Embryonic Stem Cell Differentiation: A Novel Approach to Gene Targeting in Myeloid Cells

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Abstract

Mouse embryonic stem cells (ES cells) are derived from the inner cell mass of 3.5 day blastocysts. ES cells remain totipotent when cultured on embryonic fibroblasts or in the presence of Leukaemia inhibitory factor (LIF). However, under defined culture conditions they are able to differentiate into multiple cell lineages and when reintroduced back into a host blastocyst, possess the remarkable ability to contribute to all adult tissues in the mouse. These unique features of ES cells allow genes to be deleted in mice and when combined with Cre/LoxP technology provide an ideal system for conditional deletion of genes where total 'knockout' would be lethal. One such gene is integrin α_v where knockout mice die in utero or perinatally. Integrin α_v has been implicated in angiogenesis and cell adhesion as well as in the phagocytosis of dying cells by macrophages and dendritic cells, a process that is important in the resolution of inflammation and tissue remodelling.

The main aim of this thesis was to examine the role of integrin α_v in phagocytosis. To do this, two complementary approaches were used. Firstly, ES cells with disrupted integrin α_v genes were differentiated into myeloid cells and the effects on phagocytosis by DCs assessed. Secondly Cre was delivered to macrophages cultured from integrin α_v targeted mice, to delete integrin α_v immediately prior to phagocytosis assays.

This thesis demonstrates that macrophages can be generated from the culture of ES cells. Although low in number these macrophages show similar morphology and surface phentoype to bone marrow derived macrophages. Importantly, these ES macrophages readily phagocytosed latex beads and apoptotic cells. In addition, ES cells produced cells with a dendritic cell phenotype that were capable of apoptotic cell phagocytosis and maturation. Generation of these myeloid cells from ES cells was strongly dependent on serum and the parent ES cell line. Dendritic cells could be generated from integrin α_v disrupted ES cells. These DCs retained the ability to

phagocytose apoptotic cells suggesting that integrin α_v is not essential for this process.

This study also investigates the properties of Cre fused to two transduction proteins. Although Cre retained its recombinase activity as a fusion protein these were unable to translocate into cells. However Cre could be delivered to both primary macrophages and cultured epithelial cells using a replication deficient adenovirus allowing deletion of the targeted genes. Intriguingly, the apoptotic cell phagocytosis was unaffected by lack of integrin α_v but could no longer be inhibited by the integrin antagonistic peptide RGD.

In conclusion, the capacity of ES cells to differentiate to myeloid cells combined with the ability to deliver Cre to silently targeted myeloid cells provide powerful systems for studying the role of specific genes in phagocytosis. Use of these approaches with integrin α_v demonstrates that this gene is not essential for apoptotic cell phagocytosis. However the ability of specific antagonists to inhibit phagocytosis show that integrin α_v is intimately involved in this process in normal cells.

Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Aileen M Smith

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Dedication

This thesis is dedicated to my family

Abbreviations

β_3	Integrin β_3 subunit
β_5	Integrin β_5 subunit
βH1	Embryonic Globin
α_v	Integrin α_v subunit
AdCre	Adenoviral Cre
AdGFP	Adenoviral Green Fluorescent Protein
ANTp	Antennapedia Homeodomain
APC	Allophycocyanin
BMDM	Bone Marrow Derived Macrophages
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary Deoxyribonucleic Acid
CMFDA	5-Chloro-methyl-Fluorescein Diacetate
DC	Dendritic Cell
DC $^{\alpha v -/-}$	Integrin α_v disrupted Dendritic Cells
DIA	Differentiation Inhibiting Activity
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiotheitol
EB(s)	Embryoid body/bodies
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ES cells	Embryonic Stem Cells
ES Mϕ	Embryonic Stem Cell derived Macrophage
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FSC/SSC	Forward Scatter/Side Scatter
G418	Geneticin
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMFI	Geometric Mean Fluorescence Intensity
His tag	Histidine Tag
HIV-TAT	Human Immunodeficiency Virus -Transactivator of transcription
HPRT	Hypoxanthine Phosphoribosyltransferase

HRP	Horseradish Peroxidase
ICAMs	Intracellular adhesion molecules
ICM	Inner cell mass
IL-	Interleukin
IPTG	β-D-isopropyl-thiogalactosidase
JAK	Janus associated tyrosine kinases
kb	Kilobases
kDa	Kilodaltons
LB	Luria Bertani
LIF	Leukaemia Inhibitory Factor
LPS	Lipopolysaccharide
M-CSF	Macrophage Colony-Stimulating Factor
MeC	Methylcellulose
MFG-E8	Milk fat globule-EGF-factor 8
MHC II	Major histocompatibility complex II
MME	Macrophage Metalloelastase
MOI	Multiplicity of Infection
mRNA	Messenger RNA
mtPBS	Mouse tonicity phosphate buffered saline
MTSCre	His_6-NLS-Cre-MTS (membrane translocation sequence)
NLS	Nuclear Localisation sequence
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
pfu	Plaque forming units
PMN	Polymorphonuclear Leukocyte, Neutrophil
PS	Phosphatidylserine
RNA	Ribonucelic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS-PAGE	Sodium doedecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2 domain
SSC	Side Scatter
STAT	Signal transducer and activator of transcription
TBE	Tris Boric acid EDTA
TE	Tris EDTA
TSP 1	Thrombospondin
TWEEN-20	Polyethyleneglycol sorbitanmonolaurate

VCAMs	Vascular adhesion molecules
VnR	Vitronectin receptor
VP22-tk	VP22 thymidine kinase
TNF-α	Tumour necrosis factor alpha
TGF-β	Transforming growth factor beta
MIP	Macrophage inflammatory protein

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Chapter 1

Introduction

1.1 Origin of macrophages and dendritic cells

Macrophages and dendritic cells (DCs) are highly specialised cells that are involved in the orchestration of the immune response. Both cell types exist in un-inflamed tissue at low frequency. However, during inflammation, monocytes, along with other leukocytes, are recruited at high numbers, and differentiate into macrophages and DCs. Macrophages combat infection, delete damaged or unwanted resident cells and orchestrate the ongoing inflammatory response. Macrophages are also thought to have an important role during the resolution of inflammation, removing dying cells and promoting tissue repair. DCs exist in inflamed tissue as immature cells, capable of capture of potential antigen by endocytosis and phagocytosis. DCs then mature, and migrate to lymph nodes, where they become highly efficient antigen presenting cells. They become less phagocytic and instead up-regulate adhesion and costimulatory molecules, initiating immune responses to previously captured antigen (Mellman and Steinman, 2001).

However this simple model of macrophage and DC function fails to emphasise the enormous diversity and heterogeneitiy of these cells *in vivo*. Macrophages and myeloid DCs arise from a common hematopoietic progenitor, present in foetal liver and adult bone marrow. Both macrophages and DCs can be grown as relatively pure and homogeneous populations from blood monocytes or bone marrow cells, dependent on the addition of the lineage-determining cytokines macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-3. Resident macrophages and DCs often adopt phenotypes specialised to their tissue microenvironment, such as Kupffer cells (liver), osteoclast (bone), alveolar macrophages (lung), or Langheran's cells (skin). Recruited monocyte phenotype is also dictated in part by the tissue, but also by the ongoing immune response. Recruited macrophages are defined as classically activated or alternatively activated depending on whether the conditioning immune response is a Th-1 (IL-12 and IL-18) or Th-2 (IL-4 and IL-13) type response respectively (Gordon, 2003). These activation states result in macrophages that

express very different panels of receptors and other cell surface markers, and that range from highly cytotoxic, inflammatory to phagocytic, reparative and anti-inflammatory cells. Similar differences are seen in DC phenotype, and this is further complicated by the existence of seemingly fundamentally different DC populations, myeloid and plasmacytoid DCs. The exact contribution of different DC precursors (myeloid or lymphoid) to DC phenotypes and of these distinct DCs to the final immune response is still debated (Shortman and Liu, 2002). However, this is only a brief overview of the differentiation and activation of macrophages and DCs, each of these cell types also interacts with altered host cells and the implications of this in the immune response will now be considered.

1.2 Interactions with apoptotic cells

Apoptosis is a highly regulated form of cell death that is important in normal tissue turnover, embryogenesis, and in the resolution of inflammation (Kerr et al., 1972). It is characterised by a series of morphological and biochemical changes triggering responses such as the activation of caspases, moderate increases in intracellular calcium and reactive oxygen species and reduced glutathione. In contrast to necrosis, apoptosis is an active process that is distinguished by nuclear condensation, cell shrinkage and membrane blebbing (McConkey, 1998). In the latter stages of this process, changes in the molecules on the cell surface allow their swift removal by phagocytes thereby preventing an immune response.

Early studies showed that recognition and engulfment of apoptotic cells is not associated with the release of pro-inflammatory mediators (Meagher et al., 1992) and later work has emphasised that this cell clearance event acts to suppress the immune response and increase the production of anti-inflammatory cytokines. Voll and colleagues reported that co-culture of apoptotic cells with Lipopolysaccharide (LPS) stimulated macrophages resulted in a decrease in production of tumour necrosis factor alpha (TNF-α), IL-1β and IL-12 and an increase in the anti-inflammatory cytokine IL-10 (Voll et al., 1997). Similar results were later presented by Fadok and

co-workers who also demonstrated suppression of pro-inflammatory cytokines (including thromboxane B2 and IL-8) although IL-10 secretion was shown to decrease rather than increase. Production of the anti-inflammatory cytokine transforming growth factor beta 1 (TGF-β1) was also shown to increase along with prostaglandin E2 and platelet activating factor and inhibitor studies indicated that these were acting in an autocrine/paracrine fashion to suppress TNF-α secretion (Fadok et al., 1998a). Moreover, suppression of the immune response by macrophage secretion of TGF-β1 following phagocytic clearance of apoptotic cells has also recently been demonstrated *in vivo* (Huynh et al., 2002).

Evidence is now emerging that apoptotic cell uptake can also promote an inflammatory response. Lorimore and colleagues demonstrated that splenic macrophages became activated following exposure of mice to ionising radiation and that this was a consequence of the clearance of apoptotic cells rather than a direct effect of the radiation (Lorimore et al., 2001). In addition, apoptotic cell uptake by macrophages stimulated with LPS has been shown to stimulate the production of both pro and anti-inflammatory cytokines. Lucas *et al.* reported increased production of TNF-α, macrophage inflammatory protein (MIP)-1α and MIP-2 in the first few hours after ingestion of apoptotic cells that was subsequently followed by an increase in the secretion of TGF-β1 that resulted in the inhibition of the TNF-α response (Lucas et al., 2003). A possible explanation for the apoptotic cell pro-inflammatory effect is that the initial secretion of cytokines such as TNF-α may be an important signal for the recruitment of greater numbers of macrophages to sites where there is a high number of dying cells (Savill et al., 2002).

Immature DCs, like macrophages, have been shown to be capable of ingesting apoptotic cells. Apoptotic cell uptake results in the inhibition of LPS maturation of DCs, generating mature DCs that have failed to up-regulate the costimulatory molecule CD86, produce less IL-12 and are less efficient at stimulating T-cell responses (Stuart et al., 2002). Similar to the macrophage story, ingestion of apoptotic cells can also promote an immune response. DCs that have ingested

apoptotic cells can cross-present antigen to both CD4 and CD8 T cells providing they receive the appropriate activation signal (Albert et al., 1998a; Albert et al., 1998b). Apoptotic cells clearly affect the response of interacting macrophages and DCs, and appear to promote inflammation, resolution and immune tolerance. What is less clear is the exact nature of the molecular interactions between the apoptotic cell and phagocyte.

1.3 Phagocyte recognition mechanisms

The mechanism of phagocytosis of unwanted or dying cells remains a complicated process in which many receptors, bridging molecules and 'eat me' markers on dying cells are involved (Fig 1.1) (Savill et al., 2002). Of the 'eat me' signals expressed by dying cells, phosphatidylserine (PS) is perhaps the most characterised. Exposure of PS on the outer surface of the apoptotic cell is required for efficient recognition and engulfment (Fadok et al., 1992). Recently, using phage display, Fadok and colleagues cloned a gene that when expressed in lymphocytes conferred the ability to recognise and engulf apoptotic cells, although the exact mechanism by which this candidate PS receptor mediates recognition is as yet undefined (Fadok et al., 2000). However, the definition of a prototypical 'eat me' signal and receptor will allow focused studies of this mechanism of apoptotic cell recognition. The existence of 'don't eat me' signals has also been proposed, expression of the inhibitory receptor CD31 on the surface of viable cells that normally functions as a 'move away' signal to the phagocyte becomes disabled upon apoptosis, switching the signal to a more adhesive one mediating phagocytosis (Brown et al., 2002). Therefore apoptotic cells could potentially be distinguished from viable cells either by the presence or absence of cell surface molecules. However, at present the mechanisms by which macrophages and semi-professional phagocytes do this remains poorly understood.

In comparison, the receptors expressed by the phagocyte are more clearly defined. These include: the complement receptors CR3 (also known as CD11b/CD18 and

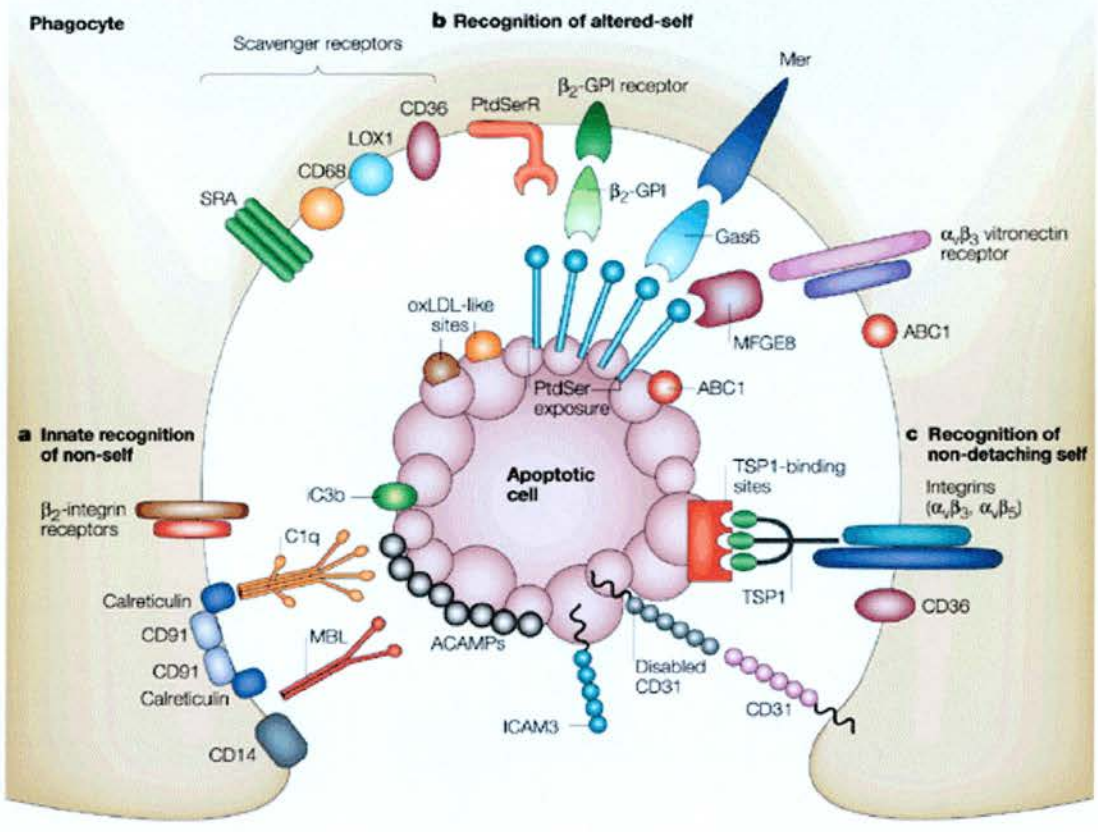


Figure 1.1: Phagocytic recognition mechanisms. Overview of the recognition molecules, bridging molecules and the 'eat me' signals involved in the phagocytic uptake of apoptotic cells. Taken from Savill 2002.

$\alpha_m\beta_2$) and CR4 (also known as CD11c/CD18 and $\alpha_x\beta_2$) that bind iC3b (Mevorach et al., 1998), CD14 that interacts with intracellular adhesion molecule (ICAM)-3/CD50 (Devitt et al., 1998; Moffatt et al., 1999), the first component of complement, C1q (Botto et al., 1998; Taylor et al., 2000), scavenger receptor A (Platt et al., 1996), CD36 (Ren et al., 1995) and the tyrosine kinase receptor, Mer (Scott et al., 2001) among others (Moynault et al., 1998; Ogden et al., 2001; Oka et al., 1998).

The vitronectin receptor (VnR) or $\alpha_v\beta_3$ integrin (CD51/CD61) was the first of the phagocytic receptors to be identified (Savill et al., 1990). Savill and co-workers proposed a role for this receptor in apoptotic cell uptake when they observed that phagocytosis was inhibited by monoclonal antibodies to the α/β subunits of the vitronectin receptor and by the synthetic tetrapeptide RGDS. Subsequently it has been shown that Thrombospondin 1 (TSP 1), secreted by macrophages binds to both VnR and CD36 to form a 'molecular bridge' between the phagocyte and the apoptotic cell to mediate uptake (Fadok et al., 1998b; Savill et al., 1992). In addition to TSP 1 another bridging molecule for $\alpha_v\beta_3$ has been proposed. The milk fat globule-EGF-factor 8 (MFG-E8) has been shown to be secreted by macrophages and bind PS on the surface of the apoptotic cell. Once engaged by PS, MFG-E8 strongly attaches to cells via its RGD motif, in particular through $\alpha_v\beta_3$ (Hanayama et al., 2002). More recently, CD36 and $\alpha_v\beta_5$ have also been implicated in the phagocytosis of apoptotic cells by immature DCs suggesting that CD36 may act cooperatively with both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Albert et al., 1998a). Furthermore, $\alpha_v\beta_3$ has also been implicated in DC uptake of apoptotic cells (Rubartelli et al., 1997).

However, despite the evidence that integrins are involved in phagocytosis little is known about the exact mechanisms by which this occurs and how critical a role they may play.

1.4 Integrins

Integrins are a large family of heterodimeric transmembrane receptors that mediate cell-cell interactions and adhesion to extracellular matrix. These surface receptors and their ligands are involved in a wide variety of process from development, hemostasis, immune response and leukocyte trafficking (Fig 1.2)(Hynes, 2002). Importantly, integrins provide a transmembrane link from the extracellular environment to the cytoskeleton that in turn, activate many intracellular signalling pathways.

Integrins are composed of α and β subunits, that each contain a large extracellular domain (>700 amino acids), a short transmembrane spanning domain and a cytoplasmic domain of ~20-60 amino acids (Travis et al., 2003). These receptors mediate signalling through the cell membrane in both directions. Binding of ligands to the extracellular domain transmits signals into the intracellular environment that influences processes such as gene expression, cellular differentiation and cytoskeletal organisation (outside-in signalling). Intracellular signals can also be transmitted to the extracellular domain that results in alterations in the ligand binding affinity of the integrin and cell adhesion (inside-out signalling). There are many different proteins that bind to the cytoplasmic tail of the integrin subunits to mediate such changes and are beyond the scope of this discussion (Liu et al., 2000a). However, some examples include talin and paxillin that bind some of the β subunits (Horwitz et al., 1986; Schaller et al., 1995), and calreticulin and calveolin-1 that bind α subunits (Rojiani et al., 1991; Wary et al., 1998). Interestingly, it is not only different proteins that can affect signalling through the integrin cytoplasmic domains but also the ligation of other integrins, a phenomenon that has been called integrin crosstalk. In particular, integrin crosstalk has been implicated in phagocytosis where ligation of $\alpha_v\beta_3$ has been shown to inhibit the phagocytic and migratory functions of $\alpha_5\beta_1$ through suppression of calcium/calmodulin-dependent kinase II (Blystone et al., 1994; Blystone et al., 1995; Blystone et al., 1999). Changes in the intracellular

environment such as increases in calcium can affect integrin avidity, leading to clustering of the integrin molecules on the cell surface. The multimerisation of integrin molecules is important in strengthening the adhesive interaction between integrins and cellular matrix or other cells. Recently it has been shown that engagement of FcγR by IgG opsonised beads leads to an accumulation of $\alpha_M\beta_2$ in the phagocytic cup suggesting that integrin avidity is likely to be important in mediating engulfment of particles and, potentially, apoptotic cells (Jongstra-Bilen et al., 2003).

At present, there are 24 distinct integrins that are composed of 8 α subunits and 18 β subunits that can be divided into sub-families according to ligand specificity (Fig 1.2)(Hynes, 2002). The β_2 integrins are specifically expressed on leukocytes and mediate binding to endothelial adhesion molecules such as intracellular adhesion molecules (ICAMs) and vascular adhesion molecules (VCAMs). The β2 integrin associates with four different α subunits, α_L (CD11a, lymphocyte function-associated antigen-1), α_M (CD11b, Mac-1), α_X (CD11c) and α_D. In addition to binding ICAMs and VCAMs, $\alpha_M\beta_2$ also binds fibrinogen and inactivated complement (iC3b) and $\alpha_X\beta_2$ to type 1 collagen and iC3b (Bouvard et al., 2001).

The β_1 family of integrins is expressed on a wide variety of tissues and involved in many biological processes that range from hematopoiesis, differentiation and migration to tumorigenesis. The β_1 subunit associates with the greatest number of α subunits, binding $\alpha_{1\text{-}10}$ and α_v but this is further complicated by the existence of alternatively spliced β1 subunits (A-D). Various extracellular matrix components are ligands for the β1 family of integrins including collagen (α_1,α_2), laminins (α_1, α_2, α_3, α_6, α_7, α_9), fibronectin (α_3, α_4, α_8, α_v) and vitronectin (α_v) (Brakebusch et al., 1997).

The most promiscuous α subunit is integrin α_v as it binds five different β subunits: β_1, β_3, β_5, β_6, and β_8. Each of these integrins binds the RGD containing ligands such

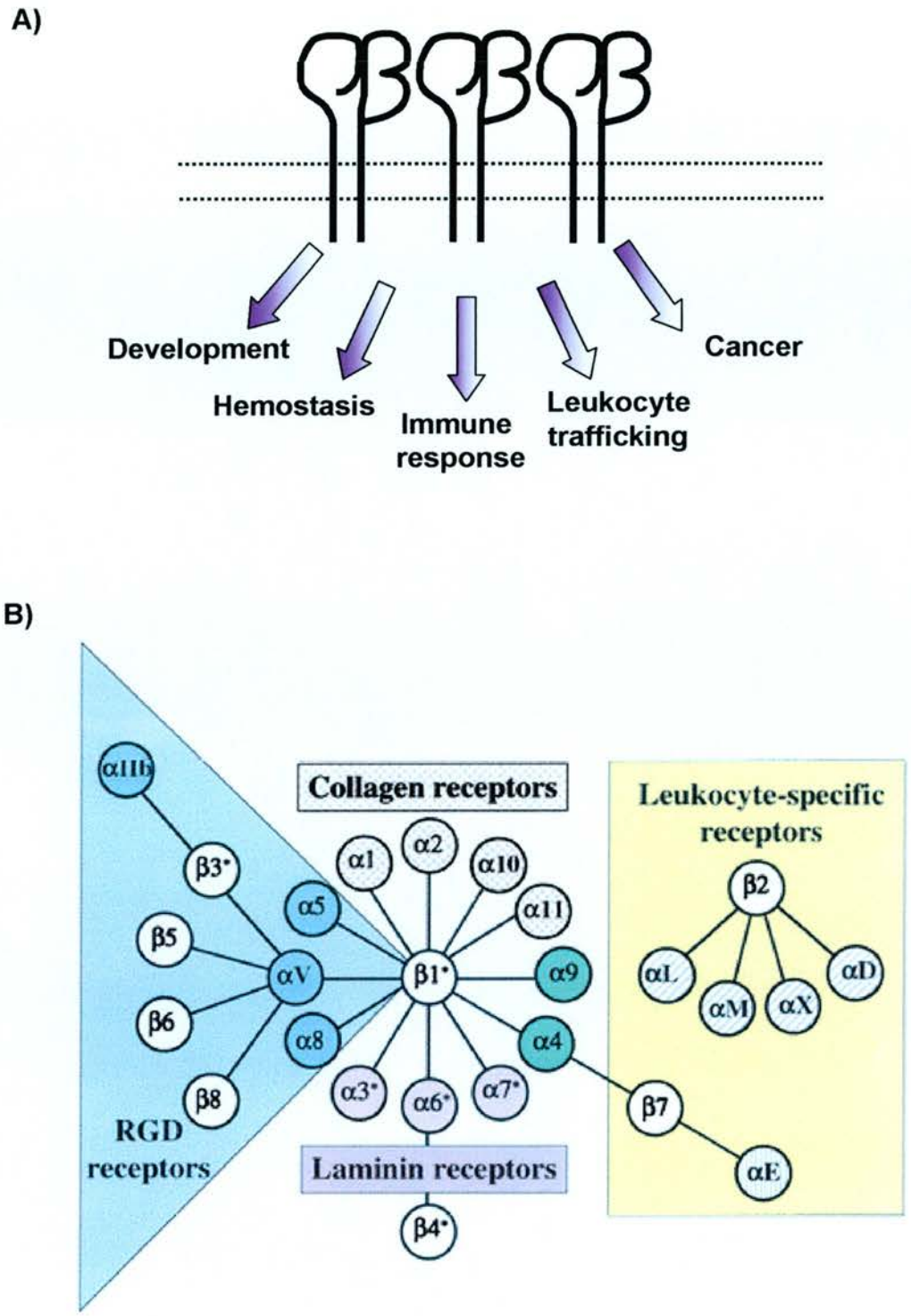


Figure 1.2: Integrin receptors. A) Integrins and their ligands are involved in a wide variety of cellular processes. B) Integrins are composed of α and β subunits that from 24 distinct integrins that can be sub-divided according to their ligand specificity. Taken from Hynes 2002.

as fibronectin, vitronectin and thrombospondin except for $\alpha_v\beta_8$ that doesn't bind vitronectin and $\alpha_v\beta_6$ that also binds tenascin-C. The α_v integrins are expressed on many different cell types including platelets that also specifically express $\alpha IIb\beta_3$ (Ginsberg et al., 1987), macrophages (Savill et al., 1990), neural cells (Delannet et al., 1994), osteoclasts (Vaananen and Horton, 1995) and epithelial cells (Huang et al., 1996) and as a result have been implicated in processes such as phagocytosis (Savill et al., 1990), angiogenesis (Brooks et al., 1994), survival (Montgomery et al., 1994), metastasis (Yun et al., 1996), wound healing (Clark et al., 1996) and migration (Klemke et al., 1994). As would be expected, due to the diversity of tissue expression and functions of α_v integrins the knockout mouse is embryonic lethal (Bader et al., 1998). Mice lacking α_v integrins die, either as a result of pericardial edema between E10-12 or perinatally from intestinal and intracranial haemorrhaging. Although the mutation was lethal development was shown to proceed almost normally in particular vasculogenesis and angiogeneisis continued (Bader et al., 1998). The phenotype of mice lacking integrin α_v has hindered further investigation of the role that these integrins may play in phagocytosis. In the absence of adult mice lacking integrin α_v, work undertaken in collaboration with Dr Adam Lacy-Hulbert has examined the role of β_3 and β_5 in apoptotic cell phagocytosis by bone marrow derived macrophages (BMDM). Interestingly, in mice lacking β_3, the levels of phagocytosis were similar to control but no longer inhibitable by the antagonistic peptide RGDS. In contrast, phagocytosis was inhibited by RGDS in mice lacking β_5 (Fig 1.3). Much of the work to date has relied upon the use of blocking antibodies, peptides, ligand binding and gain of function experiments to determine integrin involvement in phagocytosis. These experiments highlight the requirement for macrophages deficient in integrin α_v in order to gain further insight into the role of this integrin in phagocytosis.

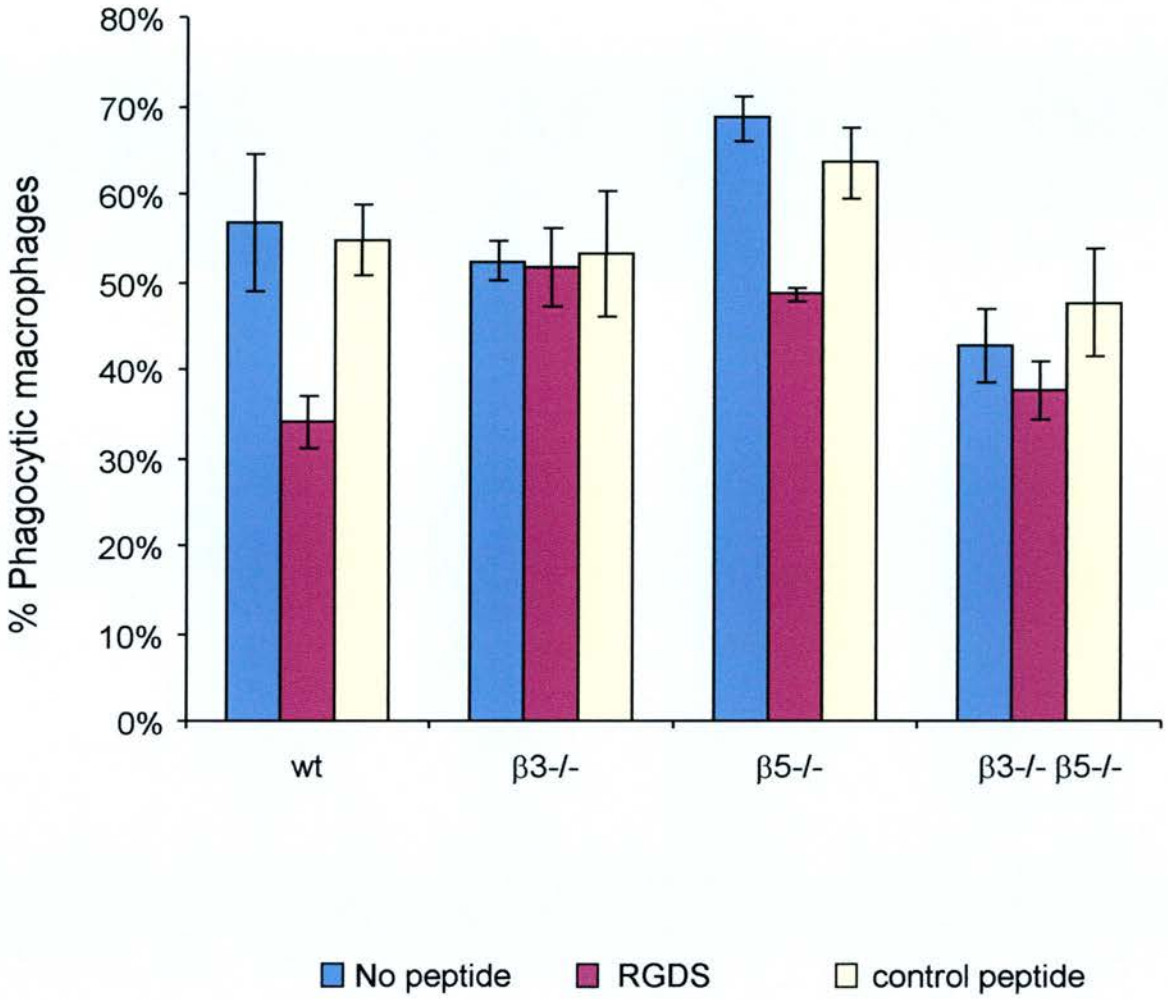
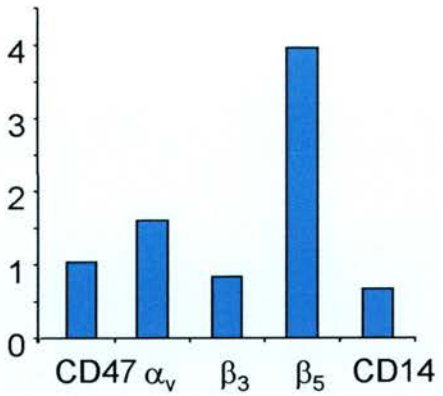


Figure 1.3: Phagocytosis of apoptotic cells by $\beta_3^{-/-}$ and $\beta_5^{-/-}$ deficient macrophages. RGD inhibits phagocytosis of apoptotic cells in $\beta_5^{-/-}$ deficient macrophages but has no effect in macrophages lacking integrin $\beta_3^{-/-}$. Data provided by Dr Adam Lacy-Hulbert.

1.5 Knockout studies for phagocytic receptors

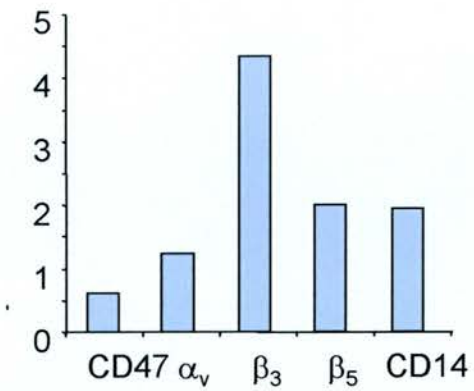
The majority of genes involved in apoptotic cell clearance in vertebrates have been defined using specific inhibitors (sugars, RGDS, PS) or antibodies (such as the blocking antibody 61D3 (Flora and Gregory, 1994) later confirmed to bind CD14 (Devitt et al., 1998). However, the role of these receptors has not necessarily been verified by other methods. Gain-of-function experiments show that expression of CD36, PSR or $\alpha_v\beta_5$ can confer increased apoptotic cell phagocytosis ability in 'non-professional' phagocytes (Albert et al., 2000; Fadok et al., 2000; Ren et al., 1995), strongly suggesting that these genes have a direct role in phagocytosis. Knockout, or 'loss-of-function' experiments have also been fruitful. Many knockout mice strains lacking apoptotic cell receptors have been generated, but clear defects in apoptotic cell clearance have been reported only in $Mer^{-/-}$ (Scott et al., 2001), $ABC1^{-/-}$ (Moynault et al., 1998), $C1q^{-/-}$ (Taylor et al., 2000) and $CD14^{-/-}$ (Chris Gregory, personal communication). Macrophages from mice deficient in SRA (Platt et al., 2000) and CD36 (A Lacy-Hulbert, personal communication) show no defect in apoptotic cell phagocytosis. Real-time PCR data from our laboratory (provided by Ailiang Zhang) has shown that in the absence of CD36, resident macrophages have increased expression of integrin β_5 messenger RNA (mRNA) and that inflammatory macrophages ($CD36^{-/-}$), similar to the $SRA^{-/-}$ macrophages have higher expression of integrin β_3 mRNA relative to BMDM (Fig 1.4). These observations that other compensatory phagocytic receptors are upregulated in the absence of CD36 and SRA and may explain why no apoptotic cell defect is observed in these mice. Furthermore in integrin $\beta_3^{-/-}$/$CD91^{-/-}$ deficient mice, CD36 appears to be upregulated on smooth muscle cells and is suppressed by retroviral reconstitution of β_3 (Weng et al., 2003) indicating that these receptors are compensating in the absence of one another.

More striking is the observation that many of these knockout mice show little or no ill effect from defective apoptotic cell clearance. Development is generally normal, and in most cases adult mice appear capable of normal resolution of inflammation.



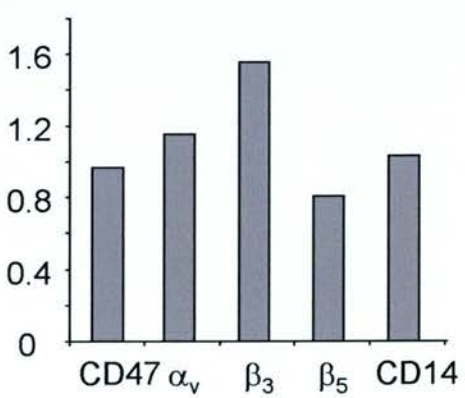
Resident peritoneal macrophages

CD36 $^{-/-}$



Inflammatory peritoneal macrophages

SRA $^{-/-}$



Resident peritoneal macrophages

Figure 1.4: Receptor expression in CD36$^{-/-}$ and SRA$^{-/-}$ macrophages. Phagocytic receptor expression profiles for macrophages from CD36 and SRA deficient mice. Data presented as relative to BMDM and provided by Ailiang Zhang.

$Mer^{-/-}$ mice show an over-responsive immune system, and develop autoimmunity, but it is unclear whether this is due to a defect in apoptotic cell clearance or a lack of the strong anti-inflammatory signalling through the Tyro-Axl-Mer kinase family. One of the better- characterised knockouts is the $C1q^{-/-}$ mouse. These mice show defective apoptotic cell clearance in inflamed sites, and develop autoimmunity with persistence of apoptotic cells in the kidney. However, autoimmunity only develops on susceptible backgrounds, suggesting that other factors play a role.

Normal apoptotic cell clearance in mice deficient in SRA and CD36, despite the evidence for the involvement of these molecules by antibodies, inhibitors or transfection experiments emphasises the need for knockout experiments to complement other studies. How can this be done for genes that have many roles in the organism, and result in lethal phenotypes when deleted? This thesis explores two strategies to study these genes in *in vitro* experiments:

1. To generate macrophages/DCs from genetically altered stem cells.
2. To conditionally delete genes in mature macrophages.

1.6 Embryonic Stem cells

The unique properties of Embryonic Stem (ES) cells have made them invaluable in understanding how genes function *in vivo*. They can be maintained as totipotent cells in culture, and genetic alterations readily carried out by homologous recombination. Modified cells can then be differentiated *in vitro*, or re-introduced into blastocysts contributing to all adult tissues. They therefore provide an excellent system for deleting genes and studying the consequences in isolated cell types or the whole animal.

ES cells were first isolated in 1981 (Evans and Kaufman, 1981; Martin, 1981) and the methods used to derive these pluripotent cells, to date, remain relatively unchanged (Robertson, 1987). Pre-implantation blastocysts are plated intact onto a feeder layer and several days later the inner cell mass (ICM) cells disaggregated and

plated onto fresh feeder layers. These cells then give rise to colonies of undifferentiated cells and various differentiated cell types. The undifferentiated colonies can be picked, dissociated and expanded in culture to generate continuous ES cell lines. The pluripotency of these cells was confirmed by their ability to differentiate into various cell types when cultured in the absence of feeder layers and to form teratomas when re-introduced into mice (Evans and Kaufman, 1981). More recently, it has been shown that ES cells originate from the epiblast or primitive ectoderm. Isolation of early epiblasts yielded ES cells at higher frequency than culture of intact blastocysts and allowed the derivation of ES cell lines not only from the 129 strain of mice but also from non-permissive strains such as CBA (Brook and Gardner, 1997). Perhaps one of the most remarkable features of ES cells is that after long-term culture and manipulation *in vitro*, they remain capable of contributing to the germ line following their incorporation into chimeric mice (Bradley et al., 1984).

1.7 Self-renewal of ES cells

ES cells can be routinely expanded in culture to generate relatively homogeneous populations of undifferentiated cells and unlike other primary cells they appear to be immortal showing no signs of crisis or senescence (Smith, 2001). Co-culture of ES cells with a feeder layer was initially thought to be essential for the maintenance of undifferentiated ES cells. Subsequently, it was discovered that conditioned media from buffalo rat liver cells containing a factor with differentiation-inhibiting activity (DIA) could be substituted for feeder cells (Smith and Hooper, 1987). Fractionation of this conditioned media identified the cytokine Leukaemia Inhibitory Factor (LIF) to be sufficient for the inhibition of ES cell differentiation (Smith et al., 1988a; Williams et al., 1988). In addition, feeder layers also express LIF that is produced in response to paracrine signalling from co-cultured ES cells (Rathjen et al., 1990). LIF supports self-renewal of ES cells by engaging the heterodimeric receptor complex that consists of the LIF receptor and the ubiquitous receptor gp130 (Davis et al., 1993; Yoshida et al., 1994). Signalling through this complex activates Janus associated tyrosine kinases (JAK) that phosphorylate and bind proteins containing

Src homology 2 (SH2) domains (Burdon et al., 2002). The signal transducer and activator of transcription (STAT) factors bind the phosphorylated JAK kinases and it has been shown that activation of STAT 3 in this way is essential for the self-renewal of ES cells (Niwa et al., 1998).

Oct-3/4 has also been shown to play an important role in self-renewal and in the differentiation and de-differentiation of ES cells (Niwa et al., 2000). Oct-3/4 is a member of the POU family of transcription factors and is expressed by pluripotent cells during embryogenesis and also by ES cells (Pesce et al., 1999). Manipulation of this transcription factor in ES cells showed that a reduction in Oct-3/4 expression caused ES cells to de-differentiate towards trophectoderm and overexpression towards differentiation to the endoderm and mesoderm lineages. It has been proposed that in part, Oct-3/4 may act as a 'gatekeeper' that prevents de-differentiation of ES cells to trophectoderm thereby maintaining the pluripotency of the ES cells (Niwa et al., 2000). In the same study it was also found that cells expressing the Oct-3/4 transgene were dependent on LIF and upon withdrawl of the cytokine differentiated in the normal manner. Therefore it has been suggested that both intrinsic expression of Oct-3/4 and the activation of STAT 3 by LIF are required to maintain the pluripotent ES phenotype (Smith, 2001).

Just recently, a third factor involved in the pluripotency of ES cells has been described (Chambers et al., 2003; Mitsui et al., 2003). The homeoprotein, Nanog, was identified through functional library screening and digital differential display comparing EST libraries from mouse ES cell and various somatic tissues. Deletion of Nanog resulted in the loss of pluripotency in ES cells and in the ICM (Mitsui et al., 2003) and seems to maintain pluripotency independently of LIF/gp130 (Chambers et al., 2003). Identification of the interactions of this homeoprotein with target genes will be fundamental in understanding how pluripotency is maintained in these cells.

1.8 ES cell differentiation

ES cells spontaneously differentiate in the absence of LIF and upon the removal of feeder support (Evans and Kaufman, 1981; Martin, 1981; Robertson, 1987). Cystic embryoid bodies (EBs) are formed and differentiation closely mimics that of the developing embryo where heart and blood cell-containing structures similar to the visceral yolk sac develop (Doetschman et al., 1985). When left in culture for longer (> 12 days) further differentiation occurs, leading to the formation of mesoderm derivatives (Robertson, 1987). In addition, ES cell differentiation can also be induced using retinoic acid and 3-methoxybenzamide (Smith, 1991) and it appears to possibly play a role in biasing differentiation towards cells of the neuronal lineage (Bain et al., 1995; Strubing et al., 1995).

Many different cell lineages have been produced using various methods of differentiation, some of which are summarised in table 1.1. At present there is no way to generate pure populations of a specific cell type through the differentiation of ES cells although several approaches have been used to enrich for the cell type of choice. Perhaps, one of the most widely used approaches is to culture the differentiated cells in cytokines appropriate for the cell type of interest. Wiles and Keller were the first to describe the defined culture conditions that allowed the differentiation of ES cells to hematopoietic cells (Wiles and Keller, 1991). Using the appropriate culture conditions it was demonstrated that hematopoietic cells could be enriched for and expanded in culture for a period of weeks. This method has been used extensively to generate cells of the hematopoietic system either by inducing EB formation in methylcellulose or suspension culture (Fairchild et al., 2000; Tsai et al., 2000; Wiles and Keller, 1991).

A second method that has been used successfully for the generation of myeloid cells, other than macrophages, is to differentiate ES cell upon a stromal cell layer either in the presence or absence of cytokines (Nakano et al., 1994; Potocnik et al., 1997). In particular, this approach has been used to generate lymphoid precursors that can give

rise to B cells and more recently has been used to generate dendritic cells (Senju et al., 2003).

Table 1: Summary of cell types differentiated from ES cells.

Adapted from Smith, 2001.

CELL TYPE	METHOD OF DIFFERENTIATION	REFERENCE
Yolk sac endoderm	Suspension	(Doetschman et al., 1985)
Yolk sac mesoderm	Suspension	(Doetschman et al., 1985)
Definitive hematopoietic	Methylcellulose + cytokines	(Wiles and Keller, 1991)
Lymphoid	Stromal layer	(Nakano et al., 1994)
Lymphoid precursor	Suspension	(Potocnik et al., 1997)
Dendritic cell	Suspension + cytokines	(Fairchild et al., 2000)
Dendritic cell	Stromal + cytokines	(Senju et al., 2003)
Endothelial cell	Plated on collagen	(Yamashita et al., 2000)
Cardiomyocyte	Hanging Drop	(Maltsev et al., 1993)
Adipocyte	Suspension + retinoic acid	(Dani et al., 1997)
Neuronal	Suspension + retinoic acid	(Bain et al., 1995; Strubing et al., 1995)
Oligondendrocyte	Suspension + cytokines	(Liu et al., 2000b)
Mast cells	Methylcellulose + cytokines	(Tsai et al., 2000)

A third approach of selecting a particular population of cells is to generate ES carrying a selection marker under the control of a promoter specific to the cell type of interest. The first report using this method came from Klug et al who differentiated ES cells carrying the α-cardiac myosin heavy chain fused to the *neo*^{r} gene that allowed the selection of cardiomyocytes when the differentiated cells were cultured in geneticin (G418) (Klug et al., 1996). Subsequently, this method was used to generate purified neural precursors using the lineage-restricted marker *Sox-2* fused the *β-geo* gene which not only served as a selection marker but also as a reporter (Li et al., 1998). Billon et al have further refined this approach by using ES cells expressing *Sox-2* fused to the *β-geo* gene to positively select for the neural precursor cells and *Oct-4* fused to the hygromycin-thymidine kinase gene to negatively select for undifferentiated cells. This resulted in an increase in the purity of the neural

precursor cells that were then further differentiated into oligodendrocytes (Billon et al., 2002). However, this approach is limiting in cell types where several subsets of cells arise from the lineage-restricted markers such as in the hematopoietic system, although a recent report has described enhanced hematopoietic differentiation of ES cells conditionally expressing STAT 5 (Kyba et al., 2003). Moreover it appears that generating pure populations of specific cell types may rely on the genetic manipulation of lineage-restricted markers and transcription factors and with the further delineation of specific pathways that govern the development of different cell types this approach will most certainly be exploited in the future.

Therefore, differentiation of ES cells in the presence of conditioning cytokines such as M-CSF or GM-CSF can be used to generate populations of myeloid cells. Coupling this system to genetic manipulation of ES cells would provide an ideal system for the production of genetically modified phagocytes, avoiding the need to generate 'knockout' mice.

1.9 Gene targeting in ES cells

However, the most powerful use of ES cells is the generation of genetically modified or 'knockout' mice. Homologous recombination allows the insertion of foreign DNA into the genome between two arms of homologous DNA. Using a replacement vector, chromosomal sequences can be replaced by the vector sequences by two reciprocal recombinations involving the flanking regions of homologous DNA. The first successful example of this was achieved using the selectable hypoxanthine phosphoribosyl transferase (HPRT) gene locus (Doetschman et al., 1987; Thomas and Capecchi, 1987). Gene targeting is this way causes complete deletion of the gene. However, as discussed above, this may result in a severe phenotype and embryonic lethality making it impossible to assess the gene function *in vivo*. These obstacles have been overcome by conditional gene targeting, using the bacteriophage-derived Cre/*LoxP* recombination system that has been shown to be applicable both *in vitro* (Sauer and Henderson, 1988) and *in vivo* (Lakso et al., 1992;

Orban et al., 1992). The Cre recombinase of the bacteriophage P1 recognises a 34bp consensus sequence called *LoxP* (Hoess et al., 1982; Sternberg et al., 1981). The *LoxP* motif consists of a core spacer sequence of 8bp and two 13bp palindromic flanking sequences. A single recombinase molecule binds each palindromic sequence of the *LoxP* site and mediates recombination within the spacer region to bring the *LoxP* sites together. The core spacer sequence defines the orientation of the *LoxP* site: if a segment of DNA is flanked by two *LoxP* sites in the same orientation, Cre excises the intervening segment of DNA leaving a single *LoxP* site behind (Fig 1.5a). Two *LoxP* sites in opposite directions allow the intervening DNA to be inverted (Fig 1.5b).

Target genes can be modified by homologous recombination in ES cells so that *LoxP* sites flank the gene or gene segment of interest. Importantly, when these ES cells are used to generate targeted mice the gene of interest is 'silently targeted' and functions normally. Targeted mice can then be crossed with mice expressing Cre in the tissue of interest allowing manipulation of the gene in a cell type specific manner that is known as conditional gene targeting. Conditional gene targeting, first demonstrated by Gu and co-workers, used mice that contained the promoter and the first exon of the DNA polymerase β gene (*polβ)* flanked with *loxP* sites. When crossed with mice that specifically expressed Cre in T cells the *polβ* gene was inactivated in the T cell population only (Gu et al., 1994). This powerful strategy has revolutionised gene-targeting approaches. There are many Cre expressing mice available such as the CD19-Cre mouse that allows tissue specific deletion in B cells (Rickert et al., 1997). Partial gene inactivation can be obtained using the 'balancer' strain that expresses Cre under the control of the nestin gene (Betz et al., 1996) and a Cre transgenic strain, called 'deleter' mediates deletion in all cells including the germ cells (Lakso et al., 1992; Schwenk et al., 1995). However, this system relies upon the identification of tissue specific promoters that can limit its potential. Temporal control over Cre can also be achieved by induction either at the transcriptional level

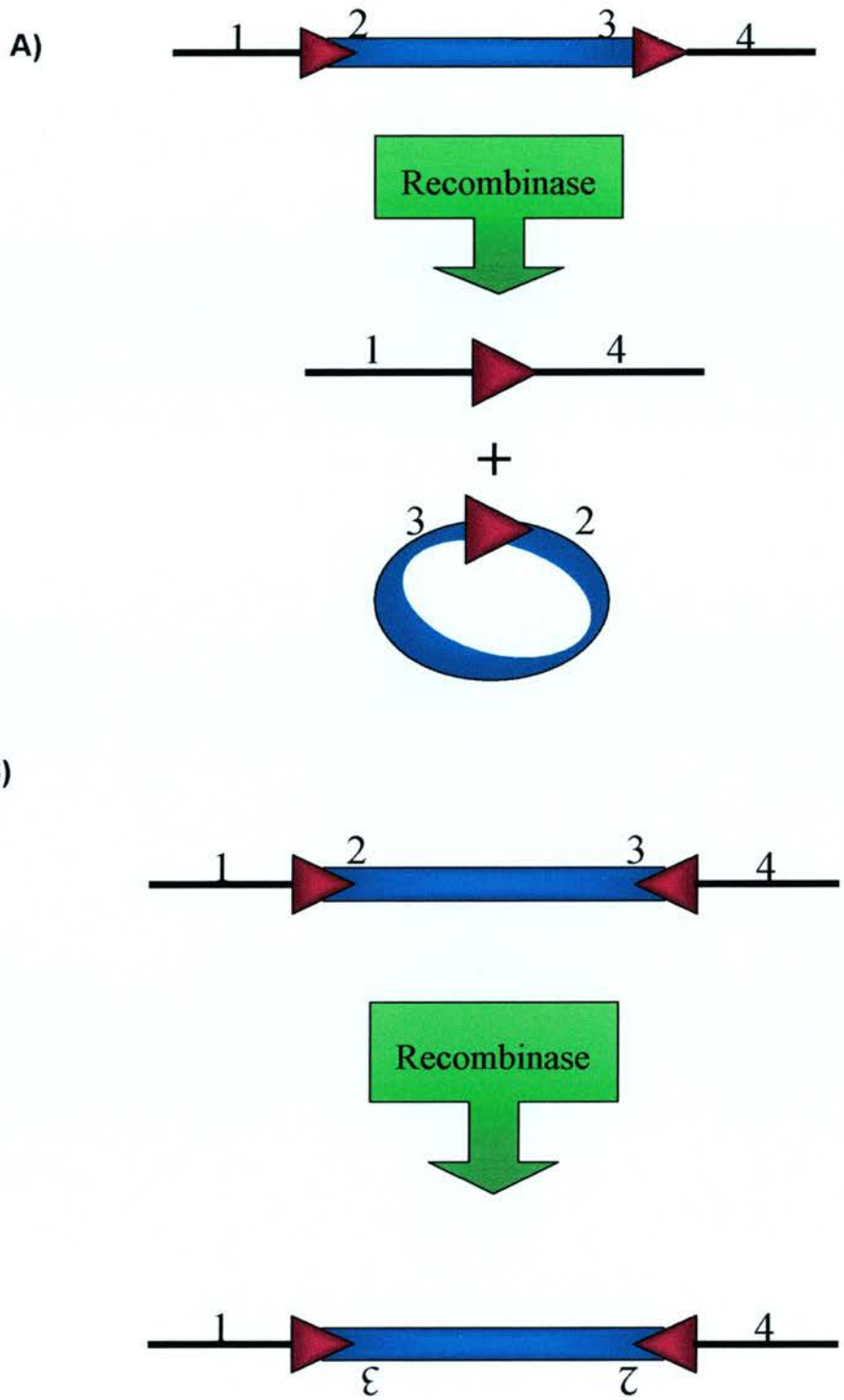


Figure 1.5: Cre/LoxP recombination. Cre recombinase mediates the deletion of intervenening segments of DNA flanked by two LoxP sites that are in the same orientation (A) and inverts the DNA if they are in opposite directions (B).

or at the post-translation level using inducers such as tetracycline (Gossen and Bujard, 1992) or tamoxifen (Metzger et al., 1995a) whereby Cre expression can be activated by administration or withdrawl of the inducing agent.

