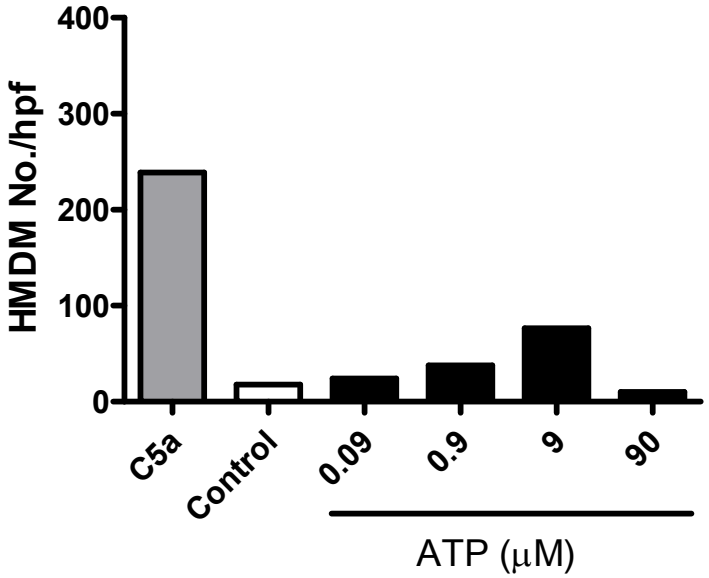


Figure 7.15 Migration of day 2 HMDM to ATP.

Two days differentiated HMDM on Petri dishes were used in chemotaxis assay, and four concentrations of ATP were examined. C5a (50ng/ml): positive control; Control: assay medium. Data presented are the mean numbers of migrated ESDM/hpf. Data from one experiment are presented.

Chapter 8 Discussion

8.1 Summary of results

Although mostly obtained using an *in vitro* model, results from this study suggest that stressed, pre-apoptotic Burkitt's lymphoma cells could contribute to tumour micro-environmental conditioning by regulating immune cell infiltration as well as direct trophic effect on other viable tumour cells and possibly stromal cells. High density stress in which glucose was rapidly consumed could trigger BL2 cells to undergo apoptosis. Cell-free stress supernatants harvested from high density stressed, pre-apoptotic BL2 cells could selectively regulate immune cell infiltration, specifically, recruit mononuclear phagocytes but inhibit neutrophil infiltration, suggesting that under stress, pre-apoptotic cells may have already started to generate signals that prelude their imminent demise. Analysis of the chemoattractive ability of stress supernatants for THP-1 cells by enzyme digestion and antagonist blockade suggested a role of nucleotides in the recruitment process of THP-1 cells. Since ATP and UTP have been identified as mononuclear phagocyte chemoattractants released from apoptotic cells (Elliott, Chekeni et al. 2009), and ATP is one of the nucleotides most studied in its role in immune cell migration, the possible role of ATP in stress supernatants as a chemoattractant was investigated further. This study indicated that ATP could be one of the chemoattractants for mononuclear phagocytes released from stressed cells; however, the mechanisms of ATP release under stress have not been identified. Furthermore, ATP was shown to activate neutrophil migration and the fact that stress supernatants which contained ATP, paradoxically, inhibited neutrophil chemotaxis, demonstrating the possible presence of potent 'keep-out' signals. These signals were not identified.

The stress supernatants could also promote the survival and proliferation of both tumour cells and mononuclear phagocytes, demonstrated that stressed BL2 cells may

have direct trophic effects on other cells in the environment. This effect was not contact-dependent. The identity of the bioactive factor(s) in stress supernatants was sought and several candidate factors were examined, including ATP, lactoferrin and S1P, however none of these had the appropriate properties. The identification of the heat-stable bioactive factor requires further investigation.

The possible association of the above micro-environmental conditioning effects with the spontaneous apoptosis in BL2 cells was excluded by studying responses of BL2 cells which over-expressed bcl-2 and therefore had inherent resistance to spontaneous apoptosis. Supernatant harvested from stressed BL2/bcl-2 cells demonstrated similar micro-environmental conditioning effects as unmodified BL2 cells, including regulating immune cell infiltration as well as a direct trophic effect. However, the question as to how the release of nucleotides and the unidentified bioactive factor(s) from pre-apoptotic BL2 cells was coupled with the intracellular pre-apoptosis program has not been answered.

On the other hand, an optimal protocol which can effectively generate relatively pure macrophages from murine embryonic stem cells was established. The purity of ESDM was assessed by morphology and macrophage markers as well as self-renewal assay which excluded the possible contamination by undifferentiated ES cells. ESDM exhibited the ability to migrate to pre-apoptotic cells and to phagocytise apoptotic cells; therefore, they and their genetically manipulated forms can be applied to studies of migration to apoptotic cells and clearance of apoptotic cells and their molecular mechanisms. Furthermore, gradual changes in the chemotaxis and phagocytosis ability of ESDM as they matured were observed. Mature ESDM were better at phagocytosing apoptotic cells whereas their precursors were better at migrating towards them.

8.2 Roles of ATP in the tumour microenvironment

8.2.1 Effect of ATP on immune cell migration

8.2.1.1 ATP as an extracellular signalling molecule

ATP is well known for its function as a fundamental intracellular component which serves not only as energy currency to sustain the cell's viability and functions but also as basic building blocks for the synthesis of DNA and RNA. However, ATP also functions as an extracellular signalling molecule in purinergic signalling, in both an autocrine and paracrine manner thereby playing an important role in modulating cellular functions in both the immune system and cancers (Burnstock, Fredholm et al. 2010).

Released ATP can be rapidly hydrolysed to AMP, ADP and adenosine by four categories of ectonucleotidases involved in purinergic signalling (Yegutkin 2008) (Table 8.1). However, the expression pattern of the ectonucleotidases varies between cell types thereby defines the different ATP metabolism and signalling mechanisms of each cell type.

Extracellular ATP and its metabolites exert a wide range of cellular effect through different receptors. Two types of purinergic receptors have been cloned and characterised: 1) P1 receptors are G protein-coupled receptors (GPCRs) and selective for adenosine binding with four subtypes (A1, A2A, A2B and A3); 2) P2 receptors are selective for ATP and ADP and some of them also bind to UTP or UDP. Two types of P2 receptors are distinguished: P2X receptors are ATP-gated ion channels with seven subtypes ($P2X_{1-7}$) and P2Y receptors are GPCRs with eight subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) (Ralevic and Burnstock 1998).

Table 8.1

Ectonucleotidases in mammalian	Family members	Substrate	Product
ectonucleoside triphosphate diphosphohydrolase (ENTPD) family	ENTPD 1-6 and ENTPD 8 < ENTPD1 (also known as CD39)>	ATP or ADP	AMP
ectonucleotide pyrophosphatase/phosphodiesterase (ENPP) family	ENPP1-3	ATP	AMP and pyrophos-phate
alkaline phosphatase family	4 members	ATP, ADP and AMP	adenosine
ecto-5'-nucleotidase (also known as CD73).	1 member	AMP	adenosine

8.2.1.2 Effects of ATP on macrophage migration

Monocytes/macrophages have machinery for both autocrine and paracrine purinergic signalling. Multiple P2 receptor subtypes are expressed on monocytes and macrophages, including $P2Y_{1,2,4,6,11,12}$ and $P2X_{1,4,5,7}$. $P2Y_{13}$ is only expressed on monocytes. The expression and activity of ectonucleotidases, including CD39 and CD73 are also detected on monocytes and macrophages (Koziak, Sevigny et al. 1999; Beigi and Dubyak 2000). The role of ATP as a chemoattractant has been studied widely (Bours, Swennen et al. 2006), but this remains controversial. In this study, ATP was demonstrated to induce the chemotaxis not chemokinesis of THP-1 cells and the optimal concentration to induce migration was 0.9 μM. A higher concentration of ATP, such as 90 μM, did not induce a chemoattractive response (Figure 4.1). The result is consistent with the findings of Elliott et al. (Elliott, Chekeni et al. 2009). They demonstrated that ATP is a chemoattractant released from apoptotic cells for mononuclear phagocytes in a dose-dependent manner in vitro and the optimal concentration was 0.1μM. Checkerboard experiments in which equal concentrations of ATP are added to both sides of the chamber induced no migration therefore

excluded the possibility of random migration without direction, namely chemokinesis. The effect of ATP as a chemoattractant for mononuclear phagocytes released from apoptotic cells was also proved in an *in vivo* air punch model. The involvement of ATP receptor P2Y2 was demonstrated both *in vitro* knock-down by shRNA and *in vivo* by using P2Y2-/- mice.

