

Conditional Immortalisation of Myeloid-Precursors to Model Innate Immunity

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Abstract

The prevalence of fungal infections is on the rise due to the increase of immune suppressed individuals. Neutrophils are key immune cells in the fight against fungal infections. The study of neutrophil biology is hampered by the short lived nature of the cells and the fact that they cannot be easily genetically modified. In this thesis, I generate and characterise myeloid precursor cell lines that can be genetically manipulated and differentiated into functional neutrophils. These in vitro generated neutrophils were adoptively transferred into live animals and tracked during inflammatory responses. Clec7a, a cell surface β -glucan receptor found on myeloid cells, and its role in immune response to fungal infections has been well characterised in macrophages and dendritic cells but less so on neutrophils. In this thesis, a model for elucidating the role of Clec7a on neutrophils was developed using primary cells and was able to show that Clec7a deficiency on neutrophils impairs recognition of zymosan and *C. albicans* but that this impairment was largely overcome by serum opsonisation of the particles. The in vitro generated neutrophils were comparably tested and, although the cells have their limitations, they largely supported the conclusions found using primary cells.

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Contents

Abbreviations	15
Chapter 1.....	18
INTRODUCTION	18
1.1 Fungal infections	19
1.1.1. Fungal Morphology	19
1.1.2. Prevalence.....	22
1.1.3 Classes of Infection	23
1.1.4. Current Treatments	25
1.2. Immunity to Fungal Infections	27
1.2.1 Haematopoiesis and Innate Immune Cells with Major Roles in Antifungal Defence	29
1.2.2. Pattern recognition receptors.....	38
1.3. Complement Cascade and Fungal Immunity	46
1.4. Immortalisation of Primary Cells	49
1.5. Studying Neutrophils	51
1.6. AIMS	53
Chapter 2.....	54
Materials and Methods	54
2.1: Reagents.....	55
2.1.1. General Reagents	55
2.1.2. Cell Culture.....	56
Cell Lines	56

2.1.3. Cell Stimulants and Activators	58
2.1.4. Dyes and Colourimetric Indicators.....	59
2.1.5. Molecular Biology	60
2.1.6. Kits.....	60
2.2: Viral DNA Constructs.....	61
2.3: Antibodies	62
2.4. General Methods	66
2.4.1. Mice.....	66
2.4.2. Antibody Staining of Cells for Flow Cytometric Analysis	66
2.4.3 Flow Cytometry.....	66
2.4.4 Cytospin Preparation of Cells.....	67
2.4.5. Cell Culture Methods	68
2.4.6. Molecular Biology Methods.....	69
2.4.7. Growth of <i>C. albicans</i> SC5314.....	71
2.4.8. Fluorescent Labelling of Zymosan and <i>C. albicans</i> SC5314.....	71
2.5. Generation and Characterisation of Conditionally Immortalised Cell lines	73
2.5.1. Preparation of pMXs-IP:FL-ER-Hoxb8 retrovirus for conditional-immortalisation of neutrophil precursors	73
2.5.2. Purification of lineage (lin) ⁻ cells from mouse bone marrow and pre-stimulation for infection	73
2.5.3. Retroviral infection and cell propagation of pre-stimulated lin ⁻ cells.....	74
2.5.4. Cell growth and maintenance of MyPH8 cells	75

2.5.5. Retroviral infection of MyPH8-B6 cells	75
2.5.6. Differentiation of MyPH8-B6 cells into neutrophils.....	76
2.5.7. Purification of MyPH8-B6 derived neutrophils.....	76
2.5.8. DNA/RNA cell cycle analysis.....	77
2.6. <i>In vivo</i> Experimental Methods	77
2.6.1. Zymosan peritonitis	77
2.6.2. IL-6 ELISA.....	77
2.6.3. BIOgel peritonitis	79
2.6.4. <i>In vivo</i> adoptive transfer model.....	79
2.7. Functional Assays.....	81
2.7.1. Measurement of intracellular calcium ion changes.....	81
2.7.2. 3'-(<i>p</i> -aminopheny) Fluorescein (APF) Assays	82
2.8. Protocols for Human Neutrophil Collection and Functional Assays	85
2.8.1. Collection of Salivary Neutrophils.....	85
2.8.2. Collection of Human Blood Neutrophils	85
2.8.3. Collection of Inflammatory Neutrophils via the Skin Window Technique	86
2.9 Statistical Tests.....	87
Chapter 3.....	88
Generation and Characterisation of Conditionally-Immortalised Myeloid Progenitor Cell Lines	88
3.1. Introduction	89
3.2. Generation of Retrovirally Transduced Cell Lines from Mouse Bone Marrow.....	91
3.3. Generation of Cell Lines from 129S6/SvEv Mice.....	94

3.4. Generation of Cell Lines from C57BL/6 and Balb/c mice	97
3.5. Differentiation Studies on Myeloid Progenitor Cell Line	98
3.6. Purification of Viable MyPH8-B6-derived Neutrophils	114
3.7. Recognition and Response of <i>in vitro</i> Generated Neutrophils to the Yeast Particle Zymosan	116
3.8. Measurement of Intracellular Calcium Ions	121
3.9. Transduction of MyPH8-B6 Progenitors to Generate Cells with Uniform Transgene Expression	125
3.10. Adoptive Transfer of Neutrophils into Live Animals	128
3.11. Conclusion	130
Chapter 4: Analysis of the Role of Clec7a (Dectin-1) in Fungal Particle Recognition on Primary Neutrophils.....	134
4.1. Introduction	135
4.2. Establishing Parameters for <i>In Vivo</i> Exploration.....	137
4.4. <i>Ex Vivo</i> Study of the Function of Clec7a on Primary Neutrophils	146
4.5. Role of Clec7a and Complement Opsonisation in β -glucan Recognition by Peripheral Blood Neutrophils	152
4.6. Investigations into the Role of Clec7a on Primary Inflammatory and Whole Blood Neutrophils Using a Clec7a Specific Agonist.....	156
4.7. The Effect of Clec4n (Dectin-2) Blockade on Yeast Particle Recognition by Inflammatory Neutrophils	159
4.8. Application of <i>In Vitro</i> APF Model to Human Neutrophils.....	162
4.9. Conclusion.....	171

Chapter 5: Modelling Innate Immunity Using Neutrophils Derived From conditionally-Immortalised	
Cell Lines	175
5.1. Introduction	176
5.2. Generation of <i>Clec7a</i> ^{-/-} and <i>Itgam</i> ^{-/-} Conditionally-Immortalised Neutrophil Precursor Cell lines	177
5.3. Transduction of Conditionally-Immortalised Neutrophil Precursors with Clec7a	177
5.4. Assay Development: Zymosan Binding	183
5.5. Assay Development: Respiratory Burst	185
5.6. <i>In Vitro</i> Study of the Function of Clec7a on Neutrophils Derived from Conditionally- immortalised Cell Lines	187
5.7. Reconstitution of <i>Itgam</i> ^{-/-} Cell Line with Human CD11b	191
5.8. Restored Function of CD11b on Reconstituted Cell Lines	193
5.9. Conclusion	195
Chapter 6: Discussion	197
Bibliography	210

Figures

Chapter 1: Introduction

1.1. Haematopoiesis	30
1.2. Different models for the generation of ROS	35
1.3. Schematic of the main Clec7a isoforms	45
1.4. Different models for the generation of ROS	48

Chapter 3: Generation and characterisation of conditionally-immortalised myeloid progenitor cell lines

3.1. Flow cytometric analysis of lineage depletion of mouse BM cells	93
3.2. Viral construct used to transduce lin^- BM cells for the production of a neutrophil progenitor cell line	94
3.3. Cytospin preparations of lin^- cells transduced with viral construct containing Hoxb8	96
3.4. Cell surface expression of CD117 and Fc ϵ RI on 'mast cell-like' progenitors	97
3.5. Cytospin preparation of differentiated MyPH8-B6 cells.....	101
3.6. Cell surface expression of Ly-6B.2.....	101
3.7. Differentiation of MyPH8-B6 precursors over 5 days with 7 different cytokine combinations.....	102
3.8. Percentage of differentiated cells in S and G2/M phase of the cell cycle	104
3.9A. Nuclear DNA analysis of SCF, SCF+G-CSF and G-CSF differentiated cells.....	105
3.9B. Nuclear DNA analysis of SCF+IL-5 and IL-5 differentiated cells	106

3.9C. Nuclear DNA analysis of SCF+GM-CSF and GM-CSF differentiated cells	107
3.10. Expression of CD117 on progenitors and differentiated cells	110
3.11A. Expression profile of MyPH8-B6 progenitors, cells differentiated in SCF and cells differentiated in SCF+G-CSF.	111
3.11B. Expression profile of MyPH8-B6 progenitors, cells differentiated in SCF and cells differentiated in SCF+G-CSF.	112
3.12. DNA/RNA Profile of MyPH8-B6 derived cells	113
3.13. Percentage of cells that are either live or dead and neutrophils before and after purification with the dead cell removal kit and anti-CD117 depletion	115
3.14. Flow cytometric plots showing the percentage of MyPH8-B6 neutrophils that have associated with either non-opsonised or opsonised zymosan, at either 4°C or 37°C.	118
3.15. Graphical analysis of the data shown in Figure 3.14 showing the percentage of MyPH8-B6 neutrophils that have associated with either non-opsonised or serum opsonised zymosan, at either 4°C or 37°C.	119
3.16. Zymosan binding and ROS production of MyPH8-B6 differentiated neutrophils. ...	111
3.17. Intracellular calcium ion measurements of MyPH8-B6 derived neutrophils.	120
3.18. Global intracellular calcium changes within a single cell after introduction to a single zymosan particle with a micropipette	123
3.19. Viral DNA construct containing a calpain-1-EGFP fusion domain	124
3.20. Flow-cytometric plots showing the GFP expression of MyPH8-B6 progenitors.....	126
3.21. Differentiation time-course of MyPH8-B6 cells transduced to express GFP	126

3.22. Adoptive transfer of in vitro generated neutrophils into C57BL/6 animals	127
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Chapter 4: Analysis of the Role of Clec7a (Dectin-1) in Fungal Particle Recognition on Primary

Neutrophils

4.1. A-C. Flow-cytometric plots showing peritoneal cells from 129S6/SvEv mice.....	139
4.1. D-E. Flow-cytometric plots showing peritoneal cells from 129S6/SvEv mice.....	140
4.2. Neutrophil and monocyte/macrophage infiltration into the peritoneal cavity before and after acute zymosan peritonitis	141
4.3. Flow-cytometric plots showing peritoneal cells from 129S6/SvEv mice and 129S6/SvEv.Clec7a ^{-/-} mice before and after acute zymosan peritonitis.....	144
4.4. Characterisation of high-dose zymosan peritonitis in Clec7a-deficient mice.....	145
4.5. Expression of Clec7a and CD11b on the primary neutrophils from wild type and Clec7a ^{-/-} mice.....	148
4.6. Flow-cytometric plots showing the association of neutrophils with labelled zymosan or <i>C.albicans</i> and the resulting production of ROS as measured via APF fluorescence ...	149
4.7. Flow-cytometric plots showing the association of inflammatory monocytes with labelled zymosan or <i>C. albicans</i> and the resulting production of ROS as measured via APF fluorescence	150
4.8. Graphical analysis of the recognition and response to zymosan and <i>C. albicans</i> of primary neutrophils and monocytes.....	151
4.9. FACS plot showing the identification of neutrophils from heparinised whole blood using draq 5 (DNA dye) and Ly6G staining	153

4.10. FACS plots showing individual samples of whole blood neutrophil association with labelled zymosan or <i>C. albicans</i> and the resulting production of ROS as measured via APF fluorescence.	154
4.11. Graphical analysis of the recognition and response to zymosan and <i>C. albicans</i> of neutrophils in whole blood.	155
4.12. Blood and inflammatory neutrophil responses to curdlan microparticles.....	158
4.13. Expression of dectin-2 on the inflammatory neutrophils and monocytes of wild type mice.	160
4.14. Graphical analysis of the recognition and response to zymosan of primary neutrophils and monocytes from Clec7a deficient mice	161
4.15. Identification of human salivary neutrophils	164
4.16. Histograms showing the production of ROS via APF fluorescence in salivary neutrophils	165
4.17. Graph showing the percentage of salivary neutrophils that have associated with non-opsonised zymosan.....	166
4.18. Identification of human peripheral blood neutrophils and their recognition and response to curdlan microparticles and zymosan	167
4.19. Histograms showing the production of ROS via APF fluorescence in neutrophils derived from the skin window technique	169
4.20. Cytospin preparation of 'skin window' neutrophils and their association with zymosan.....	170

Chapter 5: Modelling Innate Immunity Using Neutrophils Derived From conditionally-Immortalised Cell Lines

5.1. Cell surface expression of Clec7a and CD11b on 4 day <i>in vitro</i> generated neutrophils produced from conditionally-immortalised neutrophil progenitor cells line.....	180
5.2. Four day differentiation flow cytometry plots of MyPH8-B6: <i>Clec7a.A</i> , MyPH8-B6: <i>Clec7a.B</i> and MyPH8-B6: <i>pMXs-IZ</i>	181
5.3. mRNA and expression of Clec7a on <i>in vitro</i> generated, Ly-6G ⁺ , neutrophils derived from conditionally-immortalised cell lines.....	182
5.4. Zymosan binding time course of neutrophils derived from conditionally-immortalised cell lines.....	184
5.5. Neutrophils derived from MyPH8-B6 cell line were stimulated with fluorescently labelled opsonised zymosan particles.....	186
5.6. Graphical analysis of the recognition and response to zymosan and <i>C. albicans</i> of MyPH8-B6 and MyPH8-B6: <i>Clec7a</i> ^{-/-} neutrophils.....	188
5.7. Graphical analysis of the recognition and response to curdlan mp, zymosan and <i>C. albicans</i> of <i>in vitro</i> derived neutrophils	190
5.8. Expression of CD11b on primary cells and <i>in vitro</i> generated neutrophils derived from transduced <i>Itgam</i> ^{-/-} cell lines	192
5.9. Graphical analysis of the recognition and response to zymosan and <i>C. albicans</i> of <i>in vitro</i> neutrophils	194