Cre can also be delivered to cells by exogenous application, such as a virus or purified protein. Adenovirus expressing Cre recombinase (Anton and Graham, 1995a) has been used for the efficient transfection of a variety of cell types *in vitro* (Hoves et al., 2003; Prost et al., 2001a). Parts of the viral genome are replaced with the Cre recombinase gene generating a replication deficient virus that is still capable of host infection. This is a highly efficient method of transfection and is particularly attractive for hematopoietic cells such as macrophages as they are heterogenous, making the identification of good unique, active, tissue-specific promoters difficult. More recently however, the properties of a number of viral derived peptides have exploited for the cellular delivery of proteins and macromolecules thus providing an alternative delivery method to adenoviral mediated gene transfer.

1.10 Cell-penetrating peptides

The cell membrane is inherently impermeable to macromolecules that lack specialised membrane receptors or transport proteins. Generally most proteins or peptides are internalised through receptor mediated endocytosis or uptake by transporters. In the past few years there has been an increasing number of peptide sequences reported to cross the cell membrane and deliver 'cargo' proteins to the intracellular environment. These sequences are usually termed "cell-penetrating peptides" or "protein transduction domains". Three peptide sequences in particular HIV-TAT, the Antennapedia homeodomain and the Herpes Simplex virus protein VP22 have been well characterised for the delivery of a variety of different proteins although several other peptides with similar capabilities exist eg, SN50 (Lin et al., 1995), pep-1 (Morris et al., 2001). The ability of the HIV-TAT protein to cross the cell membrane was first reported in 1988 by Frankel & Pabo (Frankel and Pabo, 1988). TAT is a transcription-activating factor that is 86 amino acids in length and is

involved in the replication of HIV. Frankel & Pabo demonstrated the cellular permeability of TAT by delivering the purified peptide to cells containing the reporter gene chloramphenicol acetyltransferase (CAT) that was under the control of the HIV-1 long terminal repeat. When the TAT peptide entered the cells it resulted in an increase in the expression of CAT from the HIV-1 promoter. Subsequent studies using truncated versions of the TAT peptide have demonstrated that residues 37-72 and 48-60 are involved in the translocation of the peptide although the truncated version containing residues 48-60 appears to be more efficient (Fawell et al., 1994; Vives et al., 1997).

The Antennapedia homeodomain (ANTp) has also been shown translocate across the cell membrane (Derossi et al., 1994). This specific sequence of 60 amino acids in length is composed of three α helices with one β turn between helices 2 and 3. Derossi *et al.* have produced several mutant versions of this homeodomain and classified their ability to enter cells in culture. Deletion of a tryptophan and a phenylalanine residue within the third α helix resulted in the loss of translocation indicating that the third helix was responsible for cell membrane permeability (Derossi et al., 1998). A 16 amino acid peptide between residues 43-58 has been shown to be the minimal unit required to cross the cell membrane and is also known as pentratin-1.

The most recently described cell-penetrating peptide is VP22, a DNA binding protein from the Herpes Simplex virus type 1. While investigating the properties of the structural proteins that make up the tegument region of the virus Elliot and co-workers discovered the intercellular trafficking properties of VP22 (Elliott and O'Hare, 1997). The last 34 amino acids of this protein have been shown to be critical for the cell permeability of this protein, the properties of which are discussed in greater detail in chapter 5.

It is currently unknown how each of these peptides cross the cell membrane. Translocation of each of these peptides has been shown to be energy independent and

therefore does not involve classical receptor mediated endocytosis (Derossi et al., 1994; Elliott and O'Hare, 1997; Vives et al., 1997). However, it is not clear if each of these peptides use the same mechanism of cellular entry or not. Comparison of the sequences (Table 1.2) has shown that each peptide is rich is arginine and lysine amino acids rendering them highly basic and it is thought that this may be important for contact with the negatively charged lipids of the cell membrane, mediating cellular entry (Schwarze et al., 2000).

Table 1.2: Comparison of amino acid sequences of characterised cell-permeable peptides PTD, protein transduction domain. Taken from Schwarze, 2000.

PTD	Amino acid sequence
HIV-TAT	Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg
VP22	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Glu
ANTp	Arg-Gln-Iso-Lys-Iso-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys Trp-Lys-Lys

The one major difference between each of the peptides described is the size of the 'cargo' that can be delivered to cultured cells. The ANTp homeodomain in particular, appears to be capable of only transducing proteins of less than 100 amino acids in length (Derossi et al., 1998). However, both the TAT and the VP22 peptides have been implicated in the delivery of much larger proteins such as β-galactosidase, green fluorescent protein (GFP), p53, and the inhibitor protein $p27^{kip1}$ (Brewis et al., 2000; Fawell et al., 1994; Nagahara et al., 1998; Phelan et al., 1998). Moreover the TAT and VP22 peptides retain the ability to be exported from one cell and taken up by a neighbouring cell although spread of the VP22 fusion proteins appears to be greater than for TAT fusions (Elliott and O'Hare, 1997; Ensoli et al., 1993). Another property that is shared between these peptides is that they are all delivered to the cytoplasm and the nucleus of the transduced cell (Derossi et al., 1998; Elliott and O'Hare, 1997; Vives et al., 1997) in the absence of any other additional transfection reagents, making them ideal for investigating cellular responses where traditional transfection methods are insufficient or undesirable. Primarily these peptides have

been used to deliver functional proteins to the intracellular environment but are being increasing used in conjunction with viral delivery vectors to evaluate their potential for therapeutic gene delivery. At present, VP22 and ANTp have been fused to replication deficient adenoviral and lentiviral vectors and their ability to deliver proteins both *in vitro* and *in vivo* examined (Gratton et al., 2003; Lai et al., 2000; Wills et al., 2001). These viral/peptide fusions appear to enhance viral cell entry and improve gene expression at lower titres of virus and increase the distribution of the delivered proteins. Therefore indicating that cell-permeable peptides may, in the future play a role in the efficient delivery of gene therapy vectors.

1.11 Aims

The aim of this project was to generate genetically altered myeloid cells to determine the function of key genes in apoptotic cell phagocytosis. Integrin α_v was chosen, principally because of the difficulty in obtaining an integrin α_v knockout by conventional methods. Two approaches were used:

1. Genetically modified ES cells were differentiated to macrophages and dendritic cells.
2. Cre was delivered to macrophages cultured from mice carrying a "floxed" (flanked by two *LoxP* sites) integrin α_v gene.

Chapter 2

Materials and methods

2.1 Embryonic Stem cell culture

Embryonic stem cell culture reagents that were routinely used are listed in appendix I.

Three ES cell lines were used during the course of this work. The EFC-1 ES cells used for the initial differentiation cultures and the CP1 ES cells used for titrating the LIF activity were kind gifts from Professor Austin Smith, University of Edinburgh. The E14 ES cell line (used previously by Dr Adam Lacy-Hulbert for the targeting of the integrin α_v gene) and E14 ES cells carrying a disrupted integrin α_v gene were used in the differentiation studies.

2.1.1 Preparation of mitotically inactivated feeder layers

STO fibroblasts were cultured in ES cell medium lacking LIF. The cells were grown in 162cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified incubator. Once confluent, one flask was trypsinised and sub-cultured to maintain growing stocks and a second flask was used to prepare STO feeder plates for ES cell culture. Mitomycin C was made as a stock solution of 1mg/ml with sterile PBS. Confluent monolayers were treated with Mitomycin C at a final concentration of 10μg/ml in culture medium for 2-3 hours at 37°C. During this time 6cm tissue culture dishes were treated with 0.1% gelatin for at least 30 minutes. The cells were then washed three times with phosphate buffered saline (PBS), trypsinised and plated into 6cm culture dishes at a density of $1.2x10^6$ cells per dish. The fibroblasts were allowed to adhere to the surface of the dishes for at least 2 hours before ES cells were added

2.1.2 Leukaemia inhibitory factor conditioned media

LIF conditioned media was produced by transfecting cells with the pCAGGSLIF plasmid (a kind gift of Prof Austin Smith, University of Edinburgh). $5x10^6$ COS-7 cells were electroporated (EQUIBIO electroporator, Flowgen) with a double pulse of 750V/25μF followed by 100V/1500μF and 20μg of pCAGGSLIF plasmid

(Appendix II). The cells were then cultured for four days and the supernatants harvested, filter sterilised and stored at –20°C in 0.5ml aliquots.

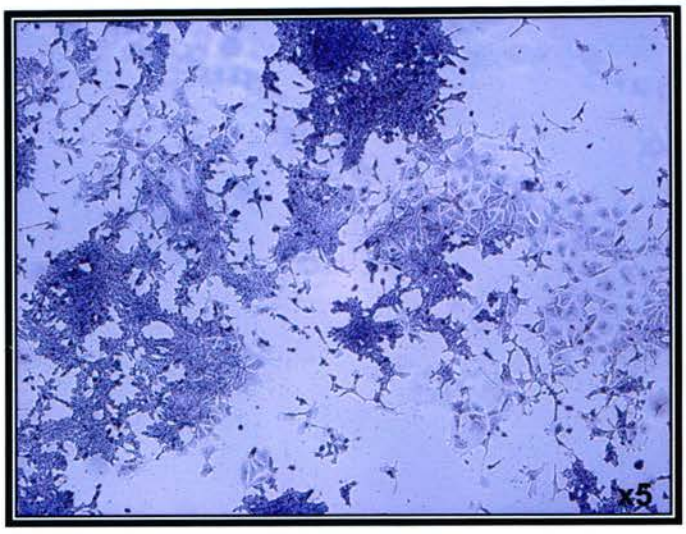
The CP1 ES cell line was used to titrate the LIF activity. The ES cells were plated onto gelatinised 24 well plates at density of 10^4 cells per well. The LIF conditioned media was then added at various dilutions ranging from 1:100 to 1:500,000 with one well containing no conditioned media and one containing LIF of a known activity. The plates were incubated for 4 days and the cells fixed and stained with Leishman's stain for 5 minutes. Prior to examination under a light microscope the cells were washed with H_2O and left to air dry. The stem cells appeared as small round colonies of tightly packed cells that were intensely stained whereas the differentiated cells formed larger spread out colonies with diffuse staining (Fig 2.1). Typically, stock conditioned media diluted 1:1000 was sufficient for maintaining ES cells in an undifferentiated state.

2.1.3 Routine culture of Embryonic Stem Cells

ES cells were routinely cultured on gelatin-coated plates or on mitotically inactivated STO fibroblasts in a humidified 37°C incubator with 5% CO_2. Tissue culture dishes (6cm) were treated with 0.1% gelatin for at least 30 minutes prior to use. ES cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% foetal bovine serum (FBS), 4500mg glucose, 10mM sodium pyruvate, 1% non-essential amino acids, 2mM L-glutamine and 10^{-4}M β-mercaptoethanol. Leukaemia inhibiting factor (LIF) was added at a 1:1000 dilution of stock conditioned media.

ES cells were grown until approximately 80% confluent (48-72 hours after plating). The medium was removed and the monolayer washed twice with 1X mouse tonicity phosphate buffered saline (mtPBS). 1ml of 0.05% trypsin-diaminoethanetetra-acetic acid (EDTA) solution was added to the monolayer and incubated at 37°C, once the cells had detached culture medium was added to quench the trypsin. The cell suspension was then counted and centrifuged at 1,000rpm for 5 minutes and the cells resuspended and plated at a density of $1x10^6$ cells per dish.

CP1 ES cells cultured in the absence of LIF



CP1 ES cells cultured in the presence of LIF

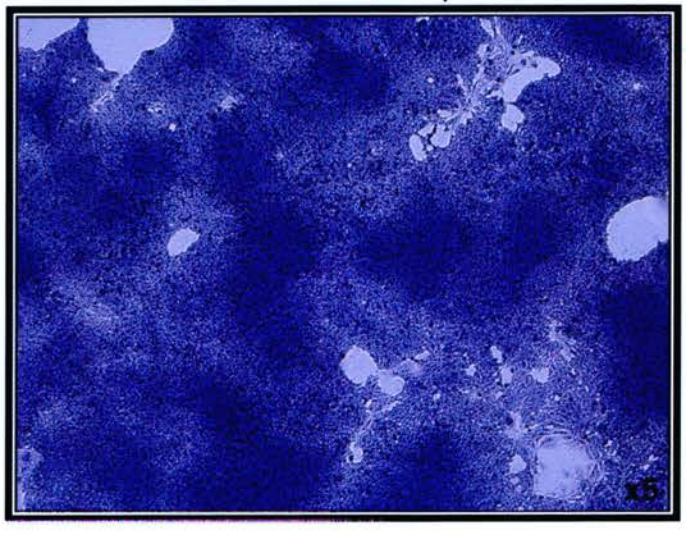


Figure 2.1:LIF titration. LIF conditioned media was titrated using the CP1 ES cell line. Cells cultured in the absence of LIF differentiated and showed diffuse staining with Leishman's stain whereas in the presence of LIF the ES cells grew in tightly packed colonies and showed intense staining.

2.1.4 Differentiation of Embryonic Stem cells

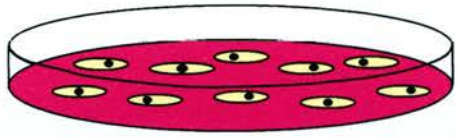
Prior to differentiation ES cells that had been grown on feeder layers were plated onto gelatin coated plates and passaged twice before use. The basic differentiation protocol is as outlined in figure 2.2 however, several conditions were optimised as indicated in chapter 4. EBs were formed by plating ES cells into bacterial grade Petri dishes in differentiation media composed of Iscoves, 0.9% MeC unless otherwise stated, 10% FBS (appendix I), 340μM monothioglycerol, 10μg/ml insulin, 10% L929 or GM-CSF conditioned media (Section 2.1.5 &6), recombinant murine IL-3 at concentrations stated in text, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. 300-1000 ES cells were plated per dish depending on the cell line used and the cultures placed at 37°C in a 5% CO_2 incubator for 14 days. After 14 days EBs were disrupted by incubation in a solution of 0.25% collagenase, 20% FCS and PBS at 37°C for 1 hour with gentle agitation. The EBs were then passed through a 21 gauge needle three times to allow dissociation to a single cell suspension. The cells were then plated onto tissue culture dishes in differentiation medium lacking MeC. Differentiated progeny appeared 2-3 weeks later and the cultures were fed as required.

2.1.5 L929 conditioned media

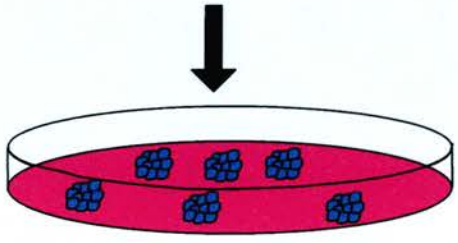
L929 mouse fibroblast cell line was cultured in DMEM/F12 supplemented with 10% FBS, 100U/ml penicillin and 100μg/ml streptomycin. Cells were routinely sub-cultured at 1/30 twice a week. To harvest M-CSF containing supernatant cells were grown until confluency and left for a further 4 days. Media was then harvested, sterile filtered and aliquots stored at –80°C.

2.1.6 GM-CSF conditioned media

The GM-CSF hybridoma cell line was a kind gift from Professor David Gray, University of Edinburgh. Hybridoma cells were cultured in RPMI supplemented with 5% heat-inactivated FBS, 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 10^{-4}M β-mercaptoethanol. Cells were selected in media containing G418 at a concentration of 1mg/ml and maintained at a concentration of 7.5-40x10^4

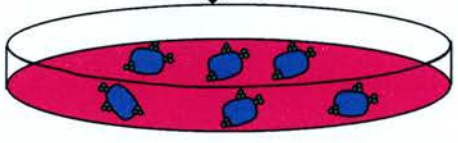


ES cells cultured on gelatin coated plates to eliminate contaminating feeder cells



ES cells cultured in bacterial grade petri dishes in Methyl Cellulose (MeC) supplemented with: 10%FCS, 10% L929 CM or 15% GM-CSF CM, IL-3, Insulin and monothioglycerol.

14 days



Differentiated hematopoietic cells emerge from embryoid bodies

Day 14 embryoid bodies are either plated or disrupted by incubation in 0.25%collagenase/10%FCS/PBS for 1 hour. EB are then passaged through a 21 gauge needle six times. Cells are counted and plated directly onto either tissue culture or bacterial grade Petri dishes.

Embryoid body-derived cells are then analysed for:

Morphology **Surface Phenotype** **Gene Expression**

Figure 2.2: Overview of the ES cell differentiation protocol. ES cells were differentiated for 14 days in methylcellulose supplemented with the appropriate cytokines to drive differentiation towards cells of the myeloid lineage. At day 14 the EBs were either plated or disrupted and the differentiated cells expanded in culture for ~21 days before analysis of morphology, surface phenotype and gene expresssion.

cells/ml by passaging at 1:3 three times per week. To harvest the supernatant, cells were washed three times and resuspended in media lacking geneticin (G418) at a density of 30-40x10^4 cells/ml in 20mls and placed in a 162cm^2 flask. Cultures were grown for a further 4-5 days until the cells were dense (0.75-1x10^6 cells/ml) but the media not exhausted. The supernatant was then collected by centrifugation, and aliquots stored at –80°C.

2.1.7 Flow cytometry

All antibodies were purchased from Pharmingen unless otherwise stated and the following primary conjugated antibodies used: F4/80-APC (Clone CI:A3-1 Caltag), F4/80-PE (Clone CI:A3-1 Caltag), F4/80-FITC (Clone CI:A3-1 Serotec) CD11a-PE (Clone 2D7), CD11b-PE (Clone M1/70), CD11c-APC (Clone ML3), CD11c-PE (Clone ML3), CD14-PE (Clone rmC5-3), CD40-PE (Clone 3/23), CD54-PE (Clone 3E2), CD80-PE (Clone 16-10A1), CD86-PE (Clone GL-1), I-A^b-FITC (Clone AF6-120.1) and GR-1-PE (RB6-8C5).

Cells were harvested, washed in PBS and resuspended in flow buffer (appendix I) before blocking in 10% mouse serum for 20 minutes. Cells were then labelled with antibodies at final dilution of 1:150, on ice and in the dark for 1 hour. Unbound antibody was washed off with flow buffer and the cells pelleted by centrifugation at 330*g* for 3 minutes. The cells were then resuspended in 200µl of flow buffer and analysed using FACSCalibur (Becton-Dickinson) and FlowJo® software. All samples were compared to the relevant isotype control.

2.1.8 Immunofluorescence (ES derived cells)

ES derived cells were fixed with 4% paraformaldehyde for 20 minutes and washed with PBS. Cells were blocked in 10% mouse serum before staining with 1:1000 dilution of phycoerythrin conjugated F4/80 and FITC conjugated CD11b (Pharmingen).

2.1.9 ES DC maturation

ES DCs were harvested by gentle washing and re-plated into fresh tissue culture plates containing differentiation medium for 2-3 hours. Cultures were then stimulated with 1μg/ml LPS for 24 hours. The following day the non-adherent cells (mature DCs) were collected by centrifugation (1000rpm, 5 minutes), plated into fresh differentiation media (no LPS) and cultured for a further 48 hours. Fresh media was also added to the culture dishes that had been stimulated allowing more mature DCs to be harvested.

2.1.10 Phagocytosis assays

2.1.10.1 Isolation of human neutrophils

Human neutrophils were isolated on my behalf by various members of the laboratory. Human neutrophils were extracted from peripheral blood of healthy volunteers as described previously (Haslett et al., 1985). Blood was drawn into citrated 50ml falcon tubes and spun at 350*g* for 20minutes. Plasma was removed and 6mls of 6% dextran added to the remaining fraction. Tubes were inverted and allowed to sediment for 20minutes. During this time a Percoll (Amersham, Buckinghamshire, UK) gradient was prepared. 100% Percoll was made to 90% by the addition of 4ml of 10XPBS to 36mls 100% Percoll. Using this 90% Percoll gradients were made from fractions containing 79%, 68% and 55% Percoll by the addition of 1XPBS without Ca^{2+}/Mg^{2+}. 2.5mls of the 68% fraction was overlayed onto 2.5mls of the 79% fraction in a 15 ml falcon tube. The leukocyte-enriched fraction was harvested from the top layer after the dextran sedimentation of the blood and pelleted at 350g for 6 minutes. The pellet was resuspended in 2.5mls of the 55% Percoll, overlayed onto the pre-made gradient and centrifuged at 720*g* for 20 minutes. Peripheral blood mononuclear cells (lymphocytes and monocytes) were harvested from the top interface and neutrophils from the bottom interface. Cells were washed twice in PBS without Ca^{2+}/Mg^{2+} before use. This yielded highly pure human neutrophils (>90%). Autologous serum was made by clotting 10ml of plasma in glass tubes by the addition of 200μl of 1M $CaCl_2$ at 37°C.

2.1.10.2 Generation of apoptotic neutrophils (PMNs)

Human neutrophils were allowed to undergo constitutive apoptosis by aging overnight in Iscoves medium supplemented with 100U/ml penicillin, 100μg/ml streptomycin and 10% autologous serum. Prior to overnight aging, cells were stained using a green cell tracker dye (5-chloromethylfluoreescein diacetate, CMFDA, Molecular Probes, Leiden Holland) at a density of $20x10^6$ cells/ml in serum free Iscoves plus 10μl of 2mg/ml CMFDA for 15 minutes at 37°C. Cells were then diluted to $4x10^6$ cells/ml in Iscoves with autologous serum. After aging cells were 40-80% apoptotic as assessed by cytospin morphology.

2.1.10.3 Flow based phagocytosis assay

Flow cytometry based phagocytosis assays: ES derived myeloid cells were incubated with fluorescently labelled apoptotic cells at a ratio of ~4:1 apoptotic cells: ES derived cells for 30 minutes or 2 hours. Assays were carried out either by adding apoptotic cells to the culture plates or performed in U-bottomed 96 well plates. Myeloid cells were counterstained with F4/80-PE or APC before flow cytometry analysis. Phagocytosis gates were set by comparing labelled cells in the absence of apoptotic cells with cells that had interacted with apoptotic cells at 37°C (gating described in grater detail in chapter 3). Some phagocytosis studies were carried out in the presence of the inhibitory peptide GRGDSP (Gly-Arg-Ala-Asp-Ser-Pro) or the control peptide GRADSP (Gly-Arg-Gly-Asp-Ser-Pro) at 0.5mg/ml. Cells were incubated with inhibitory peptide during the interaction with apoptotic cells. Cells were then analysed as described above.

2.1.11 Magnetic cell sorting of differentiated cells

Differentiated cells were removed from the culture plates by gentle washing with culture media. Cells were then harvested by centrifugation, re-suspended in MACS buffer (appendix I) and incubated with CD11b PE antibody at 1:50 dilution for 15 minutes at 4°C. Cells were washed twice with MACS buffer and 10^7 cells resuspended in 80μl of MACS buffer plus 20μl of MACS anti-PE Microbeads (Miltenyi Biotec) and incubated at 4°C for 15 minutes. Following labelling with

magnetic beads the cells were washed in MACS buffer and applied to a positive selection column (Miltenyi Biotec). Both the retained fraction (macrophages) and the flow through were collected and plated in culture. Before plating, the cells in each fraction were analysed by flow cytometry for the surface marker F4/80 as described previously.

2.1.12 Genomic DNA isolation

Genomic DNA (gDNA) was extracted from ES cells by resuspending approximately 10^7 cells in 3mls of lysis buffer (appendix I) before incubation at 55°C overnight. The DNA was precipitated with 6mls of cold ethanol containing 900µl of 5M NaCl and incubated at room temperature for 15 minutes. The DNA was then centrifuged at 2000rpm for 10 minutes and the DNA transferred to a clean tube containing 1.5ml of 70% ethanol using a yellow tip (DNA strands were rolled around tip). The DNA was then transferred to a clean tube containing 500µl of TE buffer and incubated at 37°C overnight.

PCR amplification was performed on genomic DNA using integrin α_v primers 4f6 (ggtgactcaatgtgtgaccttcagc) and 5b3 (cacaaatcaaggatgaccaaactgag). The cycle parameters were: 1 cycle of 96°C for 5 minutes followed by 40 cycles of 94°C for 30 seconds, 64°C for 30 seconds, 74°C for 1 minute and 1 cycle of 74°C for 5 minutes.

2.1.13 RNA isolation

RNA was extracted using the Trizol method (Life Technologies). Typically cells were resuspended in Trizol at a concentration of 10^6 cells per ml and frozen at –80°C until required. RNA was isolated by incubating the samples at room temperature for 5 minutes followed by the addition of 0.2ml chloroform per 1ml of Trizol. Samples were gently inverted and centrifuged at 12000*g* for 15 minutes, 4°C. The clear aqueous phase was removed and the RNA precipitated by the addition of 0.5ml isopropanol per 1ml of Trizol. Samples were incubated for 10 minutes at room temperature and then centrifuged at 12000*g* for 10 minutes at 4°C. The RNA pellet was washed with 75% ethanol and centrifuged at 7500*g* for 5 minutes at 4°C. After

air- drying the pellet RNA was dissolved in RNAse free water at 20µl per 1ml of Trizol. Contaminating DNA was removed by treatment with RNAse free DNAse and the concentration and purity determined by measuring the absorbance at 260/280nm.

2.1.14 cDNA synthesis

Complementary DNA (cDNA) synthesis was performed using Superscript™ (Life technologies). 1µg of total RNA was added to oligo dT primer at a concentration of 0.5µg/µg of RNA and made up to 12µl with nuclease free water. Samples were then heated to 70°C for 5 minutes and chilled on ice. A master mix was prepared containing 4µl of 5x RT buffer, 1µl dNTP mix, 2µl of 0.1M dithiothreitol (DTT) and 1µl (200 units) Superscript reverse transcriptase for each sample. Samples were briefly spun, 7µl of master mix added and incubated at 42°C for 1 hour. The reaction was terminated by incubation at 70°C for 15 minutes and then on ice for several minutes before storage at –20°C.

cDNA was amplified as follows; a 50µl reaction volume was used for each PCR composed of: 1X Taq buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.1µM of each primer, 1µl of diluted template DNA (pg), 0.5µl of Taq and 100ng of template DNA. The cycle parameters were: 1 cycle of 96°C for 2 minutes, followed by 30 cycles of 96°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 1 minutes/kb of product and finally 74°C for 5 minutes. Primer sequences, annealing temperatures and expected product sizes are shown in table 2.1.

Table 2.1: RT-PCR primers, annealing temperatures and expected product sizes.

Primer	Primer sequence 5'-3'	PCR Annealing Temperature	Product Size
MMEf	CACTGTCGACTCACATTCTTGGGAAGG	55°C	600bp
MMEr	TTAGTCCACGTTTCTGCCTCATCA		
3f2 (α_v)	TATGCCAAAGATGCACCACTGGAG	60°C	300bp
7b1 (α_v)	TCTGCCACTTGGTCCGAAATGAGC		
βh1f	AGTCCCCATGGAGTCAAAGA	55°C	265bp
βh1r	CCTGTGAACTCTGGTATCAG		
HPRTf	GTTGGATACAGGCCAGACTTTGTTG	50°C	350bp
HPRTr	GAGGGTAGGCTGGCCTATAGGCT		
β-actinf	TAAAACGCAGCTCAGTAACAGTCCG	48°C	350bp
β-actinr	TGGAATCCTGTGGCATCCATGAAAC		

2.1.15 Real-time PCR

RNA was isolated as previously described. All reagents were purchased from Applied Biosystems (Perkin-Elmer, Foster City, CA). cDNA synthesis was performed using Taqman multiscribe reverse transcriptase kit. A total reaction volume of 50µl was used, composed of: 200ng of RNA sample, 1X Taqman reverse transcriptase buffer, 5.5mM $MgCl_2$, 500µM of each dNTP (deoxy dNTP mix), 2.5µM Random Hexamers, 0.4U/µl RNAse inhibitors, 1.25U/µl of reverse transcriptase and RNAse free water to 50µl. The cycle parameters for the cDNA synthesis were 1 cycle of 25°C for 10 minutes followed by 1 cycle of 48°C for 1 hour and an inactivation step of 95°C for 5 minutes.

The primers and probes to be used in the real-time PCR reaction were pre-mixed (FAM mix) and stored at –20°C until use (table 2.2). For each gene, 90µl of each primer (25pmol/µl) and 300µl (5pmol/µl) probe were made up to a total volume of 2mls to create the FAM mix. The real-time PCR reaction was carried out in a total volume of 25µl composed of: 12.5µl of Taqman master mix, 7µl of FAM mix,

1.25µl of 20X 18S (internal control), 2.5µl of cDNA at 1:5 dilution and RNAse free water to 25µl. 1.25µl of 10X gene mix was used in place of the FAM mix for the assay on demand kits. The PCR reactions were carried out using an ABI PRISM® 7700 sequence detection system.

Table 2.2: Real-time PCR primer and probe sequences.

Gene	Primer/Probe	Sequence
CD14	Forward	CAGCAAACTTGACCCCTGATTT
	Reverse	AGATGGACAACTTTCAGGAGTGAGT
	Probe (FAM)	ACCCTCAGGATCCACACGGAAGGG
CD47	Forward	GTGCAAAAATCTCAGTCTCAG
	Reverse	CCATGGCATCGCGCTT
	Probe (FAM)	CAATGGCATTGCCTCTTTGAAAATGGA
Integrin α_v	Forward	GGCCGTGGGAGACTTCAAT
	Reverse	CCAAAGTCCTTGCTGCTCTTG
	Probe (FAM)	TGACGGCATTGAAGATTTTGTCTCAGGAGTT
Integrin β_3	Forward	CATCCAGACCCTGGGTACCA
	Reverse	AGCCAATCCGAAGGTTGCTA
	Probe (FAM)	TTGGCCTCTCAGATGCGCAAGCTT
Integrinβ_5	Forward	GGAGAAGCTGGCAGAGAACAAT
	Reverse	AGGGCTGTAAAATTCTTGTAGAGCATAT
	Probe (FAM)	TCAACCTCATTTTTGCTGTGACGA
C1q	Forward	GTGGTGGAGAGGACACAGCTTAT
	Reverse	AGGGACACACAGCGTTTAAAGATAC
	Probe (FAM)	CAAACTGCTACAAAGCCAGCAAGAGCTCC
TSP 1	Forward	GAGCATCTTCACCAGGGATCTG
	Reverse	TTGTCATTGACATCTCCCTTTGC
	Probe (FAM)	CCAGCGTTGCCAGGCTCCGA
SRB	Forward	GCACGCACGGCACATG
	Reverse	CTCACTTGGCAGCAGGTCTGT
	Probe (FAM)	CCACGCATGTGCAAAAACAACTCAGG
Assays on demand gene expression kits from Applied Biosystems: Primer/Probe mixes for CD36, phosphatidylserine receptor and scavenger receptor A, ABC-1 and low-density lipoprotein receptor.		

RNA levels relative to ribosomal RNA were calculated using the difference between the "detection cycle number" for the gene and the ribosomal RNA.

2.2 Macrophage Culture

2.2.1 Bone marrow derived macrophages

Bone marrow derived macrophages were cultured from either integrin α_v floxed or DOM (carrying floxed stop β geo reporter see 5.3.3) mice bred in house at the University of Edinburgh animal facility. Femurs were removed from mice and dipped in 70% ethanol before being placed in macrophage media (DMEM F/12 supplemented with 10% FBS and 100U/ml penicillin, 100μg/ml streptomycin, 10% L929 conditioned media). Bone marrow was flushed from femurs and resuspended in 10mls of macrophage media. Cells were either plated at $0.4x10^6$ cells per well of a 24 well plate or at a cell density of $0.4x10^6$ cells/ml in Teflon pots (Savillex, USA). Cells were fed on days 2 & 5 of culture and used at day 7. Phenotyping was carried out as previously described.

2.2.2 Adenoviral gene transfer

Adenoviral Cre & GFP were gifts from Dr F Graham & Dr M Hitt, McMaster University, Ontario, Canada. Batches of each virus were kindly prepared by Dr Sallenave, University of Edinburgh. COS-7 cells and bone marrow derived macrophages were grown in 24 well plates and treated with either virus in 500μl of their respective culture media for 24 hours. Once infected, the virus containing media was removed from the cells and replaced with fresh media and the cells cultured for a further 24-48 hours. Virus was used at between 25-500 plaque forming units (pfu)/cell which is equivalent to a multiplicity of infection (MOI) of 25-500. Stock virus titres of $8.96x10^{10}$ plaque forming units (pfu)/ml for AdCre and $6.68x10^{10}$ pfu/ml for AdGFP were used.

2.2.3 Genomic DNA isolation

400µl of lysis buffer (appendix I) was added to each well of a 24 well plate and the plates incubated at 55°C overnight to extract genomic DNA from macrophages. To prevent evaporation, plates were placed on a platform in a Tupperware container with water in the bottom. The following day DNA was precipitated with 800µl of ethanol containing 12µl of 5M NaCl, and the plates incubated for 15 minutes at room temperature. Plates were then centrifuged at 2000rpm for 10 minutes and the ethanol gently removed before washing the DNA 3 times in 400µl of 70% ethanol. After the last wash, plates were left upside down to air dry for 30 minutes before the DNA was resuspended in 200µl of TE buffer at 37°C overnight.

2.2.4 Plate based phagocytosis assay

Phagocytosis assays were also performed in 24 well plates: Fluorescently labelled apoptotic cells were incubated with day 7 macrophages (+/- adenoviral Cre) at a ratio of 4:1 apoptotic cells:macrophages for 2 hours in 500µl of DMEM/F12. Assays were carried out in the presence or absence of the inhibitory peptide GRGDSP or the control peptide GRADSP at a concentration of 0.5mg/ml. After 2 hours, further phagocytosis was prevented by the addition of ice-cold PBS, and unbound apoptotic cells removed by vigorous washing with PBS before fixation with 200µl of 4% paraformaldehyde. Three hundred cells were counted over 3 fields for each condition and the percentage of phagocytosis calculated.

2.3 Generation of DNA constructs (molecular techniques)

Routinely used buffers and solutions are listed in appendix I.

2.3.1 Transformation of bacteria with plasmid vectors or ligated products

TOP 10 competent cells (Invitrogen, Paisley UK) were used for all transformations. The competent cells were thawed on ice and 10ng of plasmid or ligated product added. Cells were then gently mixed and incubated on ice for 30 minutes, heat-

shocked for 90 seconds at 42°C and placed back on ice for a further 2 minutes. 1ml of Luria Bertani (LB) broth (appendix I) was added and the cells placed at 37°C for 45 minutes in a shaking incubator ~150rpm. Transformations were then centrifuged at 1000rpm for 30 seconds, the cells resuspended in 100µl of LB broth and plated onto LB agar plates containing the appropriate antibiotic. Plates were incubated at 37°C overnight to allow colony growth.

2.3.2 DNA purification

DNA was extracted using either mini or maxi prep kits from Qiagen (West Sussex, UK) as per manufacturer's instructions. Briefly, single bacterial colonies were used to inoculate larger cultures of LB broth containing the appropriate antibiotic. The bacterial cultures were grown overnight in a 37°C shaking incubator at ~250rpm. Cultures were cleared by centrifugation and resuspended in buffer P1, lysed with buffer P2 and neutralised with buffer P3. Cellular debris was then removed by centrifugation at >13,000rpm for 30 minutes and the cleared lysate applied to a DNA binding column. After several washes the DNA was eluted from the column and precipitated using iso-propanol. The DNA was then washed in a 70% ethanol solution to remove contaminating salts and the pellet air dried before re-suspension in an appropriate volume of Tris-EDTA (TE) buffer.

2.3.3 Restriction digest and modifying enzymes

Restriction enzymes were purchased from Promega or New England Biolabs. DNA was digested using the appropriate restriction enzyme at a volume of <10% of the final reaction volume in 1X optimal reaction buffer supplied with each enzyme and bovine serum albumin (BSA).

Ligation of digested DNA was performed at 1:3 or 1:10 vector:insert molar ratio. Typically 100ng of vector was incubated with the appropriate amount of insert in 1X ligase buffer and 1U of T4 DNA ligase (Promega) for ~3 hours at 22°C.

Blunt-ended fragments for the cloning of the bacterial expression vector pETVP22Cre were generated either using Klenow or Mung Bean Nuclease (New England Biolabs). The 5'overhang generated by restriction digest with *Hind*III was filled by incubating the DNA in 1X Klenow buffer containing 40μM of each deoxynucleotide triphosphate (dNTP), 0.1mg/ml BSA and 1U of Klenow polymerase at room temperature for 10 minutes. The reaction was stopped by incubation at 75°C for 10 minutes.

The *Nco*I site from the pET11d vector was removed by restriction digest with *Nco*I followed by treatment with Mung Bean Nuclease (endonuclease). The digested DNA was incubated at 30°C in 1X reaction buffer (supplied with enzyme) with 1U of nuclease for 2, 5, 10, or 20 minutes. The reaction was stopped by the addition of 2μl of DNA loading buffer and snap freezing in an ethanol/dry ice mix. The DNA was then run on an agarose gel and the DNA purified using a gel extraction kit (Qiagen).

2.3.4 Agarose gel electrophoresis

Agarose gels were routinely used between 0.8%-2% (w/v) depending on the size of the fragments to be separated. Agarose was added to 1X Tris Boric acid EDTA (TBE) solution diluted from a 10X stock, to give the final concentration of agarose (w/v), and this was heated in a microwave until dissolved. Once cool, 0.5μg/ml ethidium bromide was added and the agarose mix poured into a gel cast and left to set. Samples were loaded in 2-5μl of loading buffer depending on the sample volume. Generally 5μl of 1kb or 100bp ladder was loaded and the gel run at 150V for approx 30 minutes.

2.3.5 Polymerase chain reaction (PCR)

Primers were purchased from Hybaid or MWG Biotech. Pfu polymerase (Promega) was used for each of the cloning steps (PCR products B&C) except for A which is described in table 2.1. A 50μl reaction volume was used for each PCR composed of:

1X pfu polymerase buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.1μM of each primer, 1μl of diluted template DNA (pg), 0.5μl of pfu polymerase and 100ng of template DNA. The cycle parameters were: 1 cycle of 96°C for 2 minutes, followed by 30 cycles of 96°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 1 minutes/kb of product and finally 74°C for 5 minutes. Each of the primer sequences and annealing temperatures is given in table 2.3.

Table 2.3 Primer sequences and annealing temperatures used for the construction of pnLacZLoxP and VP22Cre.

Primer	Primer sequence	PCR annealing temperature
ATGNLSLOXP f	TCGAGCCATGGGGCCCAAGAAGAAACGC AAAGTGGGGAGCATAACTTCGTATAATGT ATGCTATACGAAGTTATCG	1 cycle of 90°C and cooled at a rate of 1°C/minutes to allow primers to anneal
ATGNLSLOXP r	GATCCGATAACTTCGTATAGCATACATTA TACGAAGTTATGCTCCCCACTTTGCGTTTC TTCTTGGGCCCCATGGC	
LoxPegfpn1f	GGATCCATAACTTCGTATAATGTATGCTAT ACGAAGTTATCCATGGTGAGCAAGGGCGA GGAGCTGTTCACCGGG	64°C
EGFP3r	GAGCTGTACAAGTAAAGATCTCGC	
LacZ f	AGCATGGAAGATCTCGTCGTTTTACAACGTCGTG	60°C
LacZ r	CTGCAGGTCCAGATCTGTACAGGTGGCTTGGTCG	
VP22Cre f	GGCGGCCGCTCCAATCTGCTGACCGTGCAC	67°C
VP22Cre r	CTCTAGATCGCCATCTTCCAGCAGGCGCAC	

2.3.6 Sequencing.

Sequencing was carried out by Oswel DNA service. DNA was sequenced with the primers as stated in chapter 5 and the sequences are described in table 2.4.

Table 2.4 Sequencing primers.

Sequencing Primer	Primer sequence 5'-3
VP221f	GGCCACGGCGACTCGA
BGH r	TAGAAGGCACAGTCGAGG
pET11D f	AATACGACTCACTATAGGG
T7 TERMINATOR	GCTAGTTATTGCTCAGCGG
LZLP 1f	CCATTGACGCAAATGGGC
LZLP 1r	CCTCCTCGCTATTACGCCAGCT
LZLP 2f	AATCCTGCCTCTTGGTCC
LZLP 2r	GGTGGTGCAGATGAACTTCAG

2.3.7 Site-Directed mutagenesis

Site-directed mutagenesis was performed using the primers LZLPSDM1 (aggagcgcaccatcttcttcaaggacg) and LZLPSDM2 (gccctgagcaaagaccccaacgagaag) purchased from Hybaid. The mutagenesis reaction mix was composed of: 1X pfu turbo DNA polymerase buffer (Stratagene Europe, Amsterdam, The Netherlands), 0.2mM dNTPs, 1.0μM of each primer, 1μl of pfu turbo DNA polymerase and 100ng of template DNA. The cycle parameters were: 1 cycle of 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 65°C for 2 minutes/kb plasmid and 1 cycle of 75°C for 7 minutes. The mutagenesis reaction was then digested with *Dpn*I for 1hour at 37°C before transformation in TOP10 electrocompetent *E.coli*.

2.3.8 COS-7 cell line

COS-7 cells were maintained as sub-confluent cultures at 37°C in a 5% CO_2 humid incubator in 165cm^2 flasks containing DMEM supplemented with 10% FBS, 2mM L-glutamine, 1x non-essential amino acids, 100U/ml penicillin and 100μg/ml streptomycin. Cells were passaged at 1:10 three times per week.

2.3.9 Amaxa transfection technology

COS-7 cells were transfected using Amaxa transfection technology (Amaxa Biosystems, Cologne Germany). Cells were washed in PBS, trypsinised, centrifuged and the pellet resuspended at $1.5x10^6$ cells/ml in the optimised buffer, buffer V (supplied by Amaxa). Typically between 2-5μg of plasmid DNA was incubated with 1ml of cell solution and placed in a transfection cuvette. Cells were then placed in the Amaxa electroporator and pulsed using program A27, the transfected cells were then placed in $75cm^2$ flasks in 15mls of RPMI supplemented with 10% FBS, 100U/ml penicillin and 100μg/ml streptomycin. Twenty four to 48 hours later the cells were analysed for expression of the plasmid.

2.3.10 β-galactosidase staining

Cells transfected with β-galactosidase expressing constructs were washed in PBS and fixed in 2%formaldehyde/0.2% glutaraldehyde for 10 minutes at room temperature. The fixative was then removed and the cells washed in PBS and incubated in β-galactosidase stain (appendix I) overnight at 37°C.

2.3.11 Immunofluorescence

COS-7 cells transfected with VP22Cre were fixed in ice-cold methanol for 2 minutes. Cell were then blocked with PBS containing 10% FCS for 20 minutes before staining with 1:1000 dilution of a rabbit polyclonal anti-Cre antibody (Novagen, supplied by CN Biosciences Nottingham UK) for 1 hour. Cells were washed twice in PBS and Cre expression detected with an Alexa 568 primary conjugated goat anti-rabbit secondary (Molecular Probes, Leiden Holland) at a 1:500 dilution.

2.4 Preparation of recombinant protein

Routinely used buffers and solutions are listed in appendix I.

2.4.1 Purification of recombinant proteins from *E.Coli*

BL21 (DE3) pLysS competent cells were transformed with the bacterial expression plasmids and plated onto LB agar plates containing the appropriate selection antibiotics (chloramphenicol and ampicillin or kanamycin). The following day a single colony was used to inoculate 50ml of LB broth plus antibiotic that was grown overnight in a 37°C shaking incubator (~250rpm). The overnight culture was then diluted 1:20 in 950ml of LB broth plus antibiotic and grown to an A_{600} ~0.8-1.0, 0.8mM β-D-isopropyl-thiogalatosidase (IPTG) was added to induce the culture which was then grown for a further for 3 hours. The bacterial cells were harvested by centrifugation at 4000rpm for 10 minutes at 4°C and the pellet stored at –70°C overnight or until required.

On the day of purification the pellet was thawed on ice and lysed using a solution of BugBuster reagent (5ml/g wet cell paste)(Novagen) and benzonase (1µl/5ml BugBuster reagent)(Novagen). A protease inhibitor cocktail was added at this stage to prevent breakdown of the protein. Cleared lysate was obtained after centrifugation at >13,000rpm for 30 minutes. 5mls of the cleared lysate was incubated with 5mls of Ni-NTA agarose (Qiagen) on a rotating platform for 1 hour at 4°C before packing into a column. The column was then washed 5 times with 5mls of wash buffer (50mM NaH_2PO_4, 300mM NaCl and 5mM Imidazole pH 8.0) before elution with 10mls of elution buffer (50mM NaH_2PO_4, 300mM NaCl and 250mM Imidazole pH 8.0). The eluted fraction was collected in 1ml volumes and protein levels determined. The fractions containing the highest levels of protein were dialysed against PBS followed by DMEM/F12 before storage at –80°C.

2.4.2 Protein concentration

Protein concentrations were measured using DC protein assay kit (Bio-Rad, Hemel Hempstead, UK). A "standard curve" was prepared using BSA ranging from 0.2 to 1.5mg/ml. 25µl of standards and samples were added to a 1.5ml eppendorf and

incubated with assay reagents for 15 minutes. The absorbance was read at 750nm and the concentration of the samples determined by linear regression.

2.4.3 SDS-PAGE & Western blotting

SDS reducing buffer (see appendix 1) was added to cell lysates and boiled at 96°C for 5 minutes. Denatured samples were applied to 12% agarose gels with 4% stacking gels (see appendix 1), and electrophoresed at 125 volts for approximately 1 hour in 1X running buffer. Protein was then blotted onto PVDF (Hybond-P, Amersham, Little Chalfont, UK) in an electroblotting apparatus (BioRad). PVDF membrane was pre-wetted in methanol for 10 seconds before being equilibrated in transfer buffer. The gels were placed onto the membrane and 2 pieces of filter paper, also soaked in transfer buffer, were placed on either side. Fibre pads soaked in transfer buffer were placed either side of the filter paper and the entire sandwich was placed into the gel holder cassette with the gel closest to the grey side of the cassette. The process was repeated for the second gel. The cassettes were placed in the electrode module, the tank filled with cooled 1X transfer buffer and the cooling block added. Transfer was performed at 80mA per gel for 90 minutes.

2.4.4 Coomassie staining of SDS-Page gels

SDS-PAGE gels were stained with Coomassie blue stain (appendix I) for 30 minutes on a rocking platform. Coomassie blue stain was then removed and the gels washed in several changes of destain (appendix I) until the protein bands were clearly visible.

2.4.5 Antibody detection

Blots were blocked in PBS containing 0.5% non-fat milk powder (Marvel) for 1 hour at room temperature on a rocking platform. Blots were subsequently probed with a polyclonal rabbit anti-Cre antibody (Novagen) diluted 1:5000 in PBS containing 0.5% non-fat milk powder and 0.05% Polyethyleneglycol sorbitanmonolaurate (TWEEN 20) and incubated on a rolling platform at 4°C overnight. The following day the blots were washed 3 times in PBS/TWEEN and detected with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (DAKO) for 1 hour at room

temperature. Blots were washed three times in PBS/TWEEN and the signal was detected by enhanced chemiluminesence (ECL, Amersham) and X-ray film (MR-1, Kodak, Rochester, NY).

2.5 Microscopy

All images were captured using an inverted microscope (Zeiss) fitted with a CoolSnap camera and OpenLab 3.0 image acquisition software (Improvision).

Chapter 3

Differentiation of ES cells into myeloid cells

3.1 Introduction

ES cells remain totipotent and undifferentiated when cultured in the presence of cytokines such as LIF or co-cultured with stromal cell layers that produce LIF (Smith et al., 1988b). Upon cytokine withdrawal ES cells spontaneously differentiate into many cell lineages that contribute to all three germ layers (endoderm, ectoderm, mesoderm) (Evans and Kaufman, 1981; Martin, 1981). This spontaneous differentiation can be manipulated by the addition of specific cytokines or conditioning factors into the culture system that can drive differentiation towards a particular cell type and enhance the numbers of cells obtained.

Following removal from stromal feeder layers or in the absence of LIF, ES cells form embryoid bodies (EBs), a cluster of differentiated cells that recapitulate many aspects of early embryo development (Doetschman et al., 1985). Although EBs will form spontaneously there are several ways in which they are routinely and reproducibly generated, the main three being I) suspension in liquid culture or II) culture upon a monolayer of stromal cells or III) suspension culture in a semi-solid mixture.

One method of EB formation is to culture ES cells in liquid culture either in bacterial grade Petri dishes or in "hanging drop" culture. When plated into bacterial grade Petri dishes the lack of substrate causes the ES cells to aggregate and form EB, however these EBs are not as uniform as those generated in MeC and may be prone to attaching to the plastic resulting in a reduction in differentiation (Wiles and Keller, 1991). A modification of this method, the hanging drop, is to culture low numbers of ES cells in droplets of media suspended from the lid of a culture plate for several days. The close proximity of the ES cells in these cultures promotes the generation of EBs, which can then be transferred into standard liquid culture or into MeC cultures for assays of hematopoietic precursors (Keller, 1995).

A second method of initiating differentiation is to culture ES cells on a monolayer of stromal cells. Several stromal cell lines are available which give rise to

macrophages. One in particular, the OP9 cell line does not express functional M-CSF and is used to differentiate ES cells to those of the lymphoid lineage and hematopoietic cells other than macrophages (Nakano et al., 1994; Senju et al., 2003). This co-culture method involves plating ES cells on a monolayer of stromal cells and sub-culturing them onto fresh stromal cells after days 5 and 10 of differentiation. This allows removal of undifferentiated cells and the formation of clusters of differentiated cells that can be expanded in liquid culture.

The simplest method of producing EBs is to use the semi-solid culture system reported by Wiles and Keller (Keller et al., 1993; Wiles and Keller, 1991) where MeC, (wood pulp) previously used in assays for hematopoietic precursor cell development (Schmitt et al., 1991) was used to provide a three-dimensional matrix to support the developing EBs. Briefly, ES cells were seeded at a low density in a MeC suspension containing various combinations of cytokines and growth factors in bacterial grade Petri dishes to prevent cell adherence. This allowed the formation of uniform EBs in a synchronous manner, which could then be plated into liquid culture for the expansion of hematopoietic cells.

Wiles and Keller used this system to investigate the influence of several cytokines on the ability of the differentiating EBs to generate specific cell types. After two weeks in culture either in the presence or absence of cytokines they observed that the EBs ruptured releasing a mass of differentiated cells consisting mainly of macrophages and erythroid cells and in some instances neutrophils and mast cells. The addition of erythropoietin increased the number of erythroid cells associated with the EB and when used in conjunction with IL-3 these numbers were further increased along with prolonged survival of the cells. IL-3 and IL-1 either alone or in combination were also found to enhance the numbers of EBs associated with macrophages at around day 12, although only IL-3 had an effect on the development of mast cells and neutrophils at around day 20. The presence of M-CSF or GM-CSF in the IL-3 cultures further augmented the number of macrophage producing EB therefore

suggesting that for macrophage differentiation the ideal combination of cytokines is IL-3 and M-CSF (Wiles and Keller, 1991).

Each of these methods described allows the efficient generation of EB and differentiated progeny. The method of differentiation chosen depends on the desired cell type and the questions being asked. The hanging drop method and the use of stromal cells are more commonly used for analysis of hematopoietic and lymphoid precursors (Palacios et al., 1995; Potocnik et al., 1997), whereas the MeC method has been widely used for the generation of macrophages (Clarke et al., 2000; Faust et al., 1994; Moore et al., 1998; Wiles and Keller, 1991). It is for this reason that this was the method of choice for the differentiation of ES cells to macrophages in our laboratory.

However, during the course of this work Fairchild *et al.* published the first report describing the directed differentiation of dendritic cells from ES cells (Fairchild et al., 2000). The ESF116 ES cell line derived from the blastocyst of a CBA mouse was differentiated by seeding ES cells into bacteriological Petri dishes in the absence of cytokines. After 14 days the EB were plated into tissue culture dishes and the media supplemented with IL-3 and GM-CSF. As early as 4 days after plating, cells with morphology resembling dendritic cells developed and continued to rapidly increase in number over the following 10 days in culture. The ES-derived dendritic cells grew in tightly packed clusters that could be harvested by gentle washing and used for analysis of surface phenotype, response to maturation stimuli and their ability to stimulate primary T–cell responses (Fairchild et al., 2000). This development allowed us to extend the differentiation of ES cells in our laboratory to include the generation of ES derived dendritic cells.