However, one study (Kaufmann, Musset et al. 2005) concluded differently. These authors showed that ATP only induced human monocyte migration within the concentration range of 10-100μM, which was found to induce little or no response of migration in the present studies and those of Elliott et al (2009). Kaufmann et al also performed a checkerboad analysis and a similar migratory effect was observed, suggesting a chemokinesis effect. Kaufmann et al also analysed the intracellular signalling of the migratory response by measuring the level of phosphorylated Akt. Unlike the increased level of phosphorylated Akt in fMLP-induced monocytes which showed a chemotaxis response, there was no increase in the level of phosphorylated Akt in samples treated with ATP. Phosphoinositide 3-kinase γ (PI-3Kγ) is well known to be essential for neutrophil and macrophage migration and deficiency of PI-3Kγ leads to a reduced migratory ability (Hirsch, Katanaev et al. 2000; Li, Jiang et al. 2000; Sasaki, Irie-Sasaki et al. 2000). Further studies revealed that G-protein coupled PI-3Kγ, which activates Akt by generating phosphatidylinositol 3,4,5-triphosphate, is essential for the directional orientation of neutrophils rather than the rate of migration, during their response to fMLP (Hannigan, Zhan et al. 2002). This led to the conclusion that ATP only induced non-directional migration of human monocytes. However, although PI-3K played an essential role in neutrophil chemotaxis, no studies have directly shown that PI-3K is important for the directionality of macrophage chemotaxis. Thus, examining phosphorylated Akt is not a convincing indicator of chemotaxis.

Another level of complexity of the relationship between ATP and macrophage migration was added by the discovery that macrophage chemotaxis required a navigational system depending on the autocrine 'purinergic feedback loops' of macrophages both *in vitro* and *in vivo* (Kronlage, Song et al. 2010). In response to the chemoattractant C5a, macrophages released ATP, and this, together with its metabolites activated subsequent multiple components of purinergic signalling, including $P2Y_2$ (receptor for ATP), $P2Y_{12}$ (receptor for adenosine diphosphate, ADP) and A2a, A2a, and A3 (receptors for adenosine) which were all required for effective chemotactic navigation. Pannexin 1 which was the channel responsible for ATP release from neutrophils in their autocrine navigational system was not responsible for autocrine ATP release in macrophages (Chen, Corriden et al. 2006). This finding is consistent with the previous observation that macrophage migration *in vivo* and *in vitro* was impaired in CD39-null mice which disrupted the metabolism of ATP, an essential component in 'purinergic feedback loops' required by the chemotaxis process (Goepfert, Sundberg et al. 2001). The study was consistent with data from the present work which showed the treatment of CCL2 with apyrase, which potentially interrupted the autocrine purinergic signalling, could cause some reduction in macrophage chemotaxis, although a substantial chemotaxis response could still be observed (Figure 4.3).

ATP might therefore still be a chemoattractant for mononuclear phagocytes despite all the complexity and controversy. However, since the *in vitro* data was generated using a Boyden chamber, considering the complexity of the interpretation, a direct microscopy-based chemotaxis assay in which macrophage directional migration and velocity could be recorded in real-time, as has already been applied to studies of neutrophil chemotaxis, could be adopted for further macrophage chemotaxis studies (Chen, Corriden et al. 2006). The data generated would help distinguish chemotaxis from chemokinesis more directly. In addition, since macrophage chemotaxis requires

autocrine ATP signalling for navigation, data from experiments using apyrase treatment or ATP receptor gene knock-out cells must be carefully examined before the role of ATP as a chemoattractant can be confirmed.

Another question remained to be answered is whether ATP is a short-range or long-range chemoattractant *in vivo*. ATP receptors and ectonucleotidases are widely distributed on almost all cells, despite of the different combinations of expression. As ATP is released, it can be rapidly hydrolysed by ectonucleotidases nearby in a stepwise manner, from ADP, AMP to adenosine. The instability of ATP indicates that it might not be functional over long-range. However, ATP can still function as a long-range chemoattractant if the released ATP is enclosed in exocytosis vehicles. Enclosed ATP can be carried over long distance and gradually release ATP, consequently forming an ATP gradient. Although the data presented in this study suggested that the release of ATP was independent of microparticles with sizes smaller than 0.22 μm in diameter, recent investigations have indicated that the diameter of the microparticles released from apoptotic BL cells can be up to 1 μm (Pasikowska 2010). Therefore, improved methods to isolate the whole population of microparticles in sterile condition should be adopted in order to examine the possibility of ATP release through microparticles. Another point to bear in mind is that ATP is present in blood. Although different methods vary widely in their estimates of blood ATP concentration: values from 28 nM to 11,000 nM have been reported (Gorman, Feigl et al. 2007), it remains unknown whether the concentrations of ATP released from stressed cells could successfully establish a chemoattractant gradient.

Another possibility is that since fractalkine can be released from apoptotic BL cells and its release is microparticle-associated, fractalkine can be the long-range chemoattractant while ATP functions as a short-range chemoattractant in BL microenvironment. Although ATP has been identified as a chemoattractant for

mononuclear phagocytes, another possibility should not be overlooked. It has been shown that activation of P2Y6 on human promonocytic cell line U937 could induce the release of MCP-1, which is a well-established mononuclear phagocyte chemoattractant, suggesting that it is likely that ATP could also be an inducer for a chemoattractant for mononuclear phagocytes to be released (Cox, Gomes et al. 2005).

8.2.1.3 Effects of ATP on other immune cell migration

The data presented also suggested ATP, especially at relatively high concentrations (9-90μM) - which exceed the amount of ATP contained in stress supernatants, could induce neutrophil migration *in vitro* (see Figure 4.7). ATP is known to be a 'danger signal' to recruit immune cells, such as neutrophils, when large amount of ATP, around 5mM to 10 mM, was suddenly released from damaged cells, thus inducing pro-inflammatory cell infiltration. The regulation of cell migration by ATP has been observed in various immune cells, such as mast cells (McCloskey, Fan et al. 1999), dendritic cells (Liu, Bohlen et al. 1999; Schnurr, Toy et al. 2003), eosinophils (Muller, Robaye et al. 2010) and natural killer cells (Gorini, Callegari et al. 2010). However, the role of ATP in neutrophil migration will be focused on here.