Tables

Chapter 1: Introduction

1.1. Differing characteristics of animals, plants and fungi.....	20
1.2. Different classes of antifungal agents and their proposed mechanism of action	26
1.3. Examples of PAMPs and associated pathogens	28
1.4. Examples of reactive oxygen species	34
1.5. Examples of different tissue resident macrophages and their locations in the body ..	37

Chapter 2: Materials and Methods

2.1.1. General reagents	55
2.1.2. Cell Culture	56 - 58
Cell lines	56
Media	56
Cytokines.....	57
Antibiotics	57
Other reagents	58
2.1.3. Cell stimulants and activators	58
2.1.4. Dyes and colourimetric indicators	59
2.1.5. Molecular biology.....	60
2.1.6. Kits	60
2.2. Viral DNA constructs	61
2.3. Antibodies	62

Chapter 3: Generation and Characterisation of Conditionally-Immortalised Myeloid Progenitor Cell

Lines

3.1. Summary of differentiated MyPH8-B6.....	98
----------------------------------------------	----

Abbreviations

AIM2	Absent In Melanoma 2
APF	Aminophenyl Fluorescein
ASC	Apoptosis-associated Speck-like protein containing a CARD
BM	Bone Marrow
C5aR	C5a Receptor
CNS	Central Nervous System
CR	Complement Receptor
Curdlan mp	Curdlan Microparticles
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dsDNA	Double Stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal Calf Serum
G-CSF	Granulocyte Colony-Stimulating Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSS	Hank's Balanced Salt Solution
HRP	Horseradish Peroxidase
i.p.	Intraperitoneal

i.v.	Intravenous
iC3b	Inactivated C3b
IFN- γ	Interferon-gamma
IgE	Immunoglobulin E
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LB	Lysogeny Broth
Lin ⁻	Lineage Negative
M-CSF	Macrophage Colony-Stimulating Factor
MDA5	Melanoma Differentiation-Associated protein 5
MINCLE	Macrophage-inducible C-type lectin
MMLV	Moloney Murine Leukaemia Virus
MyPH8	Myeloid Progenitor Hoxb8
NF- κ B	Nuclear Factor Kappa B
NIM-DAPI	Nuclear Isolation Medium--4,6-diamidino-2-phenylindole dihydrochloride
NLR	NOD-like Receptors
NOD	Nucleotide Oligomerization Domain
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
POM	Progenitor Outgrowth Medium
PRRs	Pattern Recognition Receptors

RIG-I	Retinoic acid Inducible Gene I
ROS	Reactive Oxygen Species
RPMI 1640	Roswell Park Memorial Institute 1640
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCARF	Scavenger Receptor Class F
SCF	Stem Cell Factor
SCFR	Stem Cell Factor Receptor
SOC	Super Optimal broth with Catabolite repression
ssRNA	Single Stranded RNA
TBE	TRIS/Borate/EDTA
TLR	Toll Like Receptor
TMB Substrate	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour Necrosis Factor
TRIS	Tris(hydroxymethyl)aminomethane
YPD	Yeast Peptone Dextrose

Chapter 1

INTRODUCTION

1.1 Fungal infections

1.1.1. Fungal Morphology

Fungi are a diverse kingdom of eukaryotic organisms. They share certain features with both plants and animals but are distinct from either (Table 1.1). In nature fungi can exist in two main forms; the hyphal form, which consists of thin filaments of fungal cells called hyphae and a mass of these filaments is known as the mycelium, and the unicellular yeast form (Adams 2004). Both hyphae and yeast can be pathological in humans (Diamond, Krzesicki et al. 1978; Lehmann 1985) and fungal species that can alternate between both these forms are known as dimorphic (San-Blas and San-Blas 1984). Conidia (non-motile fungal spores that are produced via mitosis and, if the conditions are favourable, are a means of asexual reproduction) can also be pathogenic and in the case of *Aspergillus spp*, conidia are the primary route of human infection (Dagenais and Keller 2009). Although there are thousands of fungal species only a small percentage of them are pathogenic to humans (Box 1).

	Animal	Fungi	Plant
Nuclear Envelope	Present	Present	Present
Mitochondria	Present	Present	Present
Chloroplasts	Absent	Absent	Present
Vacuoles	Absent	Present	Present
Cell Wall	Absent	Present: Non-cellulose polysaccharides	Present: Cellulose
Sterol	Cholesterol	Ergosterol	Stigmasterol, Sitosterol & Campesterol
Nutrition	Heterotrophic	Heterotrophic	Autotrophic

Table 1.1: Differing characteristics of animals, fungi and plants. Table created from information gathered from various sources (Whittaker 1969; Klionsky, Herman et al. 1990; Smits, van den Ende et al. 2001; Odds, Brown et al. 2003; Alberts 2008; Jacquier and Schneiter 2012)

Common Disease Causing Fungal Species:

❖ ***Candida albicans***: can cause superficial and systemic infections and is the primary cause of Candidiasis. Primarily targets mucosal membranes, e.g. oral cavity (thrush), genitalia and urinary tract, however dissemination into the blood and internal organs can occur usually only in immunocompromised individuals.

❖ ***Cryptococcus neoformans***: can cause systemic infections particularly in individuals that are immunocompromised or are diabetics. Primarily targets the respiratory tract and lungs but can progress into the central nervous system (CNS) leading to inflammation of the brain and the meninges.

❖ ***Aspergillus fumigatus***: can cause systemic infections, with immunocompromised patients and patients with cystic fibrosis being particularly at risk. The primary target is the respiratory tract and lungs but can affect almost all organs if it becomes invasive.

❖ ***Histoplasma capsulatum***: can cause systemic infections particularly in patients with chronic lung disease and patients that are immunocompromised. Primarily targets the lung but can disseminate into almost any organ.

❖ ***Coccidioides immitis***: can cause subcutaneous and systemic infections, with immunocompromised patients, pregnant women, and those of African-American, Asian or Filipino descent, being particularly at risk of dissemination. Primarily targets the lungs but can disseminate into other organs.

Box 1: Fungal species that commonly cause clinical infections in patients with compromised immune systems.

Table created from information gathered from various sources (Powell, Drutz et al. 1983; Wheat, Batteiger et al. 1990; Wheat 1994; Milla, Wielinski et al. 1996; Denning 1998; Hajjeh, Conn et al. 1999; Chen, Sorrell et al. 2000; Muller, Trusen et al. 2002; Douglas 2003; Crum, Lederman et al. 2004; Netea, Gow et al. 2006; Aude 2009; Moyes and Naglik 2011)

1.1.2. Prevalence

Healthy individuals are usually protected from fungal infections. The first barriers to infection are the anatomical barriers, i.e. the skin and mucosal surfaces. If these fail, then the immune system can overcome the fungal pathogens through four main mechanisms; complement, phagocytes, antibodies and cell mediated immunity. When both barriers fail then fungal infections can occur and become systemic (Richardson 2005).

The rate of pathological human fungal infections is on the rise. In the United States, the incidence of fungal sepsis increased by 207% between 1979 and 2000 (Martin, Mannino et al. 2003). This can be attributed to the increase in invasive medical practices, such as surgery and indwelling catheters that break down the anatomical barrier to fungi (Richardson 2005) and to the increase in the number of patients that have compromised immune systems, usually as a result of HIV/AIDS, the use of immunosuppressive drugs for organ donor recipients, cancer treatments and other medical interventions (Romani 2004). These patients become susceptible to opportunistic pathogens that would normally not cause disease. The mortality rate for opportunistic fungal infections exceeds 50% in some human studies and has been shown to be up to 95% in bone marrow transplant patients infected with *Aspergillus spp* (Romani 2004). It has been suggested that the impact of fungal infections on human health is highly underestimated and that some fungal diseases kill at least as many people as tuberculosis or malaria (Brown, Denning et al. 2012).

1.1.3 Classes of Infection

Fungal infections can be classified into several different groups (Kimura and McGinnis 1998):

1. Superficial Mycoses

This type of infection involves fungi that colonize the hair. The *Piedraia hortae*, fungus forms a fruiting body called an ascostroma around a hair shaft. The ascostroma is tightly attached to the hair shaft and only minimal tissue damage at the site where the ascostroma is attached to the hair shaft is observed (Kimura and McGinnis 1998).

2. Cutaneous Mycoses

This type of infection is usually limited to non-living tissues such as the surface of the skin, nails or hair. There is observable tissue damage and an immune response can be measured (Kimura and McGinnis 1998). This type of infection can be so innocuous as to leave patients unaware that they are affected. Examples include *Microsporum* (athlete's foot) and *Trichophyton* (ringworm) (Anaissie, McGinnis et al. 2003).

3. Subcutaneous Mycoses

This type of infection affects the living skin and underlying tissues and is caused by traumatic introduction of the pathogen to the dermis. It can lead to a chronic infection and the appearance of abscesses. This type of infection rarely becomes systemic (Kimura and McGinnis 1998). Examples include chromoblastomycosis which is caused by fungi such as *Phialophora verrucosa* and *Fonsecaea pedrosoi* (Bonifaz, Carrasco-Gerard et al. 2001).

4. Systemic Mycoses

Fungal pathogens that have entered the body and are able to infect internal organs are known as systemic infections. These types of infections are able to disseminate between different organs and can be fatal if vital organs are infected (Kimura and McGinnis 1998). Examples include histoplasmosis which is caused by fungi from the genus *Histoplasma* (Aide 2009) and Coccidioidomycosis, which is caused by *Coccidioides immitis* (Galgiani 1993).

5. Candidiasis

This is a class of infections caused by fungi from the genus *Candida*, usually *Candida albicans*, which is a ubiquitous and commensal fungus. It is found on the skin, nails and mucous membranes of humans. Involvement may be localized to mucosal membranes or become systemic. In healthy individuals, *Candida* infections occur in all age groups, but are most common in the new-born and the elderly (Schulze and Sonnenborn 2009; Moyes and Naglik 2011).

1.1.4. Current Treatments

Historically, research into treatments for fungal infections has been sparse. This can be attributed to observation that prior to the 1980s, the incidence of serious fungal infections was relatively low, especially in comparison with bacterial infections (Sheehan, Hitchcock et al. 1999). However, due to the increase in incidence (Chapter 1.1.2), research and development of antifungal agents has increased (Ghannoum and Rice 1999). The first agent with antifungal properties, griseofulvin, was isolated in 1939 but it was not approved for clinical use until 1958 (Sheehan, Hitchcock et al. 1999). Since then several different classes of antifungal agents have been discovered and taken into the clinic (Table 1.2). Fungal cells are eukaryotic cells that are very similar to mammalian cells in terms of their biochemical reactions (Romani 2004). This can make the treatment of invasive fungal infections difficult as drugs that do kill fungal cells can also be very toxic to human cells and can produce severe side effects in patients. For example, amphotericin B, used in the treatment of systemic fungal infections, is known to cause significant nephrotoxicity as well as fever, chills, headache and vomiting (Dismukes 2000). Other than the side effects produced by antifungal agents, one of the major obstacles to comprehensive treatment is the growing rise in resistance to current treatments (Ghannoum and Rice 1999).

Class	Example	Mechanism of Action
Allylamines	Terbinafine	Inhibits early steps of ergosterol biosynthesis, leading to an increase in membrane permeability and disruption of fungal cell.
Azoles	Fluconazole	Inhibition of cytochrome P-450-dependent 14 α -sterol demethylase, an enzyme required in the biosynthesis of ergosterol, leading to ergosterol depletion and membrane disruption.
Cell wall inhibitors	Echinocandins	Inhibits biosynthesis of cell wall components. Echinocandins inhibit 3 β -glucan synthase, which results in structural changes in the cell wall that can ultimately lead to cell death.
Morpholines	Amorolfine	Inhibits ergosterol biosynthesis.
Nucleic acid inhibitors	5-fluorocytosine	Disrupts pyrimidine metabolism as well as interfering with RNA, DNA and protein synthesis in fungal cells.
Polyenes	Amphotericin B	Interacts with sterols in the outer membrane of fungal cells leading to cellular death. Fungal cells without sterols in their outer membrane are not susceptible to polyenes and inhibitory action of polyenes can be disrupted by the addition of sterols into the growth medium of fungal cultures. Amphotericin B interactions are thought to cause pores in the outer membrane.

Table 1.2: Different classes of antifungal agents and their proposed mechanism of action. Table created from information gathered from various sources (Ghannoum and Rice 1999; Sheehan, Hitchcock et al. 1999; Onyewu, Blankenship et al. 2003).

1.2. Immunity to Fungal Infections

The human body is usually able to mount an immune response that destroys fungal pathogens that invade living tissues, which is demonstrated by the observation that risk factors for the development of systemic fungal infections include patients that have undergone solid organ transplantation, patients with HIV or AIDS and patients who for underlying medical reasons exhibit some form of immune suppression (Patel and Paya 1997; Procop and Roberts 2004; Fishman 2007; Morris, Wei et al. 2008; Walzer, Evans et al. 2008; Neofytos, Fishman et al. 2010). In order to destroy fungal pathogens the immune system must first recognise the pathogen as an invading foreign body. The innate immune system recognises pathogen-associated molecular patterns (PAMPs), which are a host of molecules common to most microbial species (Table 1.3). These PAMPs are recognised by pattern recognition receptors (PRRs) that can be either soluble or attached to plasma membranes on the cell surface or on intracellular compartments (Akira, Uematsu et al. 2006).