Differentiation of hematopoietic cells from ES cells has been widely used by many groups not only in studies examining hematopoietic precursors (Clarke et al., 2000) and transgene activation (Faust et al., 1994) but also in more functional studies such as the role of macrophages in atherosclerosis (Moore et al., 1998). Unlike cells of

the myeloid lineage ES cells are easily transfected therefore permitting over-expression, inactivation or deletion of a particular gene of interest. Subsequent differentiation of these targeted ES cells allows analysis of developmental or phenotypic changes in the desired cell type that may not have been possible *in vivo* due to embryonic lethality (Keller, 1995). Here, this particular aspect of ES cell biology has been exploited to enable the investigation of integrin α_v in phagocytosis. Until now, studies examining a role for integrin α_v in phagocytosis have been limited as mice carrying a null mutation in the integrin α_v gene die mid-gestation or perinatally (Bader et al., 1998).

The main aims of this part of the project were to:

1. To establish the technique of differentiating ES cells to myeloid cells using the EFC-1 ES cell line.
2. To utilise this method to differentiate ES cells (E14 cell line) carrying a disrupted integrin α_v gene.
3. To determine whether myeloid phagocytes derived from E14 ES cells in which α_v has been disrupted represent a suitable system for analysis of the role of integrin α_v in the phagocytosis of apoptotic cells.

3.2 Characterisation of EFC-1 ES cell differentiation

3.2.1 EB formation during hematopoietic differentiation of ES cells

The differentiation capacity of ES cells varies considerably with cell line. It was important, therefore, to use a cell line capable of reproducible differentiation when establishing this technique in our laboratory. The data presented in this chapter was generated using the EFC-1 ES cell line that is routinely used to study hematopoietic differentiation (Baird et al., 2001).

A protocol based on that of Wiles and Keller was used to generate embryoid bodies (EBs) and is outlined in figure 3.1. EFC-1 ES cells cultured on gelatin coated plates in the presence of LIF grew in tightly packed colonies (Fig 3.2). When these cells were cultured in MeC in the absence of LIF they proliferated and formed dense cell aggregates, EBs (Fig 3.2). The EBs gradually increased in size (200-500μm) and density and by day 8 clusters of differentiated cells could be seen emerging from the central EB mass. Occasionally, the development of red blood islands is observed in some EBs. Between days 12 and 14 of culture EBs reached their maximum size of ~1-2mm in diameter, and ruptured, releasing differentiated cells (Fig 3.2). At day 14 EB cultures were disrupted and plated for expansion and analysis of differentiated cells.

3.2.1 Reverse Transcriptase-Polymerase Chain Reaction analysis of differentiating EB

RT-PCR analysis has already been carried out on differentiating EB and some of the genes involved in this process are described in the literature, but it was important to determine if the genes of interest for our experiments were expressed. Gene expression in differentiating EBs (days 6 and 13) was compared to bone marrow and bone marrow derived macrophages (BMDM) at days 3, 5 and 7 of culture. The macrophage marker MME (Shapiro et al., 1992) was expressed at constant levels in BMDM from day 3 but was absent in bone marrow and early EB differentiation with

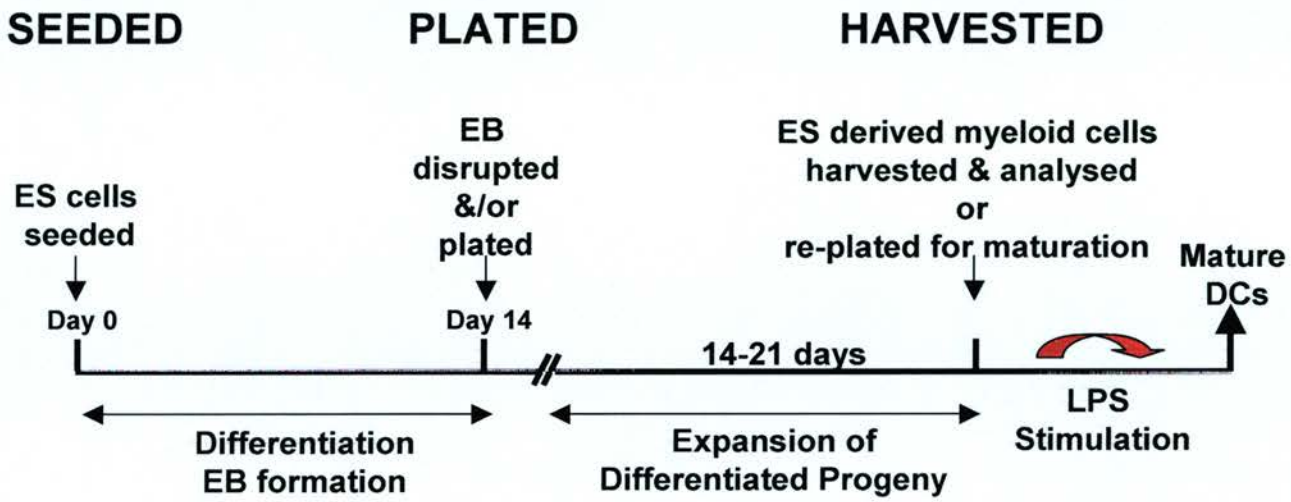


Figure 3.1: Overview of the differentiation protocol for ES derived myeloid cells. ES cells were seeded into methylcellulose cultures in bacteriological Petri dishes to allow the formation of EB. After 14 days the EB were either disrupted and/or plated into tissue culture plates and the differentiated cells expanded for ~21 days. The ES derived myeloid cells were then harvested and analysed or if DCs the cells were re-plated for maturation studies.

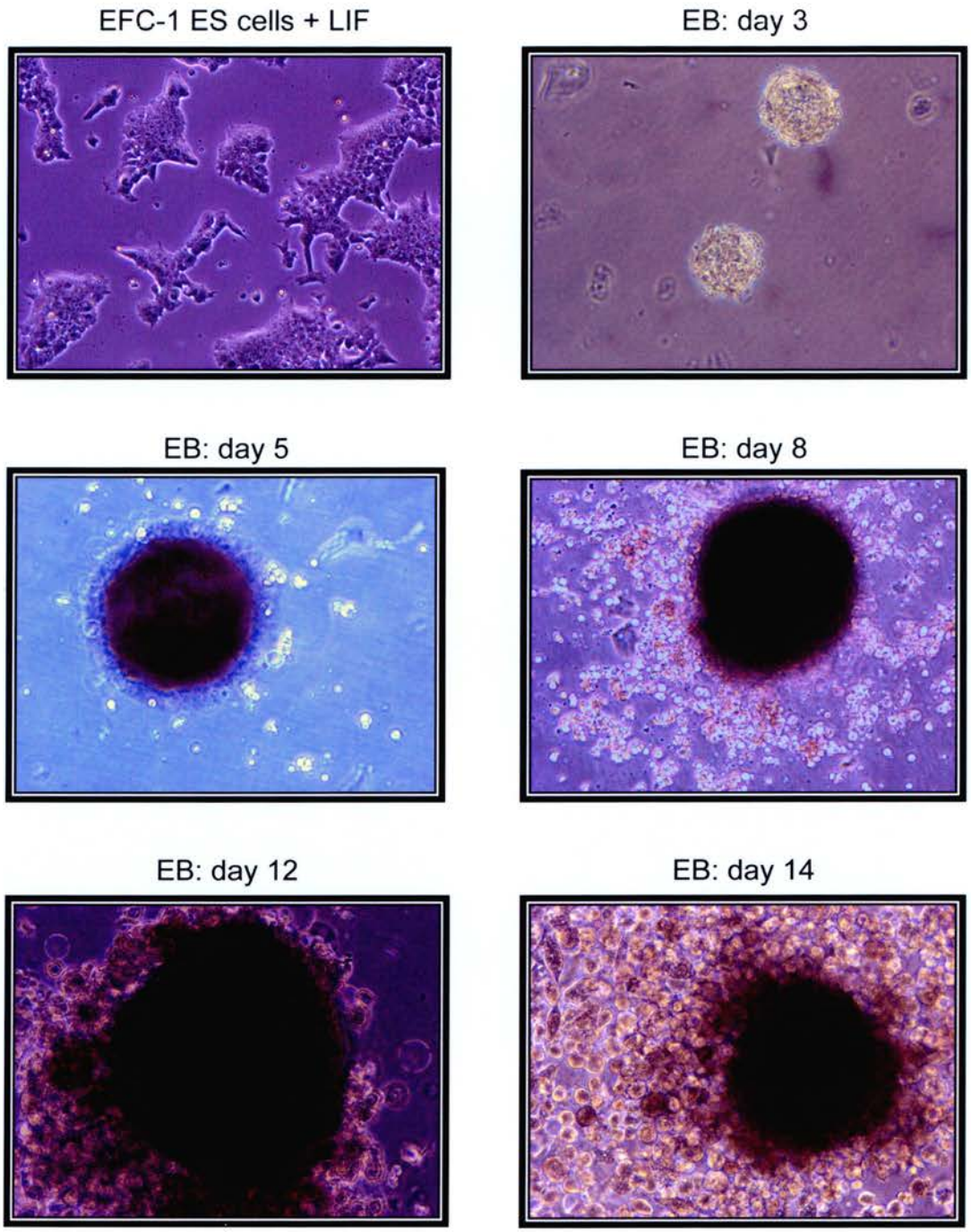


Figure 3.2: Hematopoietic differentiation of EFC-1 ES cells. Stages of EB formation in methlycellulose media containing IL-3 and M-CSF from undifferentiated ES cells to fully differentiated EB surrounded by hematopoietic colonies. Magnification x10

Figure 3.3: RT-PCR analysis of EB compared to bone marrow derived macrophages. RNA was harvested from bone marrow macrophage cultures at days 0, 3, 5 and 7 and from day 6 and 13 embryoid bodies. RT-PCR analysis was performed for MME, integrin α_v, βH1, HPRT and β-actin. (see text and abbreviations).

the onset of expression occurring between days 6 and 13 (Fig 3.3), therefore indicating that MME is not expressed by macrophage precursors. In contrast integrin α_v was present at low levels in day 6 EBs with expression increasing as differentiation proceeds (Fig 3.3). BMDM also expressed integrin α_v with the highest levels detected at day 5 of culture. Embryonic globin (βH1) expression was absent in bone marrow cultures but was expressed at high levels in day 6 EBs and was seen to decrease by day 13. Hypoxanthine phosphoribosyltransferase (HPRT) and β-actin were used as controls. The expression of MME confirmed that the differentiating EBs showed commitment to the macrophage lineage and importantly, the EBs expressed the gene of interest, integrin α_v. Therefore, EBs, were disrupted and the macrophages expanded in the presence of IL-3 and M-CSF or GM-CSF.

3.2.3 Differentiated Progeny

When plated, disrupted EBs gave rise to an assortment of cell types. Partially disaggregated EBs adhered strongly to the tissue culture plates and spread into a tight network of stromal cells (Fig 3.4a). These cell layers were often observed to beat spontaneously due to the presence of cardiac myocytes (Doetschman et al., 1985). In some cultures other morphologically distinct cell types such as adipocytes could be seen in distinct areas within the cell monolayers (Fig 3.4b). In addition to these cell dense regions, smaller more distinct populations of cells were seen physically separate from the EB surrounding layers. These presumably arose from non-adherent or highly motile precursor cells derived from EBs and showed the morphology of myeloid cells. Importantly, these putative myeloid cells showed a subtly different morphology depending on the cytokines in the culture medium; cells generated in the presence of M-CSF were adherent with a macrophage-like polar morphology (Fig 3.4c). In contrast, cells generated with GM-CSF were less adherent and exhibited a more 'spiky' dendritic like appearance (Fig 3.4d).

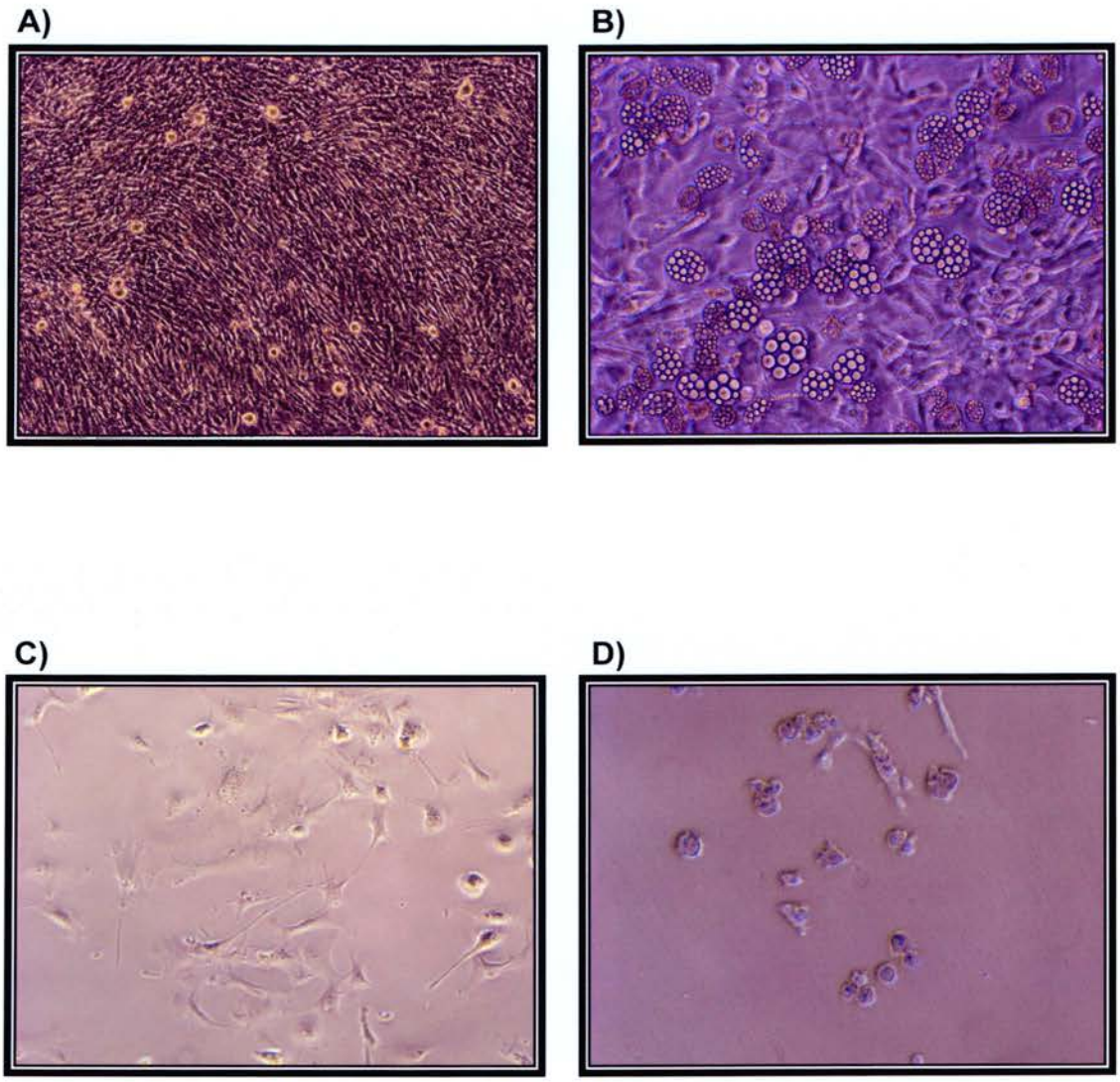


Figure 3.4: Cells generated from the differentiation of EFC-1 ES cells. EB disrupted with collagenase and plated onto tissue culture plastic give rise to various different cell types A) densely packed region surrounding EB x10 , B) adipocytes x 10 C) & D) myeloid cells grown in the presence of IL-3 and either M-CSF or GM-CSF respectively. Magnification x16.

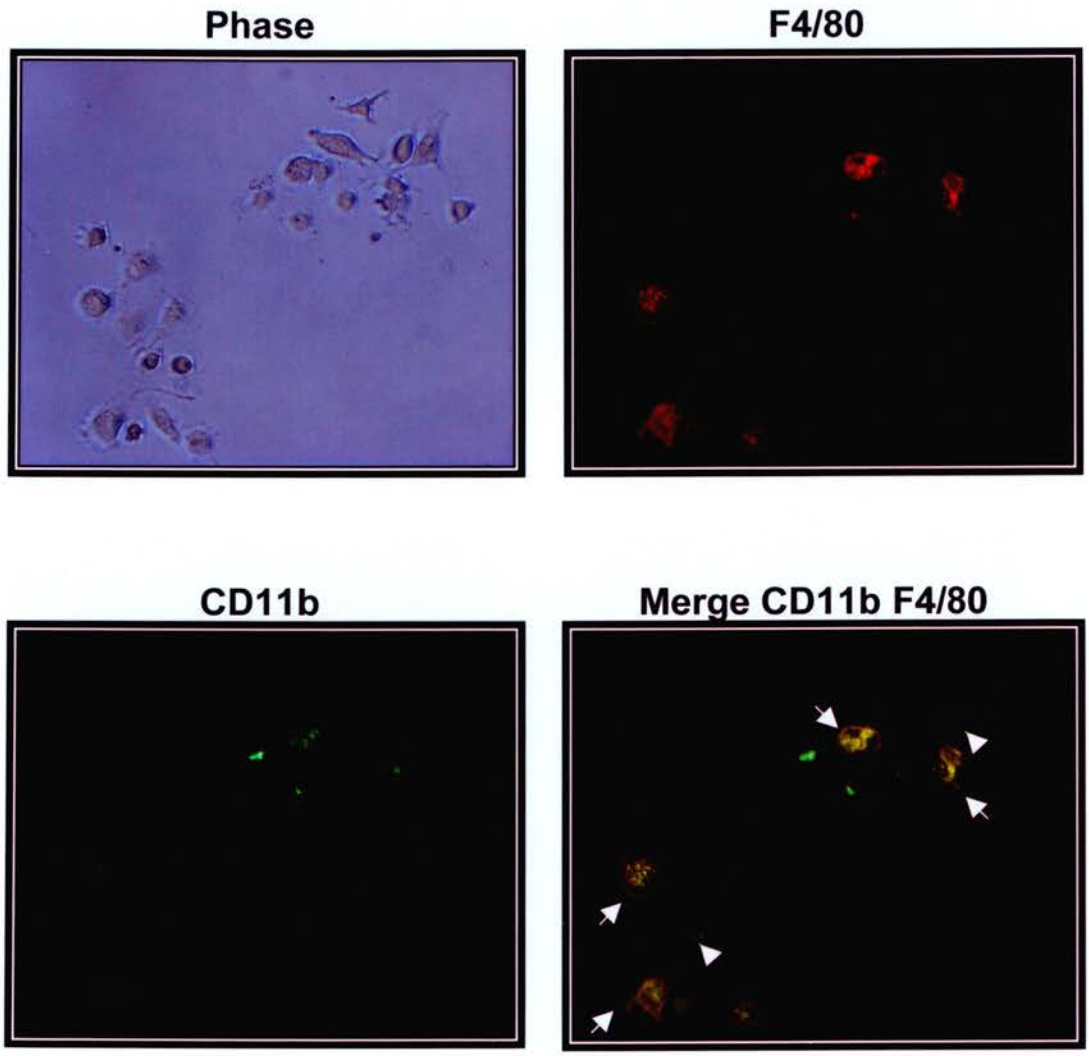


Figure 3.5: Immunofluorescence studies of macrophage surface markers. Surface expression of the macrophage marker F4/80 (PE conjugated) and the myeloid cell marker CD11b (FITC conjugated). The cultures are a mixed population of cells that express both markers (arrows) and cells that express CD11b only (arrow head). Magnification x16.

3.2.4 Immunofluorescence studies

To confirm the myeloid identity of these cells, they were stained for the macrophage/monocyte markers F4/80 and CD11b. Two populations of cells were apparent (Fig 3.5). Phase contrast revealed cells with a round phenotype and cells with a more elongated morphology. Merged images clearly showed that the rounded cells were positive for both F4/80 and CD11b and were likely to be macrophages while those that showed a more elongated morphology were only positive for CD11b , possibly representing monocytes or immature macrophages indicating that cultures are a mixed population of myeloid cells at different stages of development.

3.2.5 Surface phenotype of ES derived cells by flow cytometry analysis

Further analysis of macrophage/monocyte markers was performed using flow cytometry analysis of ES cell derived cultures. Two to three weeks after EB disruption and plating the differentiated cells were harvested using ice cold PBS/EDTA. Analysis by forward/side scatter (FSC/SSC) showed a heterogeneous population of cells with of cell debris of low FSC/SSC (Fig 3.6). Staining with F4/80 showed that 63.4% of total cells were F4/80 positive (~$5x10^3$ macrophages per EB). These F4/80 positive cells formed a distinct population identifiable by FSC/SSC. Myeloid cells derived from ES cells differentiated in M-CSF were positive for F4/80 (high & low), CD11b, CD14 and CD54 (ICAM-1) comparable to BMDM (Fig 3.6 & 3.8). However, unlike BMDM they exhibited only low levels of CD11a staining and weak staining for CD11c and Gr1 (Fig 3.6).

Cells from ES cells differentiated in GM-CSF showed a very different profile by forward and side scatter. A much more distinct population of cells was observed but this was only ~10% of the total cell population (Fig 3.7). Expression of the surface markers CD11b, CD11c and CD54 were comparable to both ES cells cultured in M-CSF and BMDM (Fig 3.6, 3.7 & 3.8). They were also positive for F4/80 but lack the high expressing population. In contrast to cells cultured in M-CSF they do not express CD14 but have greater levels of CD11a (Fig 3.7). Figures 3.6 and 3.7 show that overall the surface phenotype of myeloid cells derived from ES cells grown in

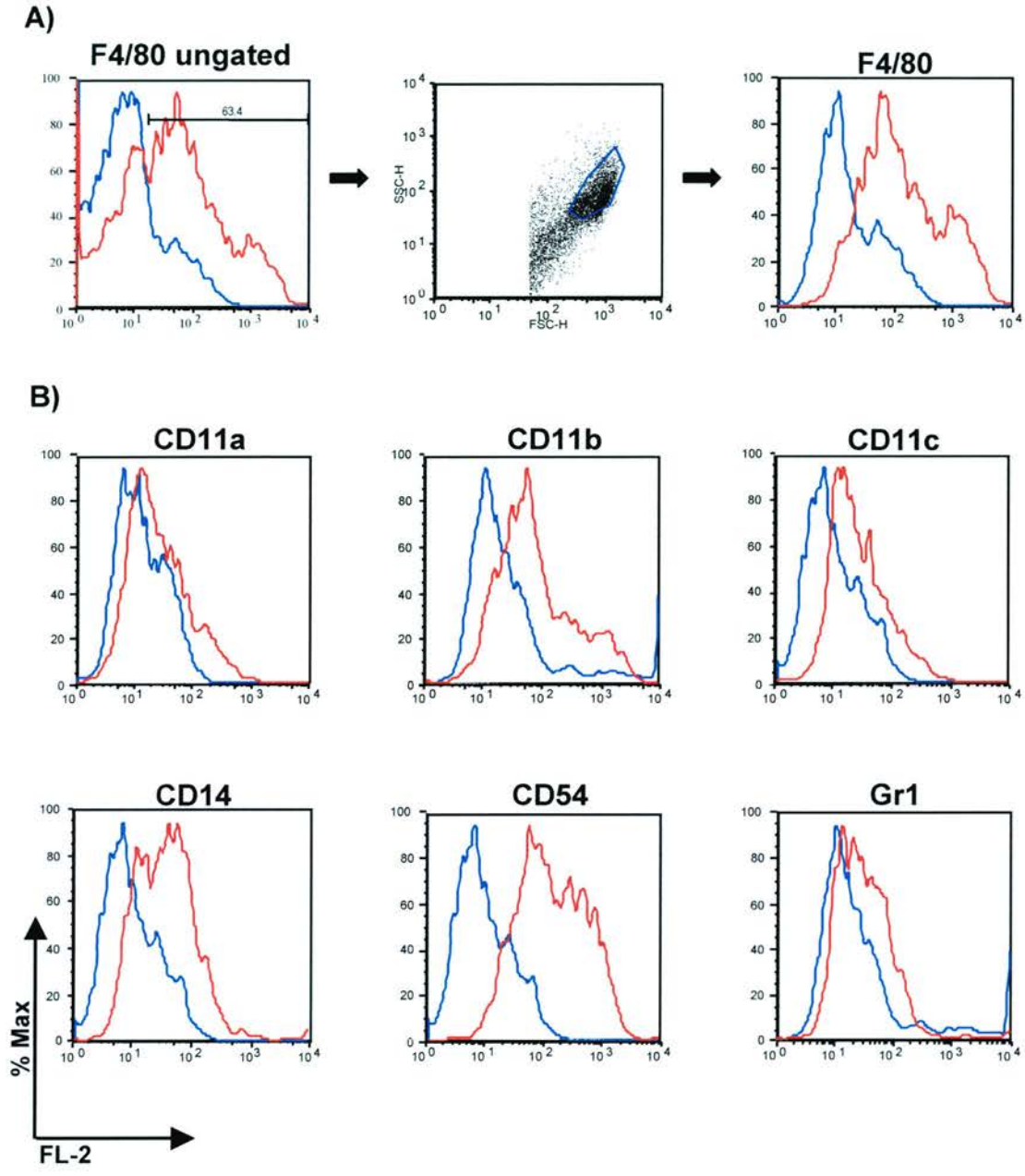


Figure 3.6: Surface phenotype of ES derived cells grown in IL-3 and M-CSF. Cells harvested from cultures grown in IL-3 and M-CSF were analysed by flow cytometry for expression of F4/80, CD11a, CD11b, CD11c, CD14, CD54 and Gr1. A) F4/80 staining of total cells allowed identification of a distinct F480 positive population (outlined in blue). This gate was used for the subsequent analysis of the other cell surface markers (B). Surface marker is shown in red and isotype control in blue.

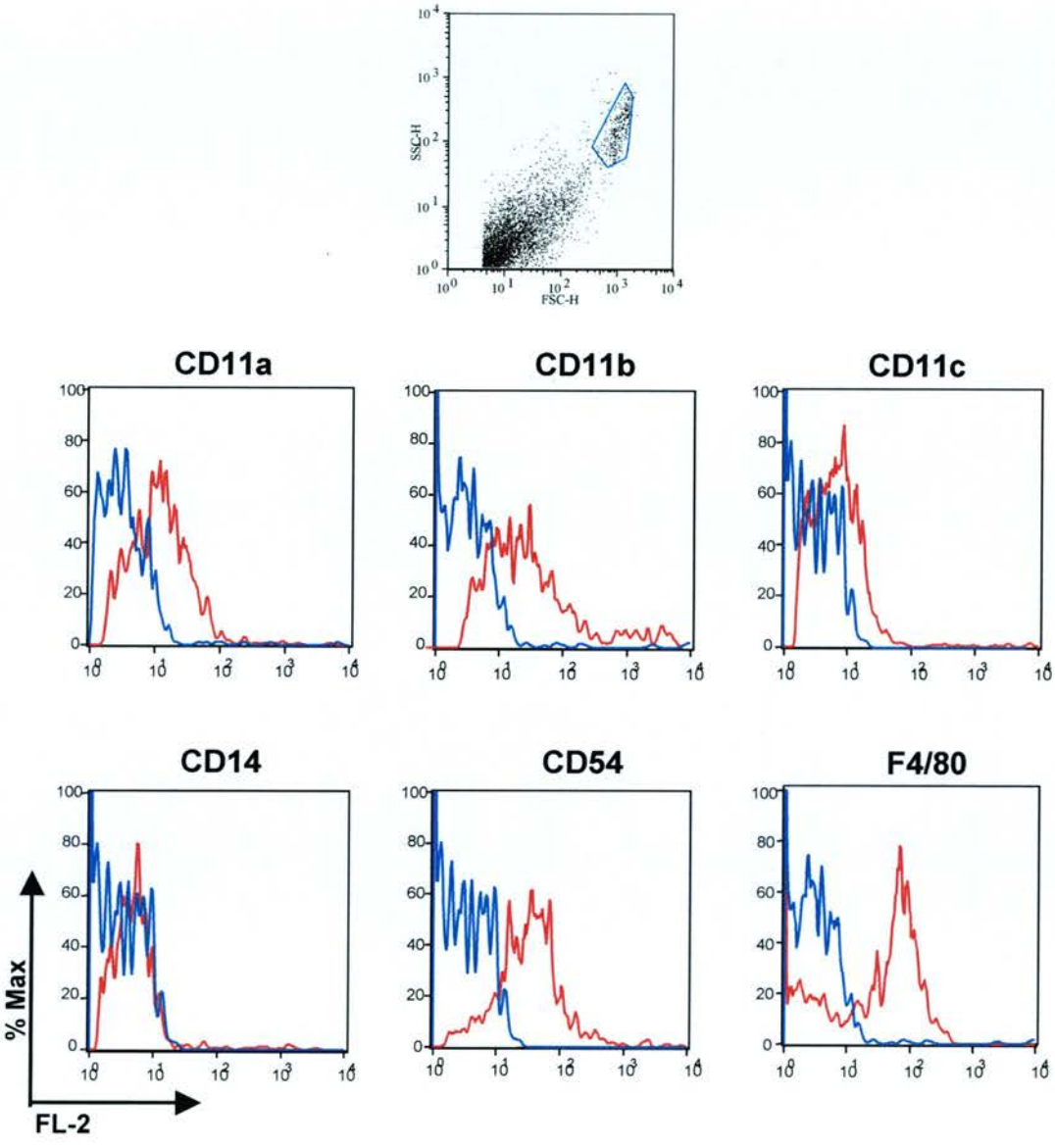


Figure 3.7: Surface phenotype of ES derived cells grown in IL-3 and GM-CSF. Cells harvested from cultures grown in IL-3 and GM-CSF were analysed by flow cytometry for expression of F4/80, CD11a, CD11b, CD11c, CD14, CD54. No surface staining was detected in the low FSC/SSC population. Surface marker is shown in red and isotype control in blue.

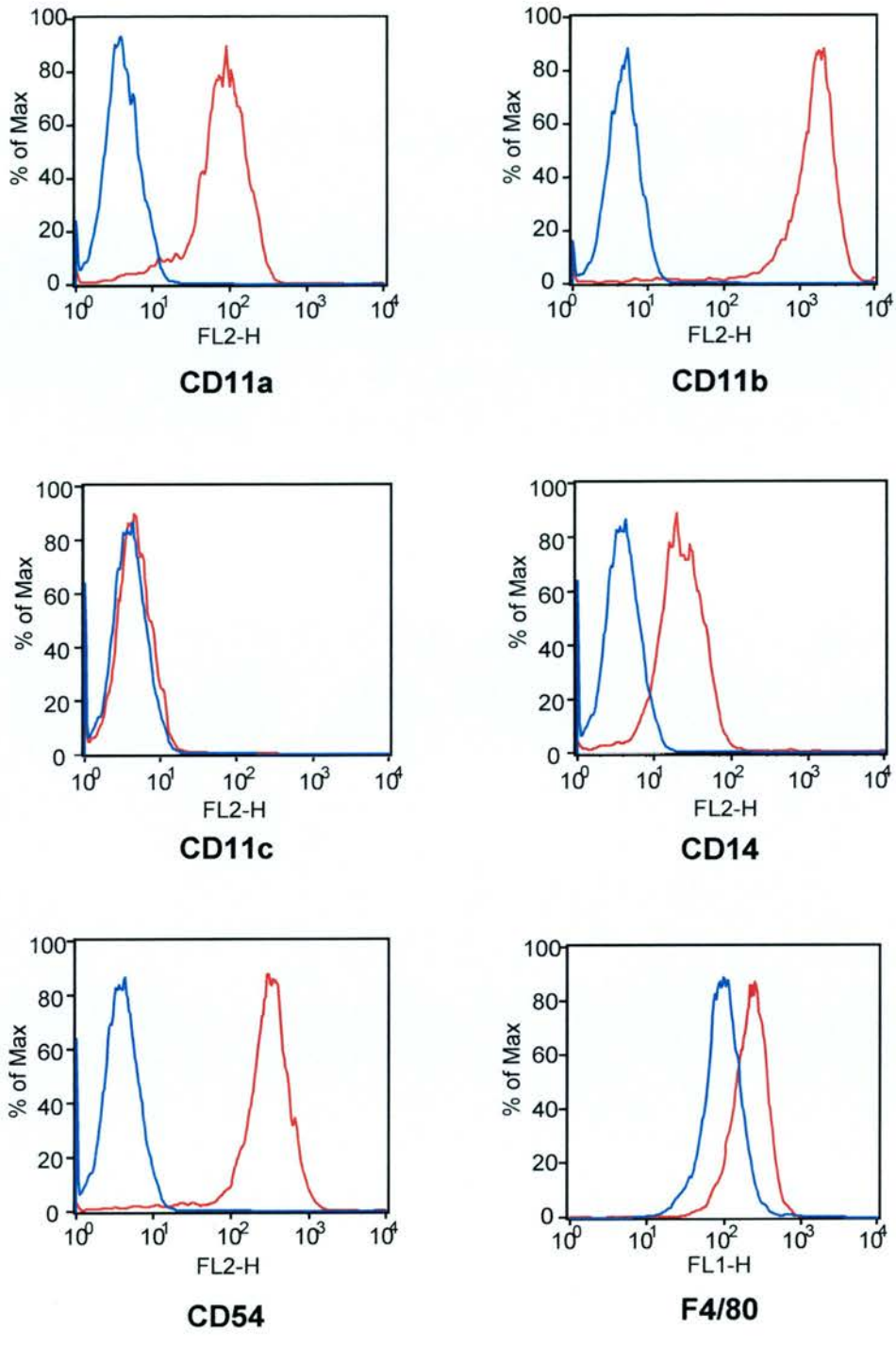


Figure 3.8: Surface phenotype of bone marrow derived macrophages. Day 7 Bone marrow derived macrophages were analysed for expression of F4/80, CD11a, CD11b, CD11c, CD14, CD54. All surface markers are shown in red and are phycoerythrin labelled except F4/80 which is labelled with FITC. Isotype controls are shown in blue.

either M-CSF or GM-CSF is similar to that of BMDM with subtle differences. However, the numbers of cells obtained were relatively low.

3.2.6 ES macrophages have the ability to phagocytose both latex beads and apoptotic cells

Macrophages play an important role in the phagocytosis of foreign or apoptotic material and the aim of this project was to investigate the role of integrin α_v in this process. Having generated a population of cells expressing macrophage markers (ES Mϕ) it was necessary to determine whether they had the ability to phagocytose. ES derived cells were incubated with 2μm FITC labelled latex beads for 30 mins and analysed by flow cytometry. ES derived cells clearly separated into a phagocytic (High FSC/SSC) and non-phagocytic (Low FSC/SSC) population, corresponding with previous analysis of F4/80 expression. 51.2% of the macrophages (high FSC/SSC) had ingested latex beads with 21.1% having ingested 1 bead (red arrow), 14.5% ingested 2 beads (green arrow) and 15.6% ingested ≥3 beads. The ability of ES Mϕ to phagocytose larger targets was also examined (Fig 3.10). ES Mϕ were cultured with fluorescent (green) apoptotic PMNs for 30 mins or 2 hours and analysed by flow cytometry. ES Mϕ were identified by F4/80 staining and phagocytosis determined by incorporation of apoptotic cell associated green fluorescence. After 30 mins interaction 80% of the ES Mϕ ingested apoptotic cells, increasing to 86% after 2 hours. These results confirm that ES Mϕ, like primary or bone marrow derived macrophages, possess the ability to phagocytose.

3.3 Optimisation of ES differentiation protocol

3.3.1 Effect of IL-3

ES cells differentiate to macrophages, expressing macrophage markers and are capable of phagocytosis but the yields obtained were low. Therefore, to increase the numbers of macrophages produced by differentiating ES cells various steps of the culture protocol were optimised. Macrophage differentiation is dependent on IL-3

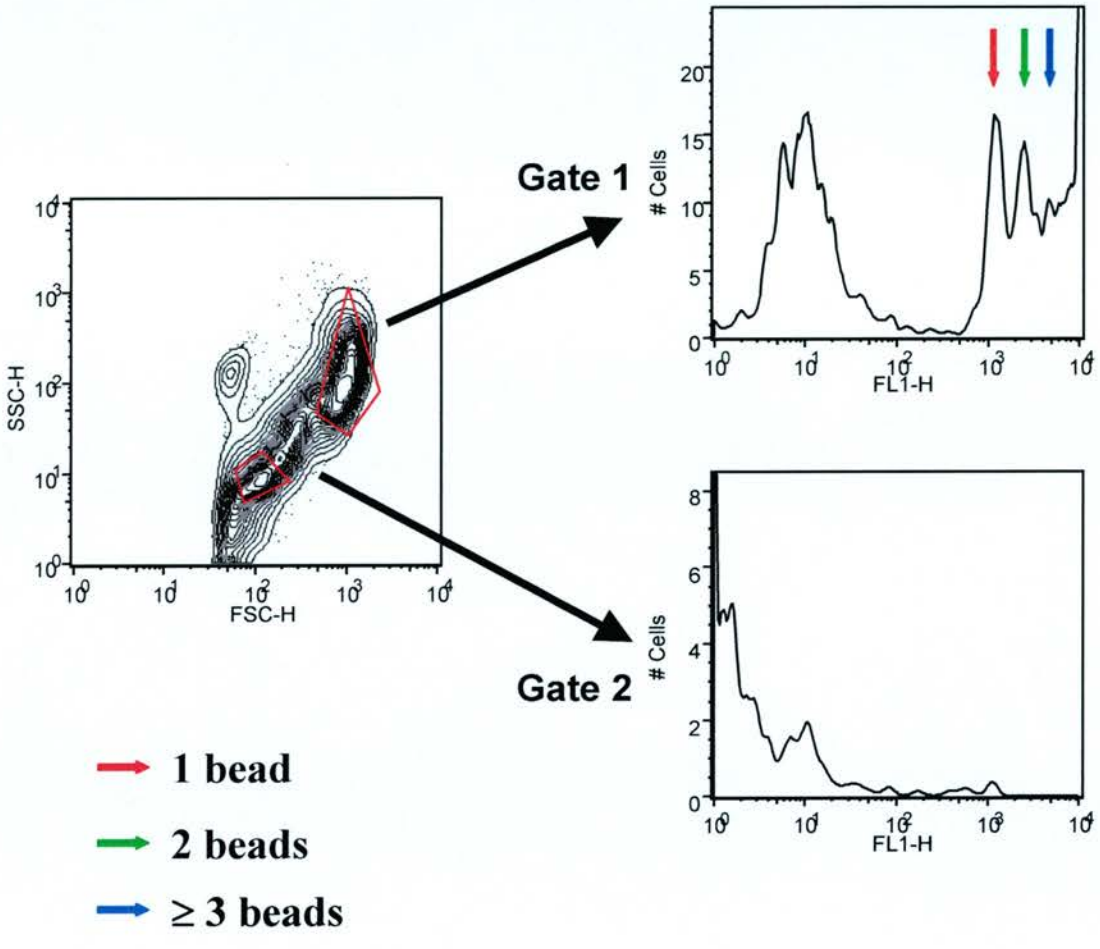


Figure 3.9: Phagocytosis of latex beads. ES Mϕ were incubated with an excess of 2μm FITC labelled latex beads for 30 mins. Cells were gated by FSC/SSC into macrophages (gate 1) and non-macrophages (gate 2) and the latex beads excluded by size, n=1.

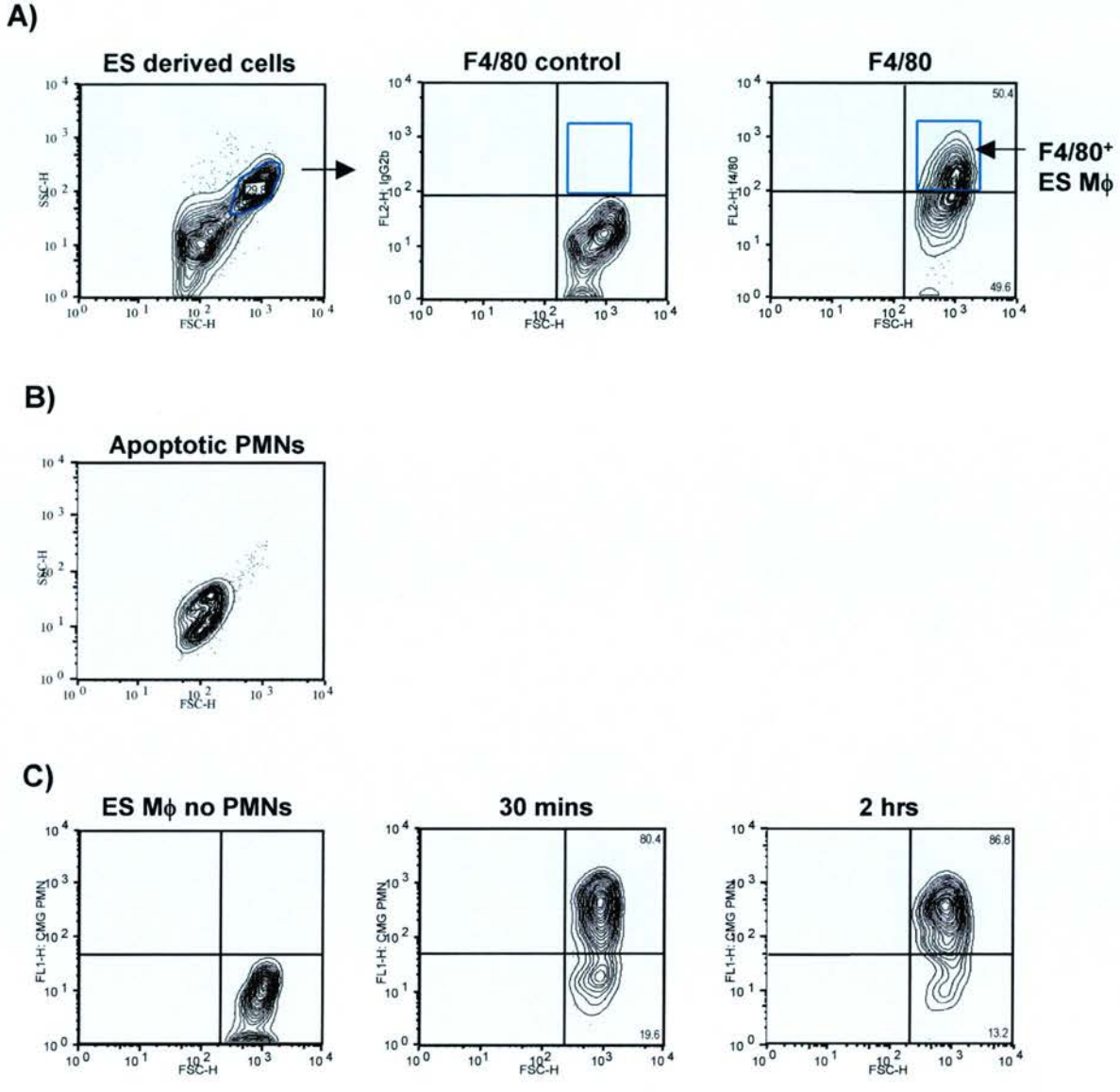


Figure 3.10: Phagocytosis of apoptotic cells. ES Mϕ were incubated with fluorescent green labelled apoptotic neutrophils for either 30 minutes or 2 hours. A) ES derived macrophages were gated as the high FSC/SSC population based on previous experiments. F4/80 positive cells were then gated (A) and unlabelled macrophages that had ingested apoptotic neutrophils were excluded from analysis (B). Phagocytosis was assessed by gating on the F4/80$^+$ ES macrophage population (C).

and M-CSF. The presence of IL-3 in the differentiation cultures greatly enhances the number of EB containing macrophages over those differentiated in M-CSF alone (Wiles and Keller, 1991). It was for this reason it was decided to vary the concentration of IL-3 used in the culture system. EFC-1 cells were differentiated in a fixed concentration of M-CSF (10% CM) and increasing amounts of IL-3 (8ng-800ng/ml). Cells were harvested 14 days after plating and analysed by flow cytometry for surface expression of F4/80 and CD11b.

Figure 3.11a shows representative flow cytometry plots for cells cultured at 8ng/ml IL-3. The majority of high FSC/SSC cells were $CD11b^{+}F480^{+}$ with 8ng/ml IL-3 giving the highest percentage (74% and 73 % respectively of gated cells) (Fig 3.11b). However, only 6% of the total cell population fell within this gate (Fig 3.11a) compared with 45% in previous differentiations. The percentage of F4/80 or CD11b positive cells in the whole population was also lower than previously observed, at 8ng/ml IL-3 only 8% of the population was positive for either marker decreasing to 3% in cultures supplemented with 800ng/ml IL-3 (Fig 3.11c). Overall, increasing the IL-3 concentration produced fewer ES Mϕ in total and reduced the macrophage proportion of the high FSC/SSC cells.

3.3.2 Disruption versus plating of EB cultured in M-CSF or GM-CSF

Differentiated cells can be expanded from EB either by disruption or plating of intact EB straight into tissue culture dishes (Wiles, 1993). This latter method has the added benefit of allowing continual harvesting of the macrophages over several weeks permitting larger numbers to be produced. In an attempt to increase the numbers of cells generated/harvested, ES cells were differentiated in M-CSF or GM-CSF plus IL-3 and the EB either disrupted or plated intact. 14 days after plating, differentiated cells were harvested using ice-cold PBS/EDTA solution and analysed by flow cytometry for surface expression of F4/80 and CD11b.

Plating of intact EB cultured in M-CSF gave rise to a higher percentage of positive cells within the macrophage gate. CD11b was expressed by 54% of the cells and

66% were expressing F4/80 compared to only 8% of cells positive for either marker in the cultures where the EB had been disrupted (Fig 3.12a). Cells cultured in GM-CSF also demonstrated a similar pattern of expression of F4/80 and CD11b. Again, plating of EB produced higher numbers of cells expressing either marker compared to those that had been disrupted (Fig 3.12b). Nevertheless, regardless of the method of plating or the cytokine used only small percentages of the whole populations lay within the macrophage gates suggesting that the macrophages were not successfully being removed from the plates.

3.3.3 Harvesting of differentiated cells from plated EB

In the experiments discussed above it was highlighted that the percentage of macrophages in the harvested cells was extremely low. This could have been due to the ES cells not differentiating as well as before or that the macrophages were not being removed from the plates. Several different approaches and cell detachment agents were assessed for their ability to lift the macrophages.

The first approach taken was adapted from that previously by reported Moore *et al.* (Moore et al., 1998). Single EB were plated into each well of a 24 well plate, after 7 days and every two days thereafter the supernatants were harvested and re-plated into fresh 24 well plates. Very few macrophage-like cells were harvested and after 2-3 harvests the EB plates stopped producing cells. The experiment was repeated but ~10 EBs were plated into wells of a 6 well plate and left for 14 days. Cells were harvested by gentle washing of the plates with culture medium and the cells re-plated into fresh plates. After 2-3 days the re-plated cells were examined by light microscopy. Unexpectedly there were no cells with macrophage morphology and instead, the cells looked like ES cells (Fig 3.13a&b).

The second approach was to compare the ability of ice-cold PBS/EDTA, trypsin and collagenase to detach the macrophages from the plates. Figure 3.13d-f shows a panel of phase contrast micrographs that give an overview of the diversity of macrophage clusters observed within the culture plates. 20-30 EB were plated per 10 cm tissue

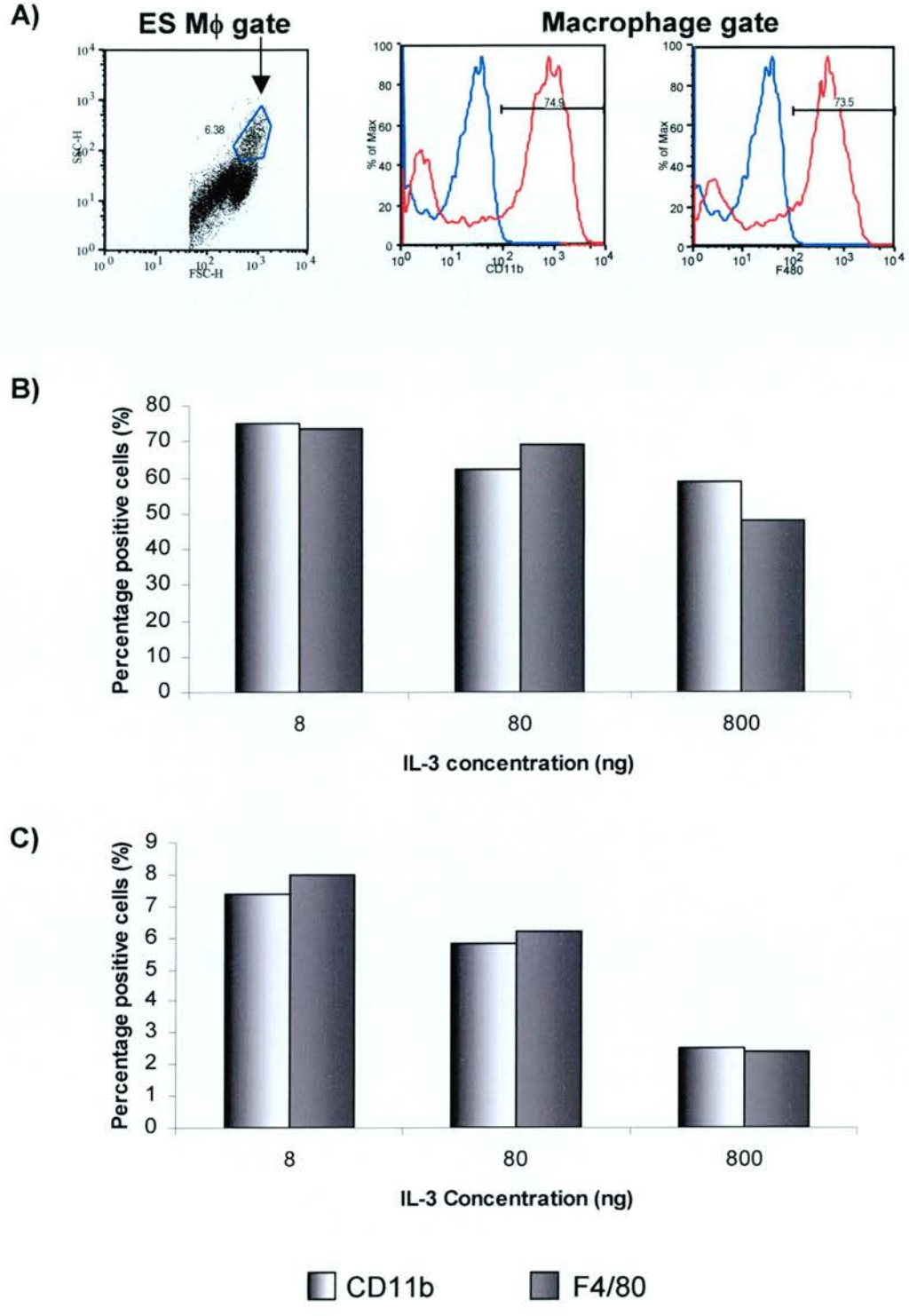


Figure 3.11: Effect of IL-3 concentration on macrophage differentiation. Differentiation of EFC-1 cells was carried out in increasing concentrations of IL-3 and the differentiated progeny analysed by flow cytometry for the percentage of F4/80 and CD11b positive cells. A) representative flow cytometry plots of cells cultured in 8ng of IL-3. Bar graphs showing the percentage of F4/80 and CD11b positive cells in the gated macrophage population (b) and the whole population (c) .