The ATP receptors expressed on neutrophils include $P2Y_{1,2,4,6,11,14}$ and $P2X_{1,4,5,7}$. They also express CD39 and CD73. Thus, neutrophils can respond to and hydrolyse ATP. On the other hand, neutrophils can release ATP during inflammation (Bours, Swennen et al. 2006). Therefore, the functions of neutrophils can be regulated by ATP in both paracrine and autocrine manner. Evidence from *in vitro* studies suggested that ATP can stimulate neutrophil adherence to endothelial cells (Rounds, Likar et al. 1999). *In vivo* study confirmed that ATP could enhance the adherence of neutrophils to blood vessels in the initial stage of inflammatory response and increase the number of recruited neutrophils (McDonald, Pittman et al. 2010). However, the question whether

extracellular ATP functions as a neutrophil chemoattractant has not yet been answered unequivocally. Different observations have been described in different models. For example, instead of affecting migration, 0.3 μM ATP induces only the aggregation of rat neutrophils (Ford-Hutchinson 1982). Micromolar concentrations of ATP were found to inhibit rabbit neutrophil chemotaxis induced by fMLP (Elferink, de Koster et al. 1992). ATP analogue, ATPγS, induced human neutrophils chemotaxis and actin polymerization (Verghese, Kneisler et al. 1996). However, recent studies indicated that ATP could induce neutrophil chemokinesis and autocrine signalling of ATP induced by chemotactic stimulus (such as fMLP) was essential in facilitating the migratory response of human neutrophils (Chen, Corriden et al. 2006). The function of ATP as a neutrophil chemoattactant has not yet been established, however, the mechanism of ATP autocrine signalling in promoting chemotaxis has been characterised. ATP can be released near the activated chemoattractant receptors and either activate its receptor $P2Y_2$, or be hydrolyzed by membrane CD39 and alkaline phosphatases (AP) into adenosine which further activates A3 receptors at the leading edge. These activated $P2Y_2$ and A3 receptors can enhance the mobilization of cellular skeleton in the migratory response and therefore facilitate the chemotaxis response towards the chemotactic source (Corriden, Chen et al. 2008). The common requirement of autocrine purinergic signalling for the navigation of macrophage and neutrophil chemotaxis suggests that there might be a common mechanism of navigational mechanism present for immune cell migration.

8.2.1.4 Chemoattractants released during different stages of apoptosis

Multiple chemoattractants have been reported to be released from apoptotic cells before loss of cellular integrity, but little is known about the stage of apoptosis. However, several studies have revealed the association between chemoattractant

release and apoptosis program. LPC is released as a 'find-me' signal for mononuclear phagocytes from apoptotic MCF-7 breast cancer cells (from other apoptotic cells as well but with less characterisation) and the release of this soluble lipid mediator is dependent on capase-3 cleaved calcium-independent phospholipase A2 (Lauber, Bohn et al. 2003). ATP as a mononuclear phagocyte chemoattractant can be released from apoptotic cells through pannexin 1 channels, and the opening of these channels is dependent on caspase-3, and caspase-7 cleavage (Elliott, Chekeni et al. 2009; Chekeni, Elliott et al. 2010). However, the release of other chemoattractive molecules, such as S1P, S19, EMAPII, fractalkine, is only described as release from apoptotic cells (Shalak, Kaminska et al. 2001; Yamamoto 2007; Gude, Alvarez et al. 2008; Truman, Ford et al. 2008). In this study, it has been demonstrated that the release of ATP is from pre-apoptotic cells and independent of apoptosis, therefore, clearly defined the stage of 'find-me' signal release, although the releasing mechanism of ATP under stress has not been confirmed. But the question whether and how ATP release from pre-apoptotic cells under stress is coupled to any components in apoptosis program is intriguing.

8.2.2 Multiple roles of ATP in the tumour microenvironment

8.2.2.1 Direct effects of ATP on tumour cells

It has been shown in human glioma cells that 100μM ATP could significantly promote their proliferation (Rathbone, Middlemiss et al. 1992; Morrone, Jacques-Silva et al. 2003). However, in my study, different concentrations of ATP (from 0.09 μM to 90μM) were examined to see their proliferative effects on BL2 cells; however, ATP did not show any direct effect on BL2 cell proliferation, indicating the direct tumour growth promoting effect of ATP does not apply to all the tumour models. However, the possible roles of ATP on tumour cells should not be ignored. The extracellular concentration of ATP can be highly elevated in tumours compared with normal tissues,

whose near to plasma membrane concentrations of extracellular ATP under resting condition are only within a 1 to 10 nanomolar range. ATP concentrations in the interstitium of various tumours have been reported to be in the hundreds of micromolar range using a chimeric plasma membrane-luciferase assay (Pellegatti, Raffaghello et al. 2008). Thus, elevated extracellular ATP in tumours may be associated with tumourigenesis. For example, human fibrosarcoma cells could release ATP to induce the upregulation of intracellular calcium level in endothelium cells which is an important intracellular regulator in the endothelial cells invasion and metastasis (Nejime, Tanaka et al. 2008).

Another indirect mechanism of tumour promotion involving ATP signalling was relevant to one of ATP receptors, P2X7. It is known that very high concentrations of extracellular ATP, for example, more than 1mM, have cytotoxic effect both in normal tissues and malignancies, involving the activation of P2X7 receptor (Wang, Wang et al. 2004; Zhang, Meng et al. 2009). However, the level of P2X7 is found significantly lowered in different types of cancer cells compared to their normal counterparts (Li, Zhou et al. 2006), suggesting a tumour protective mechanism.

8.2.2.2 Effects of ATP on tumour-associated macrophages

As a ‘find-me’ signal released from pre-apoptotic cells, ATP has a broader potential to regulate TAM than simply being a chemoattractant. The qualitative effect of ATP on macrophages is concentration dependent. High concentrations of ATP (ATP at millimolar concentration) stimulate monocytes/macrophages to release proinflammatory cytokines, such as IL-1α, IL-18 and TNF-α and the effect is suggested to be mediated by P2X7 receptor (Bours, Swennen et al. 2006). Low to medium concentrations of ATP has immunomodulatory or immunosuppressive effects on macrophages. It has been reported that pre-treatment of peritoneal macrophages with 100 μM ATP could significantly reduce the amount of IL-12 and TNF-α released

upon LPS stimulation (Hasko, Kuhel et al. 2000), while 0.1 to 1 mM ATP significantly reduced the production of IL-12p40 and TNF-α in LPS-primed human alveolar macrophages (Myrtek, Muller et al. 2008). Also, Kaufmann et al., (2005) have reported that 10 -100μM ATPγS, which is a P2Y2 antagonist, profoundly inhibited secretion of proinflammatory cytokines (TNF-α and MCP-1) from classically activated human monocytes, but increased production of IL-10, an anti-inflammatory cytokine (Kaufmann, Musset et al. 2005). In LPS stimulated human whole blood, 100 μM and 300 μM ATP inhibited the release of TNF-α but increased IL-10 release, and thereby had an anti-inflammatory effect (Swennen, Bast et al. 2005). The anti-inflammatory effect was not affected by treatment with adenosine deaminase, suggesting the effect was not due to adenosine derived from ATP degradation. Although the concentration of ATP in the tumour microenvironment has not been measured directly in BL, in other tumours concentrations in the hundreds of micromolar range have been reported. This suggests ATP is capable of driving recruited mononuclear phagocytes into an M2, 'anti-inflammatory' phenotype.

ATP has been reported to enhance the phagocytosis of latex beads by macrophages (Ichinose 1995). Recent studies indicate that exogenous ATP could enhance the binding of macrophages to apoptotic cells (Marques-da-Silva, Burnstock et al. 2011). Thus it has been speculated that ATP could also enhance the phagocytosis of apoptotic cells by recruited macrophages in BL microenvironment. Either purified ATP or supernatants from stressed BL cells could be examined for their ability to change the functional capacities of macrophages. Finally, adenosine, a metabolite of ATP whose functions are beyond the scope of this study, could as well enhance the macrophage phagocytosis ability and tune macrophage into M2 phenotype (Hask, Pacher et al. 2007). Therefore, the early release of ATP from stressed tumour cells could contribute to the macrophage polarization for subsequently clearance of apoptotic cells and pro-tumour functions which thereby conditions the tumour microenvironment.