PAMP	PRR	Pathogen
Lipopolysaccharide (LPS)	TLR4	Gram negative bacteria
Lipoteichoic acid	TLR2:TLR6 Heterodimer	Gram positive bacteria
Lipopeptides	TLR2:TLR1 or TLR2:TLR6 Heterodimers	
Lipoarabinomannan	TLR2	
Peptidoglycan	TLR2	
Flagellin	TLR5	Flagellated bacteria
Glucans	Clec7a & TLR2	Fungi
Mannan	Mannan Binding Lectin , TLR4 & Clec4n	
CpG DNA	TLR9	Fungi and Bacteria
Env Protein	TLR4	Viruses
Double stranded RNA	Melanoma Differentiation- Associated protein 5 (MDA5) & TLR3	
Single stranded RNA	Retinoic acid inducible gene-I (RIG-I), TLR7 & human TLR8	

Table 1.3: Examples of PAMPs and associated pathogens. Table created from information gathered from various sources (Schwandner, Dziarski et al. 1999; Underhill and Ozinsky 2002; Adams 2004; Crozat and Beutler 2004; Brown 2006; Dalpke, Frank et al. 2006; Netea, Van der Meer et al. 2006; Sheng, Pouniotis et al. 2006; Thompson and Locarnini 2007; Farhat, Riekenberg et al. 2008; Kawai and Akira 2009).

1.2.1 Haematopoiesis and Innate Immune Cells with Major Roles in Antifungal Defence

Haematopoiesis

Haematopoiesis is the generation of blood cells including all immune cells (Figure 1.1), which primarily takes place in the bone marrow. All blood cells are generated from a multipotent stem cell and a host of cytokines and growth factors are involved in the growth and differentiation of these cells (Robb 2007). These cells are able to self-renew and expand to enable a constant population of stem cells present for the continual production of blood cells as required in a lifetime (Reya 2003). The multipotent haematopoietic stem cell can differentiate into either the common myeloid progenitor (CMP) or the common lymphoid progenitor (CLP). These cells are primitive progenitor cells and are able to generate all blood cells. The CLP generates the lymphoid cells e.g. B cells, T cells and natural killer (NK) cells, and is also able to generate dendritic cells. The CMP has the potential to generate all myeloid cells e.g. neutrophils and macrophages, dendritic cells, red blood cells and platelets. Neutrophils and macrophages have important roles in immunity to fungal infections and they will be looked at in a little more detail.

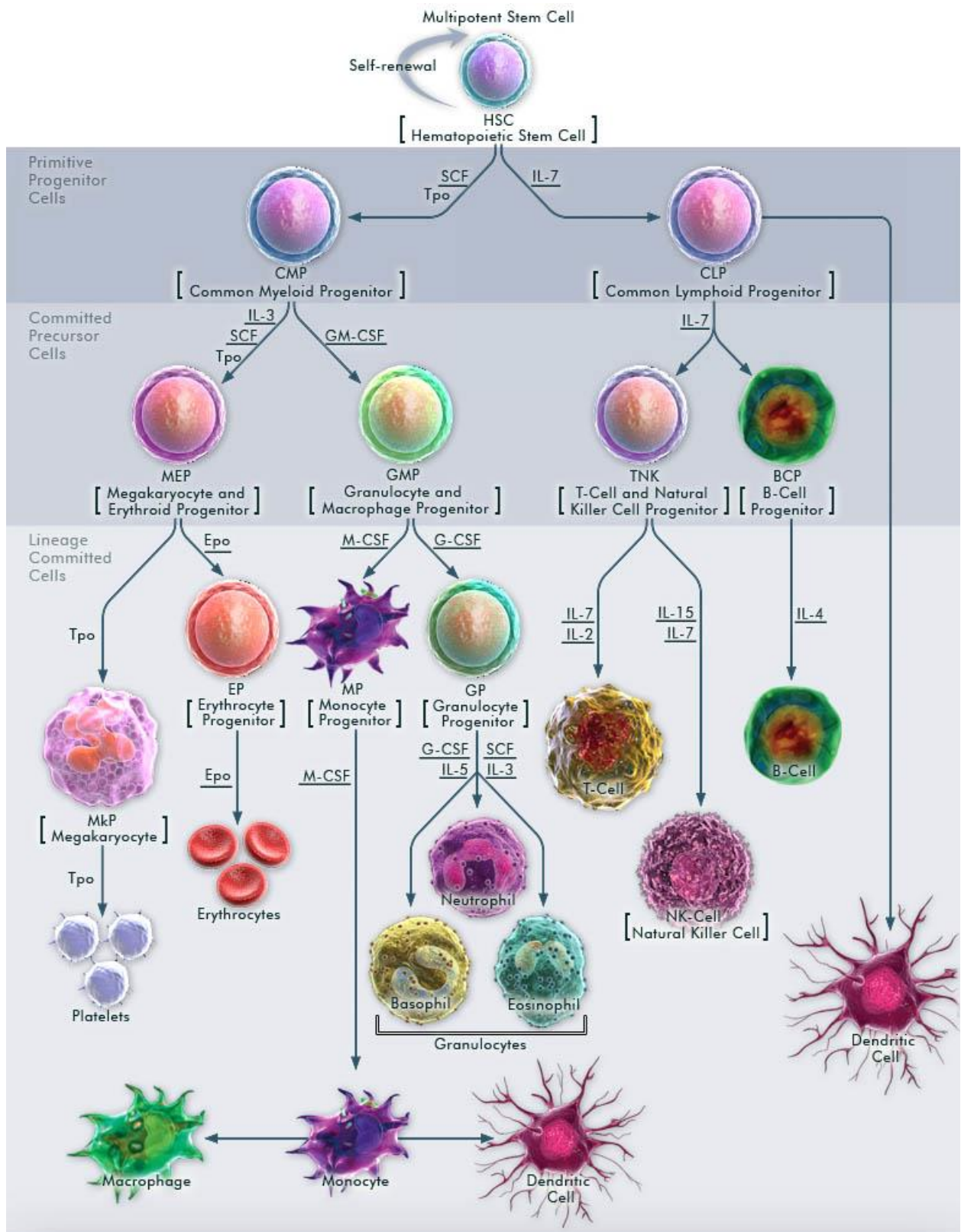


Figure 1.1: Haematopoiesis: Schematic showing haematopoiesis; the generation of all blood cells, including all the immune cells in the body, from a multipotent haematopoietic stem cell (eBioscience 2012).

Polymorphonuclear Neutrophilic Leukocytes

Polymorphonuclear neutrophilic leukocytes, commonly known as neutrophils, are an integral part of the innate immune system. Neutrophils are important in the clearance of fungal infections, and neutropenia (a severe deficiency in the number of neutrophils circulating in the blood) is associated with increased susceptibility to bacterial and fungal infections (Warnock 1998).

There are $3 - 5.5 \times 10^9$ neutrophils per litre of blood, which makes neutrophils by far the most abundant white blood cell (60-70%) in the blood. They have an irregular shaped and lobed (polymorphic) nucleus and contain granules. Neutrophils are initially generated in the bone marrow and then circulate in the blood. During an immune challenge these short lived cells migrate and extravasate to sites of infection with the help of chemoattractants such as interleukin-8 (IL-8) (released by macrophages and monocytes) (Baggiolini, Loetscher et al. 1995) and C5a (a potent anaphylatoxin produced by complement activation) (Snyderman, Phillips et al. 1970; Becker 1972).

Neutrophil Mediated Killing

Neutrophils are one of the first cells to migrate to the infection site. They circulate in the blood and follow chemoattractants such as C5a and IL-8. These proteins diffuse from the infection site and create a concentration gradient which the neutrophils are able to follow to the infection site. They also activate the neutrophil, leading to the increase of the adhesion molecule, Complement Receptor 3 (CR3) integrin, which is a heterodimer of CD11b and CD18, and a down-regulation of L-selectin (CD62L) (Jutilla, Rott et al. 1989; Humbria, Diaz-Gonzalez et al. 1994) on the cell surface which then encourages trafficking of the cells to the site of inflammation and extravasation (Witko-Sarsat, Rieu et al. 2000). Once

the neutrophil has left the blood stream and reached the site of infection it can act in several ways to promote pathogen killing.

At the site of infection, neutrophils can recognise pathogens, using a variety of cell surface associated PRRs (e.g. Toll like receptors TLRs (Table 1.4)), and are able to phagocytose them. One such receptor, a major focus of this thesis, is Clec7a or dectin-1, which has been shown to recognise beta-glucans (Brown and Gordon 2001; Brown, Taylor et al. 2002; Taylor, Tsoni et al. 2007). These PRRs are discussed in more detail below. Recognition of microbes is also largely facilitated by complement opsonisation and CR3, which will be discussed in more detail below.

Once pathogens have been phagocytosed, they form intracellular phagosomes that then fuse with azurophilic and secondary specific granules in the cytoplasm to form phagolysosomes (Ricevuti, Mazzone et al. 1993; Perskvist, Roberg et al. 2002). The contents of these granules include proteases, antimicrobial proteins, components of the respiratory burst reaction, therefore these granules are usually toxic enough to kill most pathogens (Faurischou and Borregaard 2003). Examples of the contents of these granules include myeloperoxidase, serine proteases, and antibiotic proteins (Fouret, du Bois et al. 1989; Faurischou and Borregaard 2003). Another process that takes place within the cell that results in pathogen killing is the respiratory burst (Sbarra and Karnovsky 1959). This results in a number of reactive oxygen species (ROS) being produced, e.g. superoxide anion, hydroxyl radical and hydrogen peroxide, all of which are toxic to the pathogen (Babior, Kipnes et al. 1973; Chanock, el Benna et al. 1994). Neutrophils are able to release the contents of their granules into the extracellular space in a process called degranulation. This process facilitates migration of the neutrophils by releasing enzymes that degrade the

extracellular matrix, as well as potentially killing any extracellular pathogens that have not been phagocytosed (Witko-Sarsat, Rieu et al. 2000; Faurschou and Borregaard 2003). Neutrophils have also been shown create extracellular traps that can capture and kill pathogens including hyphal fungi. The cells send out fibres that contain granule proteins and chromatin into the extracellular matrix, trapping and killing pathogens that have evaded phagocytosis (Urban, Reichard et al. 2006).

Reactive Oxygen Species and the Respiratory Burst

The term reactive oxygen species (ROS) is used to describe a range of reactive molecules and free radicals (Table 1.4) that are derived from molecular oxygen. Although they are produced as by-products of normal metabolic processes primarily aerobic respiration, they can cause considerable damage to cells and even cause cell death (Valencia and Moran 2004; Orrenius, Gogvadze et al. 2007). Phagocytes, particularly neutrophils take advantage of these destructive chemical species to kill invading microbes in the phagolysosome (Figure 1.2) (Bylund, Brown et al. 2010). When stimulated by an invading pathogen, these phagocytes undergo a process known as respiratory burst, where they increase their consumption of molecular oxygen and generate ROS (Sbarra and Karnovsky 1959; Reeves, Nagl et al. 2003). This involves the formation and activation of NADPH oxidase on the membrane of specific/gelatinase granules. This enzyme then uses cellular oxygen to produce ROS species inside the granule to await fusion with the phagosome. The phagosome fuses with the specific/gelatinase granules as well as with azurophil granules which contain myeloperoxidase (MPO) (Bylund, Brown et al. 2010). The mixing of the MPO with the ROS leads to the production of further ROS such as the hypochlorate ion and singlet oxygen (Klebanoff 1967; Klebanoff 1968; Reeves, Nagl et al. 2003)(Table 1.4).

Neutrophils deficient in MPO have been characterised as “low responders” when measuring ROS production (Aniansson, Stendahl et al. 1984). Other models of ROS production via MPO include radical diffusion, where the contents of the specific/gelatinase granules, diffuses through the cytoplasm and enters the azurophil granules, and granule fusion, where the specific/gelatinase granules and the azurophil granules directly fuse (Bylund, Brown et al. 2010).

Reactive Oxygen Species	Chemical Formula
Oxygen	O_2
Singlet oxygen	1O_2
Superoxide anion	$\cdot O_2^-$
Hydroxyl radical	$\cdot OH$
Hydroxyl ion	OH^-
Hydrogen peroxide	H_2O_2
Peroxide anion	$\cdot O_2^{-2}$
Hypochlorate anion	OCl^-

Table 1.4: Examples of Reactive oxygen species found within the cell.