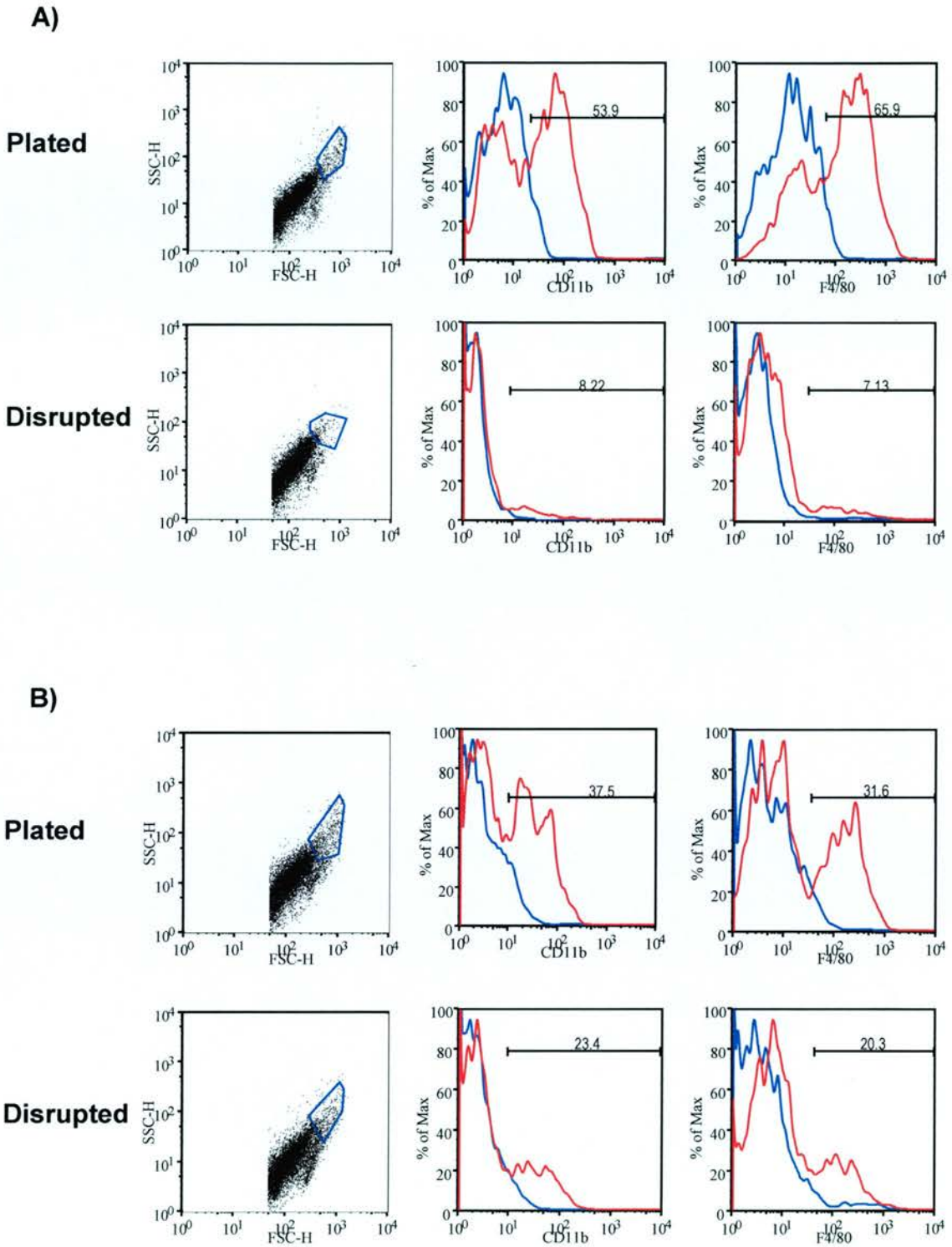


Figure 3.12: Effect of disruption versus plating on macrophage number. EB generated by differentiating EFC-1 cells in IL-3 and either M-CSF (a) or GM-CSF (b) were either disrupted with collagenase before plating or plated straight into tissue culture dishes. The differentiated progeny were analysed by flow cytometry for the percentage of F4/80 or CD11b positive cells.

culture dish and left to adhere and spread (Fig 3.13c). After 14 days in culture larger rounded cells thought to be macrophages appeared and were quite often encompassed by the plated EB (Fig 3.13d). Other areas of the cultures were colonised by very large, flat cells that seemed to provide a matrix for the macrophages (Fig 3.13e) or by clusters of macrophages that settled away from the regions where EB had plated (Fig 3.13f). To harvest the macrophages the plates were incubated with PBS/EDTA, trypsin or collagenase and passed through an 80μm cell filter to remove the larger EB. The cells were then analysed for surface expression of F4/80 and CD11b. None of the cells analysed by flow cytometry were positive for either marker (data not shown) suggesting that little or no macrophages were present in the cultures or that the cells were lost during the filtering step.

3.3.4 Magnetic cell sorting of ES derived macrophages

To circumvent the problems with harvesting and to enrich for the macrophages that were not adherent to the EB layer the cultures were sorted using magnetic beads. 14 days after plating $9x10^6$ cells were harvested from the plates using ice cold PBS/EDTA and incubated with CD11b-PE. The labelled cells were then incubated with anti-PE microbeads (Miltenyi Biotec) and bound to a magnetic cell separator (MACS, Miltenyi Biotec). Unsorted cells and both the unretained and retained fractions were analysed for morphology and surface expression of F4/80. Labelling of the unsorted population with F4/80 showed that 64% of the high FSC/SSC cells gated were indeed macrophages. Sorting of the cells with the magnetic beads clearly enriched for F4/80 positive (retained fraction) cells (Fig 3.14a). The sorted cells when plated showed strikingly different morphology (Fig 3.14b). The F4/80 positive cells grew predominately in a monolayer with some sparsely populated regions. At a higher magnification these cells distinctly looked like macrophages unlike the F4/80 negative fraction where the cells were more fibroblastic in morphology. The method of magnetic cell sorting was successful in generating relatively pure (85%) populations of macrophages however, the numbers of macrophages harvested was

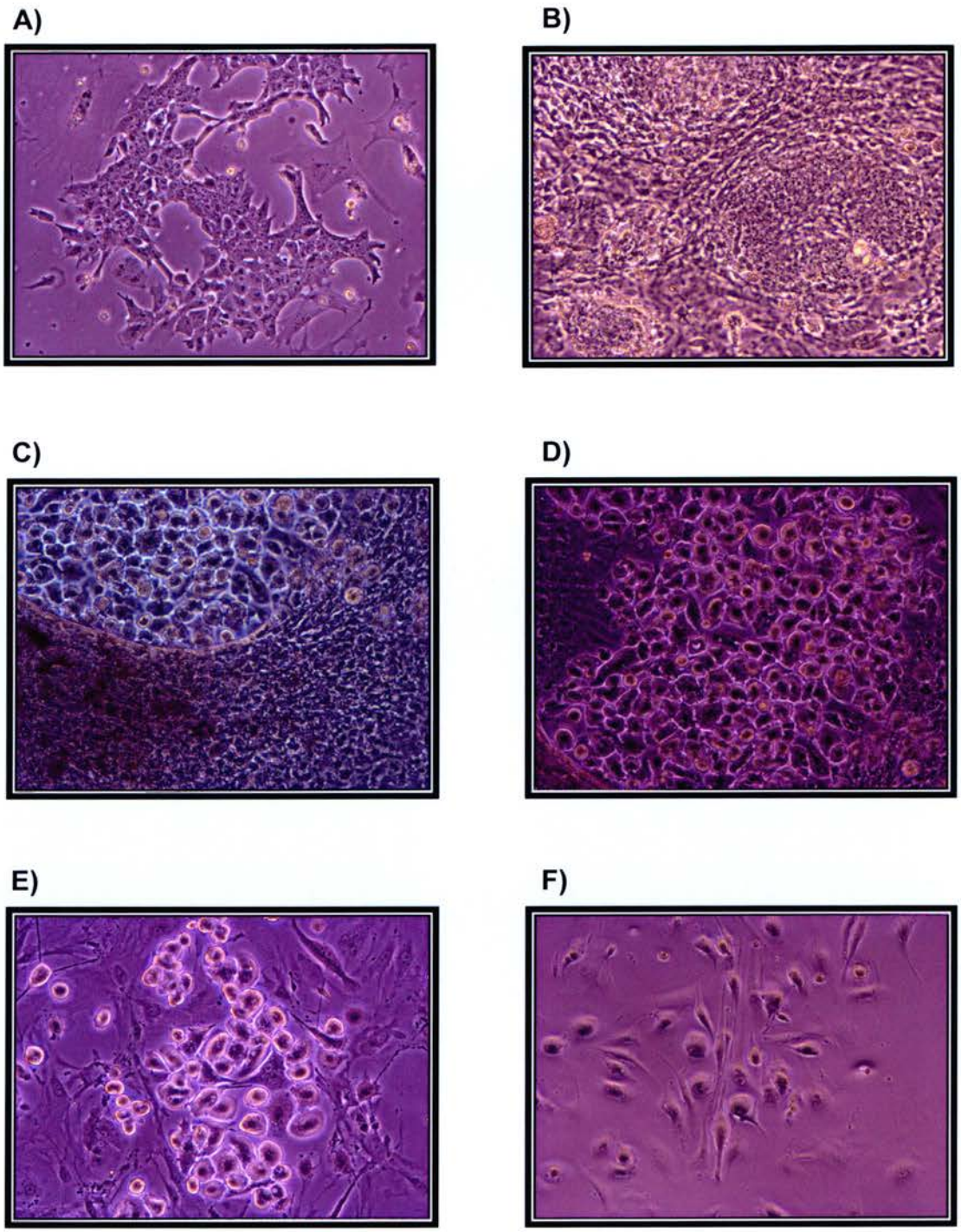
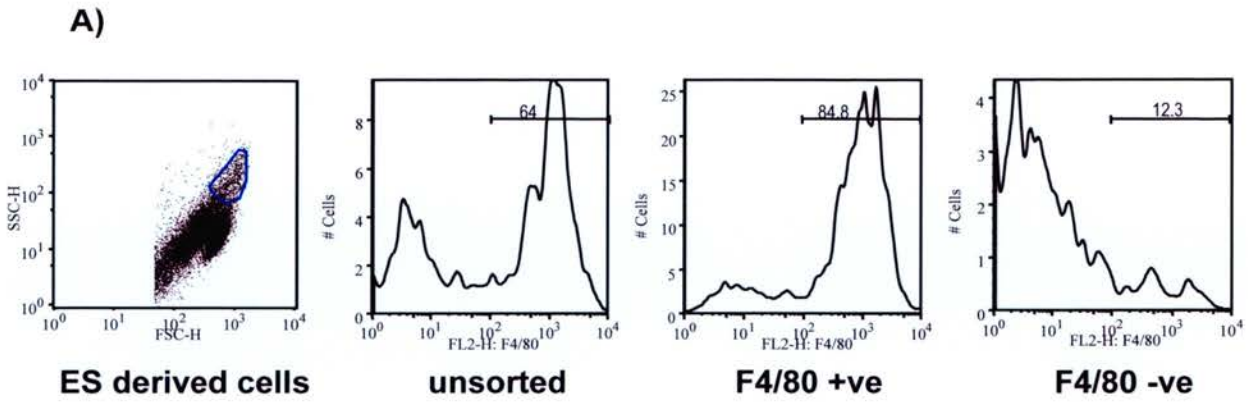


Figure 3.13: Morphology of ES-derived macrophages after plating. A & B) Cells that have been harvested in the culture supernatants and re-plated. C) Small area of a plated EB. D) example of ES macrophages encompassed by the EB. E) macrophages growing at the edge of an EB. F) ES macrophages that have adhered to the plastic and spread.



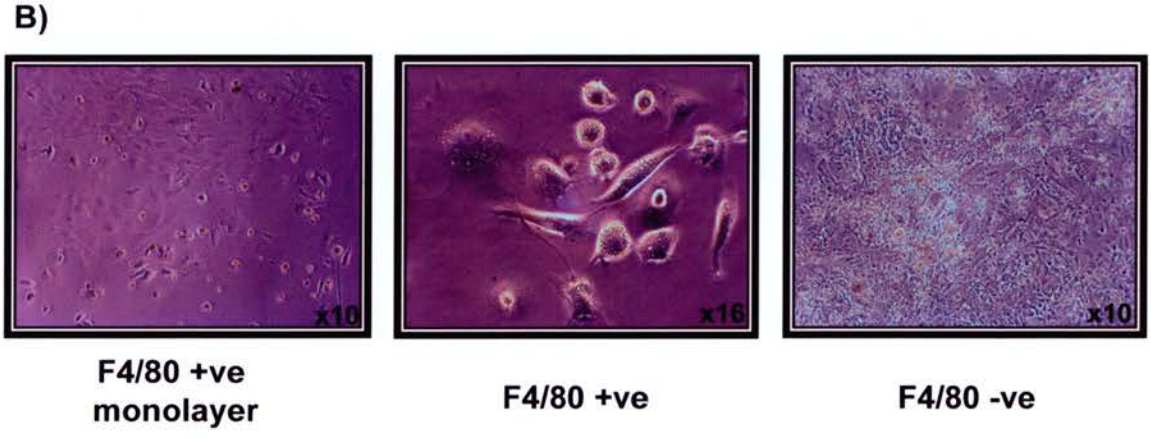


Figure 3.14: Magnetic cell sorting of ES derived macrophages. Differentiated cells were labelled with F4/80 antibody, incubated with beads and bound to a magnetic cell sorter. Unretained and retained fractions were collected and analysed for morphology (a) and surface expression of F4/80 (b).

still of some concern as only 7% of the total number of cells harvested were retained in the positive cell fraction.

3.3.5 Effect of serum change on EFC-1 differentiation

At this time the data discussed earlier in this chapter was presented as a poster at a British Society of Immunology meeting where it was suggested that changing the serum used to differentiate the cells might improve the numbers of cells generated (Paul Fairchild, personal communication). Previous differentiations had been carried out using serum that had been pre-tested to support the *in vitro* differentiation of ES cells (Stem Cell Technologies) and was substituted with serum purchased from Labtech.

In an endeavour to overcome the problems experienced with harvesting, it was also decided to try and generate dendritic cells (DC) that would be less adherent than macrophages. The EFC-1 ES cells were differentiated using a protocol based upon the method described by Fairchild *et al.* (Fairchild et al., 2000). ES cells were plated in the absence of cytokines into 10cm Petri dishes and left to form EB. After 3 days spherical EBs had appeared but by day 5 they had attached to the surface of the dishes and spread, losing their differentiation potential. The bacterial dishes used for this experiment may have been slightly "sticky" so it was decided to induce EB formation in the MeC cultures previously used.

An outline of the culture conditions is given in Figure 3.15a. ES cells were cultured either in MeC supplemented with 1000u/ml IL-3 and 25ng/ml GM-CSF from the onset of differentiation (+/+) or at the time of EB plating (–/+). At day 3 of differentiation small spherical EB had formed in both sets of cultures indicating that IL-3 and GM-CSF had no effect upon the early stages of differentiation (Fig 3.15b upper panel). By day 14 the EB had considerably increased in size and were surrounded by hematopoietic colonies. No differences were observed between the two conditions except that EB cultured in the presence of cytokines were surrounded by greater numbers of differentiated cells (Fig 3.15b lower panel). However, this

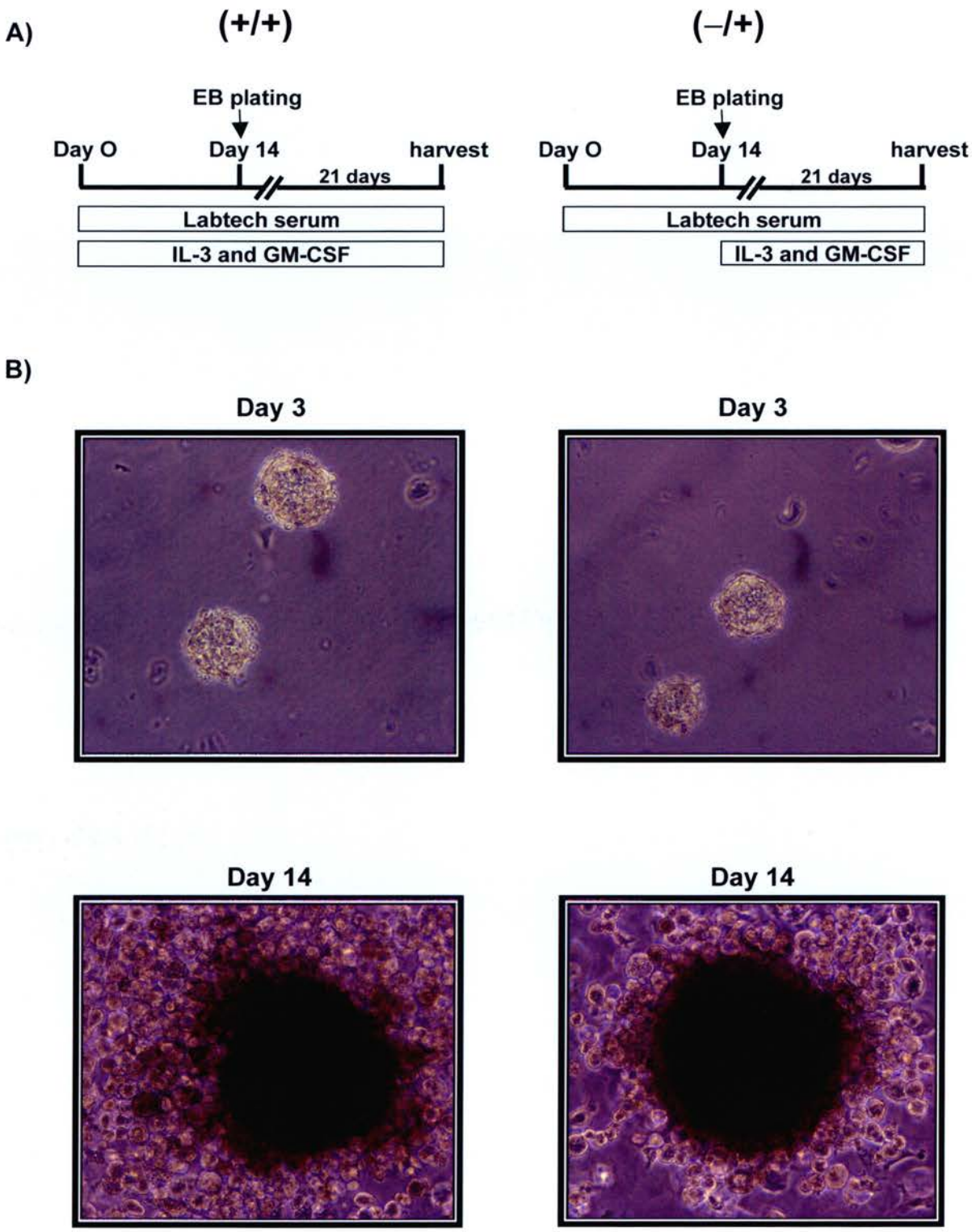


Figure 3.15: Effect of serum change on EB formation. EFC-1 ES cells were differentiated using Labtech serum in the presence or absence of cytokines. A) overview of the culture conditions for cells that were grown in cytokines from initiation of differentiation (+/+) and cells that were only exposed to cytokines at the time of plating (–/+). B) Representative phase contrast micrographs of EB at days 3 and 14 of differentiation for both culture conditions. Magnification x10.

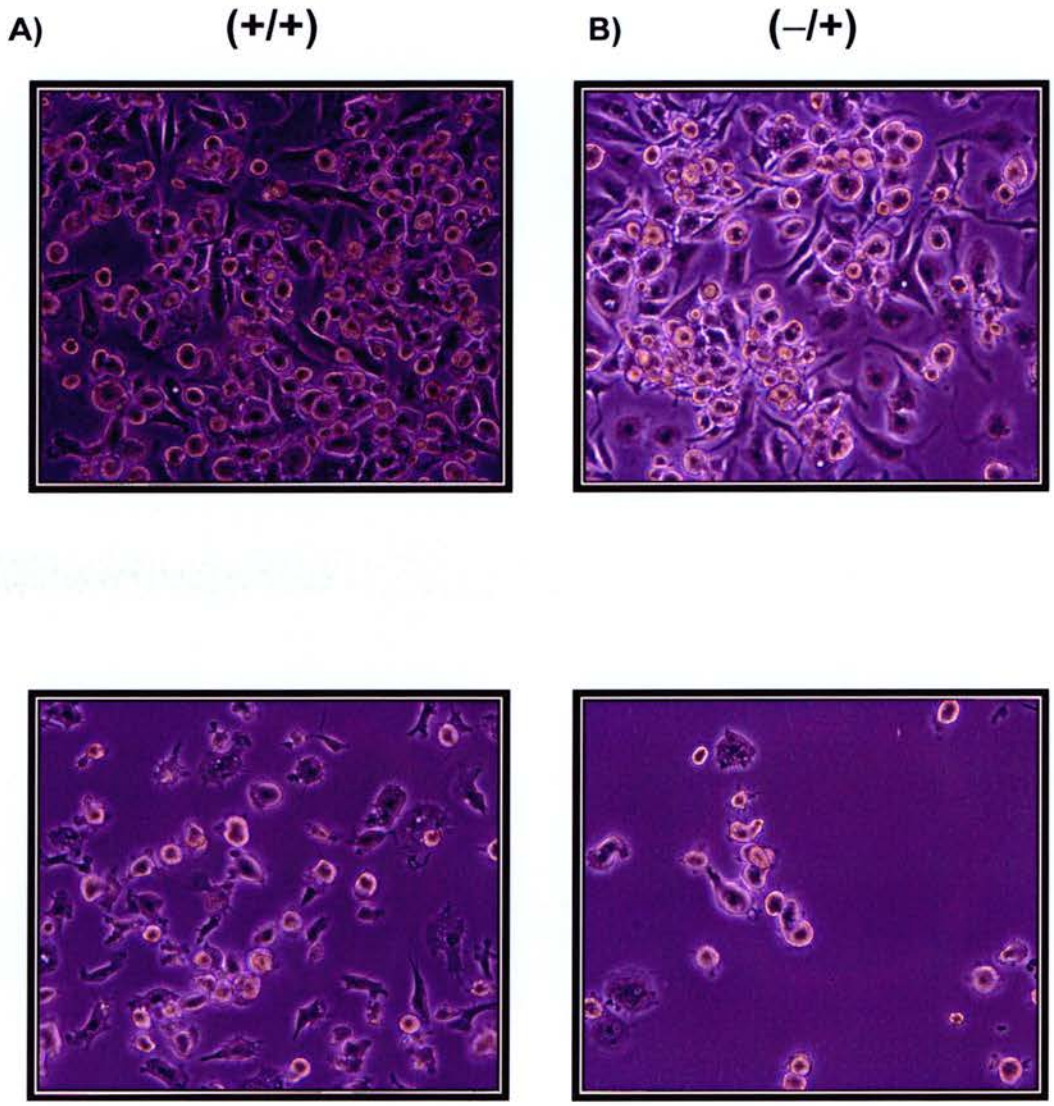


Figure 3.16: Effect of Labtech serum on differentiated progeny. EB differentiated in Labtech serum for 14 days either in the presence or absence of cytokines were plated and cultured for a further 21 days in the presence of cytokines. Representative phase contrast micrographs are shown for cells cultured in IL-3 and GM-CSF from initiation of differentiation(+/+) (A) and cells cultured in IL-3 and GM-CSF from day of plating (–/+) (B).

observation was not definitive as there was great variability in the numbers of cells surrounding any one EB independent of the culture conditions used.

The differentiated progeny arising from EB cultured in the presence or absence of cytokines were very similar but exhibited morphology markedly different to that seen in former differentiations. As before, the cultures were quite heterogeneous in that some areas of the culture plates were more densely populated than others, this was especially evident in the regions surrounding the plated EB. Initially, the cells that appeared formed a confluent monolayer encircling the plated EB, as time progressed smaller more irregular shaped cells emerged and began to aggregate loosely adhering to the monolayer (Fig 3.16a&b). Fewer cells were found in areas where there was no EB but these did become confluent if the plates were left in culture for several weeks. Figure 3.16a&b lower panels show more clearly the protrusions emanating from the smaller cells indicating a more dendritic like morphology compared to the more rounded macrophage morphology observed in earlier differentiations. To determine whether these cells were in fact DC-like, the cells were analysed by flow cytometry for surface expression of several DC markers. The loosely adherent clusters were harvested by gentle washing with PBS and labelled with F4/80, CD11c, CD40, CD80 and CD86. Analysis of the cells by FSC/SSC showed less debris in the cultures and a more distinct population of cells in both of the culture conditions compared to earlier ES Mϕ cultures. Cells cultured in cytokines from the onset generated higher numbers of cells (64.8%) that could be gated by FSC/SSC compared to the cells supplemented with cytokines at the time of plating (36.9%)(Fig 3.17). Despite this difference in gated cell number the surface phenotype of both sets of cells was identical. F4/80 was expressed at moderate levels although to a slightly greater degree in the cells that had been exposed to cytokines for longer. The differentiated cells expressed relatively high levels of the DC marker CD80 and moderate levels of CD11c but lacked surface expression of CD40 and CD86 (Fig 3.17). Expression levels for each of the DC markers were comparable between both populations and were similar to that described for un-stimulated bone marrow derived dendritic cells.

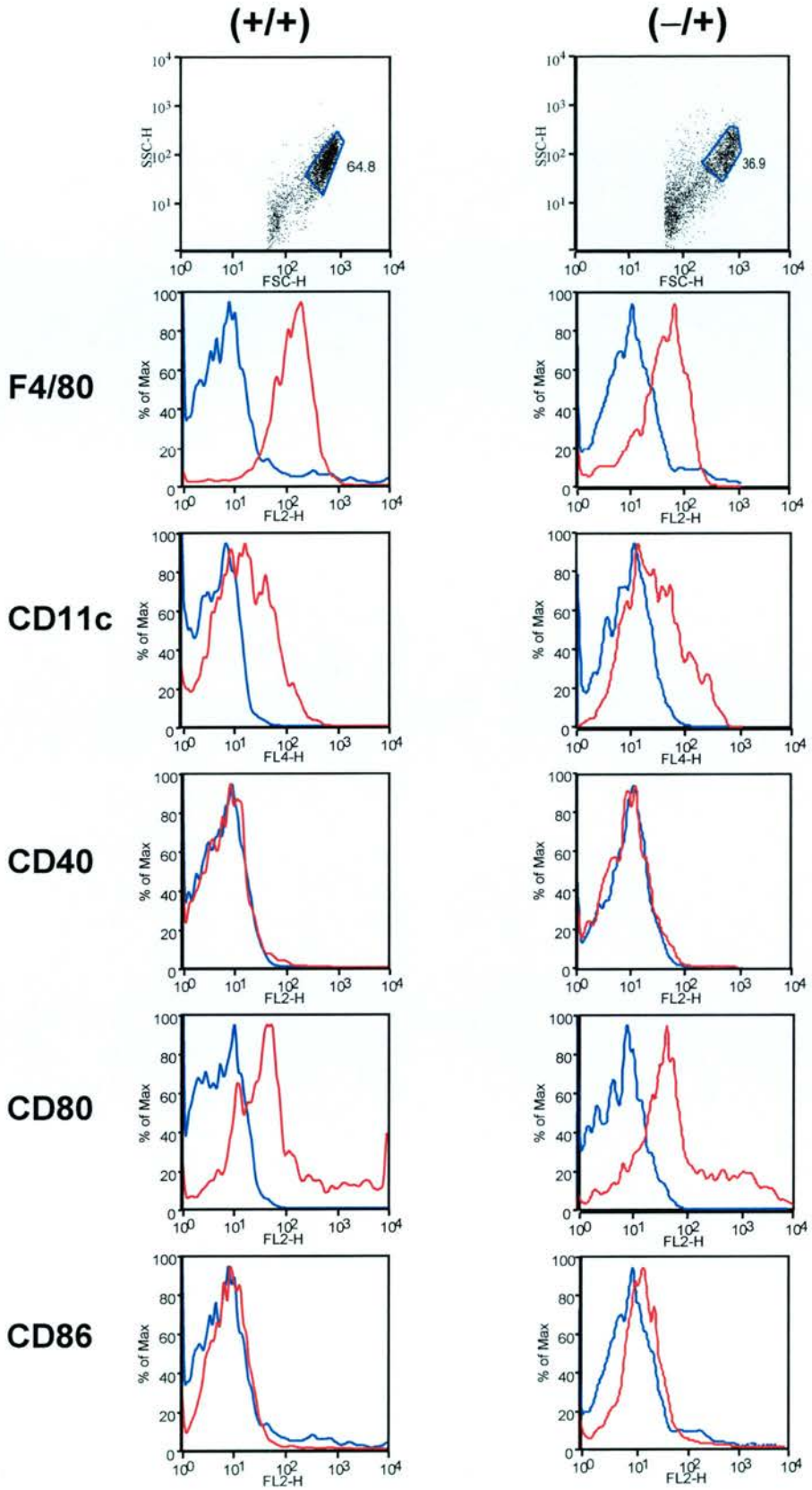


Figure 3.17: Surface phenotype of differentiated progeny cultured in Labtech serum. Cells harvested from cultures grown in either (+/+) or(–/+) cytokines were analysed for surface expression of F4/80 and the dendritic cell markers CD11c, CD40, CD80 and CD86.

3.4 Differentiation of E14 ES cells

3.4.1 Targeting of integrin α_v in the E14 ES cell line

The work presented in the first part of this chapter was carried out using the EFC-1 ES cell line that is routinely used by others for ES differentiation studies. However, prior to the beginning of this project, the integrin α_v gene was targeted with LoxP sites in a different ES cell line (E14 ES cell line) by Dr Adam Lacy-Hulbert. These were used to generate integrin α_v floxed mice in work that ran closely in parallel with the ES differentiation work presented here. The strategy used to target integrin α_v is described in figure 3.18. The targeting vector comprises of a neomycin resistance cassette flanked by two Frt sites inserted upstream of exon 4 of the integrin α_v gene. The Frt NEO cassette and Exon 4 also flanked by two LoxP sites. ES cells were transfected with the targeting vector and those containing the targeted locus selected for using G418. The targeted clones were then grown in increasing concentrations of G418 to drive recombination in the second allele (Mortensen et al., 1992). ES cells containing the double-targeted locus were transfected with the FLP recombinase to delete the neomycin resistance cassette producing a silently targeted integrin α_v gene. These ES cells were used to generate integrin α_v floxed mice. To produce ES that were lacking the integrin α_v gene the ES cells could also be transfected with Cre recombinase although this was not done for these experiments.

3.4.2 Serum batch testing for the parent E14 ES cell line

Having established and optimised the method of differentiating ES cells with the EFC-1 cell line it was important to determine if the cell line we had used in our targeting studies was also capable of generating macrophages and dendritic cells. As discussed, variations in batches of serum can affect both the growth and differentiation of ES cells. Five different batches were tested for their ability to support EB formation using the E14 ES cell line. Each batch was tested in triplicate either in the presence or absence of IL-3 and GM-CSF and 400 ES cells plated per

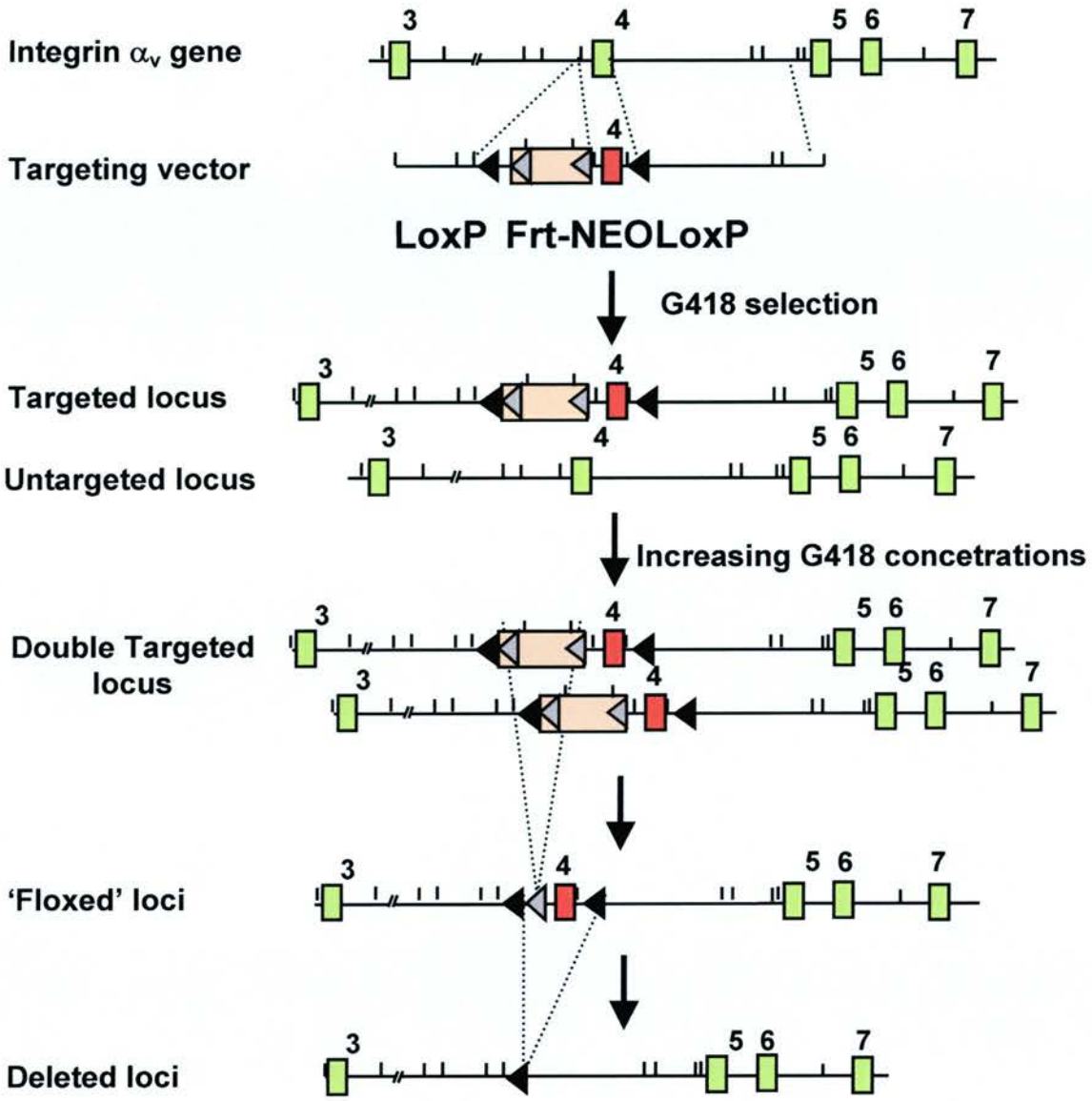


Figure 3.18: Targeting strategy for the deletion of the integrin α_v gene. Integrin α_v targeting was carried out by Dr Adam Lacy-Hulbert. E14 ES cells were transfected with the targeting vector and positive clones selected for using G418. Positive clones containing on targeted locus were then cultured in increasing concentrations of G418 to drive recombination in the second locus. Transfection of the cells with Flp recombinase removes the neomycin resistance cassette and leave double floxed loci. Subsequent transfection with Cre recombinase would allow the deletion of the integrin α_v gene although this was not done in these experiments.

condition. After 14 days the cultures were scored for the number of EB formed and the appearance of hematopoietic colonies.

The average number of EB produced for each of the five batches was between 40-70 per culture. The addition of cytokines to the system did not affect the total numbers of EB obtained except for batch 2 where the numbers of EBs increased from 45 to 72 (Fig 3.19a&b). In the absence of cytokines, hematopoietic colonies were still generated, however the numbers increase upon the addition of IL-3 and GM-CSF. Batch 2 showed the greatest increase with the numbers of EB plus hematopoietic colonies rising from 10 to 30 (Fig 3.19a&b). The ratio of total EB to those expressing hematopoietic colonies was the highest for batch 5 although the actual number of hematopoietic expressing EB was not that different from batch 2 (Fig 3.19a). Batch 4 was not affected by the presence of cytokines (Fig 3.19a&b).

Overall the addition of cytokines increased the frequency of EB associated with hematopoietic colonies. These EB were plated onto tissue culture plastic and left for a further 21 days. The differentiated cells produced were analysed for the expression of the surface markers F4/80 and Cd11b to determine which of the five batches would be used in subsequent experiments. Figure 3.20a shows how the flow cytometry data was analysed for each batch, using batch 5 as an example. The cells were harvested by gentle washing with PBS and each sample stained for F4/80 APC, CD11b FITC or with the relevant isotype controls. The cells were first gated upon by FSC/SSC and then the gated cells plotted as histograms (Fig 3.20a).

The differentiated cells arising from each of the five batches were similar in that 50-80% of each cell population expressed the two myeloid cell markers F4/80 and CD11b (Fig 3.20b). Batches 2 and 5 produced a marginally higher percentage of positive cells although no differences were observed between each of the batches. From this data it was decided to use batch 5 for future experiments as it gave the highest ratio of EB expressing hematopoietic colonies to total number of EB

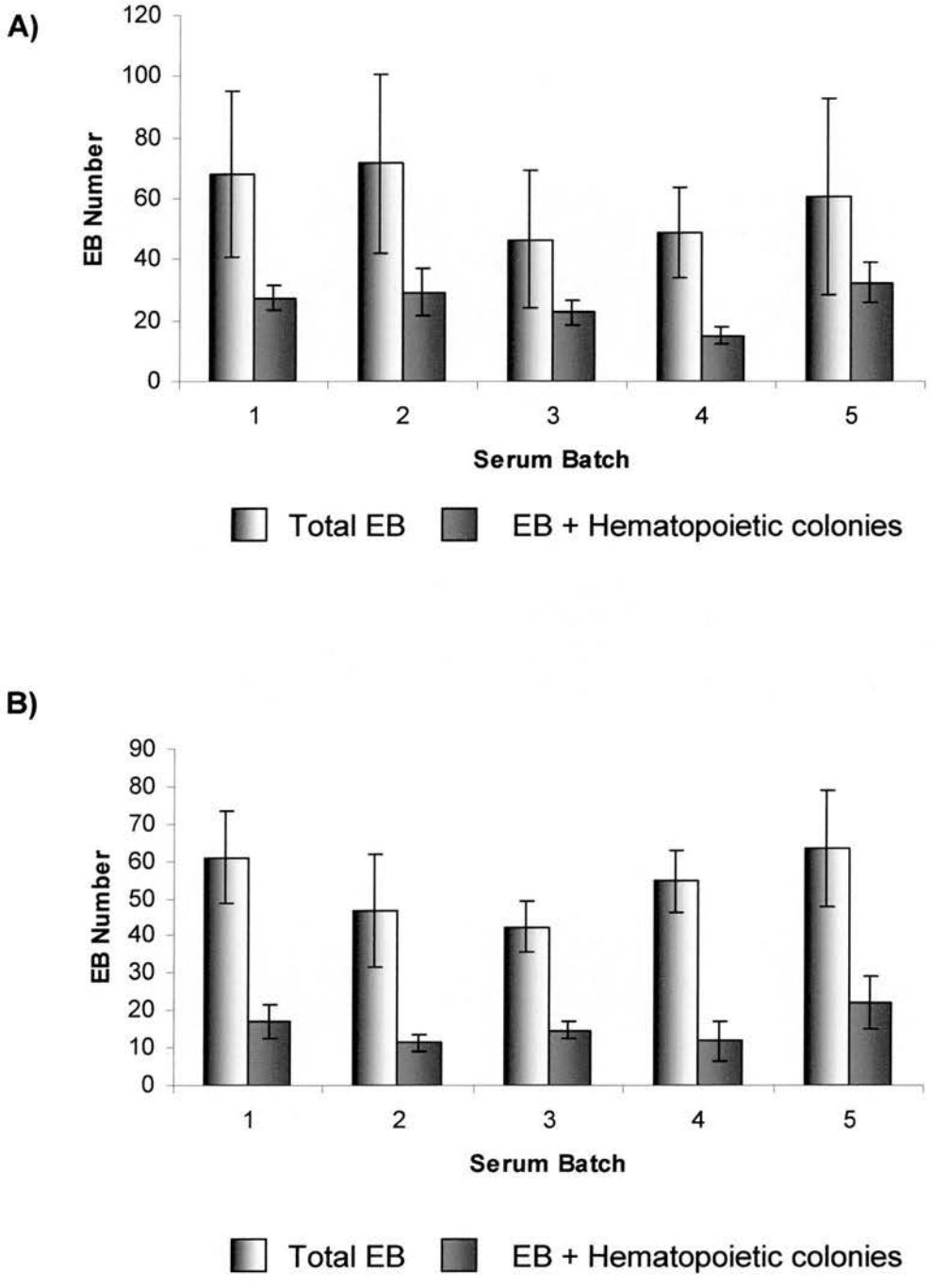
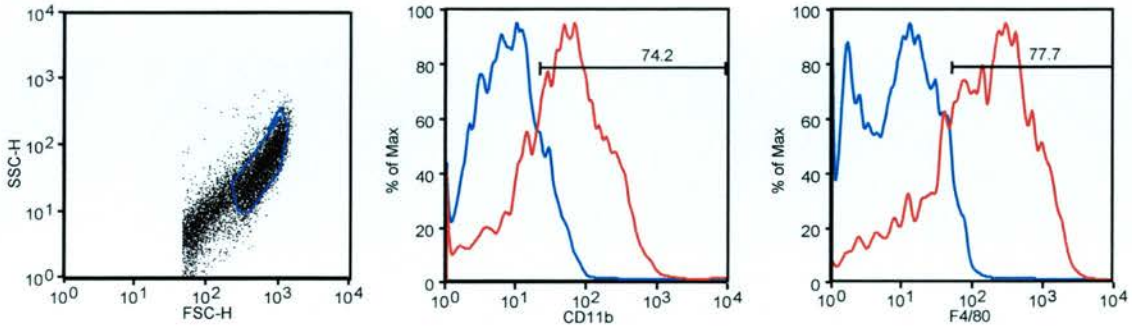


Figure 3.19: Serum batch test. Five different serums were tested for their ability to support EB formation. E14 ES cells were cultured in MeC with (a) or without (b) cytokines for each serum. At day 14 the cultures were scored for EB formation and the presence of hematopoietic colonies. Results expressed as the mean of one experiment performed in triplicate +/- standard deviation.

A)



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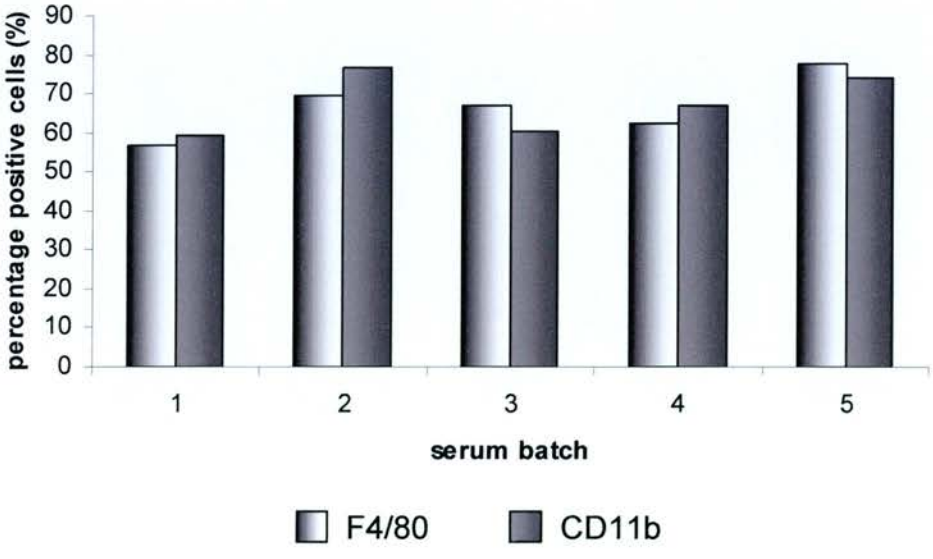


Figure 3.20: Surface phenotype of differentiated cells from serum batch test. At day 14 EB for each serum were plated in differentiation medium with cytokines and cultured for a further 21 days. The cells were then analysed by flow cytometry for expression of the surface markers F4/80 APC and Cd11b FITC. A) Representative analysis of flow data using serum 5. B) Bar graph showing the percentage of F4/80 and Cd11b positive cells for each batch.

formed and produced a slightly higher percentage of myeloid cells in the differentiated cultures.

3.4.3 Cell number titration for the parent E14 cell line

The optimal seeding density of ES cells at the initiation of differentiation varies depending on the cell line used. During the serum testing it was noted that the MeC media was becoming exhausted by day 7 of differentiation, this may have been due to the number of ES cells plated or that the EB were attaching to the culture dish promoting rapid cell proliferation. It was therefore necessary to titrate the number of E14 ES cells plated into the MeC cultures and the percentage of MeC used.

E14 cells were plated in the presence of IL-3 and GM-CSF at a cell density of 50, 100, 200, 300 or 400 cells per culture in either 0.9% or 1.1% MeC. Cultures were set up in triplicate and after 14 days scored for the number of EBs formed, the presence of hematopoietic colonies and the colour of the medium. A score of one indicated that the media was still pink at day 14, two indicated that the media was orange and three indicated that the media was exhausted. Figure 3.21 shows that the number of EB formed at each cell density was consistently higher in the 1.1% than the 0.9% MeC cultures. In contrast the number of EB associated with hematopoietic colonies was comparable between the two sets of cultures (Fig 3.21a&b). However, at a seeding density of 300 cells per dish the medium colour differed between the two percentages of MeC. At day 14 the cells plated in 0.9% MeC had depleted the medium turning it orange/yellow (score 3) in colour whereas in the 1.1% cultures the medium was still pink (score 1) (Fig 3.21c). In addition, it was also observed that in 1.1% MeC the EB remained suspended unlike in the 0.9% cultures where they appeared to be settling near the bottom of the plate. Based upon these observations it was decided only to analyse the differentiated progeny from the cultures seeded at a density of 300 cells per plate in 1.1% MeC and that these were the optimum conditions for differentiating the E14 cell line.

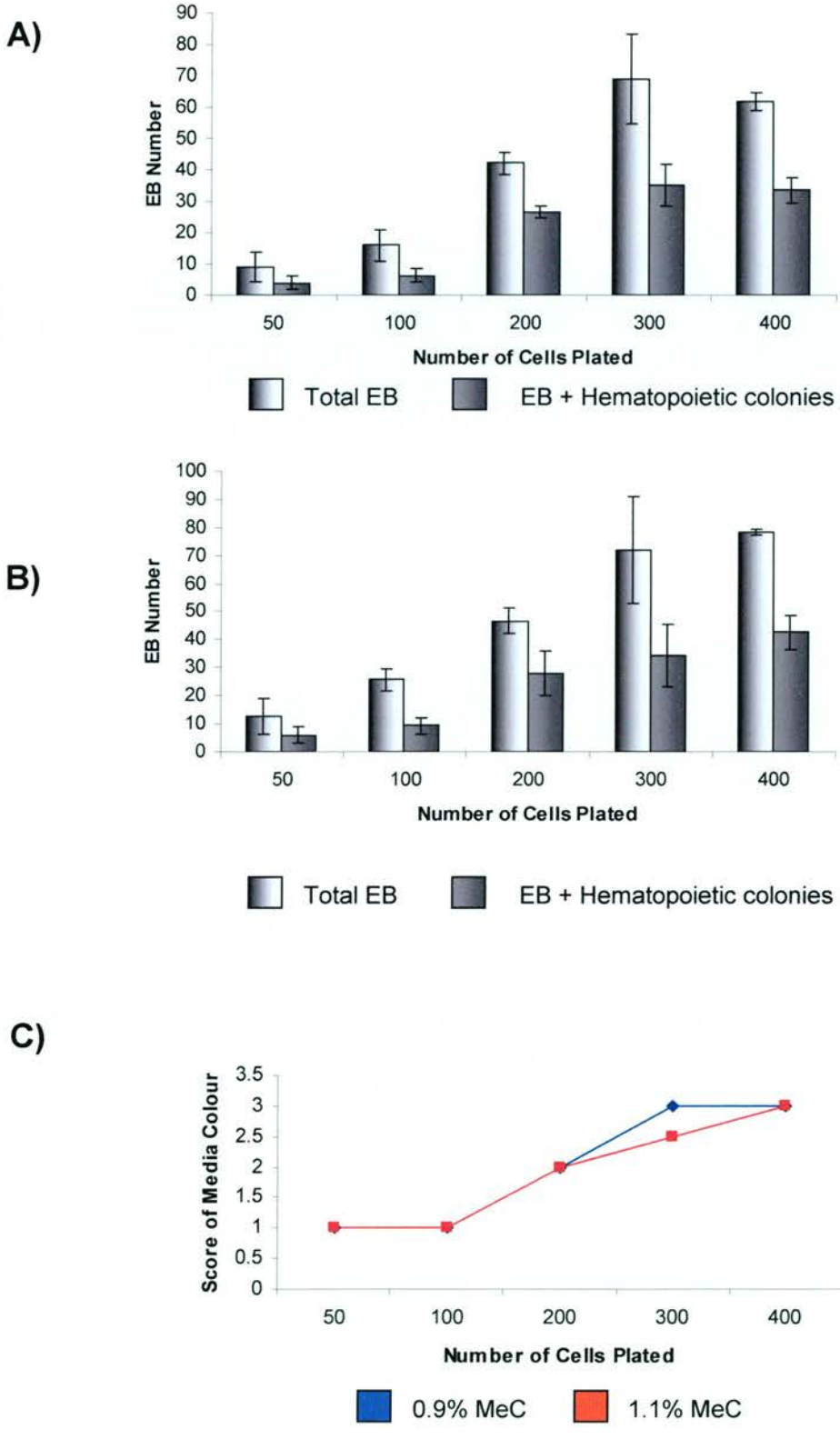


Figure 3.21: E14 seeding density titration. ES cells were plated in 0.9% (a) or 1.1% (b) MeC supplemented with cytokines and cultured for 14 days. Cultures were set up with 50, 100, 200, 300 or 400 cells plated per dish and scored for EB formation, the presence of hematopoietic colonies and medium colour. A score of 1 indicates the media was still pink, two indicates that the media was orange and a score of 3 indicates the media was exhausted. Results expressed as the mean of one experiment performed in triplicate +/- standard deviation.

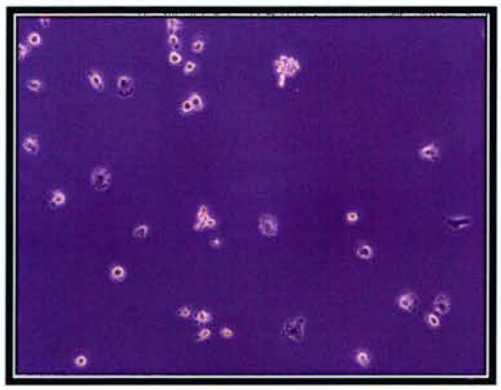
3.4.4 Morphology and Phenotype of differentiated E14 cells

Dendritic cells derived from both bone marrow and ES cells have a tendency to grow in small clusters of cells (Fairchild et al., 2000; Senju et al., 2003). E14 ES cells that had been cultured at a seeding density of 300 cells per plate in 1.1% MeC and allowed to form EBs were plated and left in culture for 21 days. Figure 3.22 shows representative pictures of the E14 differentiated progeny at days 7, 14 & 21 after plating. At day 7 the EBs had adhered to the tissue culture plastic and spread. In areas of the culture dish not colonised by EBs small numbers of irregular shaped cells were observed, these cells increased in number forming small aggregates that were loosely attached to an adherent monolayer (day 14). By day 21 the small aggregates of cells had formed larger tightly packed clusters of cells that could be harvested by gently washing with PBS. Both the adherent and non-adherent fractions were analysed by flow cytometry.

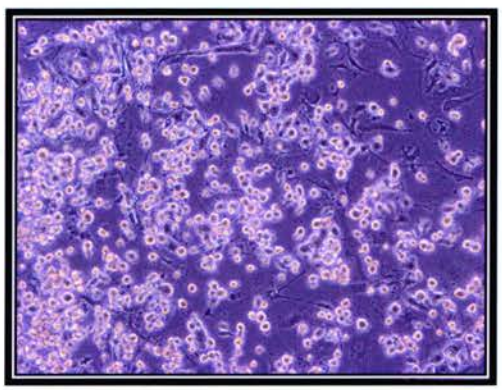
Each fraction was analysed for surface expression of CD11b, F4/80, CD86 and CD11c. Fifty percent of the non-adherent cells were $F4/80^{+}CD11b^{+}CD11c^{+}$ although the surface expression was slightly higher for F4/80 than CD11c (Fig 3.23a). Similar expression profiles were obtained for the adherent cell population. Again, 50% of the adherent cells were $F4/80^{+}CD11b^{+}CD11c^{+}$ positive (Fig 3.23b). Double staining of the adherent cells for CD11b/CD11c revealed a population of cells that were $CD11b^{+}CD11c^{low}$(Fig 3.23b). These cells may have represented a separate population of myeloid cells or may have been cells that are un-activated.

One of the defining characteristics of DCs is their ability to mature upon stimulation with LPS. The non-adherent cells showed a slightly higher percentage of CD11c positive cells than the adherent cells and were used to test the maturation ability of the E14 derived dendritic cells.