8.2.2.3 Identified roles of ATP in regulating immune responses

Large amount of ATP released from damaged cells is well recognized as a 'danger signal' which can activate NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasomes in immune cells to induce inflammatory responses, including neutrophils, macrophages and dendritic cells (Di Virgilio 2005; Schroder and Tschopp 2010). Nevertheless, the immune regulatory effect of ATP is complicated, and it has been suggested that the effect can be immuno-stimulatory or immune-suppressive depending on the concentration of ATP and the components of purinergic signalling in the environment (Boeynaems and Communi 2006). One theory was proposed that ATP played distinct roles at three stages of the inflammatory response (Bours, Swennen et al. 2006). In the first stage, large amount of ATP is released from damaged cells, which recruits leukocytes to damaged sites and initiate immune response, including inducing inflammatory activation of neutrophils and macrophages, maturation and Th_1 cell-stimulatory ability of DCs, and proliferation and activation of lymphocytes. In the second stage, ATP is partially hydrolyzed by ectonucleotidases; therefore only low concentrations of ATP and accumulating adenosine are present in the environment. Low concentrations of ATP - by activating P2Y receptors - plays an immunomodulatory role by inhibiting TNFα release and stimulating IL-10 release. On the other hand, adenosine which activates P2 receptor contributes to down-regulating the inflammatory response stimulated in the first stage. At the final stage, extracellular adenosine becomes the key factor in the process of immunosuppression and tissue healing. The expression of P1 receptors is up-regulated during the inflammatory response which subsequently enhances the immunosuppressive effect of adenosine. This hypothesis was supported by accumulated evidence (Di Virgilio, Boeynaems et al. 2009) and is consistent with the observations of macrophage responses to ATP. Considering the concentrations of ATP likely to be found in many tumours, the major influence of ATP on the tumour microenvironment would be predicted to be one of immunosuppression.

8.3 Trophic effects of stressed tumour cells

8.3.1 Further characterization of the bioactive factor

Although S1P appeared promising as a candidate for the bioactive factor in stress supernatants, it could not be detected by ELISA. However this indicates that S1P was not present in soluble form. It is possible that S1P occurs in association with microparticles which, depending on their size were not retained in supernatants under the conditions studied. This requires investigation.

Although displaying mitogenic effect, stress supernatants did enhance the viability of BL2 cells within the first 24 hours. Since group I BL cells tend to undergo apoptosis in sub-optimal conditions, such as in reduced concentrations of FCS (Gregory, Dive et al. 1991), the bioactive factor in the supernatant could possibly function as a survival factor as well as a mitogen (Collins, Perkins et al. 1994). The nature of the stimulation could be determined by analysing various relevant parameters of apoptosis in cells triggered by an inducer of apoptosis such as staurosporine, compared with cells pre-treated with supernatants from stressed BL cells. Several parameters could be measured, including caspase-3 activity, the level of bcl-2 and phophorylated-Bad.

The identification of bioactive factor with mitogenic effect should be performed further. Knowing that the factor is heat-stable, further investigation into its identify can be performed using proteinase K, DNase, or RNase to characterise it as being protein, DNA or RNA respectively. Extraction of stress supernatant with an organic solvent such as chloroform, could be informative as to the possible lipid nature of the factor. In addition, the molecules in stress supernatant could be analyzed by high-performance liquid chromatography or mass spectrometry in order to create a short-list of candidate factors before further analysis (Lutzke and Braughler 1990).

8.3.2 Direct trophic effects of stressed tumour cells

Data presented here demonstrated direct trophic effects of stressed, pre-apoptotic tumour cells on other viable tumour cells, as well as mononuclear phagocytes. Similar direct trophic effects were demonstrated *in vivo* in 1956 when it was shown that lethally damaged tumour cells by X-ray irradiation could promote tumourigenesis and progression in a mouse model (Revesz 1956). However, no mechanism has been reported to account for this observation. Nevertheless, as mentioned in the introduction, a similar phenomenon was observed in *Drosophila* (Fan and Bergmann 2008). Stress or damage induced apoptosis could trigger the proliferation of remaining cells which was termed 'apoptosis-induced compensatory proliferation'. *Drosophila*'s initiator caspase, Dronc and effector caspases DrlCE and Dcp-1 were found related to this compensatory effect but the mechanism was not completely understood. Interestingly, one study into the mechanism of compensatory proliferation indicated that under the circumstances of loss of one copy of *dronc* gene, effector caspases can still be activated, but the compensatory proliferation effect was significantly suppressed (Wells, Yoshida et al. 2006), suggesting that initiator caspase Dronc was more important than executing apoptosis program for the effect.

This finding is to some extent similar to the finding in the high density stress model reported here. The fact that supernatants from stressed, pre-apoptotic BL2 cells which were shown induced to undergo apoptosis when subsequently returned to low cell density culture (Figure 3.3) could promote growth indicated that the release of the bioactive factor(s) was independent of the complete execution of apoptosis program. Furthermore, supernatants from stressed BL2/bcl-2 cells, which were shown not to be induced to undergo apoptosis when subsequently returned to low density culture (Figure 3.4), exhibited similar growth promoting effects as stressed BL2 cells (Figures 5.4 and 5.6). This indicates that the release of the bioactive factor(s) was a process which functioned independently of bcl-2 protein. The release might be coupled to the

events upstream of bcl-2 protein in mitochondria-dependent apoptotic pathway or coupled to other mitochondria-independent apoptotic pathway if the release is apoptosis-related. Nevertheless, this direct trophic effect of stressed tumour cells might be a compensatory growth regulatory mechanism for stress- or damage-induced cell loss in normal tissue development which stressed tumour cells can harness to promote tumour growth.

8.3.3 Multiple signals act in concert to condition the tumour microenvironment in BL

This study demonstrated a selective recruitment of immune cells by stressed, pre-apoptotic Burkitt's lymphoma cells, in which mononuclear phagocytes were attracted but the migration of neutrophils was inhibited. ATP, which is present in stress supernatants, is likely to be the chemoattractant responsible for the mononuclear phagocyte recruitment, 'find-me' signal. Although it has not been determined whether ATP induces chemotaxis or chemokinesis, it has been shown to activate the migration of the neutrophils as discussed above. The presence of a neutrophil migration inhibitory factor or factors was demonstrated in stress supernatants but was not identified. It might be one of the identified neutrophil migration inhibitory factors, such as lactoferrin (Bournazou, Pound et al. 2009) and netrin-1 (Ly, Komatsuzaki et al. 2005).

The fine balance of 'find-me' and 'keep-out' signals released from pre-apoptotic cells is reminiscent of similar mechanism of apoptotic cells, since it has been demonstrated that apoptotic BL cells can also release 'find-me' and 'keep-out' signals, fractalkine and lactoferrin, respectively (Truman, Ford et al. 2008; Bournazou, Mackenzie et al. 2009; Bournazou, Pound et al. 2009). The release of molecules with similar effects at an earlier stage emphasized the importance of timing in selectively recruiting immune cells and further microenvironmental conditioning.