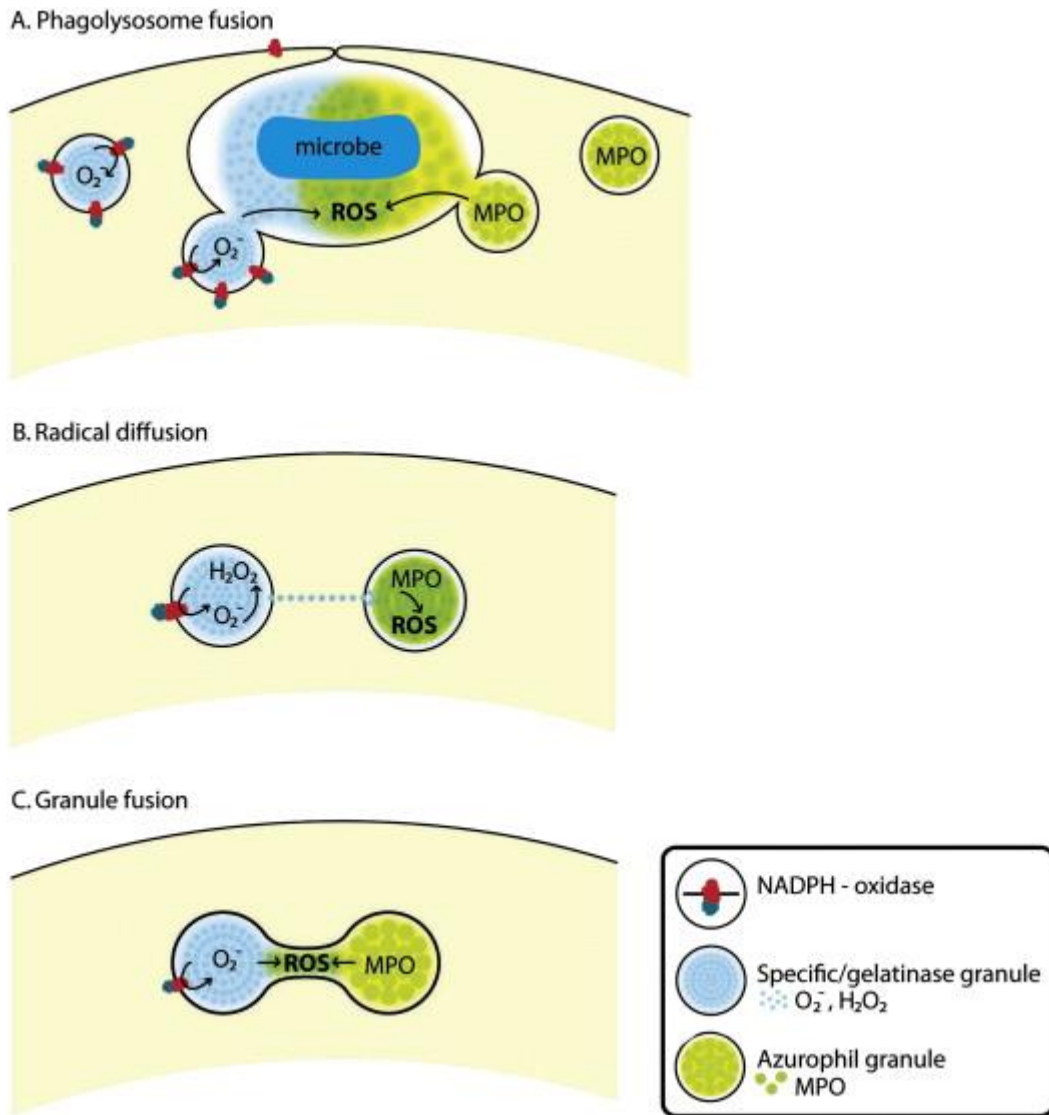


Figure 1.2: Different models for the generation of ROS. A prerequisite for the detection of ROS is that the oxygen radicals (O_2^- , H_2O_2) are present in the same compartment as MPO. This could be accomplished in the following ways: (A) fusion of both specific granules, containing NADPH oxidase, and azurophil granules, containing MPO, with the phagosomal membrane during maturation of the phagolysosome; (B) diffusion of oxygen radicals generated in granules across membranes and through the cytosol, ending up in azurophil granules to mix with MPO; and/or (C) direct fusion between specific/gelatinase and azurophil granules in the absence of phagosome formation. Figure taken from (Bylund, Brown et al. 2010).

Macrophages

Macrophages are phagocytic cells that are present in a wide variety of tissues (Table 1.5). Traditionally, macrophages were identified as originating from hematopoietic stem cells in the bone marrow (van Furth and Cohn 1968; Fogg, Sibon et al. 2006; Geissmann, Manz et al. 2010). Historically they were thought to undergo further differentiation until they leave the bone marrow and enter the blood as monocytes. After which, they then circulate in the blood for several days before they migrate to tissues and differentiate into tissue resident macrophages (Volkman and Gowans 1965). However some studies have shown that there is a separate lineage of macrophages, that develop in the embryonic yolk sac, prior to the development of hematopoietic stem cells, and that persists into maturity (Bertrand, Jalil et al. 2005; Schulz, Gomez Perdiguero et al. 2012). Other studies have shown that some tissue resident macrophages are derived from this embryonic yolk sac and localized proliferation is important for keeping these populations stable (Ginhoux, Greter et al. 2010). Recent studies have shown that tissue resident peritoneal macrophages are able to undergo bursts of localized proliferation. After acute inflammation, resident peritoneal macrophages have been shown to replenish their numbers via localized proliferation (Davies, Rosas et al. 2011). This localized proliferation has also been observed after parasitic infection with *Brugia malayi* (Jenkins, Ruckerl et al. 2011).

Macrophage	Tissue Resident
Alveolar macrophages	Alveolar sacs of the lungs
Kupffer cells	Liver
Inflammatory monocyte derived macrophages	Recruited to any site that is undergoing acute inflammation
Microglia	CNS, i.e. brain and spinal column
Osteoclasts	Bones
Peritoneal macrophages	Peritoneal cavity
Splenic macrophages	Spleen

Table 1.5: Examples of different tissues resident macrophages and their locations in the body.

As well as having a diverse role in both innate and adaptive immunity, macrophages are also responsible for a host of homeostatic processes in the body including the phagocytosis of erythrocytes and apoptotic bodies, and they are involved in other processes such as tissue remodelling and repair after injury (Erwig and Henson 2008; Cambos and Scorza 2011; Liddiard, Rosas et al. 2011).

Macrophages play a significant role in distinguishing self from non-self and are able to recognise non-self cells via a host of PRR (Taylor, Martinez-Pomares et al. 2005). When the immune system is challenged, macrophages are able to respond in several ways. They can phagocytose pathogens and destroy them in the phagolysosome and are then able to present antigen fragments to the adaptive immune system. As well as destruction via phagocytosis, they are able to release a range of pro-inflammatory cytokines including, tumour necrosis factor alpha (TNF α), interleukine-1 beta (IL-1 β), interleukin-6 (IL-6) and IL-8. Macrophages are key in the generation and recruiting neutrophils to the site of fungal

infections via their release of cytokine such as IL-8 (Baggiolini, Loetscher et al. 1995).

Macrophages also play a very important role in phagocytising apoptotic neutrophils, which is important for resolving inflammation (Michlewska, Dransfield et al. 2009).

1.2.2. Pattern recognition receptors

PRRs Important in Fungal Recognition

Having studied the literature it is clear to see that due to the complex nature of fungal pathogen PAMPs and redundancy in the immune system there are several different PRRs that are important in immunity to fungal infections. Of the TLRs, TLR1, TLR2, TLR4, TLR6 and TLR 9 have been shown to play some role in recognition and response to fungal PAMPs (See below). Of the other PRRs, Clec7a and Clec4n have also been shown to be important in recognition of fungal PAMPs (See below).

Scavenger Receptors

Scavenger receptors are a diverse family of pattern recognition receptors that recognize a host of different polyanionic ligands including pathogen-derived ligands (Mukhopadhyay and Gordon 2004). They are expressed on a variety of tissue resident macrophages and epithelial cells. These receptors are important in the clearance or “scavenging” of substances no longer of use to the host such as modified host molecules or apoptotic cells (Peiser, Mukhopadhyay et al. 2002). They have also been implicated in the recognition of microbial PAMPs (Peiser, Mukhopadhyay et al. 2002). Of relevance to fungal recognition,

the scavenger receptors SCARF1 and CD36 are thought to be β -glucan binding receptors that mediate immune response to *C. neoformans* and *C. albicans* (Means, Mylonakis et al. 2009).

Retinoic acid inducible gene-1 (RIG-I) Like Receptors

The RIG-I like receptor family are a group of PRRs that are located in the cytoplasm and that recognise, bind and initiate anti-viral response to the RNA sequences of viruses (Takeuchi and Akira 2010). There are three member of this receptor family; RIG-I, MDA5 and LGP2 (Kawai and Akira 2009). Once activated these receptors signal via interferon- promoter stimulator 1 (IPS-1) to induce the activation of several transcription factors including interferon regulatory factor 3 (IRF3) and IRF7, which leads to the production of type I interferons and NF- κ B which controls the production of inflammatory cytokines (Kawai, Takahashi et al. 2005). Mice deficient for RIG-I and MDA5 have been shown to be more susceptible to viral infection by RNA viruses compared to wild type mice (Kato, Takeuchi et al. 2006).

NOD Like Receptors

NOD like receptors (NLRs) recognise PAMPs and endogenous molecules in the cell cytosol (Martinon and Tschopp 2005; Meylan, Tschopp et al. 2006). However, NOD2 has been shown to associate with the plasma membrane and a truncated form of NOD2 that does not associate with the plasma membrane is associated with Crohn's disease (Barnich, Aguirre et al. 2005). So far at least 22 human and 34 mouse NLR proteins have been identified and the physiological function of most of these is poorly understood (Ting, Lovering et al. 2008), but some have been identified. The first NLRs identified as intracellular PRRs were NOD1, which recognises bacterial peptidoglycan containing diaminopimelic acid (Chamaillard, Hashimoto et al. 2003; Girardin, Boneca et al. 2003), and NOD2 which also detects bacterial

peptidoglycan but through a muramyl dipeptide motif (Girardin, Boneca et al. 2003; Inohara, Ogura et al. 2003).

Cytosolic DNA Sensor

Double stranded DNA in the cytoplasm, usually as a result of DNA viruses or certain bacteria, can promote anti-viral and inflammatory responses independent of TLR9 and RLR pathways (Ishii and Akira 2006; Stetson and Medzhitov 2006). Transfection of DNA into the cytoplasm of cells has been shown to result in the production of IFN- β (Ishii, Coban et al. 2006; Stetson and Medzhitov 2006), inflammasome activation (Muruve, Petrilli et al. 2008) and cell death (Stacey, Ross et al. 1993). Prior to 2009, the cytosolic DNA sensor had not been identified and the protein, DNA-dependent activator of IFN-regulatory factors (DAI), was suggested as a candidate sensor (Takaoka, Wang et al. 2007). However, it was shown to be redundant as DAI deficiency in mice does not affect immune response to dsDNA in the cytosol (Ishii, Kawagoe et al. 2008). Since then the protein, absent in melanoma 2 (AIM2), has been identified as the major receptor for cytosolic dsDNA (Fernandes-Alnemri, Yu et al. 2009; Hornung, Ablasser et al. 2009; Roberts, Idris et al. 2009). AIM2 recognition of cytosolic dsDNA results in the activation of the inflammasome via the adaptor molecule ASC and knockdown of AIM2 abrogates inflammasome activation in response to vaccinia virus (Fernandes-Alnemri, Yu et al. 2009; Hornung, Ablasser et al. 2009). Recognition of dsDNA by AIM2 has also been shown to induce pyroptosis, which is a caspase-1 dependent inflammatory form of cell death (Fernandes-Alnemri, Wu et al. 2007), via its interaction with the ASC adaptor molecule (Fernandes-Alnemri, Yu et al. 2009).

Toll like receptors

The toll like receptors (TLRs) are a family of conserved cellular receptors that recognise and mediate response to PAMPs and other ligands (Akira, Uematsu et al. 2006). Each TLR binds different agonists that results in the MyD88 or TRIF dependent NF- κ B, MAPK or type 1 interferon signalling, that induces the production of proinflammatory cytokines (Cook, Pisetsky et al. 2004; Akira, Uematsu et al. 2006; Kawai and Akira 2009). Different TLRs recognise different agonists. The main TLRs so far implicated in fungal recognition are TLR2, TLR4 and TLR9 (Romani 2004).

TLR response to fungi occurs in a species and morphotype specific manner. TLR2 signalling, which leads to increased production of cytokines such as TNF α and IL-1 β , is activated by the yeast of *C. albicans* and by the conidia and hyphae of *A. fumigatus* (Netea, Van Der Graaf et al. 2002). TLR4 recognises and responds to the hyphae of *C. albicans* and the hyphae of *A. fumigatus* but not the conidia (Wang, Warris et al. 2001). Both TLR2 and TLR4 have been shown to work cooperatively with other receptors to recognise and bind fungal particles. TLR2 works with Clec7a in order to recognise and bind zymosan (Brown, Herre et al. 2003; Gantner, Simmons et al. 2003), while TLR4 works in conjunction with CD14 to recognise *A. fumigatus* hyphae (Huffnagle and Deepe 2003). Polymorphisms in human TLR1, TLR4 and TLR 6 have been associated with increased risk of invasive aspergillosis after stem cell transplantation (Kesh, Mensah et al. 2005; Bochud, Chien et al. 2008). TLR9 has been shown to be able to recognise fungal DNA (van de Veerdonk, Kullberg et al. 2008). Human monocytes that have had their TLR blocked and TLR9 deficient mouse macrophages show less cytokine production when stimulated with *C. albicans* than cells which still have functional TLR9 (van de Veerdonk, Netea et al. 2008). TLR9 is also able to recognise the DNA of *A. fumigatus* and *C. neoformans*, resulting in the activation of myeloid cells and the

secretion of pro-inflammatory cytokines (Nakamura, Miyazato et al. 2008; Ramirez-Ortiz, Specht et al. 2008). These studies highlight the importance of TLRs in defence against fungal pathogens.

C-Type Lectin like Receptors

Clec7a

Clec7a, also known as Dectin-1, is a cell surface receptor that is broadly expressed on myeloid cells, predominantly macrophages, monocytes, dendritic cells and neutrophils (Brown, Taylor et al. 2002). It is a beta-glucan receptor and recognises 1-3 linked beta-glucans that are present in the cell wall of fungi (Brown and Gordon 2001). It consists of a carbohydrate recognition domain, an extracellular stalk region, a transmembrane domain and a signalling intracellular domain with immunoreceptor tyrosine-based activation motif (ITAM) (Ariizumi, Shen et al. 2000). Clec7a exists in two major isoforms (Heinsbroek, Taylor et al. 2006). While Clec7a.A has all domains including the extracellular stalk region, Clec7a.B is missing this stalk region (Figure 1.3). Studies have shown that a Clec7a deficient macrophages are impaired in their ability to recognise fungal particles e.g. zymosan, and respond by producing less TNF than wild type macrophages (Taylor, Tsoni et al. 2007). Clec7a deficient mice have also been shown to be impaired in their ability to clear an infection by *C. albicans* (Taylor, Tsoni et al. 2007), and although there has been some conflicting data (Saijo, Fujikado et al. 2007), data from human studies supports the evidence that Clec7a is important for clearance of *C. albicans* (Ferwerda, Ferwerda et al. 2009; Plantinga, van der Velden et al. 2009). Although the function of Clec7a on macrophages and dendritic cells have been well characterised (Brown, Taylor et al. 2002; Grunebach, Weck et al. 2002; Willment, Lin et al. 2003; Adams 2004; Herre, Marshall et al. 2004; Gantner,

Simmons et al. 2005; Underhill, Rossnagle et al. 2005; Yadav and Schorey 2006; Taylor, Tsoni et al. 2007), their role on neutrophils is less understood. However, a study has shown that blockade of Clec7a on human neutrophils resulted in a significant decrease in the ability of neutrophils to kill *C. albicans* (Kennedy, Willment et al. 2007). Interestingly, humans deficient in Clec7a are more susceptible to chronic mucocutaneous candida infections, however, the dectin-1-deficient human neutrophils were reported to have no defect in *C. albicans* recognition or killing (Ferwerda, Ferwerda et al. 2009).