Day 7 after plating



Day 14 after plating



Day 21 after plating

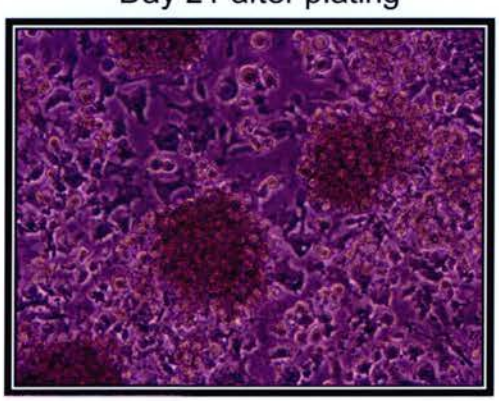
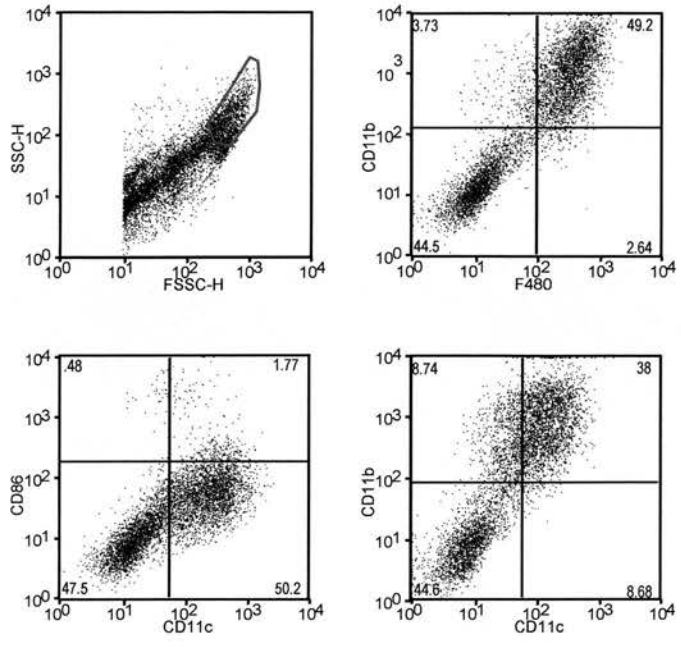


Figure 3.22: Morphology of E14 derived cells after titration of seeding density. Representative phase contrast micrographs of E14 derived cells on days 7, 14 & 21 after plating from ES cells cultured in 1.1% MeC at a seeding density of 300 cells per dish. Magnification x10

A)



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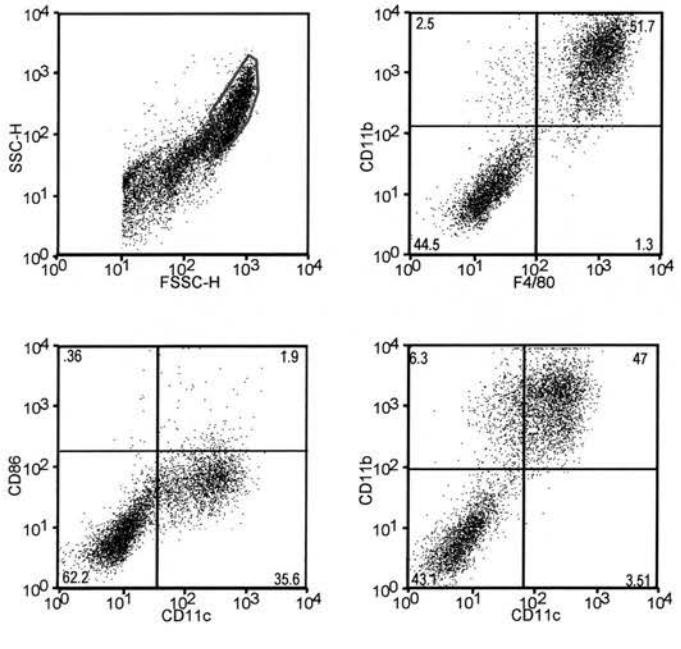


Figure 3.23: Surface phenotype of E14 derived Myeloid cells. EB cultured in 1.1% MeC at a density of 300 cells per dish were plated and cultured for 21 days. Non-adherent (a) and adherent (b) cells were analysed by flow cytometry for F4/80, CD11b, CD11c and CD86 expression, n=1.

3.4.5 Maturation of E14 ES-derived dendritic cells

Figure 3.24 gives an overview of the culture protocol used to harvest mature dendritic cells. DCs clusters were harvested from the EB containing plates by gentle washing and re-plated. 2-3 hours later, maturation was induced by adding 1μg/ml LPS. The next day the mature DCs were harvested as non-adherent cells and plated into fresh media (no LPS). The stimulated plates were also re-fed with fresh medium allowing more of the mature DCs to be harvested at 48 hours.

After re-plating the dendritic cells formed a very heterogeneous population with the DCs either in lightly adherent clusters or non-adherent in the supernatant (Fig 3.25). Figure 3.25a shows a cluster of DCs from a culture stimulated with LPS. At the higher magnification dendrites and veils of cytoplasm could be seen on the cells at the very edges of the clusters. However the mature phenotype was more easily seen in the non-adherent cells (Fig 3.25b). When examined at a higher magnification the non-adherent dendritic cells clearly showed the mature DC phenotype (Fig 3.25c).

Maturation of the DCs in this way resulted in very low yields of the non-adherent mature dendritic cells (~$4x10^5$ from three stimulated plates). The few cells that were obtained from the stimulated plates were analysed by flow cytometry for surface expression of the DC marker CD86 (Fig 3.26). The stimulated cells showed a much tighter population of cells by forward and side scatter suggesting that the maturation of the DC results in a more homogeneous population although the cultures contained a lot of cell debris (Low FSC/SSC). Surface expression of CD86 is clearly upregulated on the cells that have been treated with LPS compared to the control. Taken together the surface phenotype and the morphology of LPS treated cells indicated that the ES derived DCs can be matured and respond in the same way as their bone marrow derived counterparts.

3.4.6 Real-time PCR analysis of phagocytic receptor expression in ES DCs

The phagocytic receptors expressed by ES DCs were assessed using real-time PCR. The transcription of these receptor genes was determined by expression profiling of

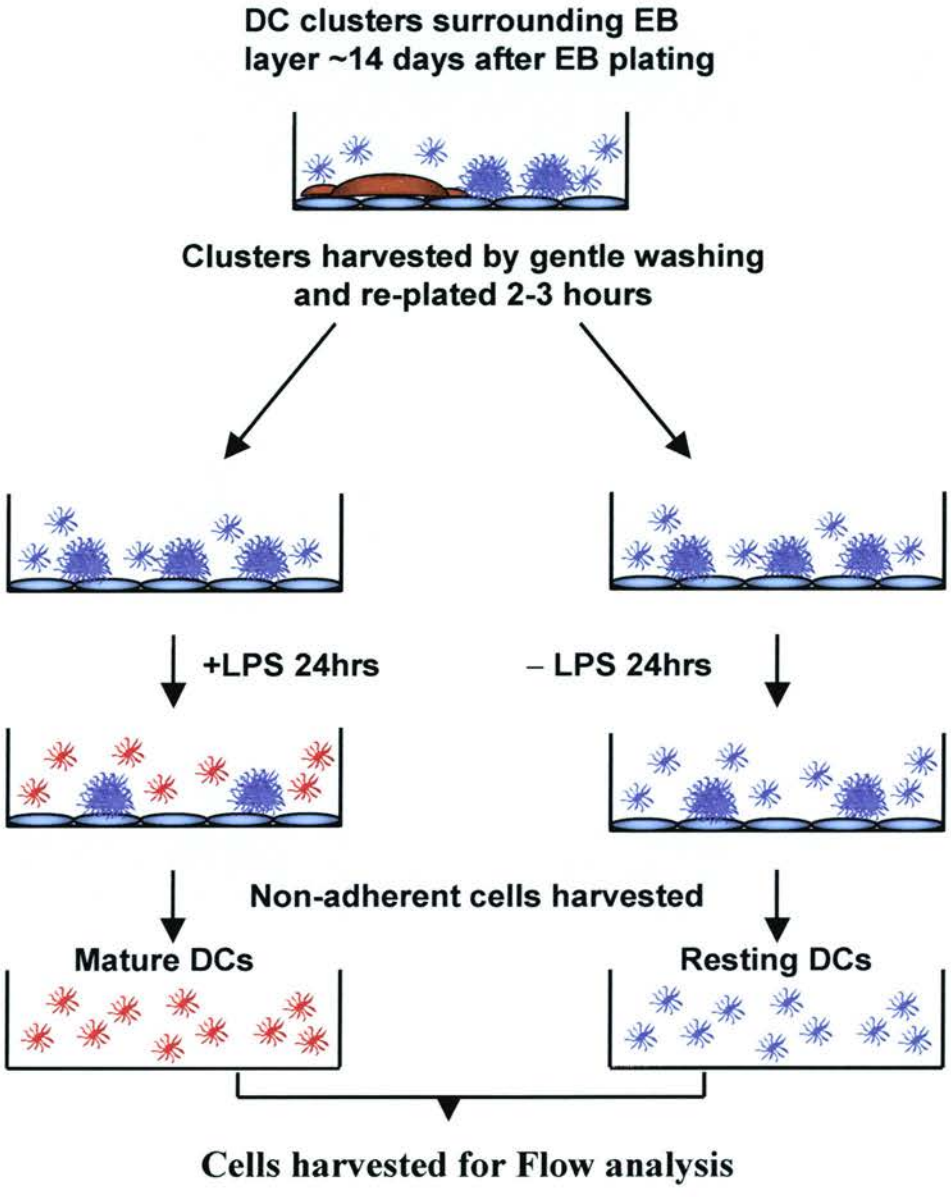


Figure 3.24: Overview of the culture method used for maturation studies. DC clusters are harvested from EB containing plates and re-plated before stimulation with 1μg/ml LPS for 24 hours. The matured DCs were then harvested from the supernatant and plated into fresh media. The stimulated plates were also replenished with fresh media and more of the mature DC collected over the next 48 hours.

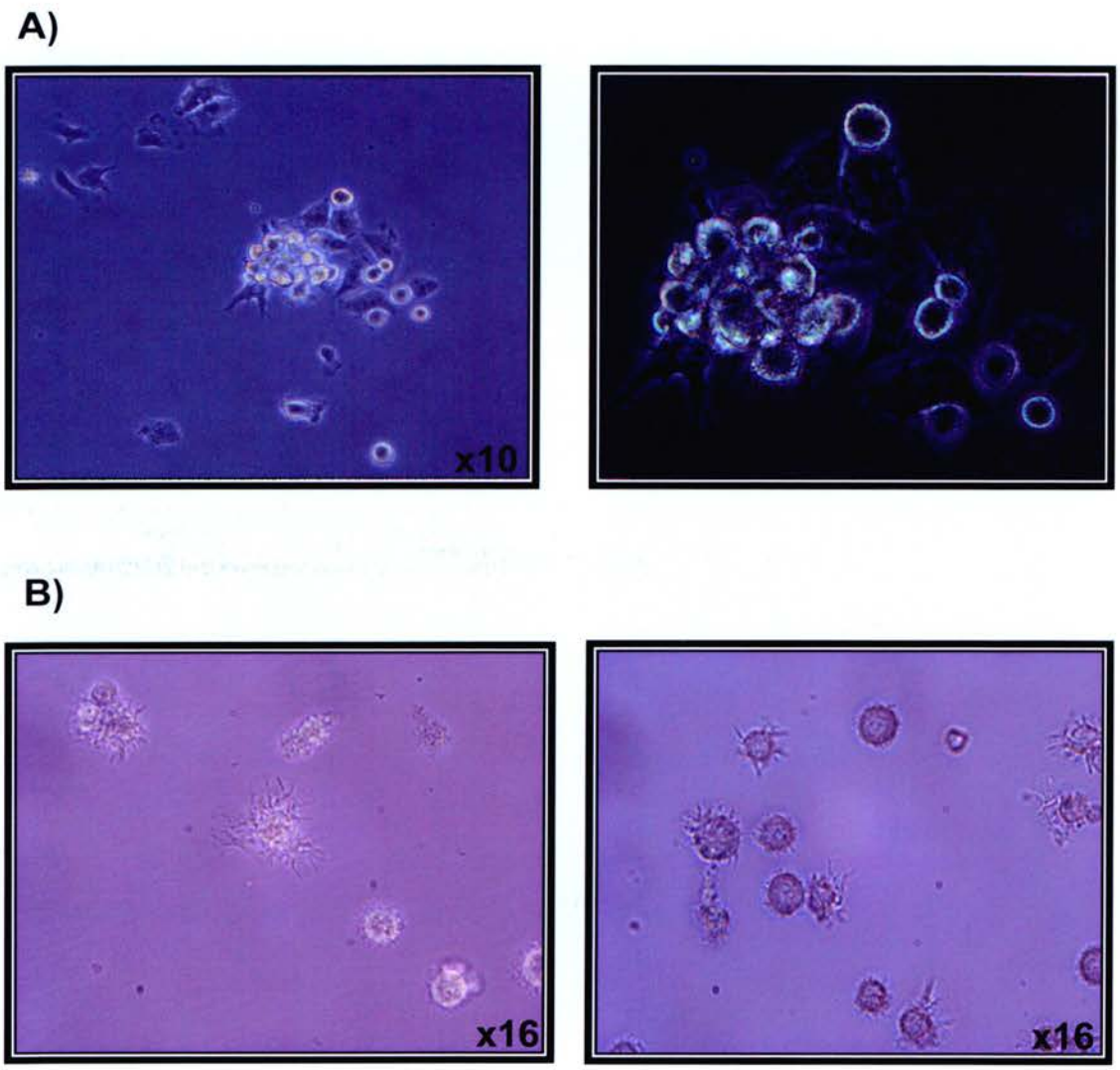


Figure 3.25: Morphology of cells stimulated with LPS.
Representative phase contrast micrographs of ES-derived dendritic cells after stimulation with 1μg/ml LPS for 24 hours. A) clusters of dendritic cells at two different magnifications in the re-plated cultures. B) non-adherent mature dendritic cells.

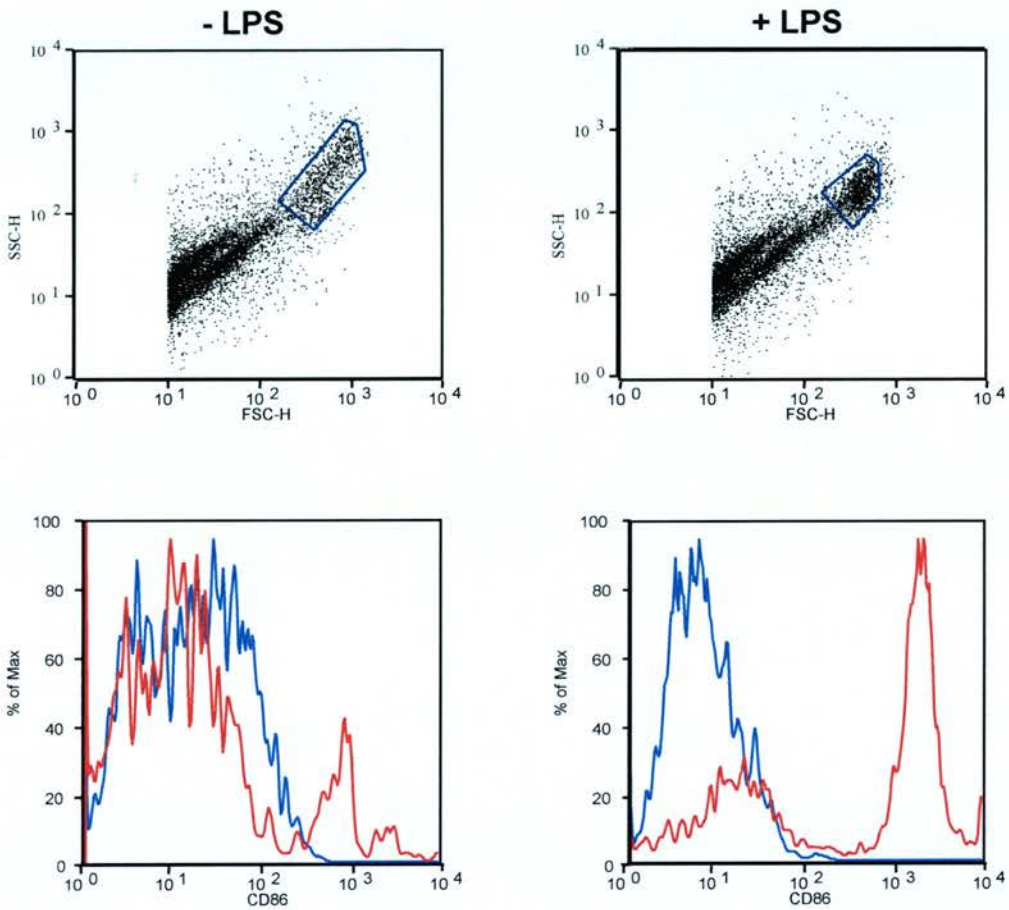


Figure 3.26: LPS Stimulation of non-adherent cells. Non-adherent cells harvested from differentiated E14 ES cells were stimulated with ± 1μg/ml LPS for 24 hours. DCs were gated by high FSC/SSC and analysed for expression of the maturation marker CD86. Isotype control shown in blue.

CD11b sorted cells. Messenger RNA levels are presented relative to expression levels in the well characterised BMDM. The majority of genes were expressed at lower levels than in the BMDM (~10%). This may reflect the internal controls used for real-time PCR, as all transcripts are measured relative to ribosomal RNA. However, CD47, integrin β_3, ABC1, and CD91 were more highly expressed in ES DCs than in BMDM, compared to other genes. CD36 showed much lower expression than in BMDM and TSP 1 showed no expression (Fig 3.27).

3.4.7 Differentiation of putatively integrin α_v disrupted E14 ES cells

The data presented in the first part of this section described the differentiation of the E14 ES cell line and the successful generation of ES derived DCs. The next step was to determine whether ES cells targeted for integrin α_v were capable of differentiation in the same way. Several setbacks were encountered when trying to culture the ES cell lines carrying the floxed loci however more success was achieved with the ES cells containing the double targeted locus including the neomycin resistance cassette (Fig 3.18). The presence of the neomycin cassette results in disruption of the integrin α_v gene effectively creating knockout cells. It was these cells that were used to investigate the role of integrin α_v in the phagocytosis of apoptotic cells by ES derived dendritic cells.

Prior to the differentiation of integrin α_v disrupted ES cells, genomic DNA was isolated and a PCR performed to confirm the presence of the neomycin resistance cassette (Fig 3.28). The primers 4f6 and 5b3 amplified a ~1 kb band that confirmed that the neomycin cassette was present, the absence of a 700bp band showed that the ES cells were targeted in both loci. The integrin α_v disrupted ES cells were differentiated in the same media as the E14 ES cell line. As previously described it was important to establish the optimum seeding density. ES cells were plated at 300, 400, 600, 800, 1000 and 1500 cells per culture in triplicate and after 14 days scored for the number of EB formed and the presence of hematopoietic colonies. The colour of the media was also scored 1, 2 or 3 with a score of 1 indicating the media

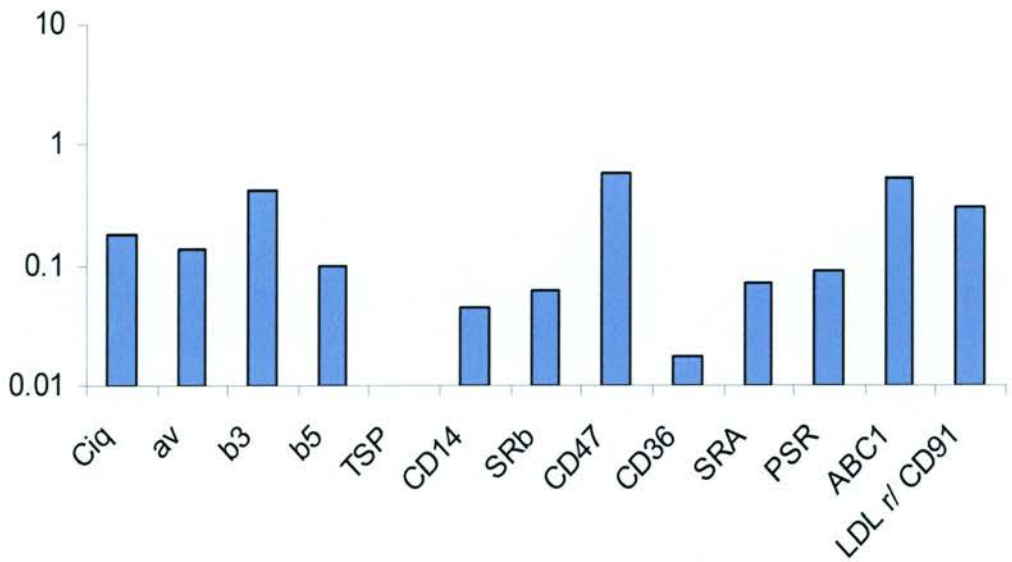


Figure 3.27:Real-Time PCR analysis of ES DCs . Real-time PCR was performed on CD11b sorted ES DCs and bone marrow derived macrophages. Data presented for each of the phagocytic receptors as expression levels of CD11b sorted cells relative to bone marrow macrophages n=1.

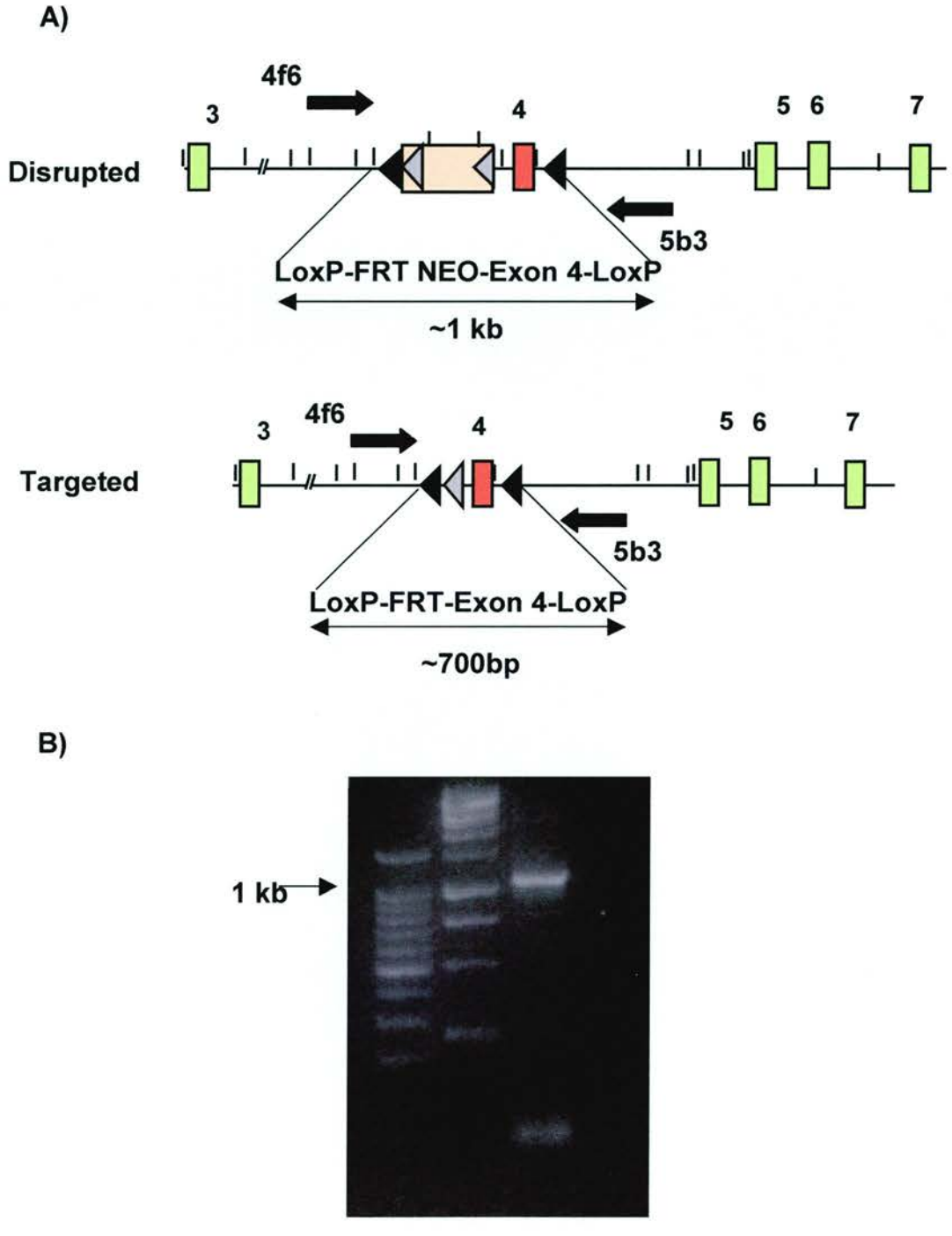


Figure 3.28: PCR of integrin α_v disrupted ES cells. A) Schematic showing the neomycin disrupted integrin α_v gene, the targeted gene, the position of the primers 4f6 and 5b3 and the expected product sizes. B) PCR of genomic DNA isolated from integrin α_v disrupted ES cells.

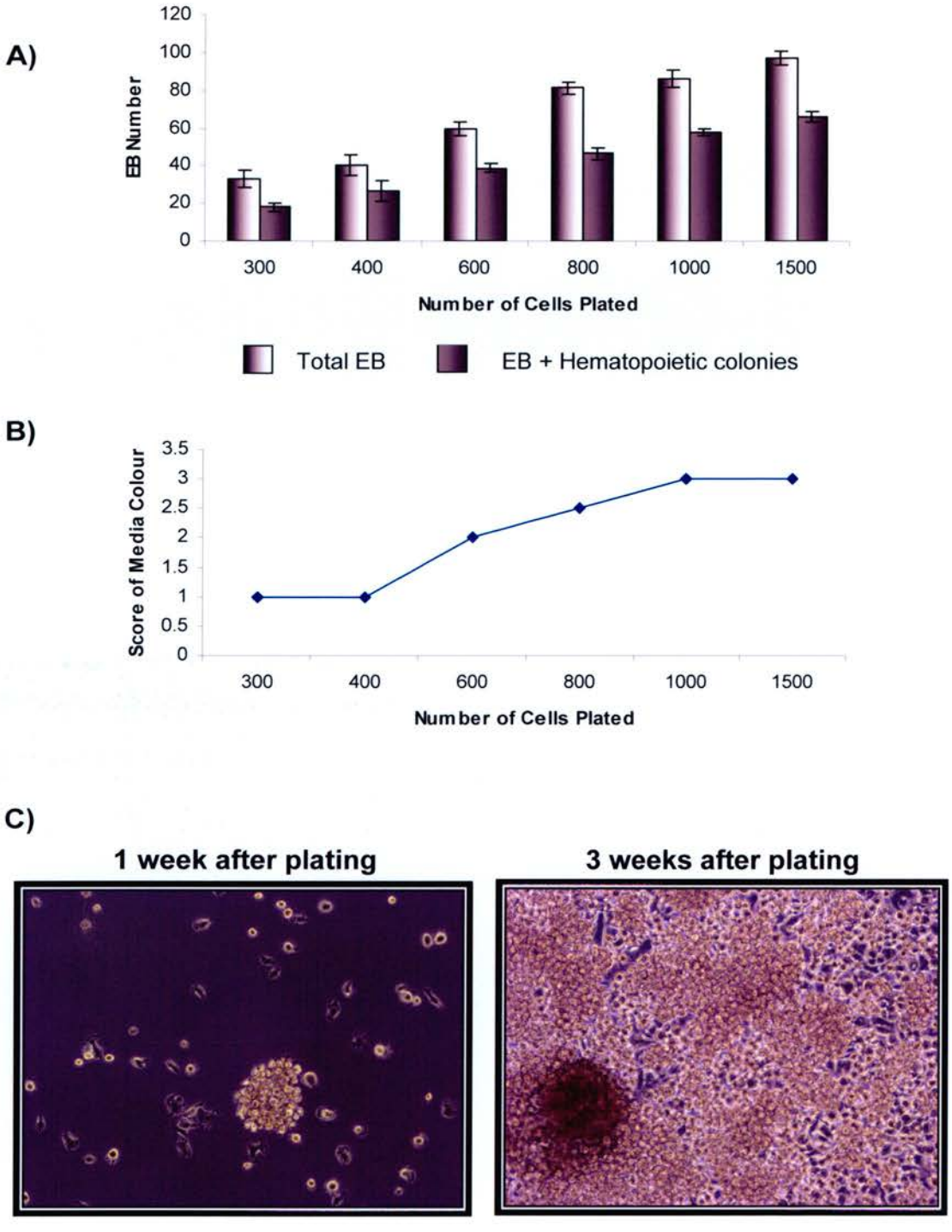


Figure 3.29: Differentiation of integrin α_v disrupted ES cells.
Integrin α_v disrupted cells were plated at seeding densities of 300, 400, 600, 800, 1000 and 1500 cells per culture to determine the optimum seeding density for ES cell differentiation (A). Results expressed as mean of one experiment performed in triplicate +/- standard deviation. The colour of the media was scored to give an indication of the maximum number of cells that could be plated without depletion of the media. A score of one indicates the media was still pink, two indicates the media was orange and a score of three indicates the media was yellow (B). (C) representative phase contrast micrographs of the differentiated progeny. Magnification x10.

was still pink, a score of 2 indicating that the media had turned orange and a score of 3 indicating that the media was yellow (exhausted). Seeding densities of 300 and 400 cells per culture generated EB but these were quite low in number (~40 EB) compared to seeding densities of 600 or 800 cells (~60 EB). The higher seeding densities of 1000 and 1500 produced a high number of EB (80-100) with over half of those associated with hematopoietic colonies (Fig 3.29a). Scoring of the media colour showed that at seeding densities of 600 and 800 the media was still orange but at 1000 and 1500 the media had become exhausted by day 14 of the differentiation (Fig 3.29b). Although the higher seeding densities produced ideal numbers of EB associated with hematopoietic colonies it was decided to use 800 cells per culture as the media was not exhausted and good numbers of hematopoietic expressing EB were obtained.

Figure 3.29c shows representative photomicrographs of the differentiated progeny arising from the differentiation of integrin α_v disrupted ES cells. One week after plating many non-adherent dendritic cells were apparent in the cultures with some forming larger lightly adherent clusters. After 3 weeks the numbers of DC clusters had increased dramatically although this varied depending on the number of EB in any one plate (~30 EB/plate). These dendritic cell clusters showed a similar morphology to the cells arising from the differentiation of wild type ES cells (Fig 3.29) indicating that disruption of integrin α_v did not affect the differentiation capacity of the ES cells. For ease, from this point onwards, DCs arising from the differentiation of integrin α_v disrupted ES cells will be termed DC$\alpha_v^{-/-}$.

3.4.8 Maturation of DC$\alpha_v^{-/-}$

DC$\alpha_v^{-/-}$ were stimulated with 1μg/ml LPS as described in figure 3.24 and analysed by flow cytometry for the DC surface markers CD11c, CD80, CD86, CD40, and major histocompatibility complex II (MHC II) (Fig 3.30). Levels of surface expression of CD11c remained unaltered in the cells stimulated with LPS although a slightly higher percentage of the cells were expressing the surface marker compared

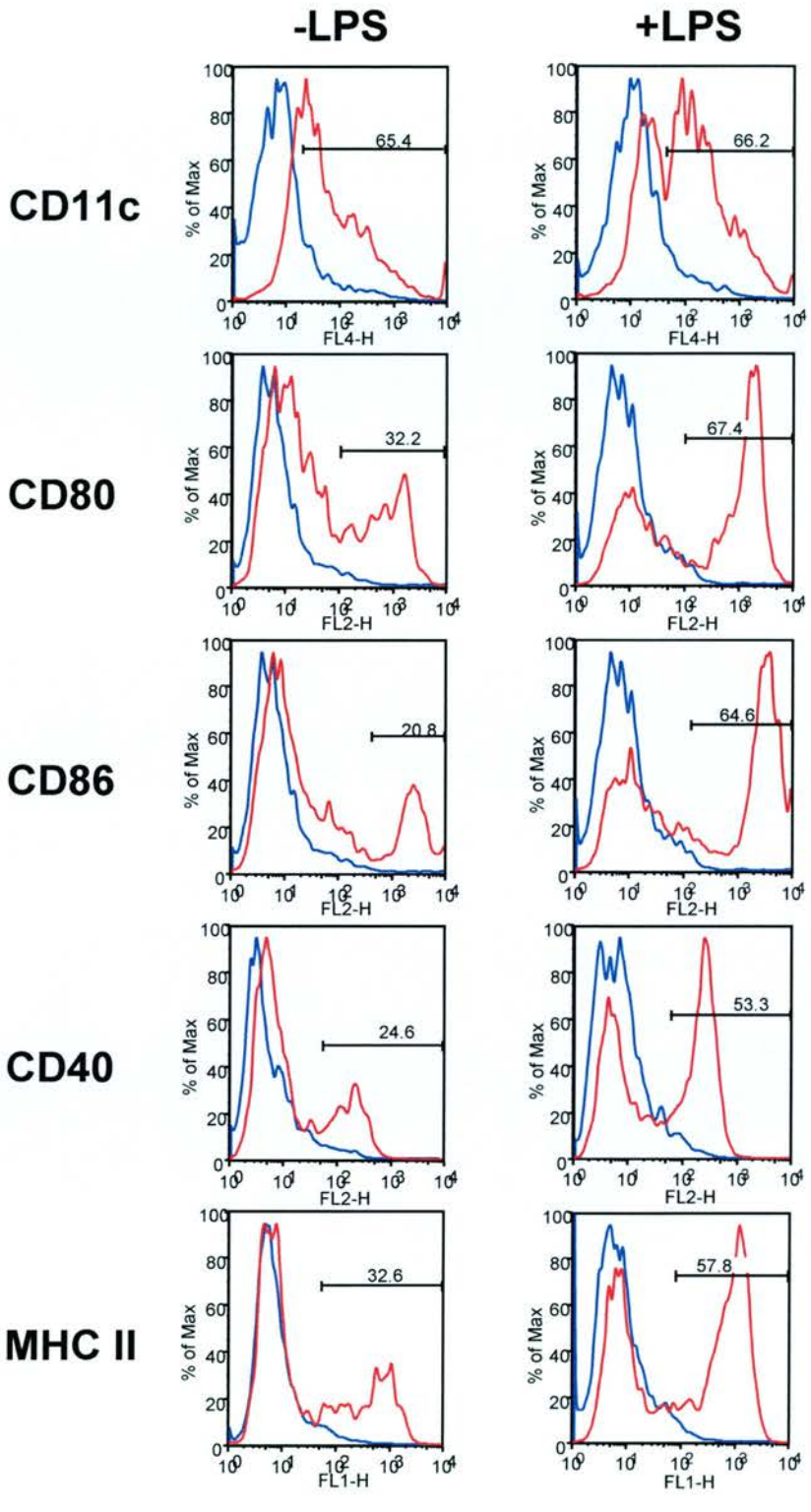


Figure 3.30: LPS stimulation of integrin α_v disrupted ES derived dendritic cells. ES derived dendritic cells were stimulated with 1μg/ml LPS for 24 hours and analysed for surface markers CD11c, CD80, CD86, CD40 and MHC II by flow cytometry.

to the resting cells. CD80, CD86 and MHC II were all expressed at comparable levels in the resting cells and each showed a marked upregulation when stimulated with LPS. CD86 expression was also similar to that observed for the DCs derived from the wild type ES cells (Fig 3.26). Unlike the other surface markers CD40 was expressed at relatively low levels in the resting cells but similarly upregulated it upon stimulation with LPS. The morphology and surface expression profiles exhibited by these DC$\alpha_v^{-/-}$ is comparable to that observed for bone marrow derived dendritic cells indicating that loss of integrin α_v does not affect the differentiation and maturation of DCs, therefore confirming that these cells can be used as a model system in which to study the role of integrin α_v in the phagocytosis of apoptotic cells by DCs.

3.4.9 Role of integrin α_v in apoptotic cell phagocytosis by DC$\alpha_v^{-/-}$

The role of integrin α_v in apoptotic cell phagocytosis was investigated using the integrin inhibitory peptide RGD. DC$\alpha_v^{-/-}$ were fed fluorescent (green) apoptotic PMNs (~50% apoptosis) in the presence of the integrin α_v inhibitory peptide RGD (0.5mg/ml) and the irrelevant control RAD (0.5mg/ml) for 2 hours. Phagocytosis was then assessed by flow cytometry with DC$\alpha_v^{-/-}$ counterstained with F4/80 APC and analysed as previously described (section 3.2.6). The level of phagocytosis in the absence of peptides was considerably lower (31.5%) than previously observed for macrophages (86%) derived from the EFC-1 ES cell line (Fig 3.31 & 3.10). Nevertheless, phagocytosis of apoptotic cells by DC$\alpha_v^{-/-}$ was inhibited by RGD (22.8%) compared to the control peptide (31.4%)(Fig 3.31). This was at variance with data obtained from BMDM from $\beta3^{-/-}$ mice (Fig 1.3) and suggested that the DC $\alpha_v^{-/-}$ culture might contain cells expressing α_v or that putatively DC $\alpha_v^{-/-}$ express low levels of α_v by mechanisms such as "read through".

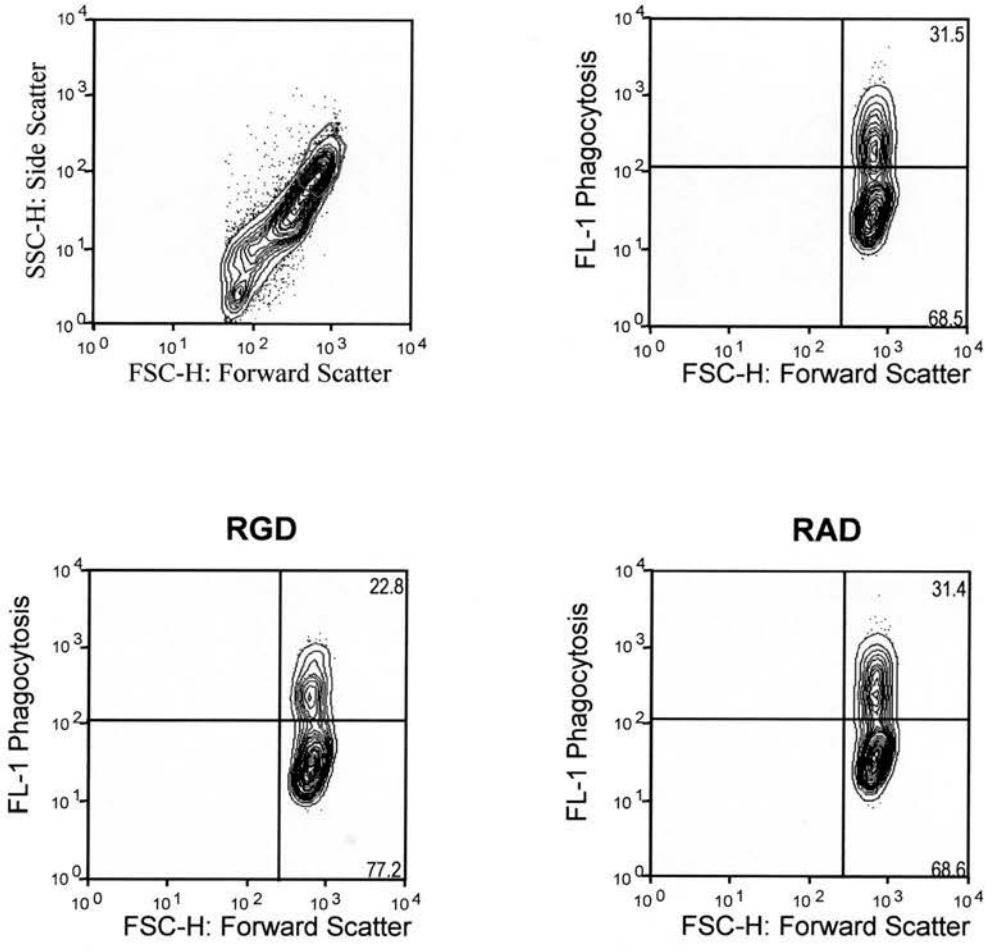


Figure 3.31: Failure of RGD to inhibit DC$\alpha_v^{-/-}$ apoptotic cell phagocytosis. DC$\alpha_v^{-/-}$ were incubated with apoptotic cells for 2 hours in the presence of the inhibitory peptide RGD and the irrelevant control RAD. DC$\alpha_v^{-/-}$ were counterstained with F4/80-APC and phagocytosis assessed by flow cytometry, n=1.

3.5 Discussion.

This chapter has focussed on the characterisation of myeloid cells generated through the differentiation of ES cells. As described by Wiles and Keller, differentiation of the EFC-1 ES cell line in MeC cultures supplemented with IL-3 and either M-CSF or GM-CSF resulted in the formation of EB, which upon disaggregation and plating gave rise to multiple cell types (Wiles and Keller, 1991). Surface phenotype alone is not sufficient to distinguish between different populations of myeloid cells and other factors such as adherence and cytochemical analysis should be considered. However, due to the relatively low numbers of cells obtained per differentiation the proportion of differentiated cells that had developed into cells of the myeloid lineage was assessed by flow cytometry. This method allowed the expression of several cell surface markers to be examined simultaneously without the need for high cell numbers and also provided an easy system for the identification of macrophages that had ingested apoptotic cells in the flow based phagocytosis assays.

The numbers of cells harvested from the differentiation cultures were significantly lower ($5x10^3$ cells per EB) than that reported by others (~$4x10^5$ cells per EB) (Faust et al., 1994). Phenotyping of the differentiated cells by gene expression or protein surface expression showed that a high percentage (~60%) of the cells generated were indeed macrophages. However, the percentage of macrophages present in the cultures may be underestimated due to the variability of the control antibody results. The level of fluorescence observed for the control antibodies was higher than expected suggesting that further titration of the amount of antibody used is required. In addition, altering the amount of serum used to block the cells or isotype controls from different sources could have been tested resulting in a higher percentage of macrophages being detected.

An inherent property of macrophages is their ability to phagocytose unwanted or foreign particles. Phagocytosis studies of ES Mϕ showed that they retained the capacity to ingest not only latex beads as previously reported (Moore et al., 1998) but

also apoptotic PMNs. The ability of ES Mϕ to ingest apoptotic cells was assessed by flow cytometry and as a result it was difficult to discriminate between apoptotic cells that had been ingested or had adhered to the surface of the macrophage. To overcome this, prior to flow cytometry, macrophages that had interacted with apoptotic cells could have been treated with trypsin to remove any cells that had adhered. Alternatively, macrophages could have been sorted by the intensity of the apoptotic cell labelling as those that had been ingested would not be as bright as those bound to the surface of the macrophage. Nevertheless the macrophages generated by differentiating ES cells were highly phagocytic rendering them ideal for investigating the receptors expressed and the phagocytic ability that they confer upon the macrophage.

To increase the numbers of myeloid cells generated by differentiating ES cells several conditions in the differentiation protocol were altered; I) the concentration of IL-3 used; II) the method of expansion of the differentiated cells; III) the method of harvesting the terminally differentiated cells; and finally IV) the effect of serum change.

The first parameter to be assessed was the concentration of IL-3 used in the culture protocol. IL-3 has been shown to be the key cytokine involved in the differentiation of ES cells to myeloid cells and when combined with M-CSF greatly enhanced the numbers of EBs associated with macrophage-like cells (Wiles and Keller, 1991). Although IL-3 is not expressed in the developing EBs it has been shown to exert a proliferative effect on early stem and hematopoietic progenitor cells (Migliaccio et al., 1988; Sonoda et al., 1988). However, increasing the concentrations of IL-3 used in the differentiation of EFC-1 ES cells failed to increase the numbers of myeloid cells produced with only ~10% of the total cells harvested comprised of macrophages.

The second condition to be altered was the way in which the differentiated cells were cultured. Two methods have been published for the expansion of differentiated cells

from EBs. EBs can either be disrupted using collagenase and the cell suspension plated (Clarke et al., 2000; Keller et al., 1993) or intact EBs plated into tissue culture plates (Moore et al., 1998; Moore et al., 2001; Wiles and Keller, 1991). Comparison of these two methods showed that plating of the intact EBs gave rise to greater numbers of myeloid cells than the disruption of the EBs regardless of the CSF cytokine used. This may have been due to the method of disruption of the EBs resulting in the loss of the myeloid precursor cells. However, as seen in the IL-3 experiment the total numbers of macrophages were still quite low (10-15% of the harvested cells). Although EB plating failed to increase the yield of macrophages the cell numbers were higher than for disrupted EBs therefore, EB plating became the method of choice for expansion of the differentiated cells.

Having determined that plating of the EBs was more successful than disruption, different methods of harvesting the macrophages were tested. A previous report (Moore et al., 1998) suggested that macrophage precursors could be harvested from EB-containing plates and re-plated to generate macrophage cultures. However, when this was attempted, no macrophages were generated and the re-plated cells looked more like undifferentiated ES cells. Morphological analysis of the cultures showed that many of the macrophage-like cells were attached to the EB layers with a few distinct clusters of macrophages separate from the EBs. To harvest these cells several cell detachment reagents were tested. Flow cytometry analysis of the cells removed from the plates showed no expression of the myeloid markers F4/80 and CD11b suggesting that either, no macrophages were present in the cultures or that the cells had been lost during filtering of the cell suspension to remove the EBs. In the cultures containing $F4/80^+$ cells, magnetic cell sorting could be used to purify macrophages. However, although this generated a relatively pure population of cells the yield was still low, therefore confirming that the underlying problem was not isolating differentiated cells but that very few of the differentiated cells were actually macrophages.

The final condition to be altered in the culture protocol was the batch of serum used. Serum can affect both the growth and differentiation of ES cells (Wiles, 1993) and it was suggested that changing the batch of serum used might improve the numbers of macrophages generated. At this time attention was also turned to generating ES derived dendritic cells (Fairchild et al., 2000) to overcome the problems experienced with the harvesting of the macrophages. Initially the EFC-1 ES cells were differentiated as described by Fairchild *et al.* but the EBs that formed adhered to the culture dish and the differentiation potential was lost. However when the EFC-1 ES cells were seeded into MeC either in the presence or absence of cytokines (IL-3 and GM-CSF) EB formation occurred as normal. No differences were observed between the EBs generated using the cytokine conditions described by Fairchild *et al* and those previously used in the differentiation cultures. Interestingly, changing the serum gave rise to differentiated progeny that were smaller in size compared to previous cultures. In addition these cells showed a surface phenotype similar to bone marrow derived dendritic cells and to ES derived dendritic cells described by others (Fairchild et al., 2000; Senju et al., 2003).

Changing the serum used in the differentiation cultures had a profound effect on the numbers of cells generated with between 40-60% of the differentiated cells falling within the myeloid cell gate (as determined by FSC/SSC) compared to ~40% in the original differentiation cultures and ~15% in the optimisation cultures. The decrease in yield and purity of the cells between the original cultures and the optimisation cultures could be due to the slight changes in the differentiating protocol or in the method of harvesting but is more likely to be due to the ES cells. The ES cells may have already become slightly differentiated or were not as healthy as those used in the initial cultures accounting for the inability to improve the macrophage number, as it is possible that the EB were not producing many hematopoietic precursors and were unable to differentiate to large numbers of macrophages. Moreover, some ES cell lines are more readily differentiated *in vitro* (Wiles and Keller, 1991) and although the EFC-1 ES cells were capable of generating macrophages, differentiation of other ES cell lines may help improve the yield. In addition, the cell number may

also have been increased as a result of switching the differentiated cell type from macrophages to the less adherent DCs. Therefore in conclusion, by changing the serum and the cytokine used in the differentiation protocol we have generated a myeloid cell that can be easily harvested, is dendritic-like in morphology and surface expression and is of interest for our work on phagocytosis.

Similar to the EFC-1 ES cell line, the E14 ES cell line (used for the targeting work) was also capable of differentiation. Prior to the differentiation of E14 ES cells carrying a disrupted integrin α_v gene the ability of the E14 ES cells to generate myeloid cells was assessed. Based on the data presented in the first part of this chapter the E14 ES cell line was cultured in IL-3 and GM-CSF to drive differentiation towards the production of dendritic cells.

As discussed previously batch variations in serum can influence the growth and differentiation of ES cells. To ensure that the Labtech serum used in the differentiation of the EFC-1 ES cell line was optimal for the E14 ES cells five batches of serum were tested. Each of the five batches produced the normal number of EBs (50-100 EB per dish) expected for cultures beyond day 6 of differentiation (Wiles, 1993). The percentage of the differentiated cells that were positive for CD11b and F4/80 were comparable for each of the batches tested. However, batch 5 (Labtech Serum) produced slightly higher numbers of EBs associated with hematopoietic cells and differentiated cells that showed expression of myeloid cell surface markers so was the batch of choice for future experiments.

The number of ES cells required to give good differentiation varies with ES cell line and any genetic manipulations that have been made. Ideally each time a new cell line or targeted cell line is used the number of ES cells required should be titrated to ensure that the optimum seeding density is used (Wiles, 1993). In the batch testing experiment the media was becoming exhausted after 14 days in culture when 400 ES cells were plated. In addition it was noted that the EBs appeared to be settling near the bottom of the dish rather than suspended in the MeC. Increasing the percentage

of MeC used in the cultures from 0.9% to 1.1% prevented the adherence of the EBs and at each seeding density a slightly higher number of EBs formed than in the 0.9% MeC. A seeding density of 300 cells per dish in 1.1% MeC was found to be optimal, as the media was not exhausted by day 14 of culture. This seeding density was considerably lower than that required for differentiation of the EFC-1 ES cell line (800 cell per dish).

The morphology of the differentiated progeny was similar to the cells derived from the EFC-1 ES cell line ~ 12 days after plating, although by day 20 the E14 derived cells had aggregated and formed large clusters of cells that were lightly attached to an adherent monolayer of cells. The morphology of these cells was highly reminiscent of dendritic cells cultured from mouse blood (Inaba et al., 1992) and showed greater similarities to the ES derived DCs described in the literature (Fairchild et al., 2000; Senju et al., 2003). Harvesting of both the adherent and non-adherent fractions showed that these E14 derived DCs were $F4/80^{+}$ $CD11b^{+}$ $CD11c^{+}$ $CD86^{-}$ and were capable of up-regulating CD86 upon stimulation with LPS. In addition stimulation of the DCs changed the morphology with more dendrites and veils of cytoplasm apparent. The surface phenotype and morphology exhibited by these cells closely resembles both bone marrow derived dendritic cells and other ES derived DCs excluding F4/80 that is only expressed on ES DCs (Fairchild et al., 2000; Lutz et al., 1999; Senju et al., 2003).

Unexpectedly, the change of ES cell line resulted in larger numbers of ES derived dendritic cells that were more comparable to those described by Fairchild *et al* than those derived from the EFC-1 ES cell line, thus providing evidence that generation of high numbers of myeloid cells from ES cells is not only strongly dependent on serum but also the ES cell line used.

The differentiation of integrin α_v disrupted ES cells provides further evidence that the E14 ES cells are better than the EFC-1 ES cells for generating myeloid cells. The number of ES cells required to give good differentiation of the integrin α_v disrupted

ES cells was higher than that for the wild type ES cells. Nonetheless, the morphology of the differentiated cells from these cultures were comparable to those for the wild type differentiations indicating that the loss of integrin α_v had no effect on the differentiation of the ES cells to myeloid cells

One of the most attractive properties of generating myeloid cells from ES cells is ability to continually harvest cells over a period of time (Fairchild et al., 2000; Moore et al., 1998; Wiles and Keller, 1991). The numbers of cells that could be obtained from the E14 ES cultures was greatly enhanced, as harvesting of the cells and replenishment of the culture media on the EB containing plates allowed cells to be harvested 2-3 times from the one culture plate over ~4 weeks. Previously this was not observed with the differentiated EFC-1 ES cells and was not assessed in the differentiation of the untargeted E14 ES cells although it is expected that these cells will also be capable of regenerating the cultures. The ability to harvest greater numbers of dendritic cells allowed a more extensive profile of maturation markers to be assessed. The upregulation of MHC class II and each of the co-stimulatory molecules CD40, CD80 and CD86 provided further confirmation that the ES derived DCs are similar to their bone marrow counterparts and other ES derived dendritic cells (Fairchild et al., 2000; Lutz et al., 1999; Winzler et al., 1997).

Expression profiling of ES DCs showed that these cells expressed many of the receptors also expressed in BMDM, a cell type in which apoptotic cell phagocytosis has been well characterised. Interestingly, ES DCs showed a relative increase in integrin β_3, CD47 and ABC1 and a reduction in expression of CD36. This suggests that ES DCs may use a subtly different recognition complex for apoptotic cells, perhaps using $\alpha v\beta 3$ but without the involvement of TSP 1 and CD36. The increased expression of CD47 is intriguing as CD47 can form complexes with $\alpha_v\beta_3$ and is involved in inflammatory signalling in DCs.

The main objective for developing ES differentiation to macrophages was to assess whether these cells could be used to investigate the role of integrin α_v in apoptotic

cell phagocytosis. Dendritic cells, like macrophages, have been shown to be capable of apoptotic cell phagocytosis and in human systems this appears to be mediated by $\alpha_v\beta_5$ (Albert et al., 1998a) or $\alpha_v\beta_3$ (Rubartelli et al., 1997). DCs derived from ES cells lacking functional α_v integrins differentiated normally and phagocytosed apoptotic cells. The levels of phagocytosis observed by the DC$\alpha_v^{-/-}$ were slightly lower than those previously observed for the EFC-1 derived macrophages although this is not unexpected as macrophages engulf apoptotic cells more efficiently than dendritic cells (Albert et al., 1998a). One of the fall-backs with using the integrin α_v disrupted DCs for phagocytosis studies is that the control cells have to be generated from a separate differentiation culture. Unfortunately, difficulties were encountered with the differentiation of the control cells and the results are therefore difficult to interpret. Ideally, ES cells silently targeted for integrin α_v should be differentiated and the α_v gene deleted using Cre at the desired time, allowing both control cells and cells lacking integrin α_v to be generated from the one differentiation culture. Previously this approach has been hampered by the inability to efficiently transfect myeloid cells with Cre but alternative methods have been tested and are discussed in the following chapters.