Since ATP can drive macrophages towards an anti-inflammatory, M2 phenotype and enhance their phagocytic ability, the early release of the ATP-dependent 'find-me' signal(s) can begin the polarization of macrophages during their migration and ensure the prompt clearance of apoptotic cell debris and the subsequent immune-suppressive, pro-tumour functions. Furthermore, macrophages have been shown with the capability to influence cell fate of survival or death. Classically activated macrophages, primed by IFN-γ and LPS exhibited ability to induce apoptosis and inhibit mitosis in mesangial cells (Duffield, Erwig et al. 2000). In the nematode *Caenorhabditis elegans*, engulfing cells were found essential in triggering apoptosis (Hoeppner, Hengartner et al. 2001; Reddien, Cameron et al. 2001). Previous work has demonstrated that IL-10 activated macrophages could secrete tumour cell growth/survival factor BAFF (B cell-activating factor of the TNF family) (Ogden, Pound et al. 2005). Since ATP was released from pre-apoptotic cells whose fate has not yet been decided, recruited macrophages might play an important role in affecting the fate of stressed, pre-apoptotic cells therefore regulating the balance of cell birth and death in the tumour microenvironment. However, further experiments are required to validate this speculation. Nevertheless, these effects, together with the direct trophic effect of stressed tumour cells demonstrated that pre-apoptotic BL cells can contribute to conditioning the tumour microenvironment in favour of tumour growth.

It is possible that the death of some tumour cells may benefit the tumour as a whole (Gregory and Pound 2010; Gregory and Pound 2011). Signals from pre-apoptotic and apoptotic BL cells may work in concert to create a pro-tumour microenvironment. Understanding the microenvironmental conditioning signals in tumours, has value in targetting therapy but also has broader implications for medicine. Considering the important tumour-promoting functions of TAM, 'find-me' signals for TAM provide promising targets for the development of antagonists to disrupt their recruitment and to weaken environmental support for tumour growth. Similarly, bringing in neutrophils

and eosinophils into tumour sites by neutralising 'keep-out' signals, such as lactoferrin, might induce inflammatory immune response leading to tumour distruction. Furthermore, failed clearance of apoptotic cells has been shown to elicit inflammatory immune responses which are responsible for some autoimmune diseases, such as systemic lupus erythematosus (SLE) (Gaipl, Munoz et al. 2007; Nagata, Hanayama et al. 2010). Therefore, blockade of the phagocytosis process in an attempt to invoke anti-tumour immune responses provides a therapeutic target which can be achieved by masking 'eat-me' signals on apoptotic cells using specific antibodies. One example is the blockade of PS which has already been demonstrated to induce tumour regression (Bondanza, Zimmermann et al. 2004). It is possible that this approach might be valuable in treatment of some autoimmune diseases. Since decreased phagocytic ability of macrophages as well as reduced levels of macrophage attraction signals were found associated with SLE, agonists that can enhance the engulfing ability of macrophages or agonists that can attract macrophages are also promising valuable therapeutic targets for treatment of SLE.

8.4 The nature of high density stress

8.4.1 Mechanism of high density stress

8.4.1.1 Main stressors in high density stress

Having excluded hypoxia as a stressor in the high density model, glucose level was measured to investigate whether glucose deprivation could be the stressor. The glucose level dropped rapidly during the first hour, and it was almost completely depleted by 4 hours, suggesting limitation of glucose supply to large numbers of cells. On the other hand, glucose deprivation could explain well the non-apoptotic cell death during longer stress, since apoptosis is an ATP-dependent process and lack of ATP could switch apoptosis to necrosis (Leist, Single et al. 1997). The induction of apoptosis after stressed cells were cultured at lower density in fresh media with sufficient glucose

therefore adequate energy supply was consistent with this interpretation. However, glucose-free media or glucose metabolism inhibitors, such as 2-deoxyglucose, could be applied in this model in order to confirm the inducing effect of glucose deprivation on BL2 cell apoptosis. Alternatively, the presence of glucose deprivation could be examined by measuring AMP-activated protein kinase (AMPK), whose activation can be induced by glucose-deprivation for temporal protection (Kato, Ogura et al. 2002; Priebe, Tan et al. 2011).

Another possible contributor in the high density stress model is oxidative stress. During high density stress, while glucose is rapidly consumed, toxic metabolites, such as reactive oxygen species (ROS), are generated. Glucose deprivation is a trigger to induce oxidative stress and transformed human cell types have been found to be more sensitive to glucose deprivation induced oxidative stress than are healthy cells (Spitz, Sim et al. 2000). ROS can damage most of the biological macromolecules, including proteins, nucleic acids and lipids (Ott, Gogvadze et al. 2007). When ROS-induced damage reaches a critical point various modes of cell death, including apoptosis, autophagic cell death, might be induced depending on the intensity of the stress (Chen, McMillan-Ward et al. 2007; Orrenius, Gogvadze et al. 2007). However, the presence of oxidative stress has to be proved and can be achieved by either measuring the damage to the macromolecules or measuring the intracellular antioxidant level.

8.4.1.2 Cellular stress responses

In order to investigate the mechanisms of the stress response after high density culture, protein levels of molecular chaperones Hsp27, Hsp70 and Hsp90 were examined by western blot. There was no change in the protein levels of Hsp70 and Hsp90, indicating that these two molecular chaperones were not induced by high density stress therefore no protective effect from them. But the protein level of Hsp27 decreased as stress continued. Interestingly, Hsp27 has been found to translocate into the nucleus

from the cytoplasm in response to stress and thereby become detergent insoluble (Arrigo, Suhan et al. 1988), which might explain the reduction in the expression level of Hsp27 since whole cell lysates were applied in the assay . However, in order to examine this interpretation, separate extraction and analysis of nuclear and cytoplasmic proteins must be performed.

Another molecule that should be examined in order to understand the cellular stress response in the high density model is p53, which is a key factor regulating cellular response to glucose deprivation and oxidative stress (Vousden and Ryan 2009). Glucose deprivation induces the activation of AMPK which subsequently activates p53. Activated p53 then performs multiple functions to sustain cell survival under stress, including cell cycle arrest at G1/S boundary by activating p21, proliferation inhibition by inhibiting Akt, growth inhibition but autophagy promotion by inhibiting mTOR. Under low level of oxidative stress, p53 can create an anti-oxidant environment by activating Sestrins, TP53-induced glycolysis and apoptosis regulator (TIGAR) and tumour protein p53-inducible nuclear protein 1 (TP53INPI), therefore lead to cellular protection and survival.

Another response that might occur in the system is the unfolded protein response (UPR) which results from the accumulation of unfolded proteins in the endoplasmic reticulum (ER stress) (Schroder and Kaufman 2005; Ron and Walter 2007). UPR can subsequently activate ER resident proteins, such as inositol-requiring protein-1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activate transcription factor 6 (ATF6) to transmit the stress signal. Since glucose starvation can induce UPR and UPR has been found to promote cell survival by secreting trophic factors (Tamatani, Matsuyama et al. 2001), UPR might be the key mediator of signal pathways induced by high density stress. However, further experiments measuring the activation of UPR targets, such as sXBP1 mRNA (a key marker for IRE activation) and the

translocation of ATF6a from the ER to the nucleus, are required in order to examine the contribution of UPR in high density stress (Cawley, Deegan et al. 2011).