Clec4n

Clec4n, also known as dectin-2, is PRR that is predominantly expressed on some DC subsets, tissue macrophages and inflammatory monocytes (Taylor, Reid et al. 2005). It couples to the immunoreceptor tyrosine-based activation motif (ITAM) containing, FcR γ , to enable signalling and it recognises α -mannans (Sato, Yang et al. 2006; Robinson, Osorio et al. 2009; Saijo and Iwakura 2011), which are a key component of fungal cell wall. Clec4n plays a role in the immune response to fungal infection, as demonstrated by the observation that *Clec4n*^{-/-} mice have an increased susceptibility to systemic *C.albicans* infection, due to decreased levels of cytokine production and Th17 response (Robinson, Osorio et al. 2009; Saijo and Iwakura 2011). This is further supported by evidence that shows that patients with deficiencies in IL-17A and IL-17F suffer from recurrent *C.albicans* infections (Puel, Cypowyj et al. 2011). The collaborative nature of PRR is demonstrated in the observation that blockade of Clec4n in *Clec7a*^{-/-} mice showed a decrease in the Th1 response (Robinson, Osorio et al. 2009). This cooperative behaviour in BMDC makes Clec4n a useful PRR to consider when exploring the role of Clec7a on neutrophils.

Clec4e

Clec4e, also known as MINCLE, is a type II transmembrane protein that is expressed on activated macrophages (Matsumoto, Tanaka et al. 1999). Like Clec4n, it couples to the adaptor protein, FcR γ , to enable signalling (Yamasaki, Ishikawa et al. 2008). This PRR is able to recognise α -mannose from fungal *Malassezia spp.* (Yamasaki, Matsumoto et al. 2009), and trehalose dimycolate, which is a mycobacterial glycolipid (Ishikawa, Ishikawa et al. 2009). Clec4e has also been shown to bind *C. albicans* yeast cells (Bugarcic, Hitchens et al. 2008) and is important in host immune response to *C. albicans* as mice deficient in Clec4e showed a significantly increased susceptibility to systemic candidiasis (Wells, Salvage-Jones et al. 2008). An extra function of Clec4e is its ability to recognise damaged cells by sensing spliceosome-associated protein 130 (SAP130), which is a soluble factor released by necrotic cells (Yamasaki, Ishikawa et al. 2008).

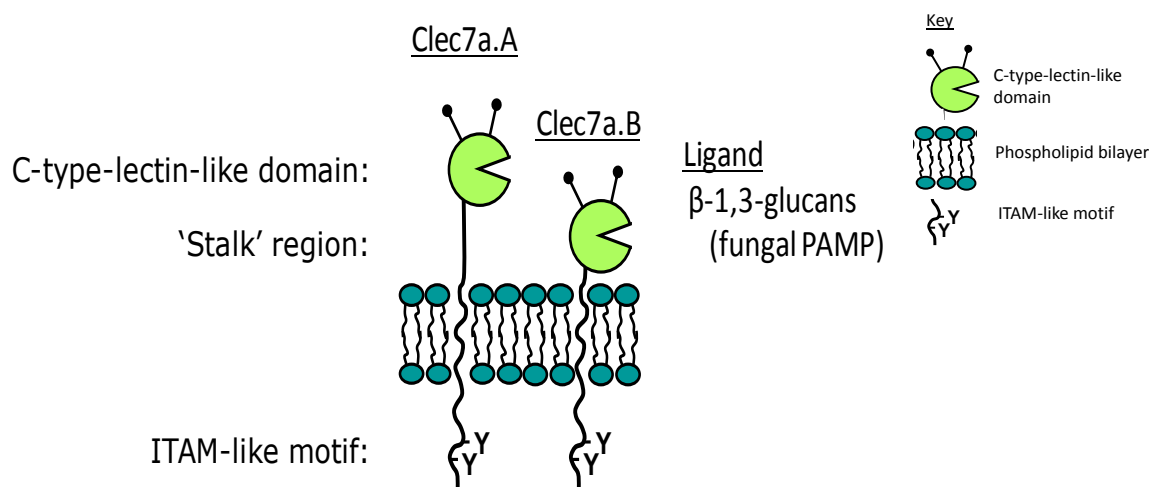


Figure 1.3: Schematic of the main Clec7a isoforms. Both isoforms consist of an extracellular c-type-lectin-like domain, which binds β -1,3-glucans, a transmembrane domain and a cytoplasmic tail with an ITAM-like motif. The isoforms differ in that Clec7a.A also contains an extracellular stalk domain that is missing in the Clec7a.B isoform.

1.3. Complement Cascade and Fungal Immunity

The complement cascade is a vital component of the immune system. It can be initiated via three main pathways which all converge and lead to the production of C3 convertase (Figure 1.2) (Dunkelberger and Song 2010). One of its effector functions is the generation of the membrane-attack complex on the pathogenic cells. This results in numerous pores being created in the cell membrane and the eventual lysis and destruction of the pathogen (Rus, Cudrici et al. 2005), although some pathogens have developed strategies to avoid this attack. An example of this is seen with *Streptococcus* group A, which has a thick peptidoglycan layer that resists penetration into the cell membrane by the membrane-attack complex (Frank 2001).

On the path to the generation of the membrane attack complex, various protein fragments are formed. One of these fragments is C3b, which becomes known as inactivated C3b (iC3b), when it covalently attaches to the pathogen (Sarma and Ward 2011). iC3b is an opsonin and this means it is able to bind to pathogenic cells and flag them for opsonisation by phagocytic cells, i.e. neutrophils and macrophages. iC3b is recognised on these phagocytes by complement receptor 1, 2 and 3 (CR1, CR2, CR3 and CR4) (Ahearn and Fearon 1989; Ueda, Rieu et al. 1994; Gasque 2004). The complement cascade can lead to the recruitment of inflammatory cells to the site of infection. C5a has been described as a potent chemoattractant, especially for neutrophils (Snyderman, Phillips et al. 1970; Becker 1972). The C5aR (CD88) is expressed on a wide range of cells including most myeloid cells (Monk, Scola et al. 2007).

Fungal cells walls are rich in mannans. The mannan binding protein (MBP) binds to the mannans in the fungal cells and this is able to activate the complement cascade, leading to the effector functions described above (Kozel 1996).

As with the case of PRR, activation of the complement cascade by fungi can be species and morphotype specific (Kozel 1996). Encapsulated *C. neoformans* is a potent activator of the complement system as, when incubated with serum, C3 fragment production increases compared to unencapsulated *C. neoformans* from approximately 2×10^6 fragments per yeast cell to 10^7 fragments per yeast cell (Young and Kozel 1993). It is also worth noting that this activation apparently occurs solely via the alternative pathway as the kinetics of C3 binding to encapsulated *C. neoformans* is not affected by ethylene glycol tetraacetic acid (EGTA), which chelates Ca^{2+} required for the classical pathway (Kozel 1996). This is in contrast to *C. albicans* where the classical, alternative and MBP pathways are thought to be involved in complement activation (Kozel 1996).

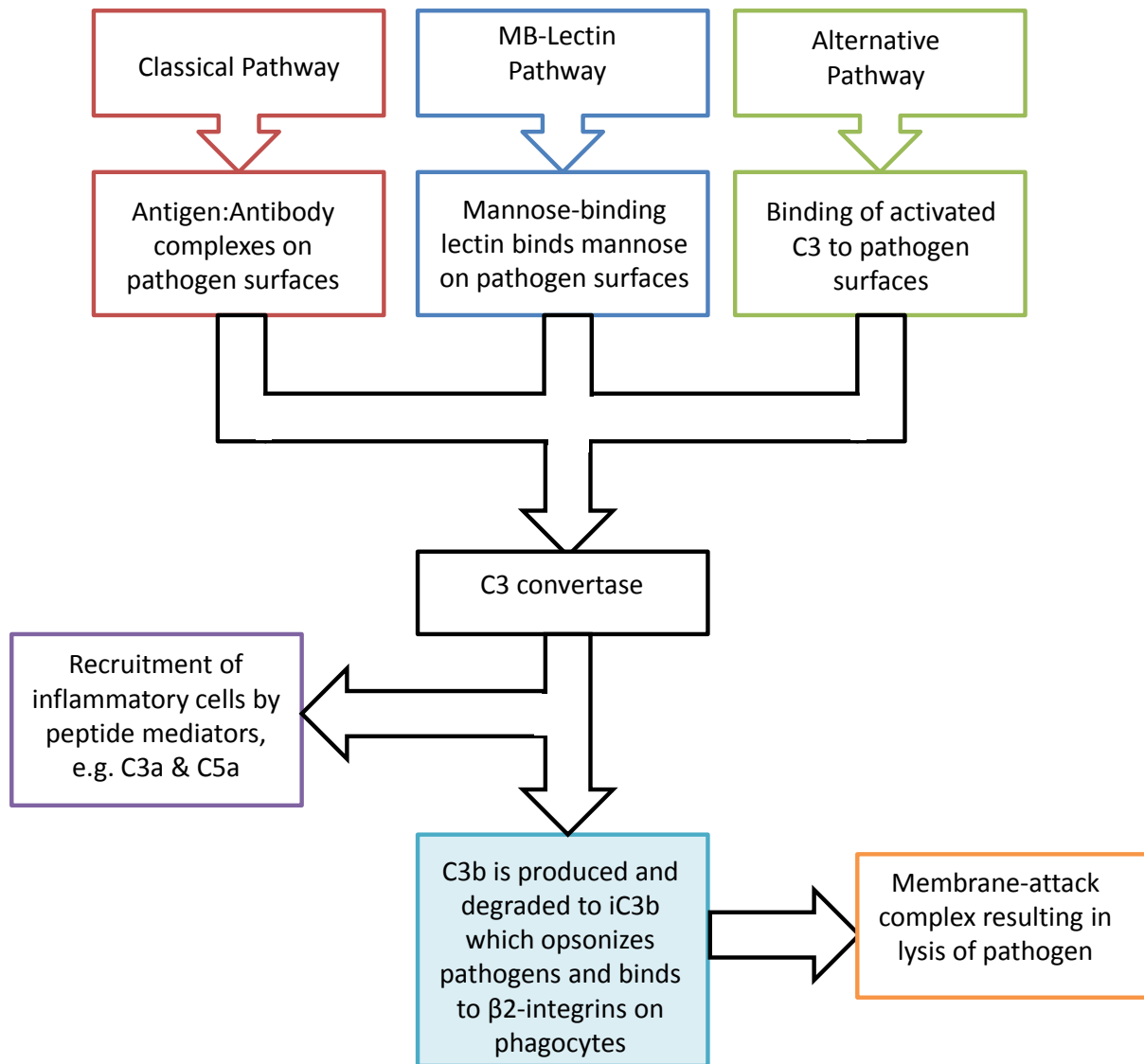


Figure 1.2: Complement cascade. The three initial complement pathways converge and result in the production of C3 convertase. This in turn leads to pathogen killing via the recruitment of inflammatory cells, the generation of the membrane-attack complex on pathogenic cells and opsonisation of pathogens that can then be recognised and phagocytosed by immune cells.

1.4. Immortalisation of Primary Cells

Primary cells can be difficult to culture, and in the case of neutrophils, the cells cannot be reliably cultured for more than a day due to their short half-life. Researchers often need to generate fresh cultures from primary cells regularly and this can be a time consuming and costly practice. One way around this is the use of immortalised cell lines that never reach senescence. There are two traditional ways of generating immortalised cells:

1. Viral gene induction

This uses the insertion of viral genes, such as Epstein–Barr virus (EBV) (Miller 1982; Oh, Oh et al. 2003) and the Simian Virus 40 (SV40) T antigens (Jha, Banga et al. 1998) into the primary cells. These genes are thought to work primarily by inactivating tumour repressor genes (e.g. p53 and Rb) (Dobbelstein and Roth 1998; Ahuja, Saenz-Robles et al. 2005; Paschos, Smith et al. 2009) and this same effect can be achieved by using siRNA targeted to these genes (Yang, Rosen et al. 2007). However, some studies have shown that SV40 is able to induce telomerase activity in infected cells (Foddis, De Rienzo et al. 2002).

2. Telomerase Reverse Transcriptase protein (TERT) expression

Another technique that is used to immortalise primary cells is inducing expression of the human TERT protein (Lee, Choi et al. 2004). This protein, a subunit of the human telomerase protein, is usually inactive in somatic cells but when exogenously expressed it maintains sufficient telomere length to keep replicative senescence at bay (Lee, Choi et al. 2004). This technique is used for cells that are particularly sensitive to telomere length i.e. human cells.