Surprisingly, in view of previous data (section 1.4, Fig 1.3) the phagocytosis of apoptotic cells by DC$\alpha_v^{-/-}$ was inhibited by the integrin antagonistic peptide RGD suggesting that integrin α_v was still being expressed. Cell surface staining was used to verify expression of integrin α_v, however no staining could be detected for either wild type BMDM or the DC$\alpha_v^{-/-}$. Therefore in the absence of confirmation of integrin α_v expression, it was concluded that the DC$\alpha_v^{-/-}$ did not represent a sufficiently rigorous system for assessment of the role of integrin α_v in the phagocytosis of apoptotic cells.

In summary, ES cells can be differentiated successfully in the presence of IL-3 and M-CSF or GM-CSF to macrophages and DCs respectively. Differentiation of high numbers of cells is dependent on the ES cell line and the serum used and upon

routine harvesting of the differentiated cells the cultures can be maintained for several weeks. These ES derived myeloid cells are highly phagocytic and the ES DCs can be matured with LPS indicating that these cells are similar to bone marrow derived myeloid cells.

Chapter 4

Generation of constructs

4.1 Introduction

The second approach that was taken to investigate the role of integrin α_v in phagocytosis of apoptotic cells was to deliver Cre to primary myeloid cells cultured from mice in which integrin α_v was targeted by flanking with LoxP sites. However, macrophages and dendritic cells are highly refractory to most transfection methods although some success has been achieved with adenoviral vectors and an integrin-targeted synthetic vector known as the LID vector (Uduehi et al., 2003). Recently, a number of proteins have been identified that can translocate across the eukaryotic cell membrane in the absence of any additional transfection reagents. These proteins can carry a variety of other macromolecules with them into many different cell types although this has never been demonstrated for macrophages. At the onset of this work only one of the transduction proteins, VP22 had been shown to mediate cellular entry of other proteins of >25 kDa in size into cells such as the COS-7 cell line and terminally differentiated skeletal muscle cells (Derer et al., 1999; Elliott and O'Hare, 1997). This discovery highlighted VP22 as a potential delivery vehicle for the Cre recombinase enzyme.

VP22 is a 38 kDa structural protein that is encoded by the UL49 gene of the Herpes Simplex virus type 1 (Elliott and Meredith, 1992). This protein has been shown to retain the unique property of intercellular transport whereby the protein is exported from the cell in which it is synthesised and imported into its surrounding neighbours (Elliott and O'Hare, 1997). This intercellular spreading of the VP22 protein has been characterised by two distinct patterns of cellular localisation. VP22 is predominately located in the cytoplasm of the cells in which it is expressed and in the nucleus of cells into which it has spread, where it binds chromatin (Elliott and O'Hare, 1997). The mechanism by which this occurs is currently unknown, although VP22 is thought to be exported via a Golgi independent process, termed nonclassical secretion, as treatment of cells expressing VP22 with Brefeldin A did not inhibit protein transduction (Elliott and O'Hare, 1997). The manner in which VP22 is transported into cells is also unclear. The ability of the VP22 protein to spread

between cells at 4°C indicated that it is an energy independent process that does not involve normal endocytosis. However, the sensitivity of VP22 import to cytochalasin D showed that uptake of the protein may in some way involve the microfilament network within the cell (Elliott and O'Hare, 1997; Elliott and O'Hare, 1998). This initial paper describing the intercellular trafficking properties of VP22 also investigated the potential of VP22 to deliver larger proteins to a cell monolayer (Elliott and O'Hare, 1997). VP22 was fused to the 27 kDa GFP protein to create a 65 kDa fusion protein. When expressed in COS-1 cells the protein was shown to exhibit a similar pattern of cellular localisation to the wt VP22 indicating that GFP did not inhibit the trafficking properties of the transduction protein.

To test the ability of VP22 to deliver the 35 kDa Cre recombinase into cells containing floxed genes, plasmids that expressed fusion proteins had to be created. This chapter focuses on the generation of plasmids for expression of a VP22Cre fusion protein in both eukaryotic and prokaryotic systems and a reporter construct that will permit analysis of the cell permeability of the VP22Cre fusion protein.

4.2 Generation of pVP22Cre

4.2.1 Cloning Strategy for pVP22Cre

A schematic representation of the cloning strategy used to construct pVP22Cre is shown in figure 4.1. The background vector used was the commercially available plasmid pVP22*myc*/His (Invitrogen)(appendix II). The plasmid contains the VP22 gene from Herpes Simplex virus under the control of the CMV IE promoter, a *myc* epitope for antibody detection, a His tag to enable purification and ampicillin and neomycin resistance cassettes for selection. Cre could be inserted into the multiple cloning site, in frame, to produce a VP22-Cre-*myc*-His fusion protein. The insert, Cre recombinase was amplified from a Cre expressing plasmid using the VP22Cre f and r primers to introduce a *Not*I restriction site at beginning of the Cre sequence and an *Xba*I site at the terminal end. These primers removed the Cre ATG sequence and

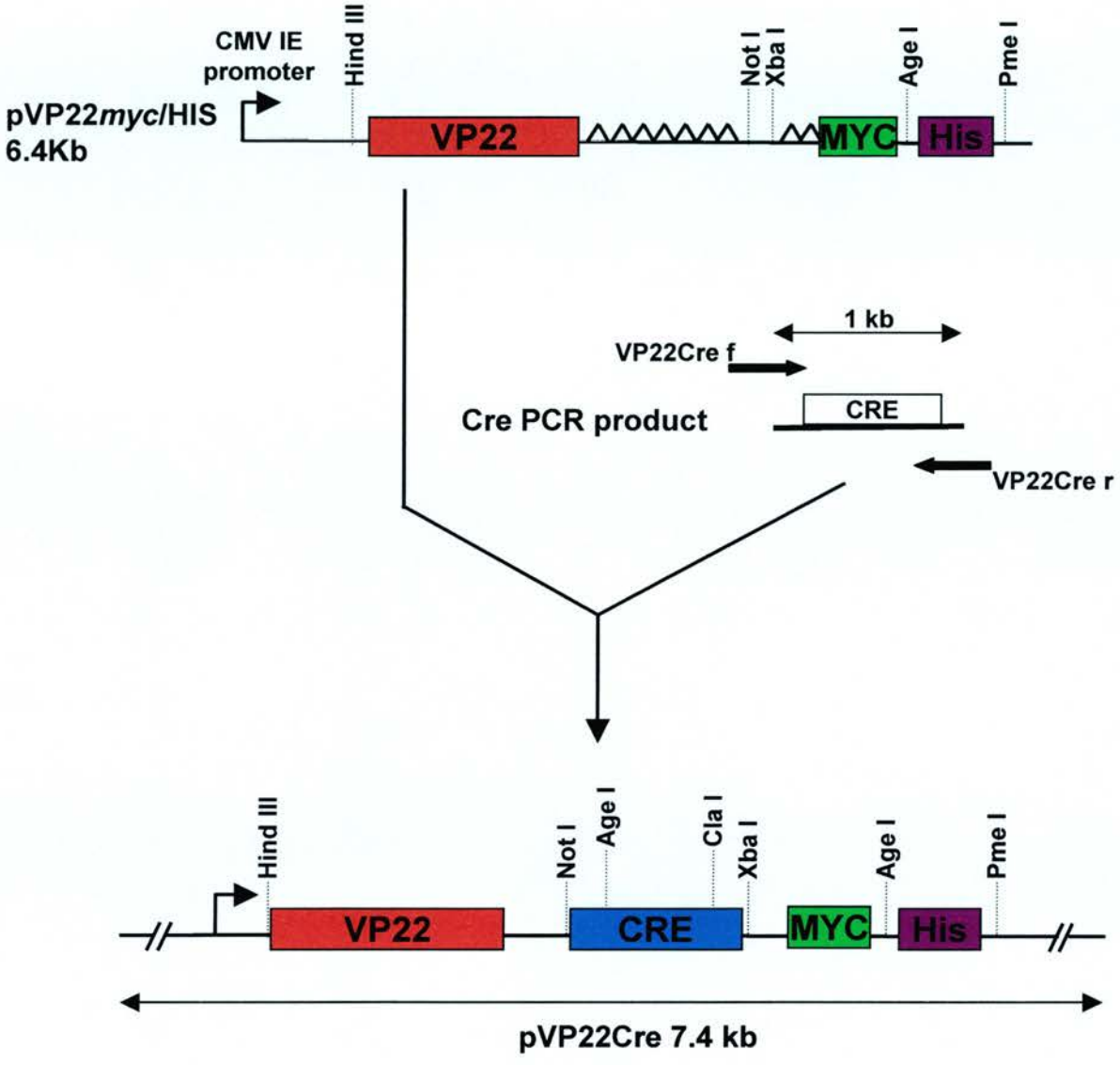
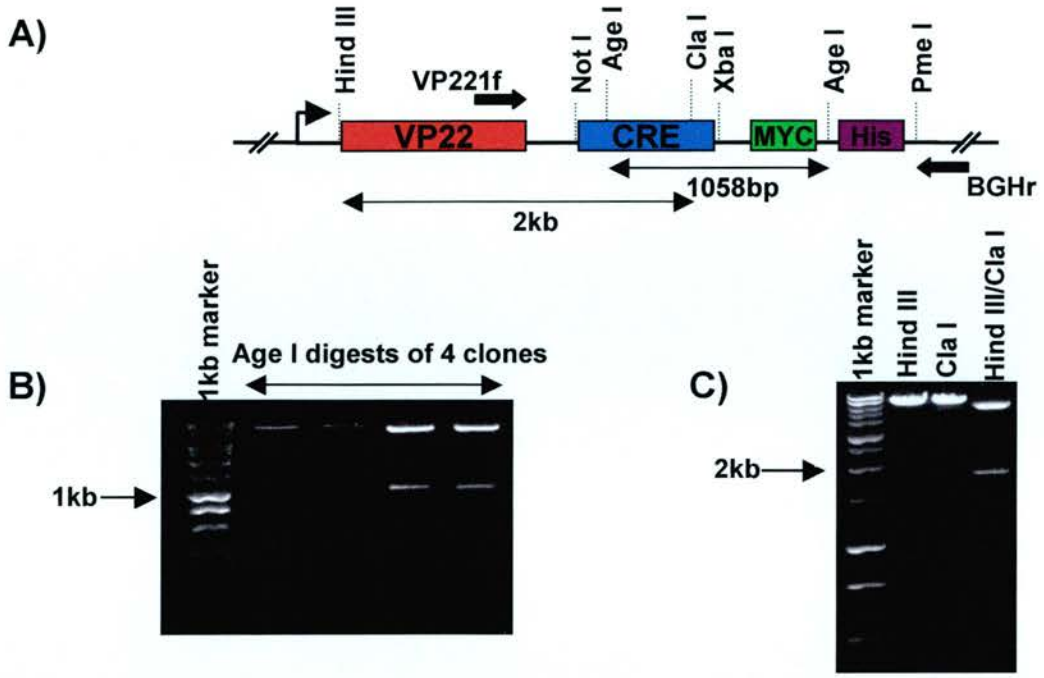


Figure 4.1: Schematic representation of the cloning strategy used to generate pVP22Cre. The Cre PCR product was amplified from Cre with the two primers VP22cre f and VP22Cre r to introduce the *Not*I and *Xba*I sites (*Not*I/*Xba*I) in the multiple cloning region of the pVP22*myc*/His vector.



D)

Sequenced with VP221f:

Not I Xba I

pVP22 *myc*/His .....TATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGCGGTT.....

Sequenced pVP22Cre.....TATCCAGCACAGTGGCGGCCGCTCCAATCTGCTGACCGTGCACCA.....

Cre GCGGCCGCTCCAATCTGCTGACCGTGCACCA.....

Sequenced with BGHr:

Not I Xba I

pVP22 *myc*/His .....TATCCAGCACAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGCGGTT.....

Sequenced pVP22Cre.....AAACAGGGGCCTGCTGGAAGATGGCGATCTAGAGGGCCCGCGGTT.....

Cre .....AAACAGGGGCCTGCTGGAAGATGGCGATCTAGA

Figure 4.2: Restriction digests of VP22Cre ligations. A) Schematic showing restriction sites and expected product sizes for *Age* I and *Hind* III/*Cla*I digests and the localisation of the sequencing primers VP221f and BGHr. Presence of the Cre insert was detected by *Age*I restriction digest (B) and the orientation of the insert determined by *Hind* III/*Cla*I double digest (C). D)Sequencing of VP22Cre with VP221f and BGHr primers aligned with the parent vector pVP22 *myc*/His and the Cre gene.

the normal Cre stop codon. Subsequently, Cre was digested with *Not*I/*Xba*I and ligated with *Not*I/*Xba*I digested pVP22*myc*/His.

4.2.2 Characterisation of VP22Cre Ligations

Restriction digests were performed on several clones arising from the ligation of pVP22*myc*/His and Cre to detect the presence and the orientation of the insert. The clones were first cut with *Age*I. *Age*I cuts once at the beginning of the Cre sequence and once in the VP22 vector releasing a 1kb fragment if the insert is present (Fig 4.2a). All but three of the clones analysed released the expected band of 1kb and were subjected to further digests to determine the orientation of the insert (Fig 4.2b). Double digest with restriction enzymes *Hind*III (VP22 vector) and *Cla*I (Cre) released a 2kb fragment when the Cre insert was in correct orientation (Fig 4.2a) or a 1kb fragment if incorrect. Six of the clones analysed linearised when digested with either *Hind*III or *Cla*I and produced the expected band of 2kb when digested with both enzymes simultaneously; one representative digest is shown (Fig 4.2c). Importantly, single digests with *Cla*I produced one band, confirming that only one of the Cre inserts had integrated. To confirm the restriction digests, the six clones were sequenced and matched to the correct pVP22Cre sequence. Figure 4.2d shows the sequence data of one of the correct pVP22Cre clones aligned with pVP22*myc*/His and the Cre PCR product. The rest of the Cre sequence was also examined (data not shown) to ensure no mutations had been introduced during the PCR reaction.

4.3 Generation of the bacterial expression plasmid pETVP22Cre

4.3.1 Cloning strategy for pETVP22Cre

Experiments describing the use of the HIV-TAT transduction protein have consistently used recombinant protein purified from bacteria allowing large quantities of protein to be produced and greater control over the amount of protein added in the culture system (Frankel and Pabo, 1988; Nagahara et al., 1998). To enable the VP22Cre to be expressed in a prokaryotic system the VP22Cre coding sequence was inserted into the bacterial expression vector pET11d (appendix II).

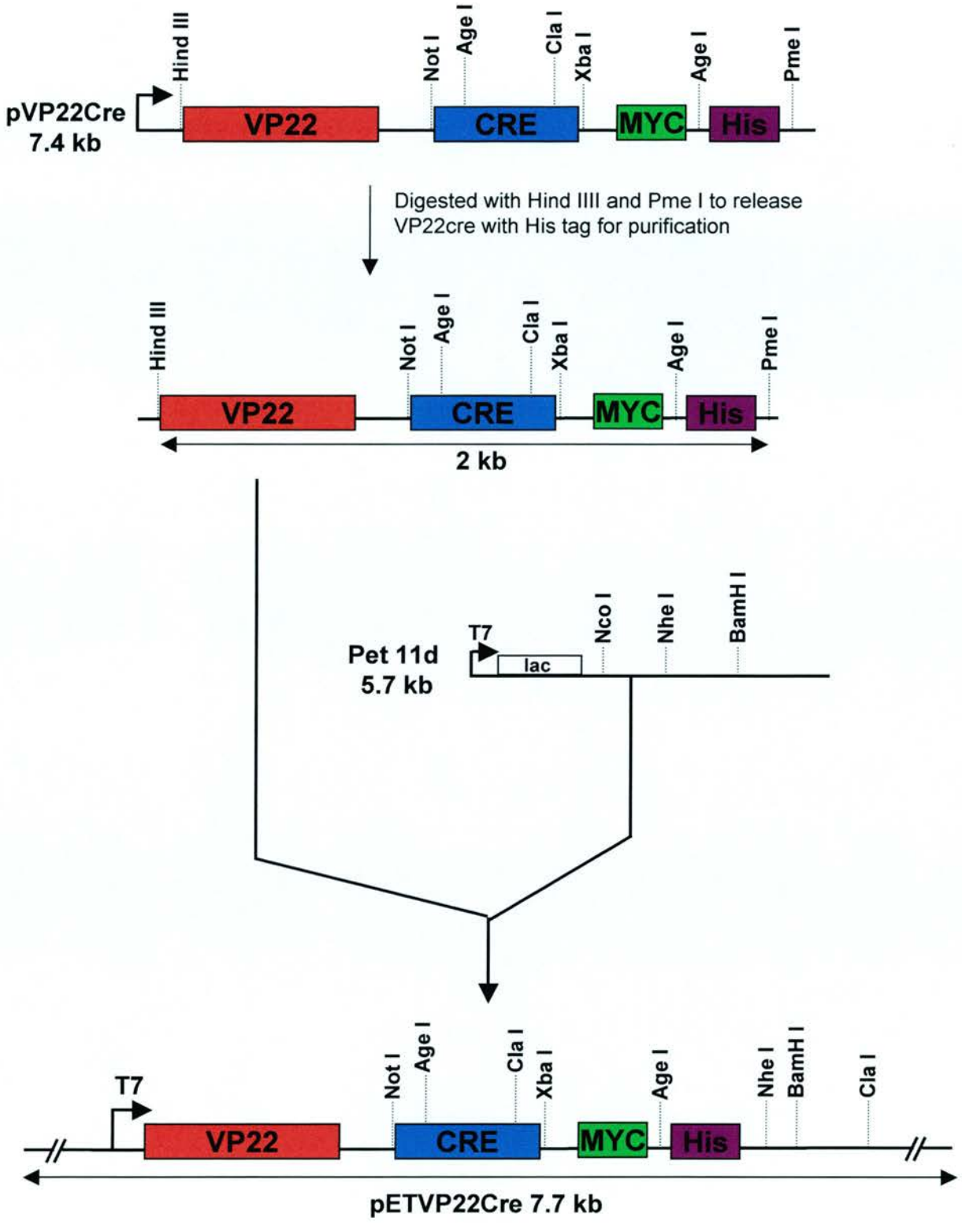


Figure 4.3: Cloning strategy used to generate pETVP22Cre. pVP22Cre was cut with restriction enzymes *Hind*III and *Pme*I to release the sequence containing the genes for VP22 and Cre and the myc/His tag for purification. After conversion to a blunt-ended fragment the VP22Cre*myc*/His sequence was cloned into the *NcoI* site of the bacterial expression vector pET11d.

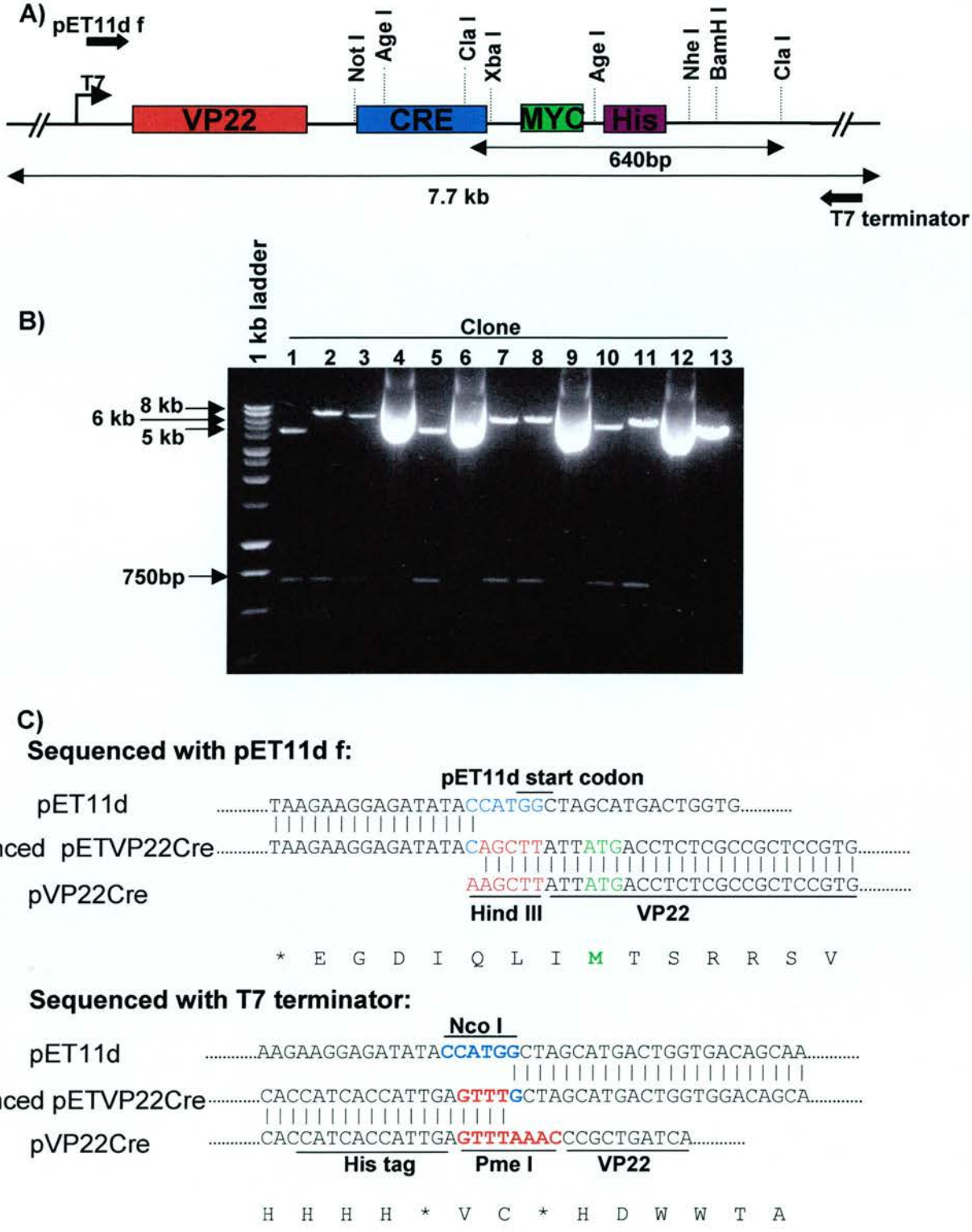


Figure 4.4: Restriction digests of pETVP22Cre. A) Schematic showing restriction sites and expected product sizes for *Cla*I digest. Presence and orientation of the VP22Cre insert was determined by *Cla*I digest (B). Correct clones 3, 7, 8, 11 produced bands of 640 bp and the remaining vector 7.1kb. C) Correct sequence of pETVP22Cre aligned with the sequences for pET11d and pVP22Cre and the translated sequence showing the translational start (M) which is the first ATG following the transcriptional start site and stop(*) codon following the His tag.

pVP22Cre was digested with *Hind*III and *Pme*I to release the 2kb VP22Cre *myc*/His fragment. The *Hind*III site was then filled in using Klenow to generate a blunt end for ligation to match the blunt *Pme*I digest end (PmeI digest leaves no overhang upon digestion). The pET11d vector was then cut with *Nco*I and treated with mung bean nuclease to create blunt-ends (Fig 4.3).

4.3.2 Characterisation of pETVP22Cre ligations

Few clones were generated from the ligations. Following restriction digest with *Cla*I several clones released a 640bp band indicating the presence and correct orientation of the VP22Cre insert (Fig 4.4b). However, the sizes of the bands for the remaining vector varied between 5 and 9kb. Mung bean nuclease can overdigest DNA and therefore clones of the correct size were selected. Four of the clones (3, 7, 8, 11) had a band of ~7kb and the correct 640bp band and were analysed by DNA sequencing. Only one of the clones when aligned with the pET11d and pVP22Cre sequences contained correct insertion of the VP22Cre*myc*/His fragment in frame (Fig 4.4c).

4.4.Generation of the reporter construct pnLacZLoxP

It was necessary to produce a reporter construct that could detect Cre activity and hence could be used to determine the efficiency of pVP22Cre transduction. Such reporters generally consist of a detectable reporter gene (such as LacZ or GFP) where expression is blocked until Cre mediated recombination, usually through a *LoxP* flanked transcriptional stop sequence. However in preliminary experiments these reporters appeared to be 'leaky' allowing some reporter expression. To prevent these problems a new reporter construct was designed. This plasmid expressed LacZ but following Cre mediated recombination, switched to expressing enhanced green fluorescent protein (EGFP). As EGFP was completely separated from the necessary transcriptional start sequences until Cre activity, the reporter would not be leaky. Furthermore LacZ would allow detection of cells that contained the reporter but had not undergone recombination, essential when calculating the efficiency of Cre delivery.

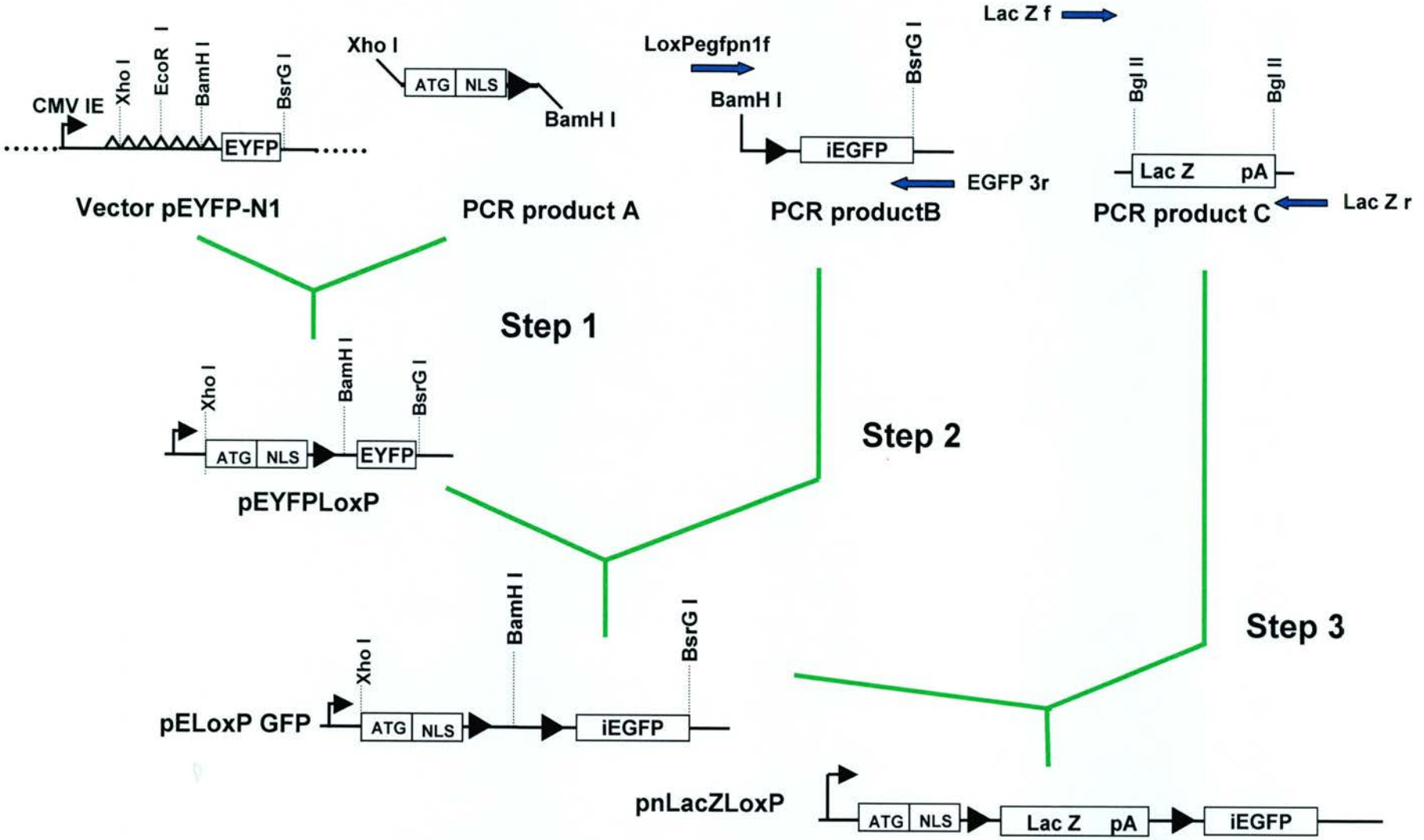
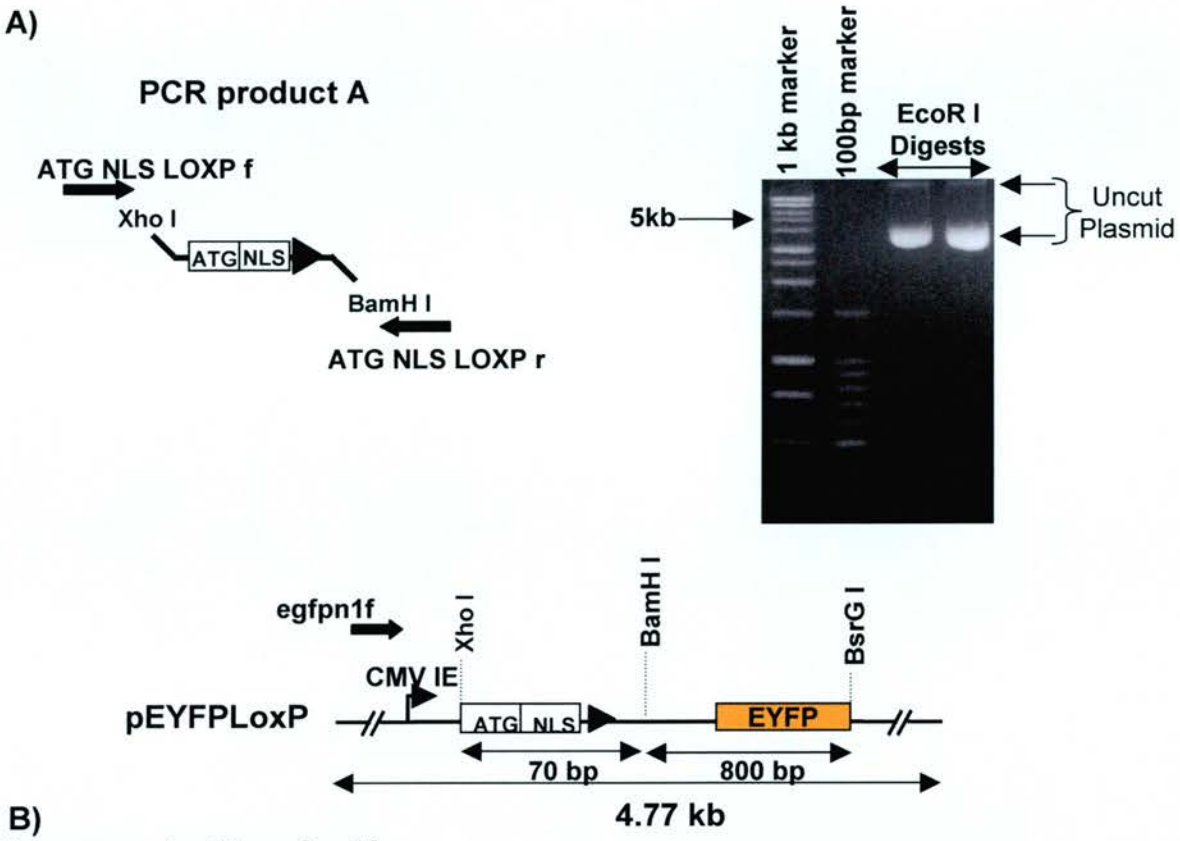


Figure 4.5: Overview of the cloning strategy used to construct pnLacZLoxP. pnLacZLoxP was created using three cloning steps. Inserts A -C were ligated in turn with background vector EYFP-N1 to generate plasmids EYFPLoxP (Step 1) and ELoxP GFP (Step 2) and finally pnLacZLoxP (Step 3).

Step 1: pEYFPLoxP

A)



B)

Sequenced with egfpn1f:

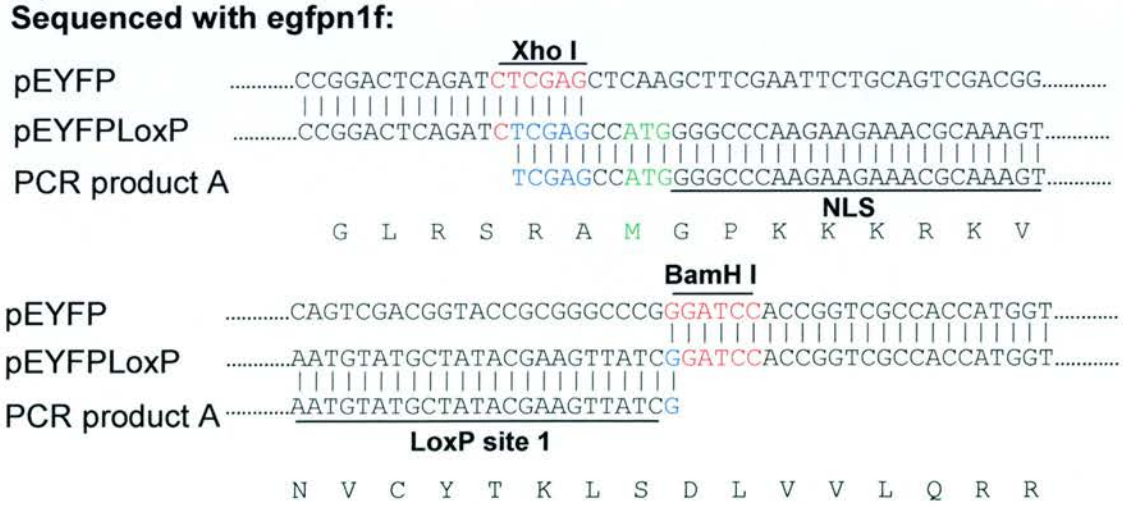


Figure 4.6: Cloning step 1; generation of EYFPLoxP plasmid. PCR product A was generated using primers ATGNLSLoxP forward and reverse. A)The short 70bp product was ligated into the *Xho*I and *Bam*HI sites of the background pEYFP-N1 vector to give plasmid pEYFPLoxP (4.77 kb). B) Sequence of pEYFPLoxP aligned with pEYFP and PCR product A and the translated sequence showing the translation start codon (M).

4.4.1 Cloning Strategy for pnLacZLoxP

Figure 4.5 gives an overview of the cloning strategy used to construct pnLacZLoxP. The background vector used was the EYFP-N1 vector available from Clontech. Three sets of primers ATGNLSLoxP f and r, LoxPegfpn1f and egfp3r, and LacZ f and r were used to create the PCR products A, B and C respectively. In turn, each of the PCR products were ligated with the background vector EYFP-N1 to give plasmids pEYFPLoxP (Step 1), pELoxPGFP (Step 2) and the final plasmid pnLacZLoxP (Step 3).

ATGNLSLoxP forward and reverse primers were used to create PCR product A containing the transcriptional start (ATG) and the nuclear localisation sequence (NLS) along-with the first *LoxP* site flanked by *Xho*I and *Bam*HI restriction sites. This 70bp sequence was then ligated with the relevant sites in the EYFP-N1 vector (4.7 kb) to give the 4.77kb plasmid pEYFPLoxP (Fig 4.5)(Step 1). Insertion of PCR product A removed an *Eco*RI site from pEYFP-N1. Ligation reactions were enriched for by digestion with *Eco*RI to remove any contaminating background vector before transformation. Plasmids that did not digest with *Eco*RI were sequenced to confirm insertion and the plasmid pEYFPLoxP was used as the background vector for step 2 of the cloning (Fig 4.6).

The second step of the cloning strategy was to introduce the gene for GFP preceded by the second of the LoxP sites. The two primers LoxPegfpn1f and EGFP3r were used to amplify iEGFP from the iEGFP expressing plasmid that contains a version of EGFP with two introns that increase expression of the gene (appendix II). The LoxPegfpn1f primer placed a *Bam*HI restriction site and the second of the *LoxP* sequences in front of the GFP to give PCR product B. This was then digested with *Bam*HI and *Bsr*GI (terminal end of GFP) to ligate with the corresponding sites in plasmid pEYFPLoxP. Restriction digests using *Bam*HI and *Bsr*GI were performed on clones arising from the ligation. Each of the clones tested linearised when digested with either of the enzymes to give the expected band of 4.9kb confirming that only one of the inserts had integrated (Fig 4.7). Double digest with both

Step 2: pELoxP GFP

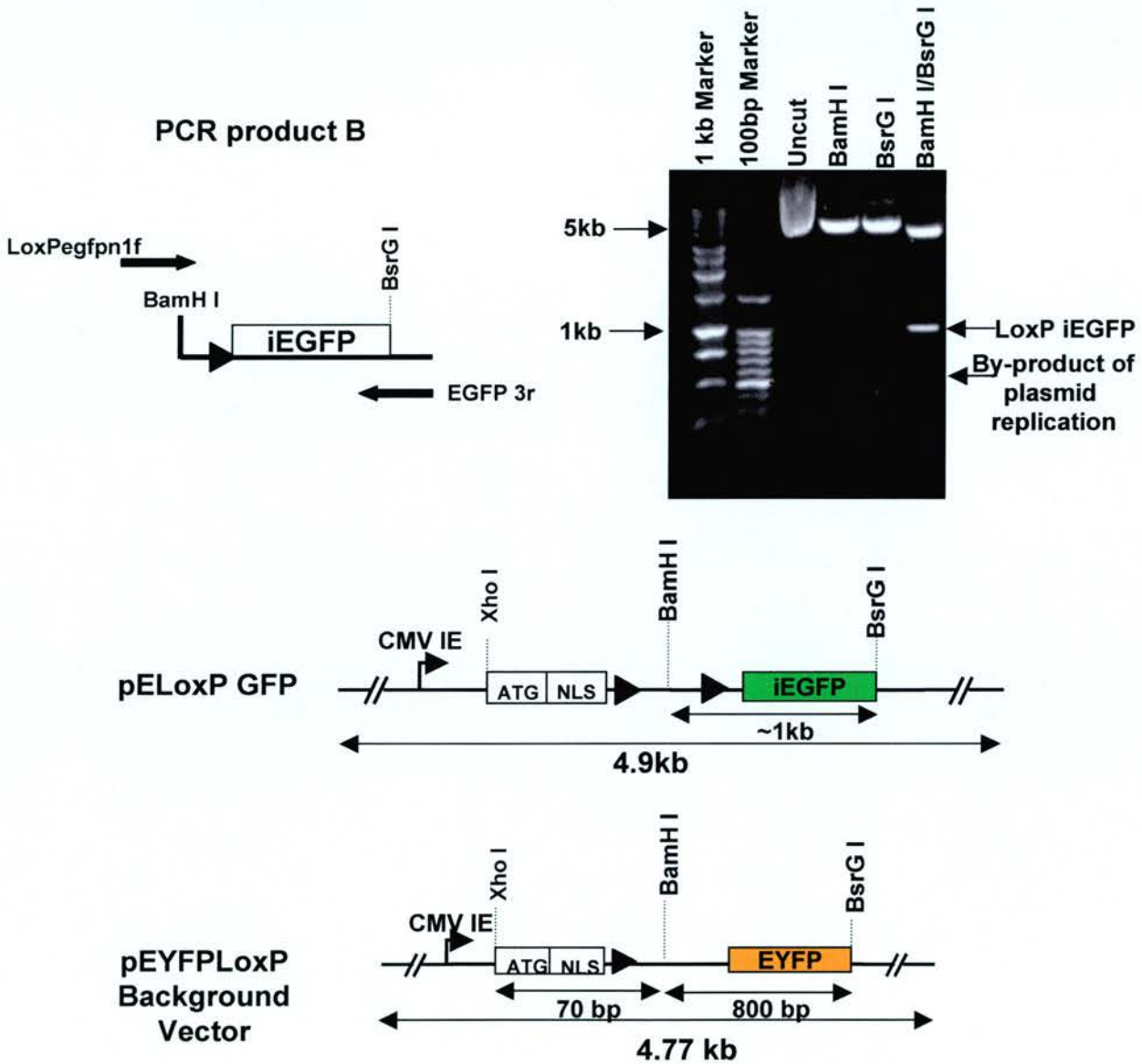


Figure 4.7: Cloning step 2; generation of ELoxP GFP plasmid. Primers LoxPegfpn1f and EGFP3r were used to introduce a *Bam*HI site and LoxP site before the GFP gene. PCR product B was ligated into the *Bam*H I/*Bsr*GI sites of pEYFPLoxP. Diagnostic digest of a representative clone arising from the ligation produced a linearised band of 5 kb if cut with either *Bam*HI or *Bsr*GI and released a 1 kb band when cut with both enzymes.

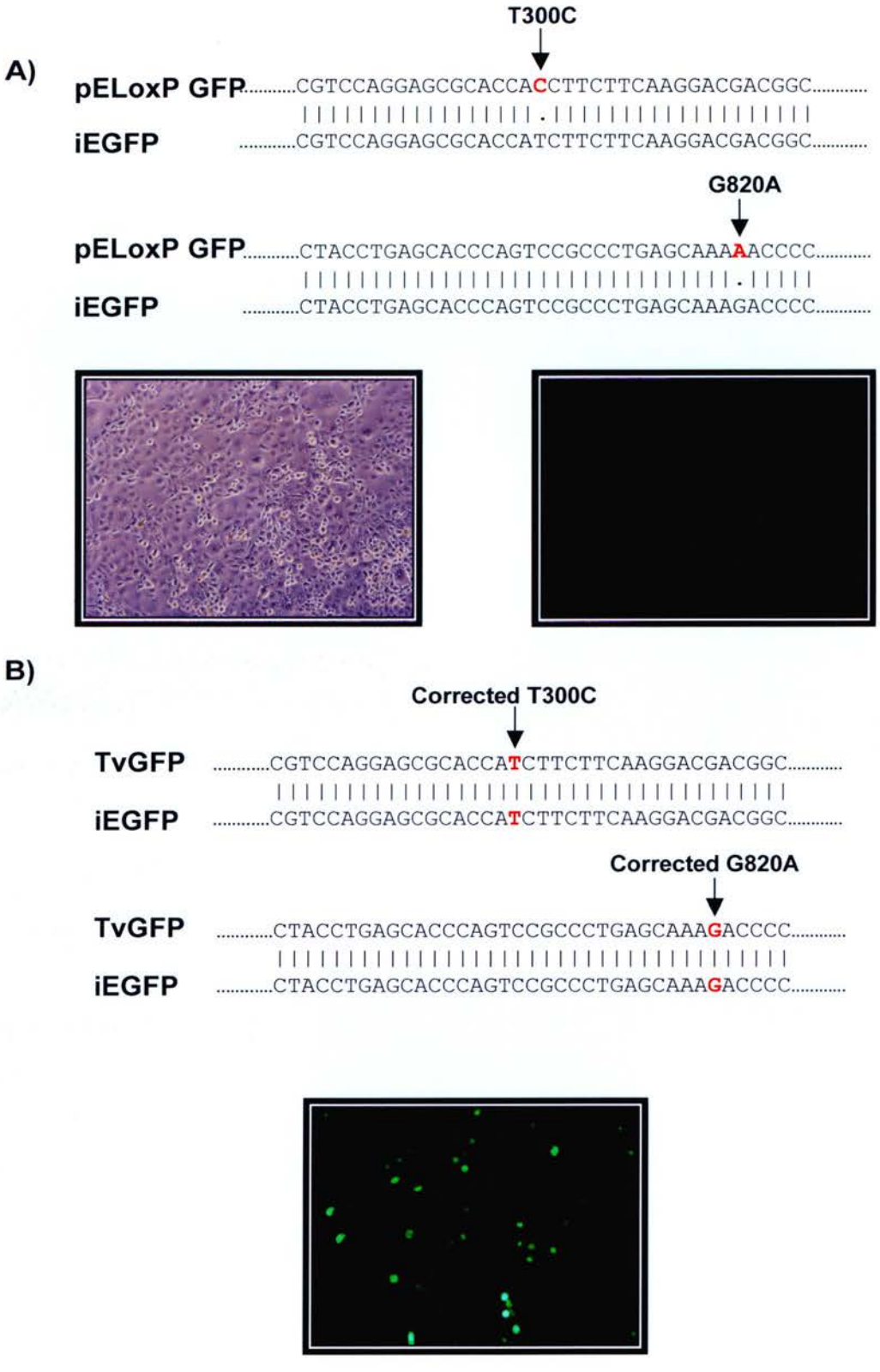


Figure 4.8: Base Changes in GFP sequence inhibit GFP fluorescence. DNA sequencing of pELoxPGFP plasmid revealed two base changes in the iEGFP sequence that resulted in loss of GFP expression (A). Base changes were altered by site-directed mutagenesis which restored GFP fluorescence. Magnification x10.

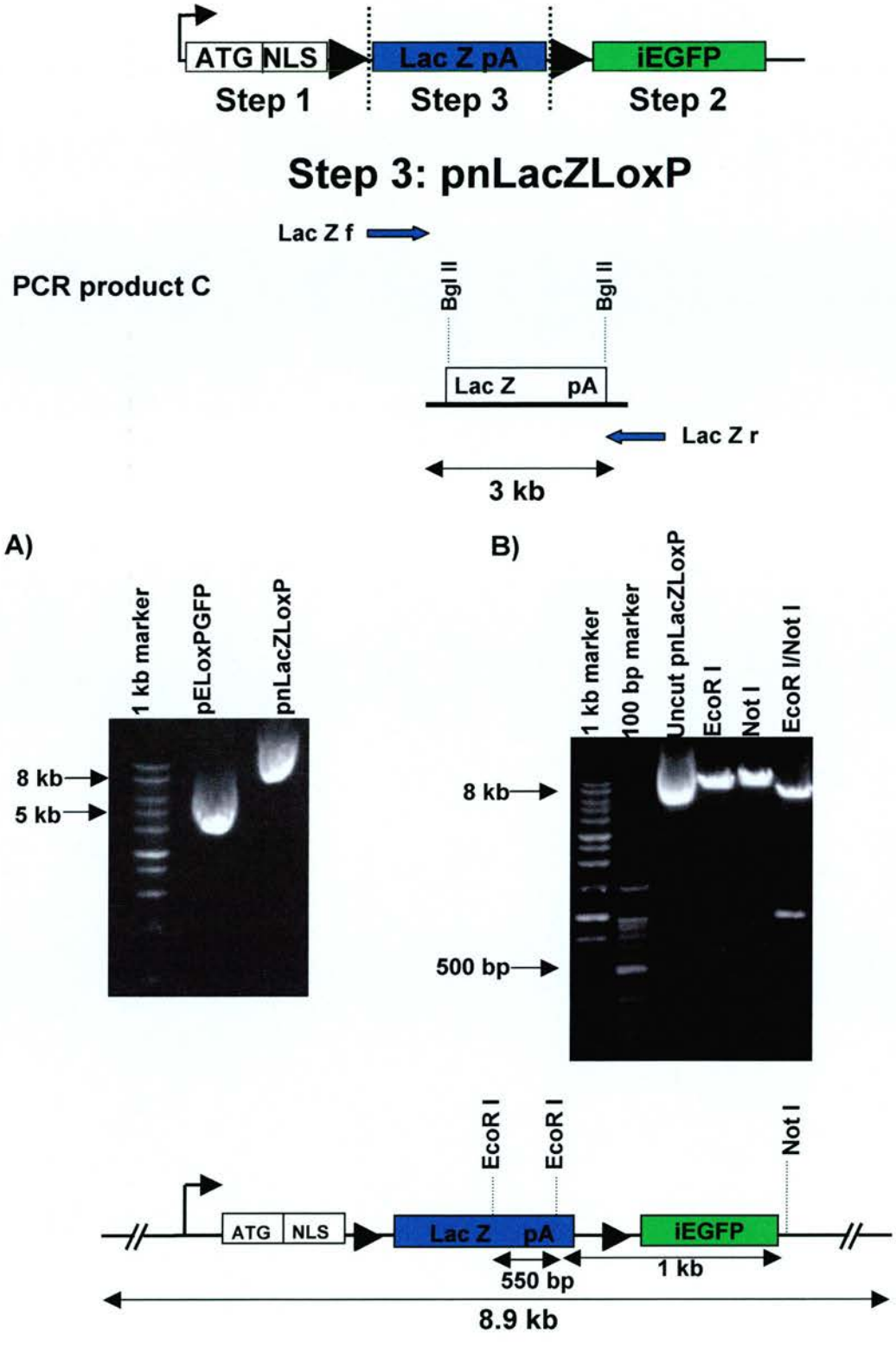


Figure 4.9: Cloning Step 3; generation of pnLacZloxP. Cloning of PCR product C with plasmid pELoxPGFP completed the final pnLacZLoxP plasmid. A) Clones containing the 3 kb insert were clearly visible on a DNA agarose gel. B) Restriction digest of the longer clones gave bands of 550 bp and 8.4 kb when cut with *Eco*RI and linearised when cut with *Not*I. Double digest released three bands of 550 bp, 1 kb and 7.4 kb.

enzymes released a band of 1.1kb (Fig 4.7) in size indicating that the LoxP GFP insert had replaced the smaller 800bp EYFP gene present in the background vector to give plasmid pELoxPGFP.

The plasmid was sequenced to check for mutations arising through PCR amplification. Two mutations were found, T300C and G820A (a thymidine to cytosine conversion and a guanine to adenine conversion) (Fig 4.8a). Both the base changes caused amino acid changes in the protein and the resulting protein showed no fluorescecnce (Fig 4.8a). Neither of the mutations were found to be present in the template DNA and therefore must have occurred during the PCR amplification of GFP. To avoid introducing further mutations by PCR it was decided to use site-directed mutagenesis to correct the base changes. Previously PCR product B had been cloned into a pGEM T vector (Promega) and this was used as a template in the mutagenesis reaction. The two primers LZLPSDM 1 and 2 were used to correct both of the base changes in one round of mutagenesis. Having successfully corrected the GFP sequence and confirmed that the GFP fluorescence was restored (Fig 4.8b) the insert was re-ligated with pEYFPLoxP to create the correct version of pELoxPGFP.

The final cloning step to generate the reporter construct pnLacZLoxP was to clone the β-galactosidase gene between the two *LoxP* sites. Again, two primers LacZ forward and reverse were used to amplify the gene from a β-galactosidase expressing plasmid (PCR product C). The background vector pELoxPGFP was digested with restriction enzyme *Bam*HI and PCR product C with the restriction enzyme *Bgl*II. Once cut these enzymes leave compatible ends allowing insertion of the LacZ gene to complete the pnLacZLoxP construct. Clones containing the LacZ insert were clearly visible on an agarose gel as they were 3kb greater in size than the pELoxPGFP plasmid (Fig 4.9a). To confirm which of the longer clones contained the insert in the correct orientation double digests with *Eco*RI and *Not*I were performed. Three of the final clones produced the expected product sizes of 550bp, 1kb and 7.4 kb (Fig 4.9b) and were checked by DNA sequencing. Figure 4.10a

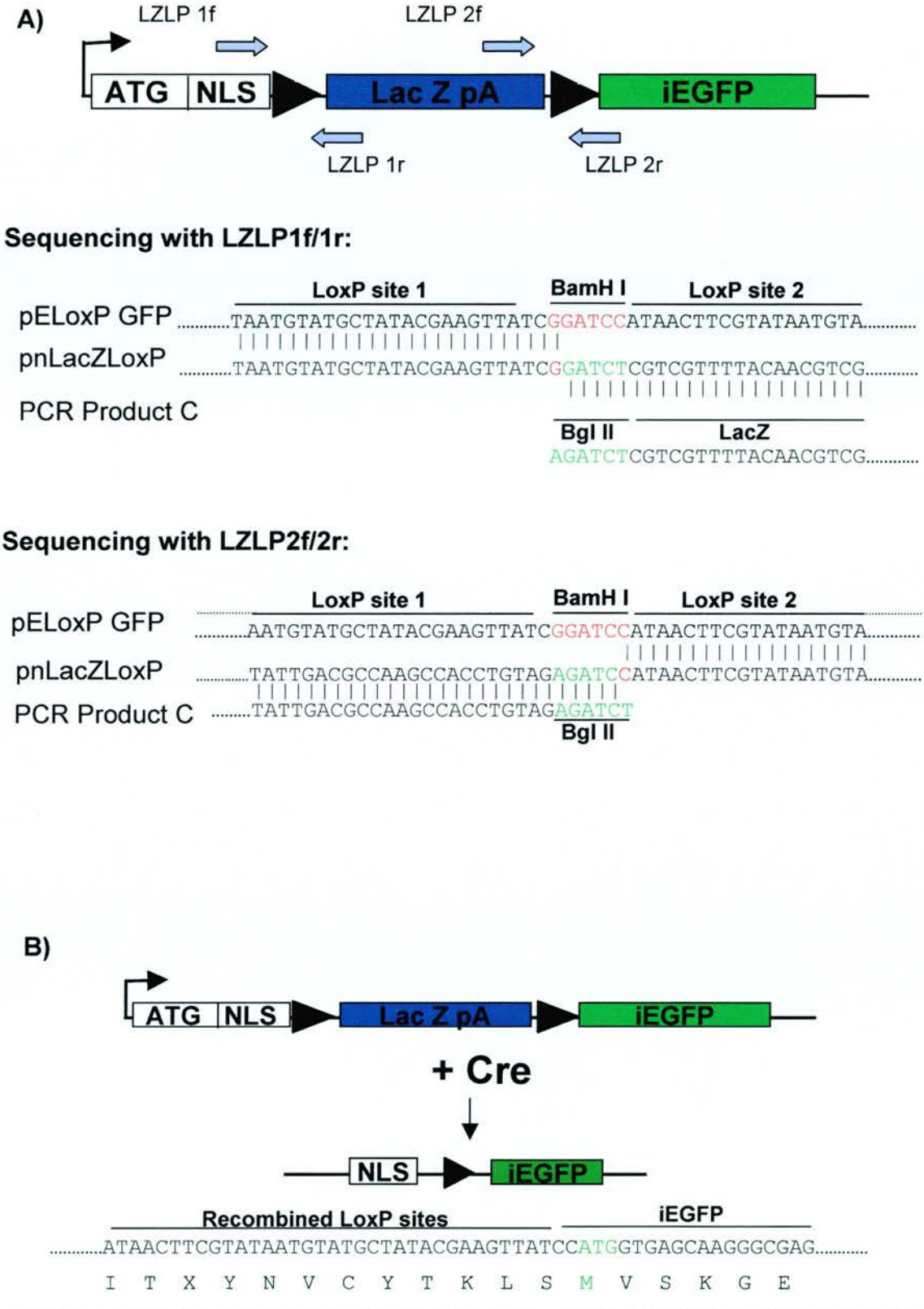


Figure 4.10: Sequencing of pnLacZLoxP. DNA sequencing with primers LZLP 1f/1r and 2f/2r confirmed the correct alignment of the β-galactosidase gene between the two LoxP sites completing the reporter plasmid (A). B) schematic and translated sequence showing the recombination of pnLacZLoxP after Cre mediated deletion and the translational start codon in the iEGFP.

shows the sequence data for the reporter construct using primers LZLP 1f/1r and 2f/2r to confirm to correct ligation of the insert between the two LoxP sites.