8.4.2 High density stress and BL2 cell apoptosis

Cellular stress responses are a self-defence mechanism, however, when the stress stimulus is beyond a certain threshold, stress signalling pathways will eventually activate the cell death pathway. The molecular events of the activation pathway leading to apoptosis after high density stress have not been identified. Some cellular stress could trigger apoptosis by the intrinsic apoptotic pathway which activates caspase-9 by way of outer mitochondrial membrane permeabilization. Other cellular stress, including heat shock and metabolic stress might directly activate caspase-2 to initiate the apoptosis programme, although in some cell types this pathway would also involve mitochondria (Green 2011). In the present studies, after 1 hour of high density stress, there was no change in mitochondrial membrane potential (Figure 3.2), therefore model is more likely to induce apoptosis by way of caspase-2 and independent of mitochondria (Bouchier-Hayes 2010). Since the role of caspase-2 in driving apoptosis was postulated to depend on the extent of its activation, it might as well explain the induction of apoptosis in only a fraction of cells by high density stress (Bouchier-Hayes and Green 2012). Furthermore, over-expression of Myc protein could induce the expression of p53 (Speidel 2009). Under acute or long-lasting unresolved stress, p53 could adopt an apoptosis induction strategy. P53 could either promote apoptosis by activating transcription of BH-3 only protein of Bcl-2 family or by acting as a BH-3 only protein (Vousden and Lane 2007). Therefore, p53 might be both a sensor and an initiator of apoptosis in response to high density stress.

Another form of cell death, autophagy, could also be induced by over-expression of Myc (Reef, Zalckvar et al. 2006), and it is reported that when the nutrients are limiting, Myc-induced lymphoma cells adopted autophagy for short-term survival before

'eating themselves to death' (Amaravadi, Yu et al. 2007; Maclean, Dorsey et al. 2008). Thus, it was also possible that during long term high density stress, cells sustained their survival by autophagy before dying by necrosis.

A group I BL cell line was employed in the present work because it retains features of cells in the original tumour. This line, BL2, possesses an intrinsically high tendency to undergo apoptosis, which could partly be attributed to the de-regulated over-expression of Myc protein and very low or no expression of anti-apoptotic protein Bcl-2 (Henderson, Rowe et al. 1991). It might also reflect a germinal centre origin of the cells, since germinal centre B cells undergo apoptosis unless they receive survival signals. In order to better understand the mechanism driving apoptosis in BL, several studies have been undertaken in B lymphoma cells. Growth factor deprivation was found to induce apoptosis in group I BL cell lines (Gregory, Dive et al. 1991). Heat shock at 42°C was found to induce apoptosis in B lymphoma cells (WEHI-231 B cells) which can be partially prevented by bcl-2 over-expression (Cuende, Ales-Martinez et al. 1993). Likewise, cold shock could potently induce group-I BL cell apoptosis when the cells were brought back to culture at 37°C (Gregory and Milner 1994). Despite the fact that these stimulators are artificial which can not be present in the natural BL tumour microenvironment, they demonstrated that these cells could undergo apoptosis in response to cellular stress of various natures. Considering the rapid proliferation of BL cells and the limitation of oxygen and nutrients which is aggravated by the de-regulated over-expression of Myc protein, a combinatiion of high density stress with limited nutrients is more likely to be a physiological stressor that drives apoptosis and conditions the microenvironment in the tumour.

8.4.3 Implications for tumour microenvironment

Although the mechanism of high density stress has not been clarified, the finding that stressed tumour cells could affect other cells in the microenvironment to promote

tumour growth provides another perspective to look at cellular stress response and the subsequent biological effects. Most studies of the cellular stress response focus on the effect on stressed cells themselves. For example, the blocking of cell cycle and the inhibition of cell proliferation by p53 in stressed cells were believed to contribute to tumour suppression (Vousden and Prives 2009). The cellular protective effect provided by the unfolded protein response was believed to contribute to tumourigenesis (Fels and Koumenis 2006). However, it is also important to remember the interaction and communication between different cells within tumours. Apart from this study, there are several very good examples of the effect of stressed cells on their neighbours. One is the compensatory proliferation in *Drosophila* as discussed above. Another one is in *Caenorhabditis elegans.* Cells under hypoxic stress could secrete a neutral tyrosinase to remotely promote cell survival (Sendoel, Kohler et al. 2010). The last one is the bystander effect induced by radiation which describes a phenomenon that cells not directly exposed to ionizing radiation die or exhibit chromosomal mutation or instability as though they have been exposed (Seymour and Mothersill 2004). Interestingly, macrophages are an important player in mediating this effect (Lorimore, Chrystal et al. 2008).

The microenvironmental effect of metabolic stressed cells might partially explain the resistance of some tumours to anti-VEGF treatment, which aims to reduce angiogenesis (Bagri, Kouros-Mehr et al. 2010; Keunen, Johansson et al. 2011).The concept of restoring the balance between pro- and anti-angiogenic factors to achieve normal vasculature in tumours has been referred to as ‘vascular normalization’. This approach, combined with traditional chemotherapy, has been shown to promote patient survival, such as patients with primary glioblastoma and prostate carcinoma and patients with breast cancer brain metastases (Goel, Duda et al. 2011).

All together, the microenvironmental conditioning effects of stressed BL cells to

promote tumour growth support the notion that in order to conquer tumours, including lymphomas, the paradigm of treatment should shift from the malignant cell-centered therapy to combining treatment targeting the interplay between malignant cells and the environment (Mantovani, Allavena et al. 2008; Burger, Ghia et al. 2009; Coupland 2011). In NHL, conventional treatments are mostly targeting the proliferating tumour cells, such as chemotherapy or rituximab, but not the microenvironment, which might explain why some malignancies are still not curable despite the advances in therapeutics. One of the new drugs targeting the microenvironment in lymphoma is an antibody against CD137 which targets T cells in the tumour microenvironment and it has shown potent anti-lymphoma effect in NHL (Houot, Goldstein et al. 2009). Thus, better understanding the tumour microenvironment may provide more targets for effective tumour treatment.

8.5 Embryonic stem cell derived macrophages (ESDM)

8.5.1 Macrophage characteristics of ESDM

The collective evidence presented from studies of their morphology, expression of macrophage markers, their phagocytic and migratory ability, argues strongly that the cells generated from murine ES cells under the conditions described were macrophages. Although 10%-20% of the cells did not stain for classical macrophage markers it is possible that this could be accounted for by reduced marker expression by dead macrophages considering the around 90% viability after detachment. However, the use of cell type-specific markers detected by monoclonal antibodies cannot be used in isolation to identify a cell type (Hume, Ross et al. 2002). It is possible that the cells which did not stain for the macrophage markers were a macrophage subset which does not express classical markers. As monocytes differentiate into macrophages, there are many phenotypic changes, including a reduction in the nucleocytoplasmic ratio (Sokol, Hudson et al. 1987), increased numbers of lysosomes and mitochondria (Cohn and Benson 1965; Kradin, McCarthy et al. 1986), and decreased levels of ER-MP12,

ER-MP20, ER-MP54, ER-MP58 expression (Leenen, de Bruijn et al. 1994). Such phenotypic features could be used to assess the macrophage characteristics of ESDM for further maturity analysis of ESDM.

A further possibility is that the cells which differentiated from ES cells included some dendritic cells. Although CD11c was long considered to be the surface marker that could distinguish dendritic cells (DC) from macrophages, it is now known to be expressed by most tissue macrophages (Hume 2008). The defining characteristic of DC is their functional ability to prime the T cell responses but this has been challenged by the finding that macrophages can also activate T cell response (Pozzi, Maciaszek et al. 2005). There are even reports suggesting that following phagocytic activation, macrophages could acquire the archetypal DC phenotype, including the specialized antigen-presenting function (Steinman and Inaba 1999; Mellman and Steinman 2001). Transcriptome analysis indicated that antigen-presenting dendritic cells were a specialized activation state of macrophages rather than a separate cell type (Hume 2006). However, in order to resolve the relationship between macrophages and DC, a closer investigation into the cells is required, by analysing cell origin, anatomical location, function and phenotype, in a systems biology approach. While recognizing the indistinct boundary between these two cell types, the properties of the macrophage-like cells generated from ES cells fit the classical definition of macrophages and the presence of classical dendritic cells was excluded by CD11c staining. This favours the notion that they are macrophages.