These techniques are not always successful however, and other avenues need to be explored to allow for the immortalisation of a wider range of cell types. Class I Hox homeodomain transcription factors have been shown to promote expansion of haematopoietic progenitors and their expression is deregulated in human and mouse myeloid leukaemia (Wang, Calvo et al. 2006). Overexpression of Hoxa9 and Hoxb6 have been used to previously immortalise haematopoietic progenitors (Calvo, Sykes et al. 2000; Fischbach, Rozenfeld et al. 2005) and Hoxb8 is known to block differentiation of primary myeloid progenitors (Blatt, Aberdam et al. 1988; Knoepfler, Sykes et al. 2001). This makes the Hox genes good candidates as immortalising factors of haematopoietic progenitor cells.

1.5. Studying Neutrophils

Studying neutrophil biology has generated a few challenges. The short lived nature of these cells means that they cannot be cultured for more than a few hours. In mouse models, this results in the large numbers of mice being culled for primary neutrophils, which can be very expensive and leads to a large loss of animal life. This then raises ethical questions about animal welfare. A key strategy in minimising animal suffering in biomedical research is the application of the three R's; **Replacement**: this refers to the use of non-animal models wherever possible; **Reduction**: this refers to using methods that enable the researcher to obtain as much information from the fewest number of animals necessary; **Refinement**: this refers to using methods that minimise and reduce animal suffering as well as increasing animal welfare. The use of cell lines to *replace* animal use is common place in biomedical research but the current technologies are limited.

There are already granulocyte producing cell lines available but there are certain problems associated with them that can make them unreliable. The HL-60 cell line was derived from peripheral blood leukocytes of a 36-year-old Caucasian female with acute promyelocytic leukaemia (Collins, Gallo et al. 1977). Although differentiation of these cells to neutrophil-like cells is possible under certain conditions, the cell line comes with certain problems including, spontaneous differentiation, difficulty in reproducing differentiation within the one lab setting and differences in differentiation success across separate labs (Fleck, Romero-Steiner et al. 2005). Another cell line, 32Dcl3, a murine myeloblastic, IL-3 dependent cell line, can produce cells that are similar to primary murine neutrophils, however, these neutrophil-like cells are unable to generate superoxide in response to appropriate stimuli (Guchhait, Tosi et al. 2003).

A more reliable and reproducible method for generating large number of neutrophils would be very useful in the study of neutrophil biology. Wang et al. 2006 attempts to address these issues by generating a conditionally-immortalised neutrophil precursor cell line (Wang, Calvo et al. 2006). This method greatly improves on what is already available as the immortalisation of the cells is conditional and can be switched off by removal of the conditional factor, oestrogen, from the culture. This removes any problems associated with constitutive presence of the 'immortalising factor' after forced differentiation and should theoretically produce more mature neutrophils (Wang, Calvo et al. 2006). However, although some work has been done, these cells have not yet been extensively immunologically characterised. If neutrophils generated from this cell line are functionally equivalent to primary murine neutrophils, the numbers of animals culled for primary neutrophils could be significantly reduced and study of neutrophil function would be greatly enhanced.

1.6. AIMS

This work plans to address the following aims:

- To generate and immunologically characterise conditionally immortalised neutrophil precursor cell lines.
- To explore the role of clec7a on neutrophils in the recognition of and response to fungal cells.
- To use neutrophils generated from a conditionally immortalised neutrophil precursor cell line to model innate immune responses.

Chapter 2

Materials and Methods

2.1: Reagents

2.1.1. General Reagents

Reagent	Supplier
Bio-Gel P-100 Gel, Fine Polyacrylamide Beads	BIO-RAD Labs
Bovine Serum Albumin (BSA)	Sigma
Chloroform	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Ethanol	VWR
Ethylenediamine Tetraacetic Acid (EDTA)	USB
Human Fibronectin	Sigma
Formaldehyde	Sigma
Fugene 6 Reagent	Roche
Hank's Balanced Salt Solution (HBSS)	Invitrogen
Heparin	Sigma
HEPES (4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid)	Invitrogen
Hexadimethrine bromide (polybrene)	Sigma
Isopropanol	VWR
Phosphate Buffered Saline (PBS)	Invitrogen

Rabbit serum	Invitrogen
Sodium Azide	Sigma
Sodium Citrate	Sigma

2.1.2. Cell Culture

Cell Lines

Reagent	Supplier	Notes
Phoenix cells	Obtained from Garry Nolan	A HEK293T, human embryonic kidney, cell line that has been transformed to enable it to package MMLV viral DNA in capsids (Swift, Lorens et al. 2001). These cells are adherent and grown in DMEM with 10% (v/v) FCS, 50 units/ml penicillin, 50 µg/ml streptomycin and 2 mM glutamine.
MyPH8-B6	Home made	Neutrophil progenitor cell line. These cells grow in suspension and are grown in OptiMem, 10% (v/v) FCS, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, 30 µM β-mercaptoethanol) with 10 ng/ml SCF and 1 uM β-estradiol.

Media

Reagent	Supplier
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen
Iscove's Modified Dulbecco's Medium (IMDM)	Invitrogen

Opti-Mem	Invitrogen
RPMI 1640	Invitrogen

Cytokines

Reagent	Supplier	Notes
Granulocyte colony-stimulating factor (G-CSF)	Peprotech	Recombinant mouse G-CSF
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Peprotech	Recombinant mouse GM-CSF
Interleukin 5 (IL-5)	Peprotech	Recombinant mouse IL-5
Interleukin-3 (IL-3)	Peprotech	Recombinant mouse IL-3
Interleukin-6 (IL-6)	Peprotech	Recombinant mouse IL-6
Stem Cell Factor (SCF)	Peprotech	Recombinant mouse SCF

Antibiotics

Reagent	Supplier
Ampicillin	Sigma
Penicillin/Streptomycin	Invitrogen
Puromycin	Sigma
Zeocin	Invitrogen

Other Cell Culture Reagents

Reagent	Supplier
β -estradiol	Sigma
β -mercaptoethanol	Sigma
Fetal Calf Serum (FCS)	Invitrogen
L-glutamine	Invitrogen

2.1.3. Cell Stimulants and Activators

Reagent	Supplier	Notes
<i>Candida albicans</i> SC5314	Obtained from Ken Haynes, Imperial College London	A strain of <i>C.albicans</i> .
Curdlan microparticles	Wako Pure Chemical Industries, Ltd.	Curdlan particles bought then converted into microparticles via sonication (Rosas, Liddiard et al. 2008).
Ionomycin	Invitrogen	Induces intracellular calcium ion increases in cells
Zymosan	Invitrogen	An insoluble preparation of particles made from the cell wall of <i>Saccharomyces cerevisiae</i> .

2.1.4. Dyes and Colourimetric Indicators

Reagent	Supplier	Notes
3'-(-p-aminophenyl) fluorescein (APF)	Invitrogen	A reactive oxygen species (ROS) indicator that becomes a fluorescent when in the presence of the $\cdot\text{OH}$, ONNO^- and $\cdot\text{OCL}$. This makes it a specific indicator of respiratory burst reactions.
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen	Cell dye that is excited by the blue laser at 488 nm and emits at 517 nm.
Cell Trace Calcein Violet	Invitrogen	Cell dye that is excited by the violet laser at 405 nm and emits at 452nm.
CellTrace DDAO-succinimidyl ester	Invitrogen	Cell dye that is excited and emits at the far red of the spectrum.
Draq5 DNA Dye	BIO Status Ltd	A cell permeant DNA dye that can be used on live cells.
Fura 2 AM	Invitrogen	Calcium ion indicator
Fura red	Invitrogen	Calcium ion indicator
NIM-DAPI	NPE Systems	The cell impermeant DNA dye DAPI in a nuclear isolation medium (NIM)
SYTO[®] RNASelect[™]	Invitrogen	A cell-permeant nucleic acid stain that selectively stains RNA.

2.1.5. Molecular Biology

Reagent	Supplier
Agarose powder	Sigma
Competant <i>E.coli</i> TOP10	Invitrogen
DNA ladder 100bp	Invitrogen
Ethidium Bromide	Sigma
Lysogeny broth (LB)	Sigma
Super Optimal broth (SOC Medium)	Invitrogen
Tris/Borate/EDTA (TBE) Buffer (10X stock)	Invitrogen
TRIzol Reagent	Invitrogen

2.1.6. Kits

Reagent	Supplier
Dead Cell Removal Kit	Miltenyi Biotec
Fluo-4 NW Calcium Assay Kit	Invitrogen
Mouse IL-6 ELISA Kit	BD Biosciences
MACS Murine Lineage Depletion Kit	Miltenyi Biotec
RNeasy Mini Kit	QIAGEN
RT-PCR Kit	Ambion

2.2: Viral DNA Constructs

Vector	Source	Description
pMXs-IP	T. Kitamura (Kitamura 1998)	MMLV derived third generation retroviral vector with internal ribosomal entry site (IRES) upstream of the puromycin resistance cassette.
pMXs-IP:FL-ER-Hoxb8	Dr. Phil Taylor	Flag (FL)-tagged estrogen receptor binding domain (ER)-Hoxb8 fusion gene cloned into pMXs-IP
pMXs-IZ	Dr. Phil Taylor/Dr. K. Liddiard (Rosas, Liddiard et al. 2008)	Modification of pMXs-IP in which the puromycin resistance cassette has been replaced with zeocin resistance.
pMXs-IZ:clec7aA.1	Dr. Phil Taylor/Dr. K. Liddiard (Rosas, Liddiard et al. 2008)	pMXs-IZ containing clec7aA.1 isoform (Heinsbroek, Taylor et al. 2006)
pMXs-IZ:clec7aB.1	Dr. Phil Taylor/Dr. K. Liddiard (Rosas, Liddiard et al. 2008)	pMXs-IZ containing clec7aB.1 isoform (Heinsbroek, Taylor et al. 2006)
pMXs-IZ:clec7aB.2	Dr. Phil Taylor/Dr. K. Liddiard (Rosas, Liddiard et al. 2008)	pMXs-IZ containing clec7aB.2 isoform (Heinsbroek, Taylor et al. 2006)
pMX- μ-calpain-EGFP	Prof. Maurice Hallet/ Kimberley Lewis	μ -Calpain-EGFP construct subcloned into pMX-IRES-GFP retroviral vector.

2.3: Antibodies

Epitope	Antibody	Specificity	Company	Notes
Ly-6G, Ly-6C	Gr-1 PE (clone RB6- 8C5)	Mouse	BD Biosciences Pharmingen	Marker that is highly expressed on neutrophils, but also on other cells, including monocytes and eosinophils (Tepper, Coffman et al. 1992; Fleming, Fleming et al. 1993; Lagasse and Weissman 1996).
Ly-6G	Ly-6G PE or PE- Cy7 (clone 1A8)	Mouse	BD Biosciences Pharmingen	Largely neutrophil restricted marker (Fleming, Fleming et al. 1993).
F4/80, r1	F4/80 PE or APC (clone A3- 1)	Mouse	AbD Serotec	'Pan-macrophage' marker (Austyn and Gordon 1981).
CD11b	CD11b FITC (clone 5C6)	Mouse	Home made	A generic myeloid cell marker, CD11b is a subunit of complement receptor 3 (Lagasse and Weissman 1996; Melo, Catchpole et al. 2000).
CD11b	CD11b APC or APC-Cy7 (clone M1/70)	Mouse	BD Biosciences Pharmingen	A generic myeloid cell marker, CD11b is a subunit of complement receptor 3

				(Lagasse and Weissman 1996; Melo, Catchpole et al. 2000).
Heat stable antigen	CD24 FITC (clone M1/69)	Mouse	BD Biosciences Pharmingen	Heat stable antigen (Aigner, Ruppert et al. 1995).
CD45.1	CD45.1 APC (clone A20)	Mouse	BD Biosciences Pharmingen	'Pan-leukocyte' marker that recognises the CD45.1 polymorphic variant (Johnson, Greenbaum et al. 1989).
C5a receptor	CD88 PE (clone 10/92)	Mouse	AbD Serotec	C5a receptor (Monk, Scola et al. 2007).
CD11c	CD11c PE	Mouse	BD Biosciences Pharmingen	Subunit of complement receptor 4, high expression of which in mice is often associated with dendritic cells, but is also seen on other cells such as NK cells, alveolar macrophages and activated monocytes and macrophages
B7.1	CD80 Biotinylated (clone 16-10A1)	Mouse	BD Biosciences Pharmingen	Co-stimulatory antigen (Bluestone 1995).

B7.2	CD86 Biotinylated	Mouse	BD Biosciences Pharmingen	Co-stimulatory antigen (Bluestone 1995).
FCεR1	FCεR1 Biotinylated (clone Mar-1)	Mouse	eBiosciences	IgE receptor 1; mast cell marker (Yamaguchi, Hirai et al. 2001).
SCF receptor	CD117 APC or Biotinylated (clone 2B8)	Mouse	BD Biosciences Pharmingen	SCF receptor (Ikuta and Weissman 1992).
Clec7a	Clec7a Biotinylated (clone 2A11)	Mouse	Home made	β-glucan receptor
Clec7a	Clec7a Alexa ⁶⁴⁷ (clone 2A11)	Mouse	AbD Serotec	β-glucan receptor
Clec7a	Clec7a (Clone 259931)	Human	R & D Systems	β-glucan receptor
Clec7a	Clec7a (Clone GE2) (Willment, Marshall et al. 2005)	Human	Home made	β-glucan receptor
Dectin-2	Clone D2.11E4 Biotinylated	Mouse	Home made	A mannose binding receptor

Ly-6B.2	Ly-6B.2 FITC or PerCP (clone 7/4)	Mouse	Home made	Neutrophil and inflammatory monocyte marker (Rosas, Thomas et al. 2010).
Ly-6B.2	Ly-6B.2 PE or Alexa ⁶⁴⁷ (clone 7/4)	Mouse	AbD Serotec	Neutrophil and inflammatory monocyte marker (Rosas, Thomas et al. 2010).
CXCR2	CXCR2 PE (clone 242216)	Mouse	R & D Systems	Receptor for the chemoattractant IL-8
CD66	CD66 PE	Human		Neutrophil marker
Fcγ RII/III (CD16&32)	Clone 2.4G2	Mouse	Home made	Used as blocking antibody to reduce background binding of antibodies to Fc receptor expressing cells.