Sequences for the pELoxPGFP plasmid (Step 2) are aligned with either end of the LacZ gene to demonstrate the final sequence for pnLacZLoxP. In the presence of Cre the β-galactosidase gene is deleted and the *LoxP* sites recombine allowing expression of the GFP. Figure 4.10b shows the recombined sequence and its translation confirming that the start codon of the GFP is in frame with the translation initiation codon and will allow expression of GFP.

4.5 Functional studies of pnLacZLoxP and VP22Cre

4.5.1 Cellular Localisation of VP22Cre

To establish if the fusion of Cre to VP22 affected Cre expression, COS-7 cells were transiently transfected with the pVP22Cre plasmid. 24 hours later the cells were fixed with ice-cold methanol (-20°C) and labelled with a rabbit polyclonal anti-Cre antibody together with a primary conjugated Alexa 568 goat anti-rabbit secondary. VP22Cre expression was predominantly nuclear but at higher magnification (x32) some cells showed punctate staining around the nuclear rim, this was particularly notable where cell fusion had occurred (Fig 4.11). High background fluorescence was also observed however there was clear distinction between expressing and non-expressing cells. Mock transfected cells were negative for the Cre.

4.5.2 Verification of plasmid function

Firstly, the activity of Cre in VP22Cre and the ability of pnLacZLoxP to act as a reporter were tested. COS-7 cells were transfected with pnLacZLoxP or a control LacZ expressing plasmid. Both plasmids expressed functional LacZ and no β-galactosidase activity was seen in the mock transfected cells (Fig 4.12). Importantly, no GFP expression was observed in the pnLacZLoxP transfected cells. High levels of Cre mediated deletion were visible in cells that had seen either pVP22Cre or the control Cre-pac (Fig 4.12).

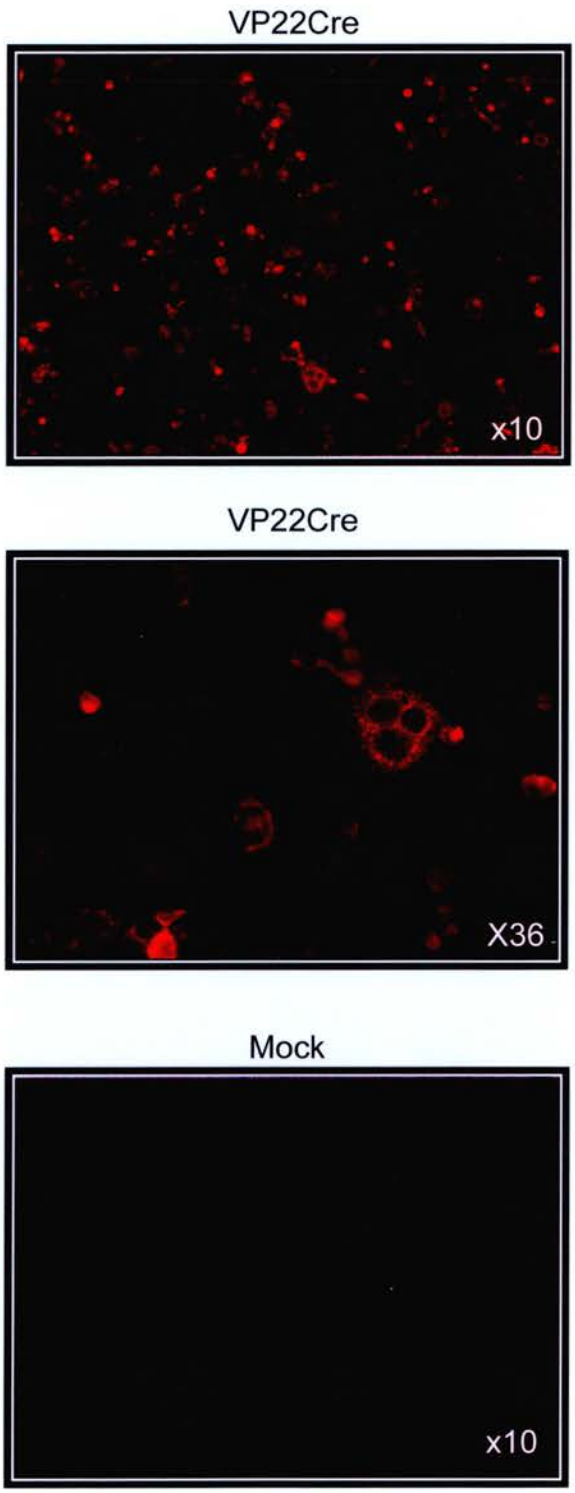


Figure 4.11: Expression of VP22Cre in COS-7 cells. Localisation of VP22cre expression in transiently transfected COS-7 cells using an anti-Cre antibody and an Alexa 568 (red) conjugated secondary antibody.

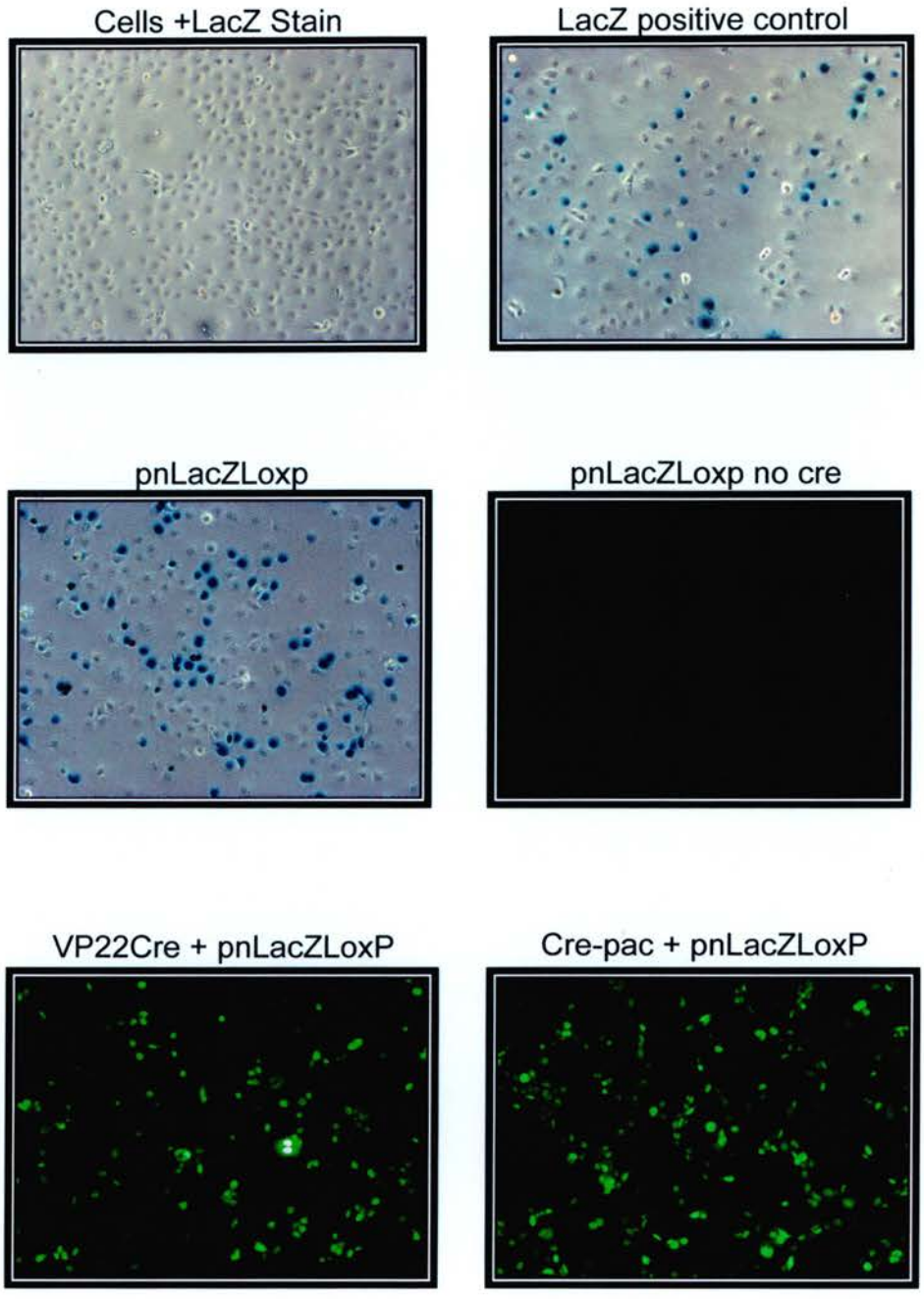


Figure 4.12: pnLacZLoxP and pVP22Cre expression. β-galactosidase expression (blue) can be clearly detected in cells transfected with pnLacZLoxP. Upon Cre mediated deletion GFP fluorescence is clearly visible.

The number of cells showing green fluorescence was slightly higher in the Cre-pac cells than in those that had been transfected with pVP22Cre, this was probably due to each plasmid being under a different promoter. However, the number of GFP (60%) positive cells was also greater than the number of cells expressing β-galactosidase (40%) suggesting that either the X-Gal stain was not sensitive enough to detect all the cells expressing the reporter, or that there was a problem with the translation or folding of the protein in some cells.

4.5 Discussion

To evaluate the potential of VP22 to translocate Cre across the cell membrane three plasmids were generated: to express VP22Cre in eukaryotic cells, to express VP22Cre in prokaryotic cells and a reporter construct that would detect Cre activity.

Antibody detection of VP22Cre in transfected COS-7 cells showed that VP22Cre exhibited a similar pattern of cellular localisation described for VP22 and other VP22 fusion proteins (Elliott and O'Hare, 1999; Phelan et al., 1998; Wybranietz et al., 1999). However, more significantly, it confirmed that fusion of the two proteins does not affect the ability of cells to express the Cre protein.

The reporter construct pnLacZLoxP was designed such that cells that were expressing the reporter could be detected by staining for LacZ and only after exposure to Cre would express GFP. Dual transfection of the reporter with pVP22Cre or a control Cre plasmid resulted in the expression of GFP confirming that pnLacZLoxP was functional as a reporter construct. More importantly, it also confirmed that fusion of the Cre recombinase to VP22 did not interfere with the ability of Cre to recognise the *LoxP* sites and mediate deletion.

The dual transfection experiments confirmed that the two plasmids can be highly expressed in the COS-7 cell line and that they function as a recombinase and as a reporter. Nevertheless, these experiments were not sufficient to determine whether

Cre affects the transduction potential of VP22. Antibody detection of VP22 is routinely used to determine intercellular spreading of VP22 fusion proteins (Aints et al., 1999; Elliott and O'Hare, 1997; Phelan et al., 1998). Detection of the VP22Cre in this way suggests that many of the cells contain the protein, however as the cells have been transfected, it is impossible to determine whether this is a result of transfection, cell division or intercellular spread of the protein. Moreover it would be more reliable to determine transduction of the VP22Cre by co-culturing cells expressing the protein with naïve cells or cells expressing the reporter construct. These experiments will be discussed in the next chapter.

Chapter 5

Cre delivery

5.1 Introduction

Conditional mutagenesis using the Cre/LoxP recombination system has become a fundamental tool in understanding how genes function *in vivo* (Lewandoski, 2001). However, the potential of site-specific recombination is limited by the difficulty in expressing the recombinase enzyme in the desired cell type and at specific stages of development. Three main strategies have been employed to gain temporal and spatial control over Cre mediated recombination *in vivo*:

I) Tissue specific expression of Cre from transgenes. The approaches generally use portions of promoters from genes expressed solely in the tissue/cell type of interest. Occasionally Cre is 'knocked into' a gene (eg. CD19-Cre) allowing increased specificity or level of expression and avoids the problems of inappropriate expression of chimerism seen in transgenics (Rickert et al., 1997). However, this method relies on finding a suitable gene in the cell type of interest, this has proven to be difficult for macrophages and dendritic cells, as many of the genes they express are shared by other myeloid cells. In addition the approaches require expression of the transgene in a particular tissue and many transgenes are silenced in the hematopoietic system. This approach has also proved to be time consuming and costly as it requires the generation of several mice.

II) Induction of Cre activity, either at the transcriptional level with inducers such as tetracycline (Gossen and Bujard, 1992) and the interferon inducible promoter Mx1 (Kuhn et al., 1995) or by controlling cellular localisation of recombinase (eg tamoxifen) (Metzger et al., 1995b; Schwenk et al., 1998). This is perhaps the most widely used method of initiating Cre recombination but is limited by the toxicity of inducers, the leakiness of the system before induction and it provides little cell type specificity.

III) Delivery of Cre into cultured cells either by transfection or by adenoviral delivery (Anton and Graham, 1995b). This can be useful *in vivo* if the

cell type of interest can be specifically targeted although has greater potential *in vitro* if the cells of interest can be readily cultured. This is the case for monocyte cells that if cultured from targeted mice and treated with Cre could be re-introduced *in vivo*. Moreover this method is far less time consuming and costly than crossing floxed mice with Cre expressing mice and allows greater control over the time and cell type specificity of recombination.

The potential for the delivery of Cre to cultured macrophages was explored. To avoid adverse responses to virus, two transduction proteins VP22 and MTS were tested alongside adenovirus.

5.2 Cre delivery using transduction proteins

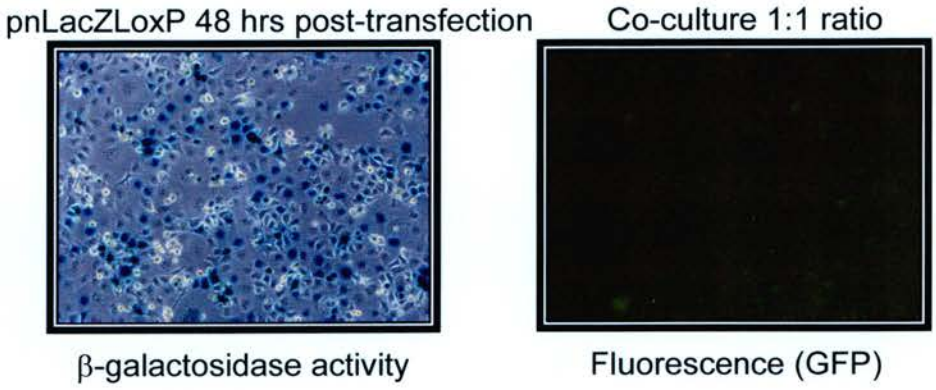
5.2.1 VP22 delivery of Cre

Many of the reports describing the transduction properties of VP22 have been carried out using VP22GFP fusion proteins and a variety of different cultured cell types (Brewis et al., 2000; Derer et al., 1999; Wybranietz et al., 1999). Much of this work has focussed on the localisation of the VP22GFP in cells expressing the fusion protein and in the cells that it has entered. Only two reports have described the use of VP22 to deliver functional enzymes that affect target cells. In the first of these reports VP22 was fused to p53 and used to induce apoptosis in a human osteosarcoma cell line (SAOS-2 p53 deficient cells). When COS-1 cells expressing the VP22 p53 fusion protein were cultured with naïve SAOS-2 cells apoptosis could be detected in the SAOS-2 cells using TUNEL staining suggesting that functional p53 had been delivered (Phelan et al., 1998). The second report used VP22 to introduce herpes simplex virus thymidine kinase (VP22-TK) to a human kidney epithelial cell line (293T) to evaluate its potential in suicide gene tharapy. Thymidine kinase makes cells sensitive to ganciclovir, and addition of VP22-TK to cultured cells increased cell death following ganciclovir treatment, again

demonstrating localisation of a VP22 fusion protein to the cytoplasm (Liu et al., 2001).

The potential of VP22 to deliver active proteins to many cell types, and to deliver these proteins to the nucleus, made it an ideal candidate for delivery of Cre. This was particularly appealing for macrophages that are resistant to many methods of transfection and due to their role in host defence are not ideally suited to viral transfection. Also, a VP22 fusion protein can be delivered by co-culturing cells or a conditioned supernatant/purified protein, therefore lending itself perfectly to the methods used for the *in vitro* culture of macrophages. The transduction ability of VP22 was tested using two cultures of COS-7 cells, one transfected with pVP22Cre and another with reporter plasmid (pnLacZLoxP). One day post-transfection VP22Cre expressing cells were plated with the reporter cells at a 1:1, 1:10 and 1:50 ratio in serum free medium and left in culture overnight. The cells were then analysed by microscopy and flow cytometry for evidence of Cre mediated recombination. The reporter construct was transfected with high efficiency with ~60% of the cells expressing β-galactosidase 48 hours after transfection. However no GFP expression was observed for any of the co-culture conditions suggesting that Cre activity was not delivered to the reporter cells (Fig 5.1a). Flow cytometry confirmed the microscopy results although showed a small peak (arrow) of GFP fluorescence that was probably caused by slight read through of the reporter plasmid in 1% of the cells (Fig 5.1b). This was only seen 48 hours after transfection and was also seen in cells transfected with reporter and cultured alone, so was independent of any Cre expression. These results indicated that the VP22Cre fusion protein expressed by the COS-7 cells was not capable of intercellular spread or was translocated into neighbouring cells but unable to function correctly following translocation. Therefore, Cre delivery from VP22Cre expressing cells was not a viable route of delivery.

A)



B)

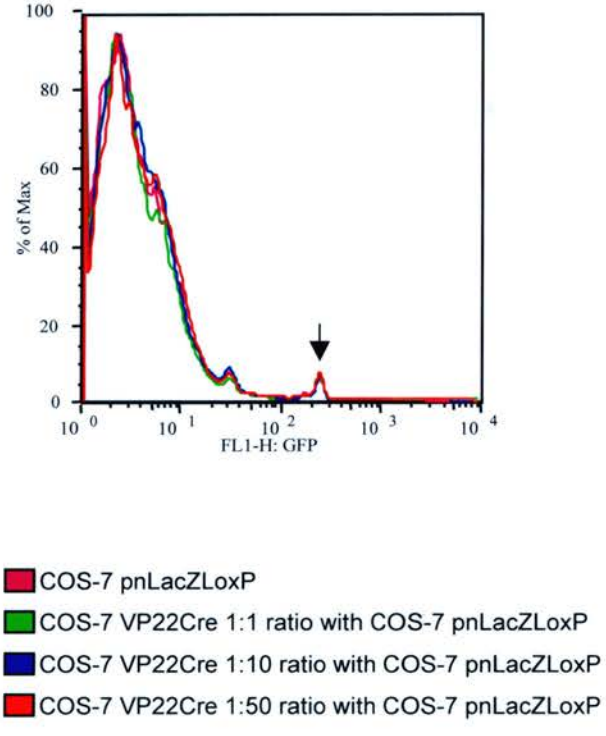


Figure 5.1: Assessment of VP22Cre transduction. COS-7 cells transiently transfected with either pnLacZLoxP or pVP22Cre were co-cultured at 1:1, 1:10 and 1:50 ratios for 16 hours. Cells were then assayed for β-galactosidase acitivity and the expression of GFP. X-gal staining indicated that ~60% of the transfected cells expressed the reporter construct however, fluorescence microscopy (A) and flow cytometry (B) showed no GFP expression indicating that there was no transduction of the VP22Cre protein.

5.2.1 Purification of pETVP22Cre

In the absence of VP22Cre translocation from transfected COS-7 cells, it was decided to generate recombinant purified protein to determine if soluble protein was capable of translocation and delivery of functional Cre into reporter cells. The 72 kDa VP22Cre protein was expressed in BL21(DE3) pLysS bacteria. Protein was prepared from bacterial cultures and His tag protein purified using a nickel affinity column. The expected 72 kDa protein could not be detected in the His tag purified fraction by Coomassie or Western blot with anti Cre antibody and was only present as a faint band in total protein fractions. Three prominent bands of 30, 25 and 18 kDa were seen and only a band of 25 kDa was purified by the His tag (Fig 5.2a&b). Very low amounts of protein were present in the eluted fraction and predominant proteins were only 20 kDa in size. The absence of the 72 k Da protein VP22Cre is most likely the result of cleavage of the protein by the bacteria. Therefore, the purification of the VP22Cre protein from bacteria was not a suitable method for generating VP22Cre protein.

5.2.2 MTSCre delivery of Cre

During the course of this work a paper was published describing the use of another transduction protein to traffic Cre recombinase into cells (Jo et al., 2001). Jo *et al* utilised the 12 amino acid membrane translocation sequence (MTS) from Kaposi fibroblast growth factor 4 that had been previously shown to permeate the cell membrane (Lin et al., 1995; Rojas et al., 1998). The expression plasmid for His_6-NLS-Cre-MTS (MTSCre) (Fig 5.3a)(a kind gift from Dr Earl Ruley, Vanderbilt University School of Medicine) was used to produce recombinant protein as an alternative to pETVP22Cre.

The 42Kda MTSCre protein was purified from BL21(DE3) pLysS competent bacteria using a nickel affinity resin (Ni-NTA agarose Qiagen). Fractions from each step of the purification procedure were run on a 12% SDS-PAGE gel and analysed by Coomassie staining and Western blot (Fig 5.3). The MTSCre protein was

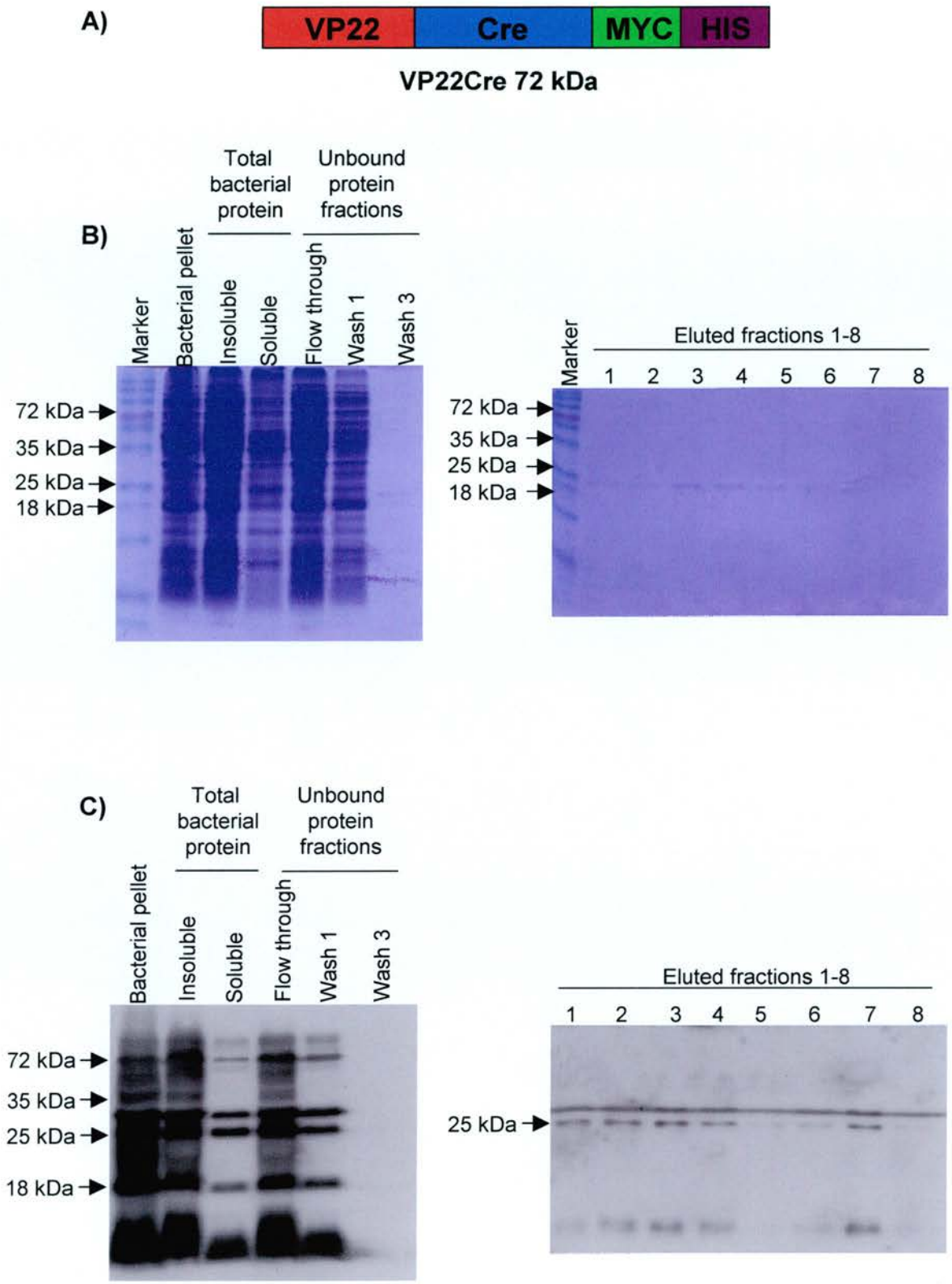


Figure 5.2: VP22Cre protein purification.
A) Schematic representation of VP22Cre protein showing VP22, Cre recombinase, myc/His tag for detection and purification. SDS-PAGE analysis of VP22Cre protein purification. B) Coomassie stained gel and C) Western blot analysis of purification fractions detected with an anti-Cre antibody. The 72 kDa VP22Cre protein was not detectable in any of the fractions.

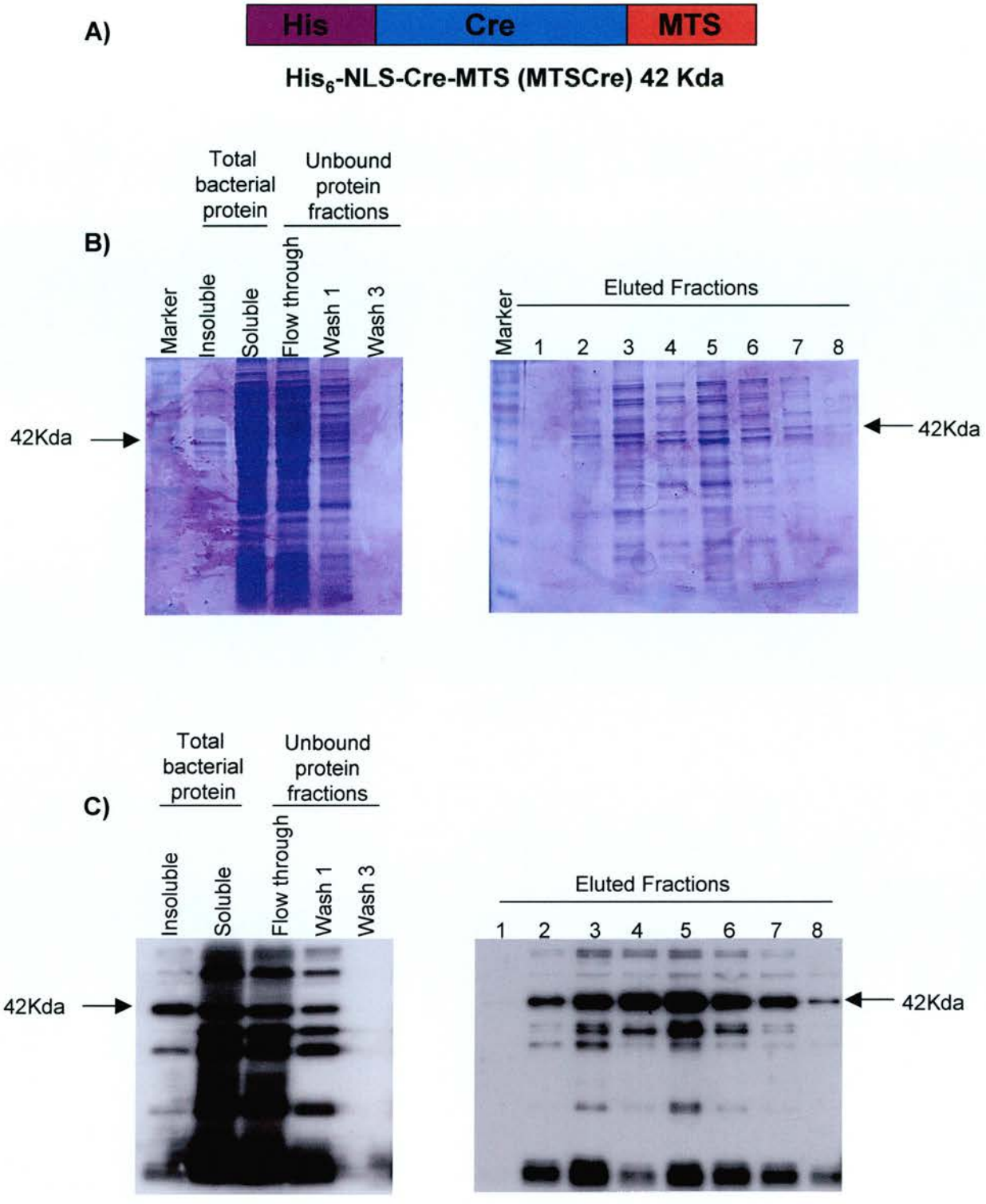


Figure 5.3: MTSCre protein purification.
A) Schematic representation of the MTSCre protein. SDS-PAGE analysis of MTSCre protein purification. B) Coomassie stained gel and C) Western blot analysis of purification fractions detected with an anti-Cre antibody. The same cell equivalent amount of protein was loaded in each lane.

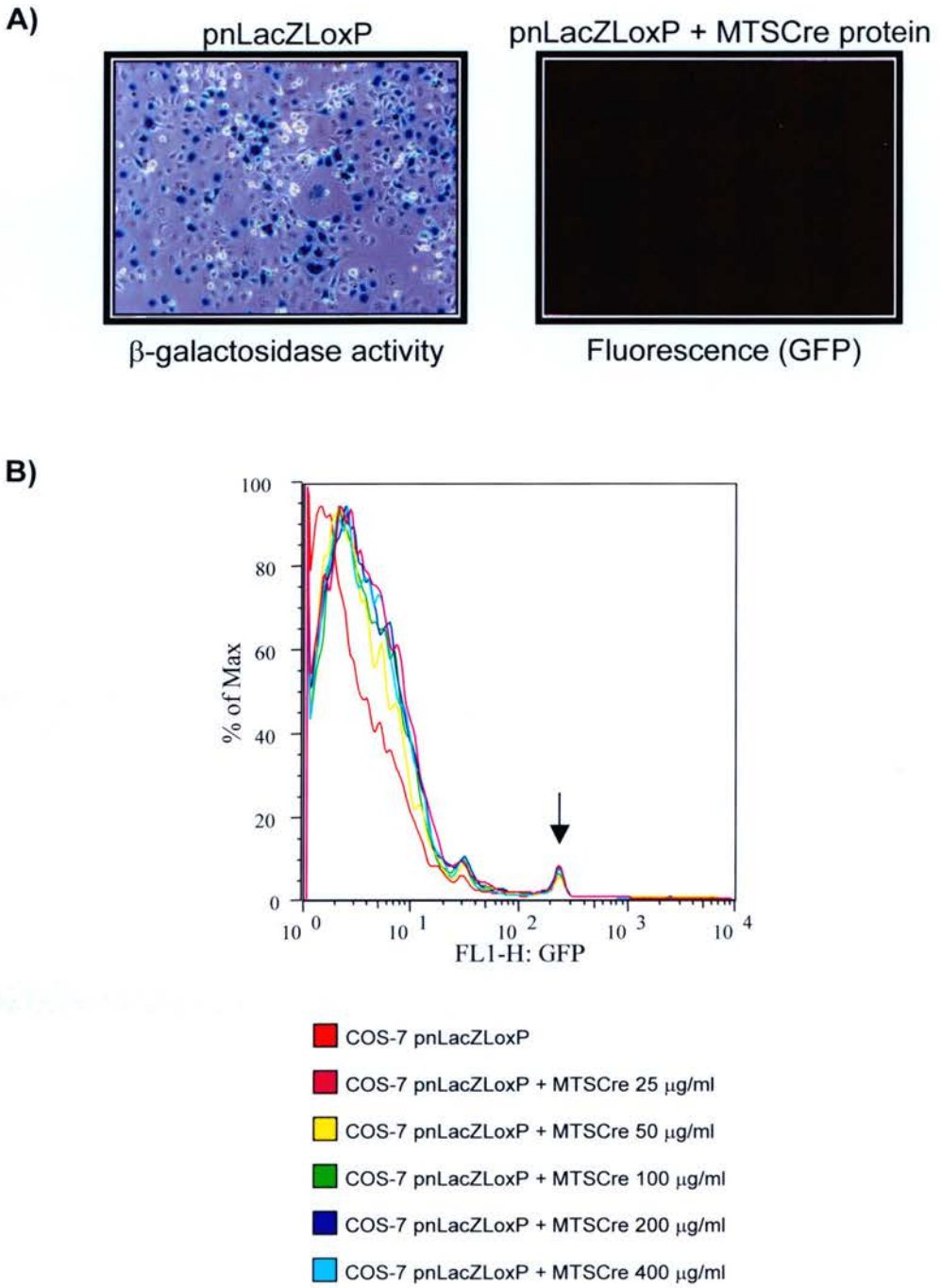


Figure 5.4: Assesment of MTSCre cell permeability. COS-7 cells transfected with pnLacZLoxP were incubated with purified MTSCre protein at 25, 50, 100, 200, 400μg/ml. X-gal staining showed ~50-60% of the cells were expressing the reporter but no GFP fluorescence was observed in any of the culture conditions as shown by microscopy (A) or flow cytometry (B).

detected using an anti-Cre antibody (Novagen). A prominent band in the Western blots, MTSCre was detected in the total bacterial protein and in the column purified fractions. MTSCre was also detected in the insoluble fraction but similar levels of protein were also apparent in the flow through and the first of the washing steps suggesting there was sufficient protein in the cleared lysate to saturate the column (Fig.5.3). Elution from the column showed that the MTSCre protein had been enriched and that fractions 2-7 contained the highest levels of the protein. However several other bacterial proteins were also co-purified and a second protein of similar size to the MTSCre protein was the predominant protein in the eluted fraction (Fig 5.3). Nevertheless high concentrations of the MTSCre protein were obtained and the protein was used without further purification.

5.2.3 Assesment of MTSCre Transduction

COS-7 cells transfected with the reporter pnLacZLoxP were used to assess whether the purified MTSCre protein was capable of inducing recombination. MTSCre was incubated with the reporter cells at concentrations of 25, 50, 100, 200 and 400μg/ml in serum free media overnight. The following day the cells were analysed by fluorescent microscopy and flow cytometry. Despite high levels (~60% of the cells) of expression of β-galactosidase no GFP fluorescence was observed in cells that had been incubated with MTSCre protein at any of the concentrations shown (Fig 5.4a). Flow cytometry analysis of the same cells showed a slight increase in autofluorescence in the treated cells but confirmed that no deletion had taken place as indicated by the lack of GFP expression (Fig 5.4b). A slight increase in fluorescence was observed in 1% of the cells (Fig 5.4b arrow) as in the VP22Cre co-culture experiments (Fig 5.1) but again this was due to slight read through of the reporter construct.

5.2.6 Interim discussion

The data presented in this section has evaluated the ability of two transduction proteins VP22 and MTS to introduce Cre into cells containing floxed genes. Co-culture of cells expressing VP22Cre fusion protein with cells expressing the reporter

construct pnLacZLoxP resulted in no detectable transduction of the protein. Several possibilities exist to explain this lack of transduction: I) Fusion of the Cre recombinase to the VP22 protein in some way inhibits the ability of VP22 to be exported and imported into neighbouring cells. A recent report by Stroch *et al.* also failed to detect intercellular spread of VP22 fused to I*k*Bα from expressing to non-transfected cells, although addition of exogenous protein from cell free extracts of cells expressing the VP22I*k*Bα protein resulted in transduction of the protein (Stroh et al., 2003). Similarly this may also be the case for the VP22Cre but suggests that the intercellular spreading of VP22 fusion proteins may be dependent on the structure of the protein that it is fused to. II) The size of the VP22Cre fusion protein may hinder intercellular transport. However, fusion of the p53 gene to VP22 (Phelan et al., 1998) produced a protein of 90 kDa that did not affect the transduction potential of the VP22. III) Cre also has intrinsic nuclear localisation ability (Le et al., 1999) and therefore it may be that a 'tug of war' exists between the Cre and the VP22 with the Cre trying to localise to the nucleus and the VP22 trying to exit the cells, resulting in absence of translocation. III) VP22Cre was capable of translocation to neighbouring cells but unable to localise to the nucleus to function. IV) The VP22Cre may be delivered not function as a recombinase in the co-cultured cells. This is highly unlikely as dual transfection of VP22Cre with the reporter construct resulted in deletion of β-galactoidase and expression of GFP, confirming that the fusion protein works as a recombinase.

Attempts to produce recombinant VP22Cre were unsuccessful. Consistent with other reports (Kueltzo et al., 2000; Normand et al., 2001; Stroh et al., 2003) expression of the VP22Cre in a bacterial expression system resulted in cleavage and loss of the recombinant VP22Cre protein. Previously it has been shown that the C-terminal half of the protein is essential for the transport function of VP22 (Elliott and O'Hare, 1997). Normand *et al.* showed that VP22 fusion proteins containing C-terminal residues 159-301 could be produced in large quantities by expression in bacteria without cleavage of the protein and that they retained the transduction properties of the full length VP22 protein (Normand et al., 2001). Due to time constraints and the

availability of the MTSCre transduction protein a truncated VP22Cre fusion protein was not generated. Although the failure to produce recombinant VP22Cre protein is likely to be linked to the use of full length VP22 it must also be considered that Cre is itself a phage protein and when expressed at high levels can be also be toxic (Loonstra et al., 2001).

Exogenous application of purified MTSCre protein on cells expressing the reporter pnLacZLoxP also showed no detectable signs of Cre activity. This was unexpected as the original work describing MTSCre as a transduction protein showed high levels (~80%) of recombination in floxed cells treated *ex vivo* with the recombinant protein (Jo *et al*, 2001). The reason for this difference remains unknown. It is possible that co-purifying bacterial proteins interfere with the MTS translocation ability. Initially it was decided that the contaminating proteins co-purified with the MTSCre protein would have little or no effect on the transduction of the protein but it is possible that these bacterial proteins could be causing the MTSCre to aggregate therefore preventing it crossing the cell membrane. Nonetheless, without further purification of the protein it remains unclear as to whether these proteins render the MTSCre ineffective.

Some controversy exists surrounding the potential of transduction proteins to cross the cell membrane. Lundberg *et al.* propose that the nuclear localisation of the transduction proteins observed in antibody detection of VP22 and TAT fusion proteins is a fixation artifact when the cells are fixed with methanol (Lundberg et al., 2003; Lundberg and Johansson, 2001). Moreover evidence is provided for binding of these fusion proteins to the cell membrane and it is suggested that the proteins are taken up by constitutive endocytosis. Another group also report that VP22 and TAT bind to the cell membrane but do not mediate membrane translocation of the diptheria Toxin A fragment (Falnes et al., 2001). However, the work describing the use of Cre recombinase fused to the MTS or TAT transduction proteins (Jo et al., 2001; Peitz et al., 2002) strongly argues against the cellular binding of these proteins as Cre is required to be expressed in the nucleus to mediate deletion of *LoxP* targeted

genes. The experiments discussed in this chapter were in no way exhaustive and cannot challenge the proposal that VP22 does not translocate across the cell membrane. Although the transduction proteins tested did not result in cellular delivery of Cre it is suggested that the intercellular trafficking of VP22 may be dependent on its fusion partner and that this work merits further investigation.

5.3 Adenoviral delivery of Cre.

Recombinant adenoviruses have been used to efficiently deliver a variety of genes to a broad spectrum of cell types (Anton and Graham, 1995b). The ability to easily manipulate the adenoviral genome and the ease with which adenovirus can be propagated in high titres renders them particularly suitable as vehicles for gene delivery (Russell, 2000). Adenovirus uptake is mediated by $\alpha_v\beta_3$ and $\alpha_v\beta5$ and expression of delivered genes is transient because the virus does not integrate into the host genome (Anton and Graham, 1995b). This is particularly important for the expression of Cre recombinase as sustained expression would result in cellular toxicity. Anton and Graham were the first to describe recombination of LoxP sites using adenoviral delivery of Cre (Anton and Graham, 1995b). Since, this approach has been widely used to gain temporal and spatial control over Cre mediated recombination both *in vitro* and *in vivo* (Prost et al., 2001b; Rohlmann et al., 1996; Shibata et al., 1997). Adenoviral gene transfer has been used with some success for the transfection of macrophages *in vitro* (Kluth et al., 2001). To overcome the difficulties experienced with Cre delivery by transduction proteins, adenovirus expressing Cre recombinase was used to mediate deletion of floxed genes in both COS-7 cells expressing a reporter and in integrin α_v targeted macrophages.

5.3.1 Adenoviral delivery of Cre to pnLacZLoxP reporter cells

The Adenovirus expressing Cre (kind gift from Dr Graham, McMaster University, Canada and batches were kindly prepared by Dr Sallenave, University of Edinburgh) is a replication deficient virus (E1 deleted). The adenoviral Cre (AdCre) was first tested on COS-7 cells expressing the pnLacZLoxP reporter at various multiplicities

of infection (MOI) and the cells analysed by fluorescence microscopy and flow cytometry for expression of GFP (Fig 5.5). The lowest dose tested (MOI 25) was sufficient to cause relatively high levels of recombination as judged by the number of cells expressing GFP (Fig 5.5a). However, at an MOI of greater than 75 the cells had started to round up and detach from the plates indicating that infection at the higher doses was toxic to the cells (Fig 5.5a). This cell death was probably a combination of the amount of virus used but also that both GFP and Cre recombinase can be toxic when expressed at high levels.

Flow cytometry of the cells showed that at an MOI of 25 ~40% of the cells were expressing GFP, at MOIs of greater than 25 ~50% of the cells were expressing GFP suggesting complete transduction (Fig 5.7b). At an MOI of greater than 50 the percentage of GFP positive cells started to level off, which was not surprising as the number of cells expressing GFP is limited by the number of cells expressing the reporter (~40-50%). Analysis of the geometric mean fluorescence intensity (GMFI) showed that increasing the MOI used did not affect the amount of GFP expressed in the cells (Fig 5.7b). This was expected, as the amount of GFP expression is dependent on the promoter driving the reporter and not on virus transduction. Having confirmed the efficiency of the AdCre (~100%) it was decided to use it on primary macrophages cultured from the integrin α_v floxed mice.

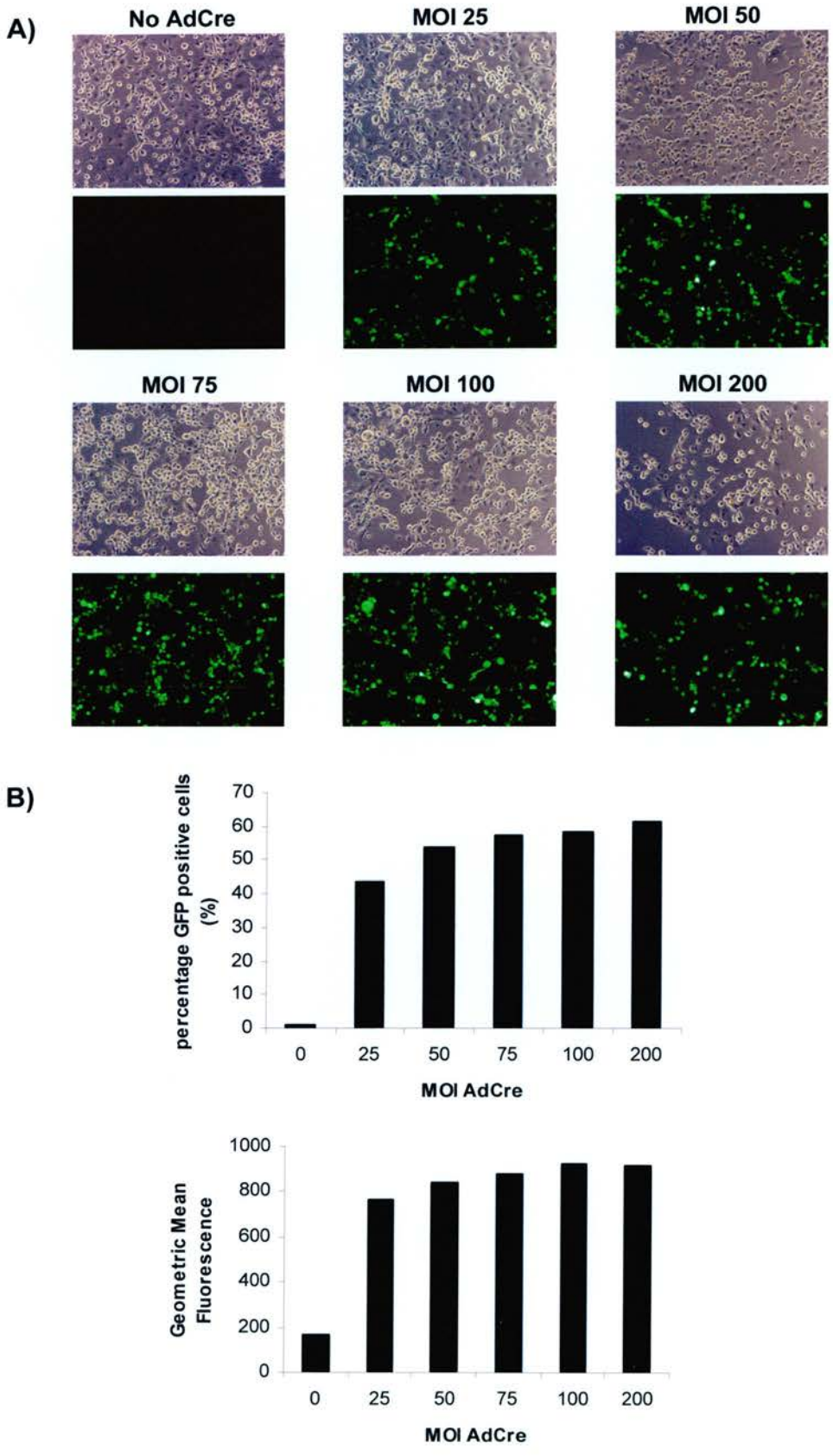


Figure 5.5: Adenoviral Cre delivery to reporter cells. COS-7 cells transfected with the pnLacZLoxP were incubated overnight with adenoviral Cre at increasing levels of infection. Cells were then analysed by microscopy (A) and flow cytometry (B) for expression of GFP. Positive cells were gated as less than 1% of control and the geometric mean fluorescence intensity measured for the positive cells. Magnification x10.

5.3.2 Adenoviral GFP titration in primary macrophages

Adenoviral transfection of human monocyte derived macrophages requires the addition of lipofectamine^{TM}(Invitrogen) to gain successful levels of transfection (Dr Sallenave, personal communication). To determine the optimum conditions for adenoviral transfer in mouse bone marrow derived macrophages (BMDM) adenovirus expressing GFP (AdGFP) was used (a kind gift of Dr Hitt, McMaster University, Canada and batches were kindly prepared by Dr Sallenave, University of Edinburgh). BMDM were cultured overnight in macrophage media containing AdGFP at MOI of 25, 75, 100, 200 or 500 ± lipofectamine. The cultures were then analysed by microscopy and flow cytometry for the expression of GFP. Cells incubated with AdGFP in the absence of lipofectamine showed a high percentage (90-95%) of GFP expression (Fig 5.8) using an MOI of 75 and above. MOI of >200 appeared to be toxic to the cells and this was reflected in the GMFI which decreased from 1100 to ~800 when an MOI of 500 was used (Fig 5.6b). In comparison macrophages cultured in the presence of lipofectamine showed lower transduction efficiency (<60%) and lower GMFI in expressing cells (Fig 5.9b). Therefore, based on the data obtained from the titration of AdGFP it was decided that an MOI of 75 or 100 of AdCre in normal macrophage media would introduce sufficient levels of the AdCre to mediate deletion of the floxed genes without inducing high levels of cell death.

5.3.3 Role of integrin α_v in apoptotic cell phagocytosis

To investigate the role integrin α_v may play in the phagocytosis of apoptotic cells integrin α_v was deleted in BMDM using AdCre infection of macrophages carrying a floxed integrin α_v gene. BMDM from mice carrying a Cre activated β-galactosidase reporter (Mao et al., 1999) were used as a control because they allow Cre mediated recombination that will not affect integrin α_v. The following day the virus containing media was removed and fresh media added and the cells cultured for a further two days. Cre recombination was assessed by PCR of genomic DNA from untreated and treated macrophages from both the integrin α_v floxed mice and control.

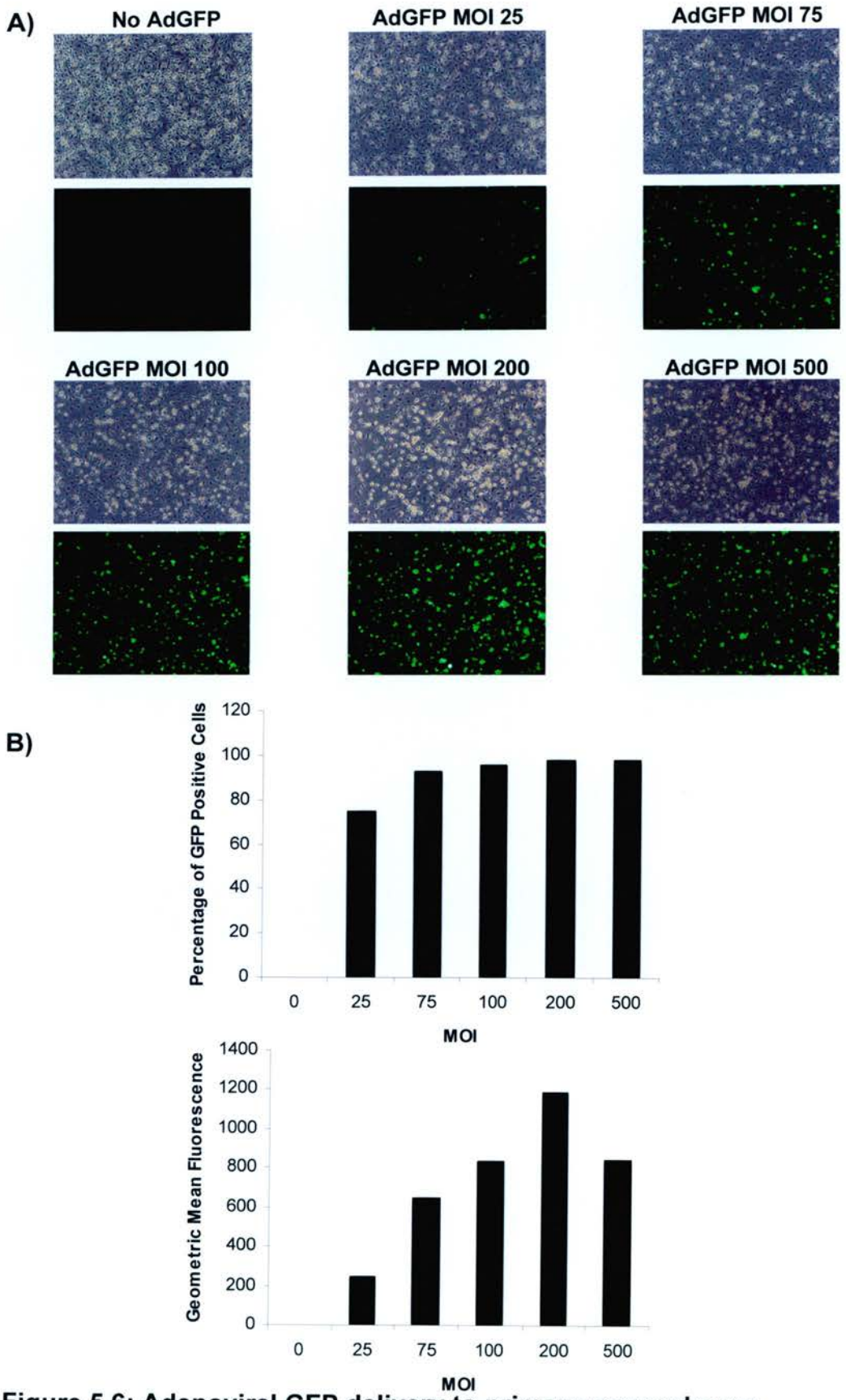


Figure 5.6: Adenoviral GFP delivery to primary macrophages.
Bone marrow derived macrophages were incubated with increasing MOI of adenoviral GFP. MOI of 75 and above is sufficient to cause ~100% transfection. Magnification x10.