When analysing the light scatter characteristics of ESDM by flow cytometry, two major subpopulations were identified on the basis of size and granularity (Figure 6.6). The two major subsets were reminiscent of the classification of two subsets in blood monocytes (Geissmann, Jung et al. 2003). It would be interesting to separate these two subpopulations using a flow cytometry-based cell sorter and see whether these cells

have any difference in phenotype and migratory ability that might enable them to be defined as different subsets of monocytes. Furthermore, in the light of the report that subpopulations of mouse blood monocytes, defined by the different expression levels of Ly6C, differ in their maturation stage (Sunderkotter, Nikolic et al. 2004), the possibility exists that the two subpopulations of ESDM, which had different expression levels of F4/80 and CD11b, might represent differences in maturity.

The observation that as ESDM matured, they gradually lost the ability to migrate towards stress supernatants and had enhanced capability to ingest apoptotic cells fits well with the classical description of monocyte recruitment into tissues, their differentiation into macrophages and their development of macrophage functions, including clearance of cell debris. However, the molecular mechanisms of this differentiation and maturation process responsible for the capability switch are unknown, and in vivo it is likely to be further complicated by stressed tumour cells releasing factors (soluble or microparticle-associated) in addition to monocyte chemoattractants that can condition monocytes and influence the differentiation process towards the production of macrophages that have acquired the ability to ingest apoptotic cells. Thus, ESDM provide a convenient in vitro model to identify the molecular mechanism of this maturation process with respect to both phenotype and functions.

8.5.2 ESDM in biology and medicine

There have been significant recent developments in studies of macrophage differentiation from stem cells. Macrophages have been generated not only from murine and human embryonic stem cells, but also from murine and human induced pluripotent stem cells which are induced pluripotent stem cells from differentiated somatic cells and exhibit the morphology, growth and differentiation properties of ES cells (Moore, Fabunmi et al. 1998; Anderson, Bandi et al. 2006; Karlsson, Cowley et

al. 2008; Senju, Haruta et al. 2009; Senju, Haruta et al. 2011). The Hox gene has already been transfected into murine ES cells to produce an immortalized myeloid progenitor cell line in an attempt to generate an endless production of macrophages when required, instead of starting new ES cell culture each time (Wang, Calvo et al. 2006; Odegaard, Vats et al. 2007). Human ES cell differentiation has successfully progressed from a co-culture system to a co-culture-free system which greatly simplifies the culture procedures. Inducible pluripotent stem cells may prove valuable in providing new therapies.

It is important to bear in mind the plasticity and adaptability of macrophages. Despite the fact that the haematopoiesis during ES cells/EB differentiation is considered to be similar, if not identical to that of the early embryo (Keller 2005), ESDM are a model generated in vitro in an artificial environment which provides broad differentiation requirements. Considering the heterogeneity of in vivo macrophages as well as the complicated interaction between cytokines and microenvironment during macrophage development, it cannot recapitulate the in vivo generation of specific subsets of macrophages. The in vitro differentiation system is highly restricted in its scope. Given the heterogeneity and plasticity of macrophages, the differentiation protocol would have to be more specific according to target organs and functions to replicate the complex interactions occurring in vivo. However, it provides a prototype for the development of future differentiation systems that can take into consideration the in situ phenotype and the microenvironment of the target organ. Therefore, before future application of ESDM in vitro and in vivo, it would be preferable to precondition ESDM in an environment that mimics the target in vivo microenvironment.

8.5.3 ESDM as a novel model for macrophages

Commonly used macrophage models have their intrinsic disadvantages. For example, tumour-derived cell lines, such as RAW 267.4, J774, and THP-1 cells, have the

potential for their tumour origin and possible tumour-specific mutations to interfere with the interpretation of experimental results. Indeed, primary tumour cells have been found to be deficient in the IFN-γ pathway (Xu, Grander et al. 1994; Wong, Krauer et al. 1997; Sun, Pabon et al. 1998) which is vital for classical macrophage activation. Gene profiling has also revealed that J774A.1 and RAW264.7 differed significantly from mouse peritoneal macrophages at the level of gene expression (Lindmark, Rosengren et al. 2004). Primary cells are also frequently used to obtain macrophages for in vitro studies (e.g. bone marrow-derived macrophages (BMDM) and elicited peritoneal macrophages); these are more like 'normal' macrophages, but they are always limited in cell numbers and have inherent resistance to genetic modifications. The acquisition of these primary cells always requires the access to living organisms. It is also demonstrated in this study that BMDM are very easily contaminated by other cell types.

The in vitro haematopoiesis that occurs in ES cells/EB differentiation in vitro has been confirmed to recapitulate the early processes of in vivo embryogenesis (Keller 2005; Wobus and Boheler 2005). Gene profiling has already demonstrated similar gene expression pattern between ESDM and peritoneal macrophages (Lindmark, Rosengren et al. 2004). Furthermore, ES cells can be genetically manipulated, including gain-of-function, loss-of-function and temporal and spatial restricted control, and relevant protocols have already been successfully established. Manipulated ES cells can be differentiated into macrophages which would be a valuable model for both in vitro and in vivo studies of macrophage gene functions. Thus, ESDM have the potential to provide an improved cellular model considering their unlimited cell number, similarity to primary macrophages and feasibility for genetic manipulation.

Despite all the advantages, this differentiation system has crucial flaws. The fundamental problem is the use of foetal bovine serum, which is a non-physiological,

undefined supplement with regards to murine ES cells. The variation in serum batches influences the reproducibility of ES cell differentiation. Therefore, in order to generate a protocol that can be employed widely, a serum-free and chemically-defined culture system must be established. Nevertheless, the demonstrated abilities of ESDM to migrate to and engulf apoptotic cells, together the genetic feasibility, provides a valuable model to study the molecular mechanisms underlying the interaction between apoptotic cells and macrophages.

8.6 Concluding remarks and future perspectives

In summary, the studies presented in this thesis indicate that in Burkitt's lymphoma, stressed, pre-apoptotic tumour cells can condition the tumour microenvironment by selectively recruiting mononuclear phagocytes and inhibiting the infiltration of neutrophils as well as secreting survival/mitogenic factors. ATP is likely to be the mononuclear phagocyte chemoattractant released from pre-apoptotic cells and other possible roles of ATP in conditioning the tumour microenvironment, such as regulating the functions of tumour-associated macrophages, direct pro-tumour effects as well as influencing immune responses, have been proposed. The possible mechanisms of cellular stress responses, including temporary cellular protection and induction of apoptosis, in high density stress have been discussed. Although the survival/mitogenic factors and neutrophil infiltration inhibitory factors have not been identified, the studies provide novel perspectives in understanding the tumour microenvironment and identifying new therapeutic targets. Targeting the 'find-me' 'keep-out' and 'eat-me' signals in the tumour microenvironment may regulate the infiltration and functions of immune cells therefore either suppress pro-tumour functions or induce anti-tumour immune responses. The discovery that stressed tumour cells have direct trophic effects on other tumour cells supports the combination treatment of cancer which targets both malignant cells and the microenvironment and the identification of the survival/mitogenic factors may provide novel therapeutic

targets.

Furthermore, a scheme for generation of ESDM has been developed and optimized and their phenotypes and functions have been assessed. The purity, availability and genetic tractability make them a valuable model to study the molecular mechanisms of macrophage functions and role of the cells in tumour development. ESDM from murine and human ES cells as well as inducible pluripotent stem cells also provide immunologically compatible cells for in vivo experiments and potential clinical treatments.