2.4. General Methods

2.4.1. Mice

129S6/SvEv and 129S6/SvEv.*Clec7a*^{-/-} mice were obtained from in house colonies belonging to Dr Phil Taylor. C57BL/6 and Balb/c mice were obtained from Harlan UK. Dectin-1-deficient (*Clec7a*^{-/-}) mice were also available backcrossed to the C57BL/6 genetic background for 8 generations. CD11b-deficient mice (*Itgam*^{-/-}) on the C57BL/6 genetic background were generated as previously described by (Coxon, Rieu et al. 1996), and cells from these mice were obtained from Prof. Marina Botto (Imperial College London). All animals were handled in accordance with institutional and UK Home Office guidelines.

2.4.2. Antibody Staining of Cells for Flow Cytometric Analysis

The cells were blocked with 'flow cytometry block' (5% (v/v) heat-inactivated rabbit serum; 0.5% (w/v) BSA; 2 mM NaN₃; 5 mM EDTA in PBS) with 2.4G2 at 4 µg/ml for 30 minutes on ice. A 96 well v-bottomed plate was chilled and after blocking 50µl of cells were placed in a well and 50 µl of 2x antibody, diluted in 'flow cytometry wash' (0.5% (w/v) BSA; 2 mM NaN₃; 5 mM EDTA in PBS), was added to the cells. The cells were incubated on ice, for 1 hour, in the dark. After incubation the cells were centrifuged (350 x *g*, 5minutes) and washed 3x with flow cytometry wash then fixed with 1% formaldehyde. For biotinylated antibodies, the cells are washed twice with flow cytometry wash after the hour incubation with the primary antibodies, then they were incubated for 30 minutes with the appropriate streptavidin conjugate before the final washes and fixing.

2.4.3 Flow Cytometry

All flow cytometric collection was performed using either the FACScalibur flow cytometer (BD), Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter) or the CyAn ADP 3 laser

Analyser (Beckman Coulter) and data was analysed using Summit (Beckman-Coulter) or FlowJo (Treestar) software. The Cell Lab Quanta SC is a blue (488nm) laser machine, with the option of a mercury lamp for UV excitation, that simultaneously performs absolute counts on the acquired samples. The CyAn ADP Analyzer has three lasers (405nm, 488nm and 642nm) and 9 colour parameters.

2.4.4 Cytospin Preparation of Cells

Approximately 5×10^4 - 10^5 cells in 100 μ l final volume were added to the assembled cytopsin cassette (Thermo-Fisher). The cells were spun for 3 minutes at 500 rpm. The cells were spun as soon as possible after loading to ensure evenly distributed cells on the cytopsin. The slide was removed from the cassette and the cells allowed to air dry. The air dried cells were then stained using the 3-colour 'Hemacolour' cell staining solutions (Merck, see below):

Stain 1: Methanol Fixative 30 seconds dipping the slide up and down

Stain 2: Eosin 10-30 seconds dipping the slide up and down

Stain 3: Methylene Blue 10-30 seconds dipping the slide up and down

The slide was gently rinsed with tap water to remove the excess stain then allowed to air dry. A cover slip was placed over the cells using DPX mounting medium (Fisher) and the cells analysed under the microscope (Leica DMLB with 40x and 100x oil immersion objectives).

2.4.5. Cell Culture Methods

Freezing Cell Stocks

Cells were frozen, using a Nalgene cryogenic freezing container, in sterile freezing medium (FCS + 5% DMSO). The cells were initially frozen at -80 °C before transfer to liquid nitrogen for long term storage.

Defrosting Cell Stocks

Frozen cells were defrosted in a 37°C water bath then 10ml of growth medium was added and the cells centrifuged (350 x g, 5 minutes). The supernatant was aspirated and the cells re-suspended in 10-20ml of growth medium.

Trypsinization of Adherent Cells

The growth medium was aspirated from the adherent cells and the cells were washed twice with sterile PBS. The PBS was aspirated and enough trypsin added to cover the cells at the bottom of the flask (0.75 ml for 75 cm³ flasks and 1.5 ml for 175 cm³ flasks). The flask was agitated and the cells incubated with the trypsin for approximately 1-5 minutes. Growth medium (10 ml) was added to the cells to stop the trypsin reaction and the cells washed with PBS ready for use.

Passaging Cell Lines

Cells were split to avoid media exhaustion and death. The timing of splitting differed but usually the cells were split every 2-3 days. Fresh medium was placed in a new flask and cultured cells added to the flask.

2.4.6. Molecular Biology Methods

Preparation of LB of Agar Plates

Pre-prepared solid LB agar was made by autoclaving of LB agar tablets (Sigma) in H₂O as instructed by the manufacturer followed by solidification by cooling. When required, solid LB agar was dissolved in a microwave and allowed to cool, but not sufficiently to allow it to set. Ampicillin was then added to a working concentration of 100 µg/ml. After gentle swirling to ensure full mixing of the ampicillin, the liquid LB agar was poured into circular plates and allowed to solidify ready for use.

Transformation of Competent E.coli with DNA

Vector DNA (<10 ng) was added to a tube containing competent E.coli TOP10 (15 µl) (Invitrogen) on ice and left to incubate for 30 minutes. The tube was then heat shocked for 30 seconds at 42°C then placed back on ice for 2 minutes. SOC medium (100 µl) (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose) was added to the cells and agitated for 30 minutes at 37°C. The cells were plated onto LB agar plates containing ampicillin and incubated at 37°C overnight.

Plasmid DNA amplification

Plasmid DNA was amplified and prepared using the QIAGEN® plasmid maxi-prep kit as directed by the manufacturer's protocol.

DNA gel electrophoresis

A 2.5% agarose gel was prepared by dissolving 2.5g of agarose powder in 100ml of 0.5 x Tris/Borate/EDTA (TBE) buffer in a glass conical flask. Ethidium bromide (0.1 µg/ml) was

added to the dissolved agarose and the flask swirled to mix. The dissolved agarose was then poured into casts with combs for the wells and allowed to set at room temperature until solid and ready for use. The gels were placed in gel electrophoresis running apparatus (Embi Tec Run-one electrophoresis cell) and running buffer of 0.5 x TBE with 0.1 µg/ml ethidium bromide was poured over the gels until covered. The markers and samples were then loaded and gels run at 100V until the appropriate resolution of bands was evident.

RNA extraction

RNA extraction using the RNeasy Mini Kit was performed on cell pellets according to the manufacturer's protocol.

TRizol Reagent:

Cells were centrifuged (350 x g, 5 minutes) and resuspended at 10^6 /ml in TRizol Reagent (Invitrogen). RNA was extracted according to the TRizol reagent manufacturer's protocol. In brief, this involved homogenization of the cell lysate via vigorous and repetitive pipetting (at this point, homogenized samples can be stored at -80°C for later use), phase separation of RNA from DNA and proteins using chloroform, RNA precipitation with 100% isopropanol and washing of RNA with 75% ethanol (ethanol diluted in DEPC water). The RNA was dissolved in DEPC water and the concentration was measured using the nanodrop spectrophotometer (Thermo Scientific).

RT-PCR

RT-PCR was performed with the RETROscript kit (Ambion) and performed according to the two-step RT-PCR company protocol. The first step involved reverse transcription of the RNA to produce cDNA. The second step involved the PCR amplification of the cDNA for

analysis. The following Clec7a primers that discriminate between the Clec7a.A and Clec7a.B isoforms (Heinsbroek, Taylor et al. 2006) were used (394 bp and 269 bp respectively):

Forward primer: 5' ACCGGATCCCAAGTGCTCTGCCTACCTAG 3'

Reverse primer: 5' GGAATCCTGTGCTTTGTGGT 3'

β 2 microglobulin primers were used as a positive control (Rosas, Thomas et al. 2010).

2.4.7. Growth of *C. albicans* SC5314

Yeast Peptone dextrose (YPD) agar plates were made in the same way as LB agar plates after mixing LB agar powder (Sigma) in H₂O (as directed by the manufacturer) and autoclaving to sterilise and dissolve. *C. albicans* SC5314 was streaked onto set YPD agar plates and incubated at 37°C overnight to enable growth and isolation of individual colonies. Single colonies were cultured in a shaking incubator for 16 h at 37°C in 50 ml of YPD broth. Live *C. albicans* was washed extensively in PBS before being fluorescently labelled (see below).

2.4.8. Fluorescent Labelling of Zymosan and *C. albicans* SC5314

Zymosan (Invitrogen) was labelled with either Alexa Fluor 405-succinimidyl ester (Invitrogen) or FITC (Sigma). For Alexa Fluor 405 labelling, 1 mg of dye was dissolved in 35 μ l DMSO. The dye solution was added to 10 mg of zymosan suspended in 1 ml borate buffer (0.2M sodium tetraborate, 0.2M boric acid, pH8.2). The resulting suspension was incubated at room temperature, in the dark and kept rotating overnight. The particles were then washed (1500 x g, 5 minutes) with PBS until all traces of the dye had been removed (minimum four washes). Fluorescent-zymosan was aliquoted and stored below -70°C until needed.

FITC labelled zymosan was provided by Dr. Phil Taylor and was produced as follows.

Zymosan at 10mg/ml was resuspended in 0.3M sodium bicarbonate buffer pH9.2 was mixed with FITC to a working concentration of between 2 and 50 µg/ml depending on desired labelling intensity and incubated on a rotator for 3 hours at room temperature. Unbound FITC was removed by repeated washing with PBS until the wash fraction was free from FITC colouring before resuspension in PBS. Fluorescent-zymosan was aliquoted and stored below -20°C until needed.

Live *Candida albicans* SC5314 yeast were labelled with Pacific Orange succinimidyl ester (Invitrogen). *C. albicans* at 3.2×10^8 /ml in 1 ml PBS was mixed with 50 µl of 1 mg/ml pacific orange dye dissolved in DMSO. After mixing, the suspension was left to incubate in the dark, at room temperature for 30 minutes, on a rotator. The labelled *C.albicans* was then washed (350 x g, 5 minutes) four times with excess PBS or medium and resuspended in medium for use.

2.5. Generation and Characterisation of Conditionally Immortalised Cell lines

2.5.1. Preparation of pMXs-IP:FL-ER-Hoxb8 retrovirus for conditional-immortalisation of neutrophil precursors

Phoenix cells (a HEK293T, human embryonic kidney, cell line that has been transformed to enable it to package MMLV viral DNA in capsids) were incubated in 6 well plates (1.5×10^6 /well) in DMEM with 10% FCS, 150 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 2 mM glutamine (37°C , 5% CO_2) overnight. The pMXs-IP:FL-ER-Hoxb8 viral DNA (See Table 2.2), or pMXs-IP empty vector, were mixed with FuGene 6 transfection reagent (Roche) in DMEM. The medium of the phoenix cells was aspirated and 2ml of DMEM with 10% FCS, 50 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, and 2 mM glutamine was added. A Fugene:DNA complex was formed by the incubation, for 5 minutes at room temperature, of 6 μl Fugene and 94 μl of DMEM (the Fugene was added directly into the medium and not allowing any to touch the sides) followed by the addition of 1 μg of DNA and a further incubation at room temperature for at least 15 minutes after mixing by swirling. The DNA:Fugene complex was then added to the phoenix cells and left to incubate (37°C , 5% CO_2) for 48 hours. The replicate supernatants were harvested, polybrene (5 $\mu\text{g/ml}$) was added and the supernatant filtered with a 0.45 μm filter. The supernatants were stored at -80°C in appropriately sized aliquots until required.

2.5.2. Purification of lineage (lin^-) cells from mouse bone marrow and pre-stimulation for infection

The femurs from mice were cleaned and the marrow was flushed out with sterile MACS Buffer (0.5% (w/v) BSA, 5mM EDTA in PBS) using a syringe and a 25 gauge needle. The cells were counted and lin^- cells were enriched using the MACS murine lineage depletion kit

(Miltenyi Biotec), following the company protocol. In brief, this involved depletion of bone marrow cells using biotinylated antibodies against the following: CD5, CD45R (B220), CD11b, Gr-1, 7/4 and Ter-119 followed by magnetic separation over a MACS column. The cells were counted, centrifuged (350 x g, 5 minutes) and resuspended in pre-stimulation medium (IMDM, 15% (v/v) FCS, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, 10ng/ml IL-3, 20ng/ml IL-6, 25ng/ml SCF) at 10^6 /ml. The cells were incubated (37°C, 5% CO₂) for 3 days prior to infection (see below).

2.5.3. Retroviral infection and cell propagation of pre-stimulated lin⁻ cells

The wells of a 12 well plate were coated with 10µg/ml fibronectin (Sigma; 2 hours, 37 °C). The pre-stimulated lin⁻ cells were counted and resuspended in progenitor outgrowth medium (POM) (OptiMem, 10% (v/v) FCS, 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, 30 µM β-mercaptoethanol) with 10 ng/ml SCF and 1 uM β-estradiol, to a concentration of 2×10^6 /ml. Cells (5×10^5 cells in 250 µl) were placed in each well and 850 µl of the viral vector, the empty vector or the mock transfected supernatant were added to each well. The cells were centrifuged (1500 x g, 90 minutes, 25 °C) to promote infection. After centrifugation, POM with SCF and β-estradiol (3 ml) was added to each well and incubated (37 °C, 5% CO₂) for 2 days.