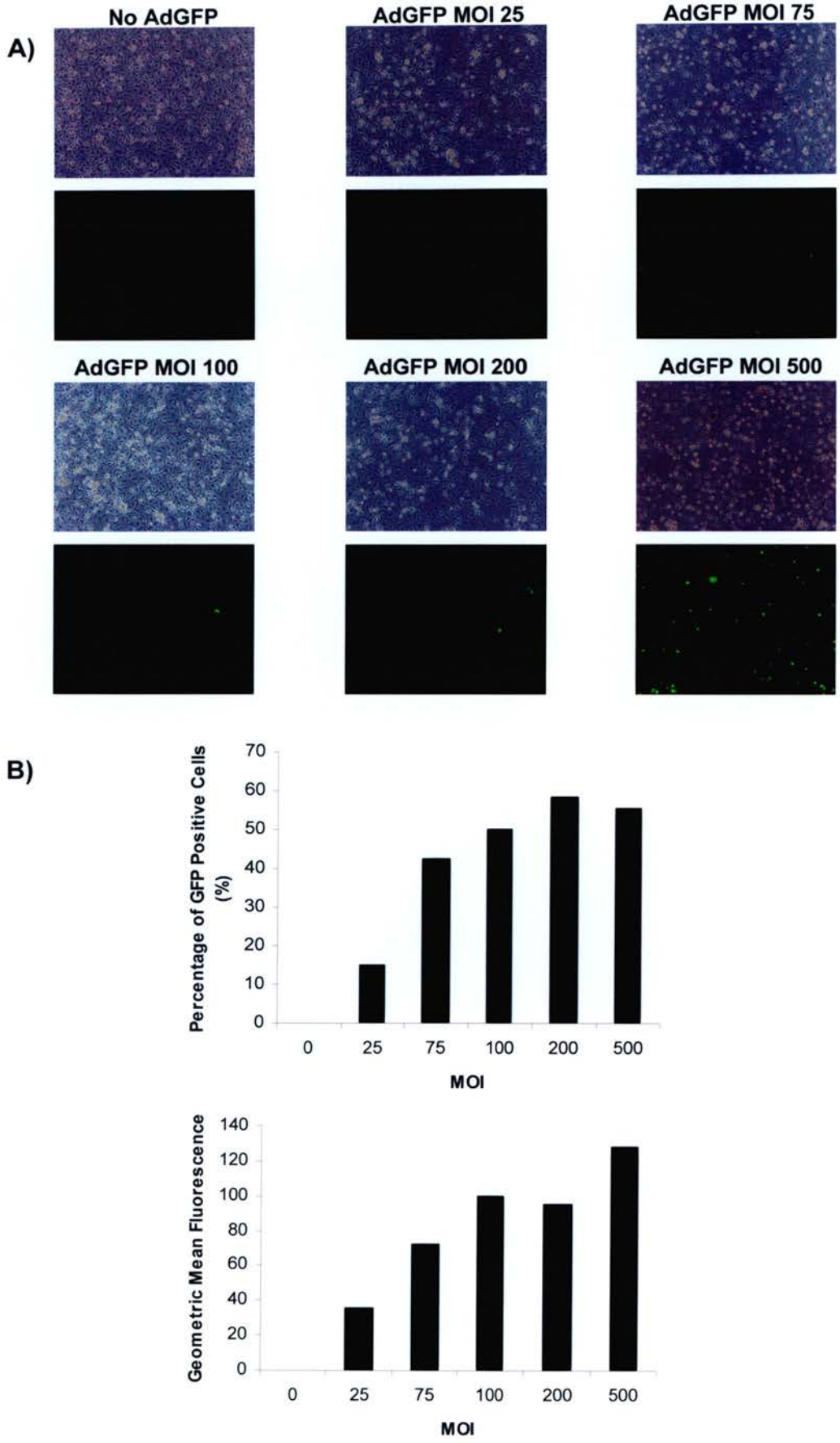


Figure 5.7: Adenoviral GFP delivery to primary macrophages in the presence of lipofectamine. Bone marrow derived macrophages were incubated with increasing MOI of AdGFP in the presence of lipofectamine. Lipofectamine appears to inhibit the levels of transfection compared to macrophages cultured in media plus AdGFP (Fig 6.8). Magnification x10.

The 4f6/5b3 primers amplified the expected 900bp product from the integrin α_v targeted macrophages (Fig 5.8). The treatment of integrin α_v floxed macrophages with AdCre resulted in recombination (~70%) as indicated by the amplification of the deleted product (250bp) but the recombination was not complete as the 900bp floxed band was also amplified. However the amount of the 900bp product was decreased compared to the untreated integrin α_v floxed cells. In each of the treatments macrophages from the control mice showed amplification of the 600bp band expected for the untargeted integrin α_v gene showing that AdCre had no effect on the normal genomic locus (Fig 5.8).

The macrophages were then fed with 24-hour aged (fluorescent) human neutrophils (~55% apoptosis) for 2 hours, GRGDSP the integrin specific inhibitory peptide or the control peptide GRADSP were added to block integrin α_v function. Interestingly the levels of apoptotic cell phagocytosis were unaffected by the loss of integrin α_v suggesting that while α_v is not essential for phagocytosis it must able to regulate the process as GRGDSP inhibits phagocytosis through integrin α_v (5.9a). Apoptotic cell phagocytosis was inhibited by GRGDSP in integrin α_v floxed macrophages (Fig 5.9). In contrast α_v floxed macrophages treated with AdCre showed no inhibition of phagocytosis in the presence of RGD compared to controls. Phagocytosis of apoptotic cells in the control floxed macrophages was also inhibited by RGD in all of the treatments confirming that loss of inhibition observed for integrin α_v deleted macrophages was not the result of AdCre treatment of the macrophages (5.9b).

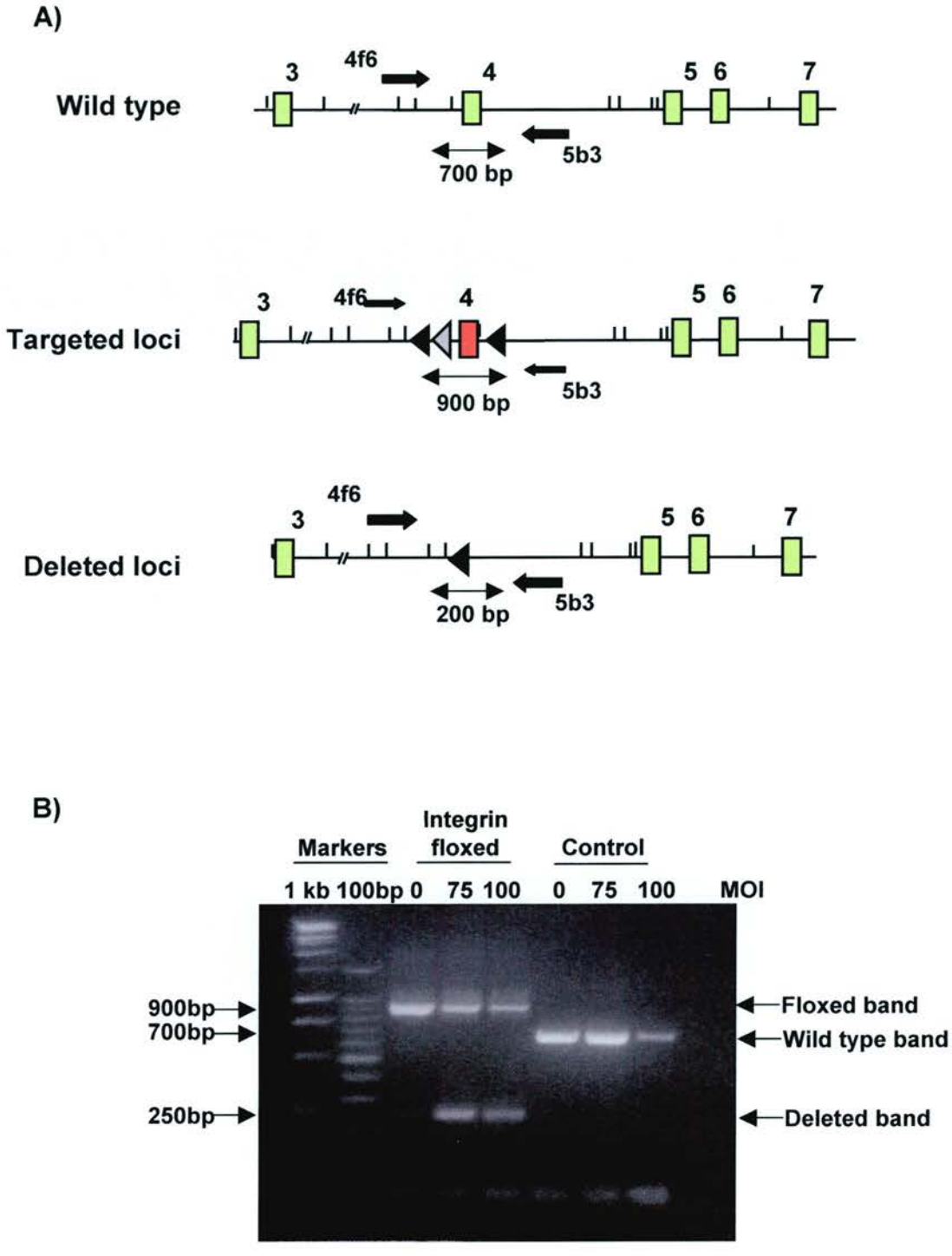


Figure 5.8: Detection of integrin α_v deletion by PCR. A) Schematic showing the positions of the PCR primers and the expected product sizes for wild type, floxed and deleted cells. B) PCR of gDNA from Integrin α_v floxed and control macrophages that were treated with AdCre at MOI of 75 and 100.

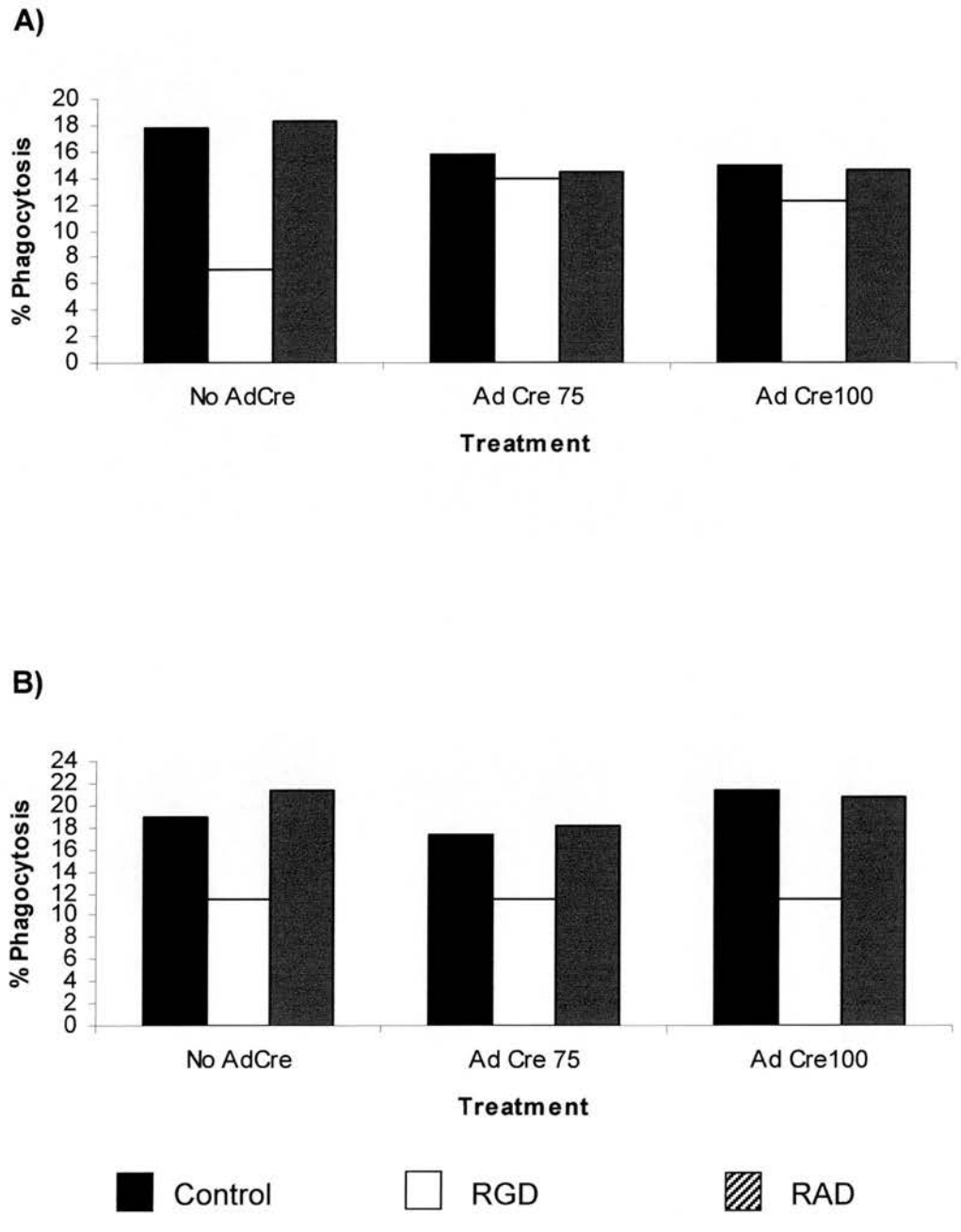


Figure 5.9: Role of integrin α_v in phagocytosis. Integrin α_v floxed macrophages (A) and macrophages from a control floxed mouse (B) were treated with AdCre at MOI of 75 and 100. Apoptotic cell phagocytosis was then assessed in the presence of the inhibitory peptide RGD or the irrelevant control peptide RAD.

5.3.4 Discussion.

Adenovirus was used as an alternative to the transduction proteins VP22 and MTS to efficiently deliver Cre to cells containing floxed DNA. Infection of COS-7 cells expressing a reporter (pnLacZLoxP) with adenoviral Cre showed that almost all of the cells that were expressing the reporter had undergone recombination within 24 hours of infection. This observation combined with the relatively low levels of infection (MOI 25) required for recombination indicate that adenoviral delivery of Cre is both a simple and extremely efficient method of deleting *LoxP* targeted genes.

A wide variety of genes and a broad spectrum of cell types have been transfected using adenovirus including human monocyte derived macrophages and dendritic cells (Anton and Graham, 1995b; Hoves et al., 2003). However, adenoviral transfection of bone marrow derived macrophages has not previously been reported. It was essential, therefore to determine if macrophages could be infected in a similar manner. Infection of bone marrow derived macrophages with an adenovirus expressing GFP showed that macrophages were readily infected, with almost 100% of the monolayer expressing GFP after 24 hours incubation with the virus. In contrast to human monocyte derived macrophages and dendritic cells that require infection under serum free conditions and in some cases the addition of a lipid transfection reagent (Hoves et al., 2003)(Dr Sallenave, personal communication), bone marrow derived macrophages could be infected in their normal media (containing FCS and L929 conditioned media). Therefore confirming that like the COS-7 cells, macrophages can easily be transfected with high efficiency using adenovirus even at relatively low MOI, allowing delivery of Cre to the targeted cells. The successful transfection of bone marrow derived macrophages using adenoviral Cre allowed the investigation of the role of the integrin α_v gene in apoptotic cell phagocytosis. $\alpha_v\beta_3$ (vitronectin receptor VnR) was first implicated in phagocytosis by Savill and colleagues (Savill et al., 1990). They demonstrated that uptake of apoptotic neutrophils was inhibited by monoclonal antibodies to either the α or β subunits of the VnR and by the tetrapeptide RGDS. Phagocytosis of apoptotic neutrophils was also inhibited by the natural RGD bearing proteins vitronectin and

fibronectin (Savill et al., 1990). Since, then $\alpha_v\beta_3$ has also been reported to be involved in the engulfment of apoptotic bodies by DCs (Rubartelli et al., 1997) although in human systems this is thought to be mediated by $\alpha_v\beta_5$ (Albert et al., 1998a). To date, much of the work implicating integrin α_v in phagocytosis has been carried out using blocking antibodies and the synthetic peptide RGD. Loss of function experiments (knockout) are difficult because integrin α_v null mice either die *in utero* or perinatally (Bader et al., 1998). However gain of function experiments have been reported. Transfection of $\alpha_v\beta_3$ into 293T cells confers increased phagocytic ability, through a complex involving CrkII-p130^{cas}-Dock180 activation of Rac1 (Albert et al., 2000).

The data presented in this chapter shows that deletion of integrin α_v in macrophages had profound implications for apoptotic cell phagocytosis. Treatment of the integrin α_v floxed macrophages with adenoviral Cre caused deletion of the floxed α_v gene. Recombination was not complete, although the levels of recombination cannot accurately be determined from the PCR. Nevertheless, loss of integrin α_v did not affect the levels of apoptotic cell phagocytosis but served to prevent inhibition of this phagocytosis by RGD. Similar results were observed in work done in collaboration with Dr Adam Lacy-Hulbert using mice carrying null mutations for β_3 or β_5 (Fig1.3) (Reynolds et al., 2002). In both knockouts, phagocytosis was comparable to control cells. However, RGDS was unable to block phagocytosis in $\beta_3^{-/-}$, but continued to block in $\beta_5^{-/-}$ cells. This would suggest that RGD is specifically inhibiting phagocytosis through $\alpha_v\beta_3$ and not through $\alpha_v\beta_5$ or other integrins. Macrophages express other RGD binding integrins, and it is unlikely that β_3 is the only target for RGDS. Indeed, at high concentrations, RGD causes detachment of macrophages, a phenomenon that occurs in the $\beta_3^{-/-}$ macrophages (Dr Adam Lacy-Hulbert, personal communication). It is important to note that RGD peptides exert a variety of biological effects and have been shown to induce anti-inflammatory, anti-coagulant and anti-metastatic activities ((Romanov, 1999; Schwarz et al., 1998; Vassilev et al., 1999)). In particular RGD peptides have been shown to induce anoikis (an

anchorage-dependent form of apoptosis) in several different cell types ((Chen et al., 1997; Levrey Hadden and Henke, 2000; Perks et al., 1999) however, this phenomenon was not observed during the time period with which the phagocytosis assays were carried out. Therefore, RGD does have effects independent of β_3, but its effects on phagocytosis are solely via $\alpha_v\beta_3$.

There are two potential mechanisms to explain these observations: Firstly, it may be that in the absence of $\alpha_v\beta_3$ other phagocytic pathways are upregulated to maintain phagocytosis. It is likely that in the BMDM cultures the macrophages are undergoing apoptosis (possibly triggered by the adenovirus) exerting a 'tonic' effect on expression of phagocytic pathways. Real-time PCR expression data from within our laboratory has shown that $CD36^{-/-}$ and $SRA^{-/-}$ macrophages have increased expression of β_3 integrins relative to control BMDM suggesting that in the absence of CD36 or SRA, other potentially compensatory phagocytic pathways were upregulated. Similarly in mice lacking β_3 and low-density lipoprotein receptor (LDLr or CD91) CD36 expression has been shown to be upregulated in smooth muscle cells (Weng et al., 2003).

Alternatively, $\alpha_v\beta_3$ may act as a negative regulator of normal phagocytosis, by regulating involvement of other integrins or receptors. $\alpha_v\beta_3$ ligands such as RGDS, antibodies or matrix proteins probably cause downregulation of phagocytic machinery. Evidence for such integrin crosstalk has already implicated β_3 in the regulation of $\alpha_5\beta_1$ phagocytosis (Blystone et al., 1994; Blystone et al., 1999). Furthermore β_1 integrins have been shown to influence the binding and uptake of apoptotic cells by leukocytes (Schwartz et al., 1999; Vernon-Wilson et al., 2003). However, to address the mechanisms involved in maintaining phagocytosis in the absence of integrin α_v further studies using a combination of blocking antibodies and knockout mice will be required.

Despite the initial promise of transduction proteins as vehicles for Cre delivery into macrophages the data presented in this chapter has shown that this approach is limited by several factors I) the ability of VP22 fusion proteins to traffic between cells is not as simple as first thought. II) the translocation potential of the VP22 fusion protein may be dependent on the fusion partner and III) the inability to produce large quantities of recombinant protein. Nevertheless, the most simple and efficient method of transfecting macrophages with Cre *in vitro* was found to be adenoviral gene transfer. One of the main advantages of delivering Cre to cells in this way is that it allows the deletion of the *LoxP* targeted genes at desired time points within the cells of interest (Wang et al., 1996). Moreover, the ability to knockout specific genes *in vitro* lends itself to the potential for re-introducing the cells back into mice to assess the role of particular genes/cell types *in vivo* and importantly it allows the investigation of gene function where knockout mutations prove to be embryonic lethal (Anton and Graham, 1995b). However there is one major drawback with this approach. Adenoviral gene transfer elicits an immune response that can result in the removal of the infected cells (Akagi et al., 1997). Several attempts have been made to overcome this response that is the result of the activation of $CD8^+$ lymphocytes against the cells expressing the virus. In particular the use of immunosuppressive drugs has permitted sustained expression of genes delivered by the virus (Kafri et al., 1998) although it is thought that further debilitation of the virus by removal of all viral coding regions may be required to resolve the immunogenicity (Yang et al., 1995).

Chapter 6

General conclusions and future work

The ultimate aim of this thesis was to produce myeloid cells lacking the integrin α_v gene to allow the investigation of the role that α_v integrins play in the phagocytosis of apoptotic cells. Due to the lethal phenotype of the α_v knockout mouse, two alternative complementary approaches were used. In the first approach, ES cells carrying a disrupted integrin α_v gene were differentiated into DCs. In the second approach Cre was delivered to macrophages cultured from integrin α_v targeted mice to allow deletion of the gene.

Prior to the differentiation of genetically modified ES cells, wild type ES cells were used to characterise the potential of this system to generate myeloid phagocytes capable of ingesting particles such as apoptotic cells. As presented in this thesis ES cells were successfully differentiated to macrophages and DCs in the presence of the cytokines IL-3 and either M-CSF or GM-CSF. Both the ES cell line and the serum used were critical factors in generating large numbers of these cells. The cytokines used did not generate pure populations of either cell type and most cultures were likely to be a mixture of both. Comparison of the fluorescence intensity profiles for ES derived cells and BMDM showed a more heterogeneous pattern of expression for each of the surface markers in the ES derived cells. This suggested that not only terminally differentiated macrophages and DCs were present in the cultures but probably many precursor cells at different stages of development. This was not unexpected as the cytokines used to drive the differentiation of ES cells are not only involved in driving differentiation towards the terminally differentiated macrophages and DCs but are also important in each stage of differentiation from pluripotent stem cells to myeloid cells to monocyte precursors (Fig 7.1).

E14 ES derived cells were able to regenerate DCs upon routine harvesting of the cultures permitting the maintenance of long-term cultures. This suggested that early precursor cells present in the cultures were probably capable of self-renewal. One possible explanation for this is the presence of stromal cells in the cultures containing plated EBs, these cells probably condition the media with factors that help maintain

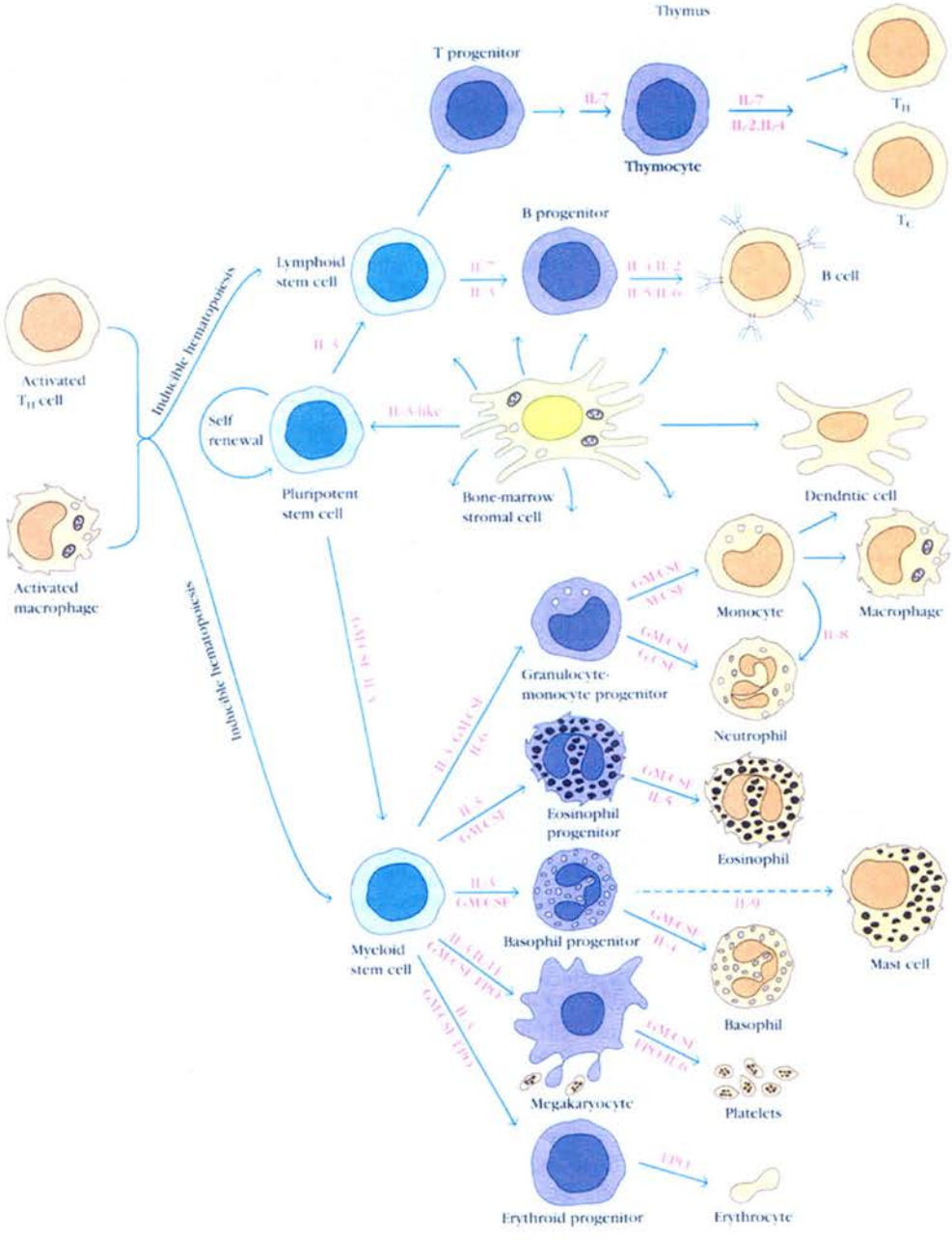


Figure 6.1: Cytokine regulation of hematopoiesis. IL-3, GM-CSF and M-CSF drive hematopoiesis from pluripotent stem cells to myeloid stem cells and finally to terminally differentiated cell types such as macrophages and DCs taken from Kuby,1997.

renewal of the precursor cells. In comparison, cultures of BMDM do not appear to regenerate from stem cells, although BMDM will proliferate in the presence of M-CSF. This may be due to the absence of the factors required to maintain renewal of progenitor cells but it is more likely that in culture the BMDM arise from a precursor cell that is more committed than those present in the differentiated ES cultures.

Surface phenotype alone is not sufficient to distinguish clearly between macrophages and DCs in culture and the morphology and functional characteristics such as adherence, phagocytic capability and the ability to up-regulate co-stimulatory molecules must also be considered. These ES derived myeloid cells were highly phagocytic and phagocytosed both inert particles such as latex beads and apoptotic cells. ES derived DCs could also be matured with LPS and while the capacity of ES derived DCs to stimulate a T-cell response has previously been shown by others (Fairchild et al., 2000; Senju et al., 2003) it was not assessed during the course of this work. Nevertheless, both macrophages and DCs generated by the differentiation of ES cells were similar in surface phenotype to bone marrow derived myeloid cells and showed some of their functional attributes.

The differentiation of genetically altered ES cells was successful in that ES cells carrying a disrupted integrin α_v gene differentiated in a similar manner to normal ES cells. The DCs arising from the differentiation of these ES cells were comparable to those derived from normal ES cells and were capable of both phagocytosis and maturation. However, DCs putatively bearing a disrupted integrin α_v gene did not exhibit phagocytic properties expected from studies of $\beta_3^{-/-}$ macrophages and expression of functional integrin α_v could not be excluded. Instead, adenoviral delivery of Cre to integrin α_v floxed macrophages proved to be a more useful method of generating α_v null macrophages. The major advantage of delivering Cre to floxed BMDM is that greater numbers of cells lacking the floxed gene can be generated relatively easily, but this method requires the generation of targeted mice that is both costly and time consuming. Alternatively, delivery of Cre to targeted ES derived cells allows the investigation of genes in specific cell types without the need to

generate a conditionally targeted mouse. One disadvantage of differentiating ES cells was that the cultures often contain several different cell types unlike the cultures of BMDM. While the use of cytokines to drive differentiation was effective in skewing the differentiation towards a particular cell type, cultures of pure cell types cannot be generated by this method. This could be overcome by the introduction of selectable markers into lineage-restricted genes (Billon et al., 2002). At present this is difficult for myeloid cells as they are very heterogeneous but with further delineation of the transcription factors involved in hematopoietic differentiation this approach could be exploited in the future.

Phagocytosis studies using $\beta_3^{-/-}$ macrophages showed that in the absence of this integrin phagocytosis was unaffected but was no longer inhibitable by the antagonistic peptide RGD. Similar results were obtained for macrophages lacking integrin α_v, however phagocytosis by DCs derived from ES cells carrying a disrupted integrin α_v gene could still be inhibited by RGD. This raised the question of whether the inhibition was a result of functional expression of integrin α_v. One way to address this would be to carry out immunohistochemistry studies. Previous attempts to detect surface expression of α_v and β_3 integrin subunits were unsuccessful with the antibodies available but different antibodies from other suppliers could be tested. Alternatively, integrin α_v expression could be assessed using Western blotting. Nevertheless, the data would be difficult to interpret without investigating the effects on phagocytosis by RGD on ES derived DCs that were expressing a normal integrin α_v gene. While wild type E14 ES cells could be used to generate control DCs, the ideal experiment would be to differentiate ES cells carrying a floxed integrin α_v gene. The floxed DCs could then be treated with adenovirus expressing Cre in a similar manner to the BMDM allowing both control and integrin α_v deficient DCs to be generated simultaneously.

The capacity to generate integrin α_v null macrophages by treating floxed macrophages cultured from targeted mice with adenoviral Cre has opened up new

avenues for investigating the role of this integrin in apoptotic cell phagocytosis. Integrin α_v is thought to bind TSP 1 alongwith CD36 to form a "molecular bridge" to apoptotic cells. Macrophages cultured from $CD36^{-/-}$ mice, similar to integrin $\alpha_v^{-/-}$ macrophages show no defect in phagocytosis (Dr Adam Lacy-Hulbert, personal communication) and this may be due to compensation by the other receptor. A previous report using $\beta3^{-/-}$ mice has shown that in the absence of this integrin, CD36 becomes upregulated on smooth muscle cells indicating a close link between these receptors (Weng et al., 2003). Crossing integrin α_v targeted mice with $CD36^{-/-}$ mice and treating the macrophages with adenoviral Cre to generate integrin $\alpha_v^{-/-}/CD36^{-/-}$ macrophages may provide greater insight into the compensatory pathways involved in maintaining phagocytosis in the absence of these receptors. Real-time PCR could also be a useful method for investigating which phagocytic receptors are upregulated in macrophages deficient in integrin α_v. This would help to determine which receptor knockout mice could be crossed with integrin α_v targeted mice or which combinations of blocking antibodies could be used to help delineate the pathways involved in phagocytosis in the absence of integrin α_v. It would also be of interest to generate integrin α_v deficient DCs from the integrin α_v targeted mice to investigate the effects of RGD on apoptotic cell phagocytosis by DCs and this may help elucidate if macrophages and DCs use different integrins for this process.

In summary, this thesis has presented two alternative methods for generating genetically modified myeloid cells. Both methods, combined with the ability to deliver Cre to these cells has provided a powerful system in which to assess the role of genes that have a lethal phenotype *in vivo*. Moreover, delivery of Cre to genetically modified cells derived from ES cells allows the investigation of genes in specific cell types without necessarily generating a conditionally targeted mouse. Importantly, macrophages and DCs generated by either of these methods could be re-introduced back into mice allowing assessment of specific gene function *in vivo*.

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Appendix I: Materials and Solutions.

All tissue culture reagents were purchased from Invitrogen (Life technologies, Paisley, UK) unless otherwise stated. All chemicals were of analytical reagent grade and purchased from Sigma (Poole, UK) unless otherwise stated. Tissue culture plastics were purchased from Costar excluding the ES differentiation culture plates.

Routinely used reagents	**Cat.No**
Collagenase-Sigma	C-0130
Culture dishes for differentiation- Stem Cell Technologies	27150
ES cell differentiation serum: Labtech serum-Labtech International, Sussex, UK	
ES cell serum-Sigma	F2442
FBS (pre-tested for differentiation)- Stem Cell Technologies	06900
Gelatin-Sigma	G1890
GRADSP-Calbiochem,	03-34-0052
GRGDSP-Calbiochem, Nottingham, UK	03-34-0035
Insulin-Sigma	I882
Leishmans's stain -BDH Poole, Dorset	350224L
LPS-Sigma	L3129
Methylcellulose -Stem Cell Technologies, Canada	M3120
Mitomycin C-Sigma	M0503
Monothioglycerol -Sigma	M6145
RmIL-3-R&D -Oxon, UK	403-ML-010

Serum for batch testing:

Batch1-Cat.No. F2442, Batch 101K8401 (Sigma)

Batch 2-Cat.No. PET 10270098, Batch 40G2027K (Invitrogen)

Batch 3-Cat.No. PET 10270098, Batch 40F9224K (Invitrogen)

Batch 4-Cat.No. PET 10106151, Batch 40Q8124F (Invitrogen)

Batch 5-Labtech serum, Batch 2539 (Labtech International)

Solutions for bacterial work

Luria Bertani (LB) Broth

Bactotryptone (DIFCO)	10g/L
Yeast extract (DIFCO)	5g/L
NaCl	10g/L

Make up to 1L with distilled water and autoclaved.

Supplemented with antibiotics where stated:

Ampicillin 50μg/ml

Kanamycin 30μg/ml

Chloramphenicol 50μg/ml

15g of Bactoagar (DIFCO) was added to 1 L of LB broth to make Agar plates.

DNA Solutions

10X TBE Buffer

TRIZMA base	108g
Boric Acid	55g
0.5M EDTA pH 8.0	40ml

Make up to 1L with distilled water and autoclaved.

TE Buffer

Tris	1.2g
EDTA	0.37g

PH 8.0 and make up to 100ml distilled water

DNA loading buffer

20% glycerol in 1xTBE

bromophenol blue

Xylene cyanol

Genomic DNA lysis buffer

Tris-Cl (pH8.0)	10mM
NaCl	400mM
EDTA (pH8.0)	2mM
Proteinase K (add fresh)	100µg/ml

Protein Buffers

SDS-PAGE- 12% separating gel

Distilled water	5.25ml
4x Tris-HCL.SDS, pH8.0	3.75ml
acrylamide/Bis acrylamide (37:5:1)	6.0ml
10% (0.1mg/ml) ammonium persulphate	50µl
TEMED (Sigma)	10µl

4% Stacking gel

Distilled water	3.05ml
4x Tris-HCL.SDS, pH 6.8	1.25ml
acrylamide/Bis acrylamide (37:5:1)	0.65ml
10% (0.1mg/ml) ammonium persulphate	25µl
TEMED (Sigma)	5µl

4X Tris.Cl/SDS pH 8.8

Tris base	91g
SDS	2g

Dissolve Tris in 300ml distilled water, pH to 8.8 with 1N HCL and filtered through 0.45µm filter before adding SDS.

Make up to 500 ml

4X Tris.Cl/SDS pH 6.8

Tris base	6.05g
SDS	0.4g

Dissolve Tris in 40ml distilled water, pH to 6.8 with 1N HCL and filtered through 0.45μm filter before adding SDS.

Make up to 100 ml

4X SDS-reducing Buffer

1M Tris.Cl pH 6.8	2ml
glycerol	3.2ml
SDS	0.64g
β-mercaptoethanol	1.6ml
distilled water	1.2ml
Bromophenol blue	trace

5X Running Buffer

Tris base	15.1g
Glycine	72g
SDS	5g

Make up to 1L with distilled water

10X Transfer Buffer

Glycine	144g
Tris base	30g

Make up to 1L with distilled water.

Coomassie Blue Stain

Coomassie Blue	0.25g
Methanol	45ml
Acetic acid	10ml
Distilled water	45ml

Add coomassie blue into methanol, then add acetic acid and water, filter before use.

Destain

Methanol	45%
Acetic acid	10%
Distilled water	45%

Miscellaneous

MACS Buffer

PBS	
EDTA	2mM
BSA (TC grade)	0.5%

FACS wash

PBS without Ca^{2+}/Mg^{2+}	
BSA	0.2%
Sodium azide	0.1%

β-galactosidase stain

Potassium ferrocyanate (0.4M Final)	100µl
Potassium ferricyanate (0.4M Final)	100µl
X-gal (40mg/ml stock)	250µl
$MgCl_2$ (2M)	10µl

Make up to 10mls with PBS

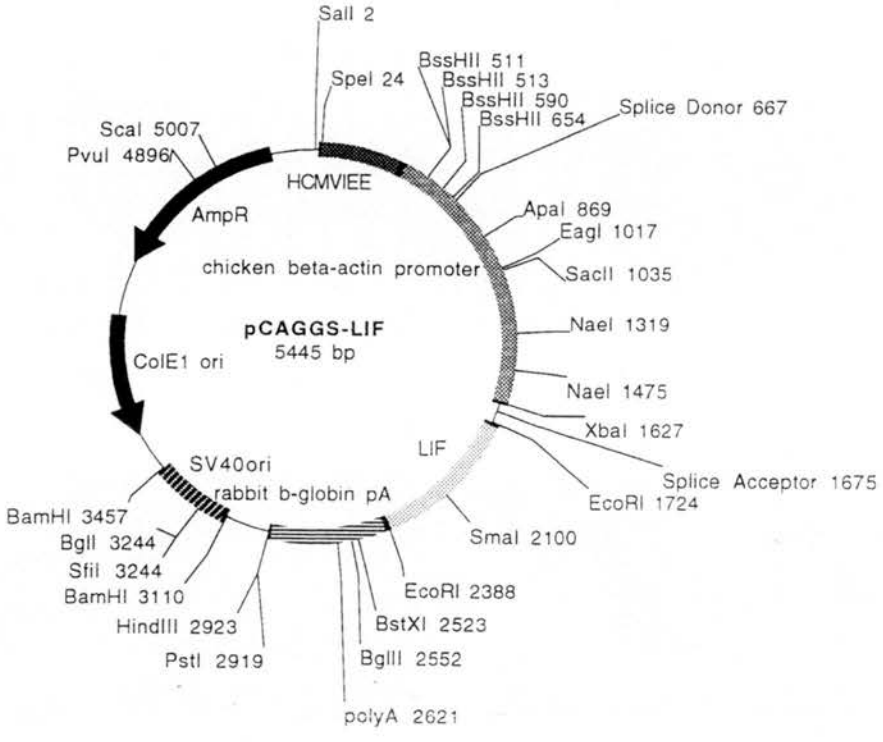
Mouse tonicity PBS

NaH_2PO_4 (4mM)	0.48g/l
Na_2HPO_4 (16mM)	2.27g/l
NaCl (150mM)	8.77g/l

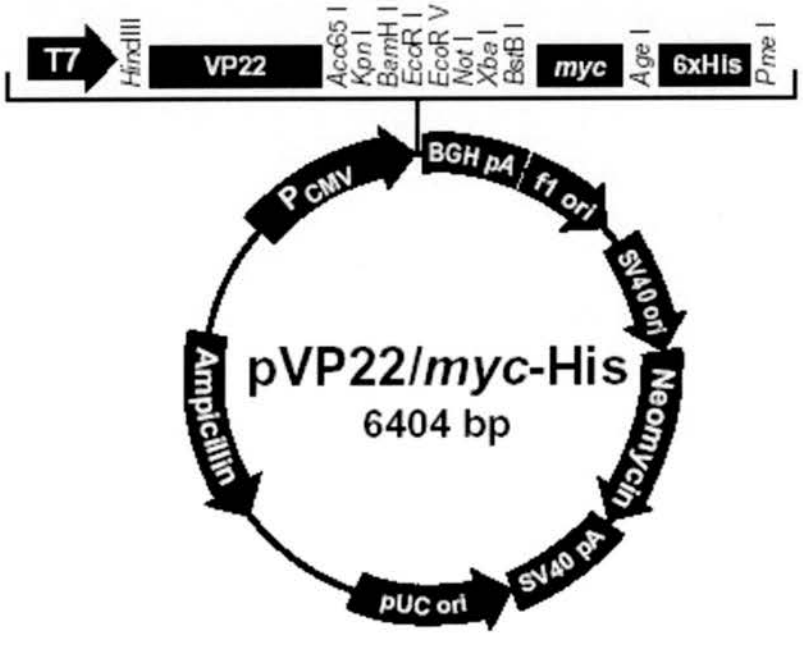
Make up to 1L with distilled water and autoclave.

Appendix II Plasmid Maps

pCAGGS-LIF 5.4 kb (a kind gift from Professor Austin Smith)



pVP22/*myc*-His 6.4 (Invitrogen, Paisley, UK)



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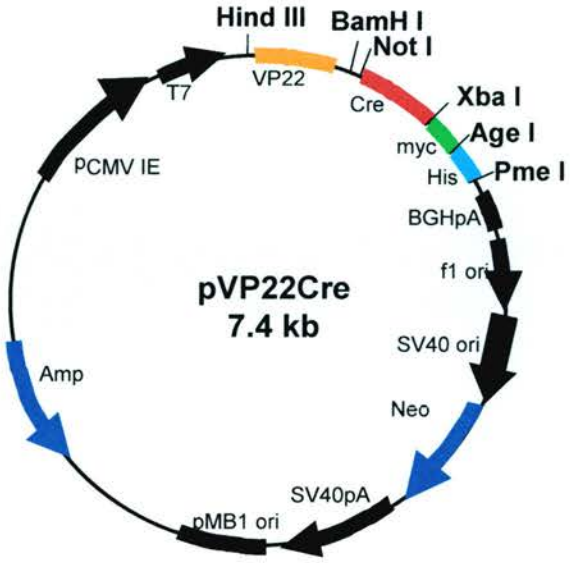
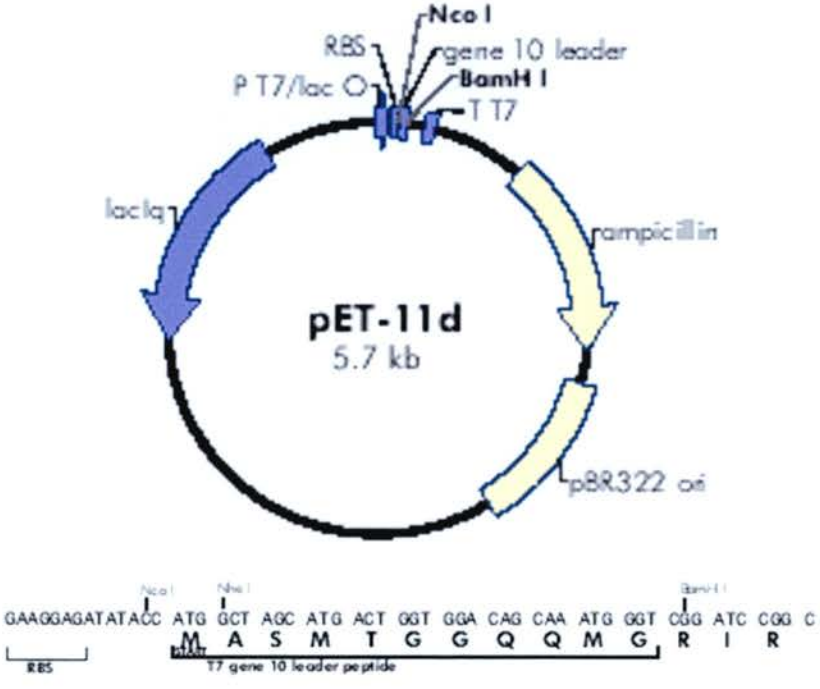


pET-11d 5.7kb (Stratagene)



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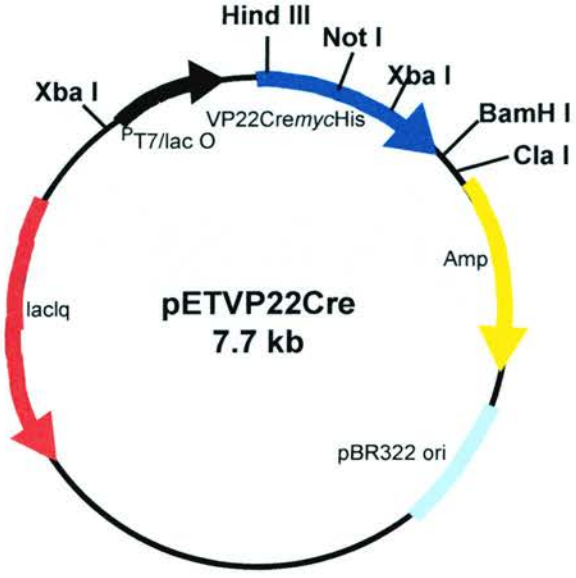
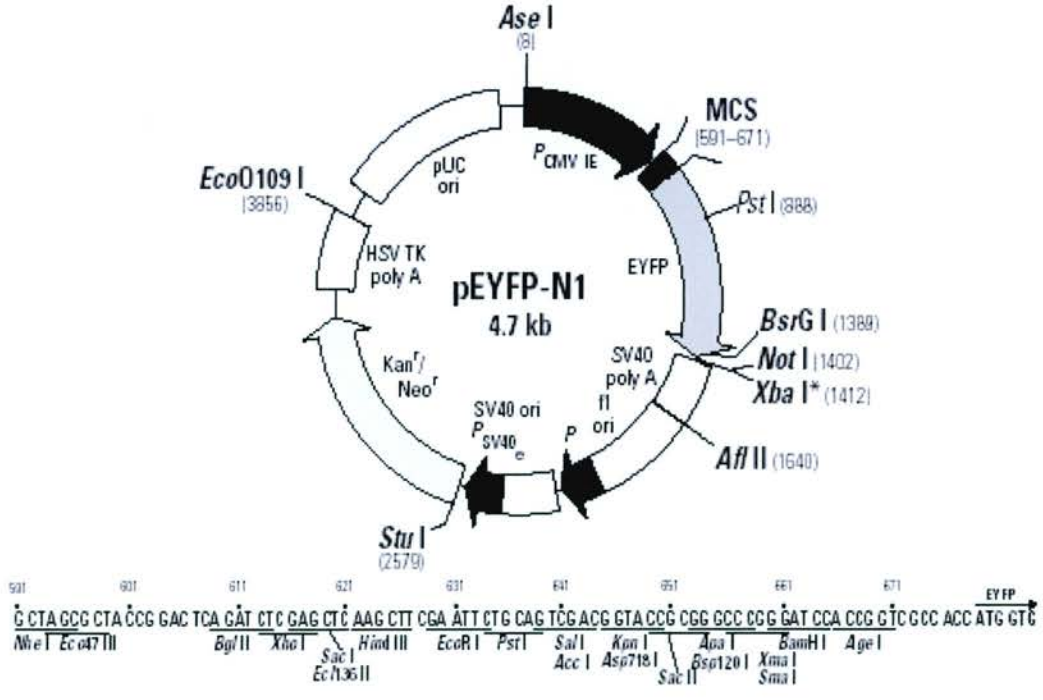
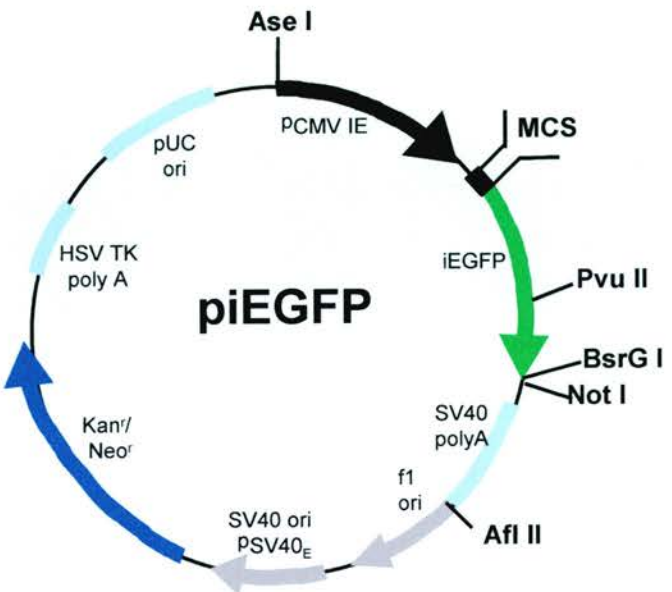


pEYFP-N1-4.7kb (Clontech Laboratories, Hampshire, UK)



piEGFP 4.9kb (constructed by Dr Adam Lacy-Hulbert)



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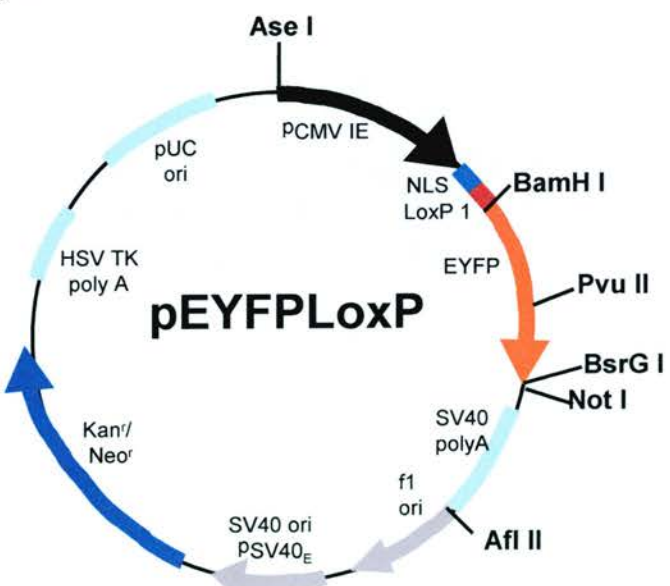

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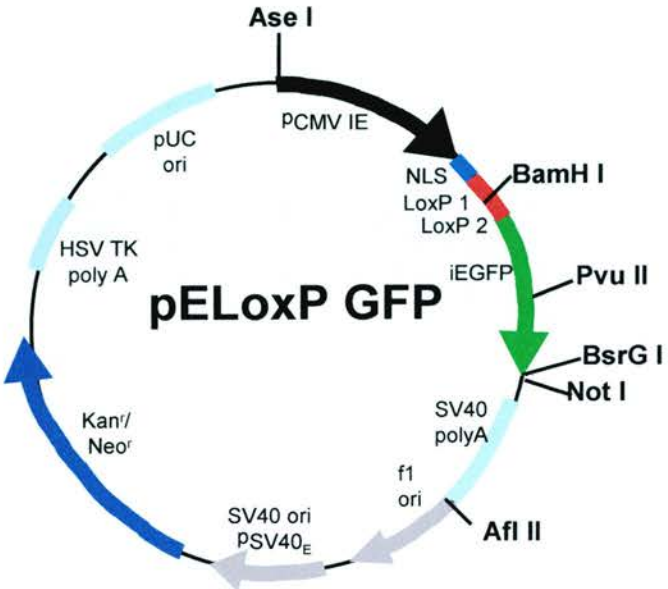

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