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Appendix 1: List of medium

Embryonic stem (ES) cell growth medium ($GMEM_{FCS}$):

1× Glasgow Minimal Essential Medium (Gibco)

10% (v/v) FCS (Globepharm) (batch tested for ES cell self-renewal)

0.25% (v/v) sodium bicarbonate (Giobo)

1% (v/v) non-essential amino acids (Gibco)

4mM L-glutamine (Gibco)

2mM sodium pyruvate (Gibco)

0.1mM 2-mercaptoethanol (Sigma)

The above media was supplemented with 100U/mL Leukaemia Inhibitory Factor (LIF) as required.

Embryonic stem (ES) cell differentiation medium ($GMEM_{Diff}$) :

1× Glasgow Minimal Essential Medium (Gibco)

10% (v/v) Diff (Globepharm) (batch tested for ES cell differentiation)

0.25% (v/v) sodium bicarbonate (Giobo)

1% (v/v) non-essential amino acids (Gibco)

4mM L-glutamine (Gibco)

2mM sodium pyruvate (Gibco)

0.1mM 2-mercaptoethanol (Sigma)

Burkitt's lymphoma cell culture medium (BL medium):

1×RPMI 1640 (Gibco)

10% fetal bovine serum (FBS) (v/v, Biosera)

2mM L-glutamine (PAA)

100 IU/ml penicillin (PAA)

100 µg/ml streptomycin (PAA)

L929 cell culture medium:

1×D-MEM: F12 with Glutamax (Gibco)

10% fetal bovine serum (FBS) (v/v, Biosera)

100 IU/ml penicillin (PAA)

100µg/ml streptomycin (PAA)

Bone marrow derived macrophages culture medium ($DMEM_{supp}$):

1×D-MEM: F12 with GLutamax (Gibco)

2mM L-glutamine (PAA)

100 IU/ml penicillin (PAA)

100µg/ml streptomycin (PAA)

20% (v/v) FBS

10% (v/v) L929 CM

MonoMac6 cell culture medium:

1×RPMI 1640 (Gibco)

10% fetal bovine serum (FBS) (v/v, Biosera)

2mM L-glutamine (PAA)

100 IU/ml penicillin (PAA)

100 µg/ml streptomycin (PAA)

OPI media supplement, HYBRI-MAX (Sigma) (contains final working concentrations of 1 mM oxaloacetate, 0.45 mM pyruvate, 0.2 U/ml insulin)

1×Amino acids non-essential (PAA)

THP-1 cell culture medium:

1×RPMI 1640 (Gibco)

10% fetal bovine serum (FBS) (v/v, Biosera)

2mM L-glutamine (PAA)

100 IU/ml penicillin (PAA)

100 µg/ml streptomycin (PAA)

Embryonic stem cell derived macrophages (ESDM) culture medium ($ESDM_{cult}$):

$GMEM_{Diff}$

15% (v/v) L929 CM

100 IU/ml penicillin (PAA)

100 µg/ml streptomycin (PAA)

Embryonic stem cell derived macrophages (ESDM) differentiation medium ($ESDM_{Diff}$):

$GMEM_{Diff}$

15% (v/v) L929 CM

1ng/ml IL-3

Chemotaxis assay medium:

1×RPMI 1640 (Gibco)

2mM L-glutamine (PAA)

100 IU/ml penicillin (PAA)

100 µg/ml streptomycin (PAA)

Appendix 2: List of buffer

Detachment Buffer:

Hank's BSS (without Ca^{2+} /Mg^{2+}/ phenol red, PAA)

5mM EDTA (Fisher)

0.2% low-endotoxin BSA (w/v, PAA))

Substrate solution preparation for self-renewal assay:

The substrate was prepared freshly as follows: 1ml sodium nitrite solution was gently mixed with 1ml Fast Red TR solution and the mixture was added to 50ml deionized water. The above diluted solution was supplemented with 1ml naphthol AS-B1 alkaline solution.

Appendix 3: Standard curves

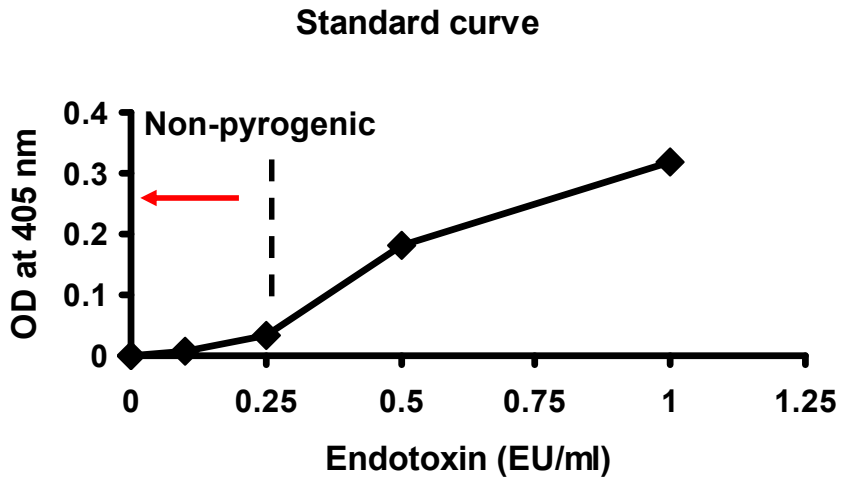

Figure Appendix 3-1 LAL assay standard curve.

The dotted line and red arrow indicates the non-pyrogenic level of endotoxin (less than 0.25 EU/ml).

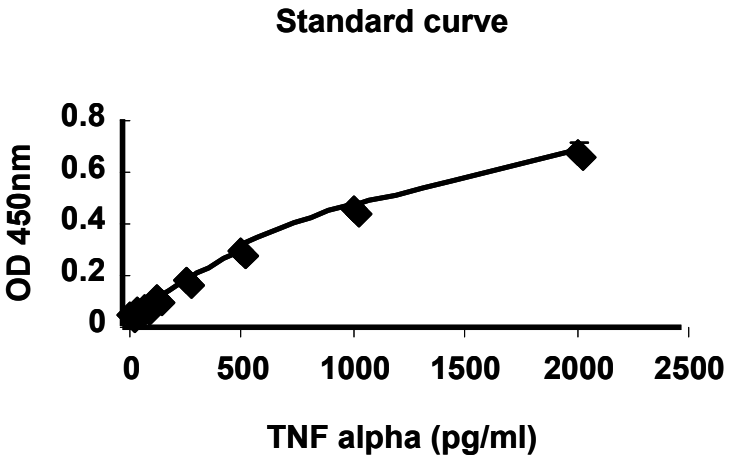

Figure Appendix 3-2 Standard curve of TNF alpha ELISA.

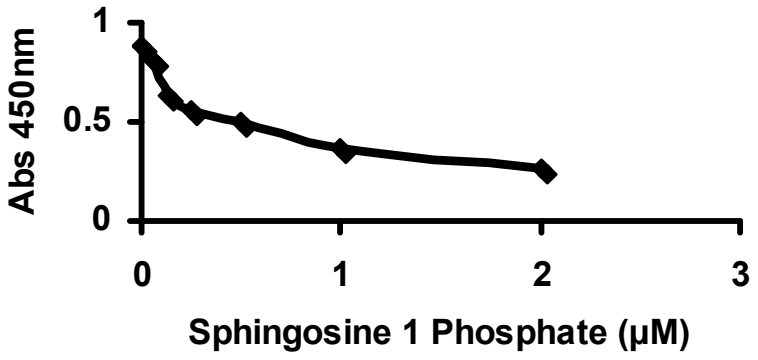

Figure Appendix 3-3 Standard curve of S1P ELISA.