After 2 days the replicates were pooled, centrifuged (350 x g, 5 minutes) and resuspended in POM with SCF, β-estradiol and 1.5 µg/ml puromycin. Previous work done by Dr. Kate Liddiard confirmed 1.5 µg/ml as the best dose for selection as lower doses showed some premature differentiation of progenitors, while higher doses were too harsh and killed too many cells. The cells were then allowed to grow over 10 days with regular replenishing of the medium. When using C57BL/6 lin⁻ cells, only the cells infected with the pMXs-IP:FL-ER-

Hoxb8 vector survived puromycin selection and on day 10 the puromycin was removed and the cells cultured in POM with SCF and β -estradiol. On addition of puromycin a large proportion of the cells died, but within 3 days growth was noticeable and by day 10 a robustly growing cell culture was evident. These cells were named myeloid progenitor Hoxb8 from C57BL/6 (MyPH8-B6) cells.

2.5.4. Cell growth and maintenance of MyPH8 cells

MyPH8 cells can be maintained in culture, in POM with SCF and β -estradiol, as progenitors, indefinitely with regular passaging (usually every 2-3 days). When passaging, cells were usually diluted at a 1:10 ratio for general maintenance of the cell line. Cell stocks were kept frozen (-80°C or in liquid nitrogen) and a new vial was defrosted and kept in culture every 3 months in order to avoid genetic drift and abnormal function of the cell line. Cells are frozen and defrosted as described in 2.4.5.

2.5.5. Retroviral infection of MyPH8-B6 cells

The undifferentiated cells were counted and 5×10^5 cells were centrifuged ($350 \times g$, 5 minutes) and resuspended in 1.5 ml neat viral supernatant containing 10 ng/ml SCF and 1 μM β -estradiol. The cells were added to a well of a sterile 6-well plate and centrifuged ($1500 \times g$, 90 minutes, 25°C). After centrifugation, 2 ml of POM containing SCF and β -estradiol was added to each well and the plates incubated for 2 days (37°C , 5% CO_2). After 2 days incubation, the cells were either selected with antibiotics (50 $\mu\text{g}/\text{ml}$ zeocin, when using the pMXs-IZ vector) or sorted with a MoFlo Legacy cell sorter (Beckman-Coulter), depending on the specific viral vector that was used to infect the cells (discussed in detail in the appropriate results sections).

2.5.6. Differentiation of MyPH8-B6 cells into neutrophils

MyPH8-B6 cells were washed three times with sterile PBS (350 x g, 5 minutes) to remove the β -estradiol. The cells were then counted and resuspended ($5 \times 10^4 - 10^5$ cells/ml) in 10ml POM containing SCF+G-CSF (both at 20 ng/ml). The cells were monitored and 5 ml medium was added to the cells every day for four days. By day four the majority of the cells had differentiated into morphologically recognisable neutrophils.

2.5.7. Purification of MyPH8-B6 derived neutrophils

MyPH8-B6 derived neutrophils were purified using a 'Dead Cell Removal Kit' (Miltenyi Biotec), CD117 depletion and subsequent magnetic separation. Cells were taken out of culture and counted. The cells were then resuspended at 10^6 cells/ml, in sterile 'FACS Block' (5% heat-inactivated rabbit serum; 0.5% BSA; 2mM NaN_3 ; 5mM EDTA in PBS) containing 4 $\mu\text{g/ml}$ anti-Fc γ RII/III (2.4G2), and incubated at 4°C for 10 minutes. An equal amount of 'FACS Block' containing 10 $\mu\text{g/ml}$ of biotinylated anti-CD117 (working concentration 5 $\mu\text{g/ml}$) was added to the cells. The suspension was mixed and incubated at 4°C, in the dark for 30 minutes. The cells were then washed three times with sterile PBS (350 x g, 5 minutes). The cells were then resuspended in dead cell removal and streptavidin coated microbeads (10^7 cells/100 μl), mixed and incubated at room temperature for 15 minutes. A suitable amount of 1x binding buffer, provided in the kit, was added to the cell suspension and the cells applied to a pre-prepared (column had been rinsed with 1x binding buffer) magnetic column within a magnetic field. The cells were allowed to flow through via gravity and the column washed three times with 1x binding buffer. Biotin labelled cells (CD117⁺ progenitors and dead cells) stay within the magnetic field while live, differentiated neutrophils pass through the field and are collected for further use.

2.5.8. DNA/RNA cell cycle analysis

A 100 µl aliquot of cells was added to 100 µl NIM-DAPI solution (NPE Systems) and the cells left to stain for 30 minutes at room temperature. The cells were then collected and analysed using the 'Quanta' flow cytometer (Beckman-Coulter). For DNA and RNA analysis, 10^5 cells were suspended in 1ml of NIM-DAPI solution containing 500nM SYTO RNASelect (Invitrogen). The cells were incubated for 30 minutes at room temperature than analysed via flow cytometry using the CyAn ADP.

2.6. *In vivo* Experimental Methods

2.6.1. Zymosan peritonitis

129S6/SvEv mice were used for all experiments designed to look at the role of dectin-1 on neutrophils *in vivo*. The mice were given i.p. injections of 2×10^7 particles of FITC-labelled or unlabelled zymosan 7 days, 3 days, 18 hours and 4 hours before the mice were sacrificed and the cells harvested via peritoneal lavage. Some mice were not injected with zymosan as controls. The cells were counted using the Cell Lab Quanta and stained with F4/80-PE, CD11b-PerCPCy5.5 and 7/4-Alexa 647 for differential counting on the FACScalibur (BD). The remainder of the cells that were not stained were centrifuged (350 x g, 5 minutes) and the supernatant collected for IL-6 analysis.

2.6.2. IL-6 ELISA

Supernatants from *in vivo* studies were tested for the presence of IL-6 as a marker of inflammation using an IL-6 ELISA kit obtained from BD Biosciences. The purified anti-IL-6 capture antibody was diluted to 1/250 in PBS and 100 µl of which was added to each well of

a 96-well protein-binding ELISA plate. The plate was sealed and incubated overnight in the fridge (4°C). The following day the capture antibody was removed and the plate washed 3 times with wash buffer (PBS with 0.5% tween). The plate was then blocked with 200 µl of blocking solution (PBS with 10% FCS) in each well, sealed and incubated at room temperature for 1 hour. IL-6 standards were diluted according to kit protocol in blocking solution during the incubation period. Experimental samples were also diluted in blocking solution. After blocking, the plate was washed 3 times with wash buffer then 100 µl of standards and samples were added to each well. The plate was sealed and incubated for 2 hours at room temperature. Standards were plated in duplicate and samples in triplicates. A working enzyme solution was prepared, just before use, according to kit protocol, that consisted of streptavidin-HRP antibody and the anti-IL-6 detection antibody diluted to 1/500. After incubation the plate was washed 5 times with wash buffer and 100 µl of the working enzyme solution was added to each well. The plate was sealed and incubated for 1 hour at room temperature. The TMB substrate solutions were brought to room temperature then an equal amount of solution A and solution B were mixed. The plate was washed 5 times with wash buffer then 100 µl of premixed TMB substrate was added to each well and the plate allowed to develop in the dark. Once satisfied with colour development, the substrate reaction was stopped with 50 µl of 2N H₂SO₄ and the optical density of the plate read (endpoint test wavelength: 450 nm; reference wavelength: 570 nm) using a Multiskan spectrum plate reader (Thermo-Fisher). A linear standard curve was calculated and used to determine quantity of IL-6 present in experimental samples.

2.6.3. BIOgel peritonitis

Preparation of BIOgel P100 (BIO-Rad Labs)

BIOgel P100 Fine acrylamide beads (BIO-Rad) were suspended in tissue culture endotoxin tested water (2% w/v) in a glass bottle. The suspension was then autoclaved. Once cool the BIOgel is ready for use and was stored at 4°C.

Elicitation of neutrophils with BIOgel P100

Mice were injected intraperitoneally with 1ml of 2% (w/v) BIOgel in pure H₂O, which led to high numbers of neutrophils being recruited to the peritoneal cavity. Approximately 16-18 hours later the mice were sacrificed and the peritoneal cavity lavaged with 5mM EDTA in PBS. The cells were strained through a 40 µm cell strainer to remove BIOgel particles. This process resulted in neutrophil preparations that were approximately 70-80% pure.

2.6.4. *In vivo* adoptive transfer model

In vitro generated neutrophils were labelled with CellTrace DDAO-succinimidyl ester (DDAO; Invitrogen) either alone or at the same time as Calcein Violet (Invitrogen).

DDAO-SE (Invitrogen) cell labelling

Cells were washed (350 x g, 5 minutes) twice with PBS then resuspended at 10⁷/ml in PBS. An equal amount of 1 µM DDAO was added to the cell suspension (working concentration 0.5 µM) and the cells left to incubate in the dark, at room temperature for 10 minutes. After incubation, the labelled cells were washed with POM four times and then suspended ready for use.

DDAO-SE and Calcein Violet Cell Labelling

Calcein violet dye was prepared by adding 42 μl of DMSO to a 25 μg vial of dye and mixing vigorously. From this stock solution 40 μl of dye was taken and added to 1.25 ml of PBS to produce a final stock solution as per company protocol. Cells were washed (350 x g, 5 minutes) twice with PBS then resuspended at 5×10^6 /ml in PBS. An equal amount of 1 μM DDAO (working concentration 0.5 μM) and 10 μl of the calcein violet stock solution was added to the cell suspension and the cells left to incubate in the dark, at room temperature for 30 minutes. After incubation, the labelled cells were washed with POM four times prior to use.

Adoptive Transfer

In vitro generated neutrophils from different genotypic backgrounds were labelled with different fluorescent dyes, mixed in a 1:1 ratio and resuspended in PBS (5×10^6 of each type per 100 μl). Adult C57BL/6 mice were injected i.v. (performed by Dr. P Taylor) with 100 μl of the mixed cells followed by an i.p. injection with 100 μl of 1mg/ml FITC zymosan (approximately 2×10^7 particles), or 1ml of 4% Thioglycollate Broth (Sigma), or appropriate volume of PBS control. After 3 hours, the mice were sacrificed and the peritoneal cells were recovered by lavage with 5 ml of 5 mM EDTA in PBS. Total cell counts were performed and differential analysis was performed by flow cytometry as described above using the CyAn ADP analyser and Summit software (both Beckman-Coulter).

Statistical Analysis of *In Vivo* Adoptive Transfers

For analysis of the behaviour of co-injected cell lines in the *in vivo* adoptive transfer model a two-tailed paired *t*-test was used and P values are displayed with the results. Analysis was conducted with GraphPad Prism (*= $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$).

2.7. Functional Assays

2.7.1. Measurement of intracellular calcium ion changes

Flow-Cytometric Assay

The calcium indicator dye Fluo-4 from the Fluo-4 NW Calcium Assay Kit (Invitrogen) was prepared according to the manufacturer's instructions. Fura red solution (Invitrogen) was added to the prepared Fluo-4 solution at a concentration of 1/500 (working concentration 1/1000). MyPH8-B6 derived neutrophils were centrifuged and resuspended in assay buffer (1x HBSS + 20nM HEPES, provided in the Fluo-4 NW Calcium Assay Kit) at 2.5×10^6 cells/ml then 0.5ml of calcium indicator solution was added to 0.5ml of cells (125,000 cells). The cells were incubated (37 °C, 5% CO₂), with the calcium indicator solution, for 30 minutes, to allow uptake of calcium indicator dyes into the cell, then placed in a 5 ml flow cytometry collection tube. Stimulants, zymosan (working concentration of 100 µg/ml) or ionomycin (working concentration of 1ug/ml), were diluted in 1 ml assay buffer to give 2x the working concentration; this was mixed with 1ml of cells and immediately analysed via flow cytometry.

Live Imaging Ca²⁺-Flux and Phagocytosis Assay

MyPH8-B6 derived neutrophils were centrifuged and 10^7 cells were resuspended in 1 ml of differentiation medium. Fura2AM (1µl) (InvitroGen) was added to the neutrophils, which were then mixed and incubated for 30 minutes (37 °C, 5% CO₂). A few drops of cell suspension were added to a glass coverslip on a heated stage (37 °C) and the neutrophils allowed to adhere to the glass. Zymosan particles were added to the coverslip and allowed to sediment among the cells. A micropipette was used to collect a zymosan particle, via a slight negative pressure, and the particle brought lightly into contact with an adhered

neutrophil. Once adherence between the cell and particle had been visually confirmed by gentle movement of the micropipette, which would disrupt the neutrophil shape once adherence was complete, the particle was released from the micropipette by removing the negative pressure and the progression of phagocytosis was monitored. Zymosan phagocytosis was observed using a Nikon Eclipse inverted microscope (Nikon) and recorded on video tape by a red sensitive camera (Watec, Japan). The cells were viewed under a 100X objective, and the excitation wavelengths (340nm and 380nm) selected using a rapid monochromator (Delta RAM, PTI, Surbiton, UK), which was connected to the microscope. The images at each excitation wavelength were collected using an intensified CCD camera (IC100 PTI, Surbiton, UK) and the ratio image calculated using Image Master software (PTI, UK).

2.7.2. 3'-(-p-aminopheny) Fluorescein (APF) Assays

3'-(-p-aminopheny) fluorescein (APF) is a reactive oxygen species indicator that is not fluorescent until it reacts with the hydroxyl radical, peroxy nitrite anion or the hypochlorite anion. To load the cells with this indicator, the cells were incubated in POM containing APF (5 μ M) at 37 °C for 30 minutes. After loading the cells with APF, 40 μ l of stimulant diluted in reaction medium (POM), was added to each well and the plate was placed in a 37 °C water bath for 15 minutes (except for when a timecourse assay was performed – see below). Cells were typically stimulated with curdlan microparticles (100 μ g/ml), zymosan (100 μ g/ml; this corresponds to approximately a 20 particle per leukocyte ratio based on the assumption of 6,000 leukocytes per μ l of whole blood), mouse serum opsonised zymosan (100 μ g/ml), live *Candida albicans* SC5314 (1:20, cell:*Candida* ratio, based on the above-mentioned

assumption) and mouse serum opsonised live *Candida albicans* SC5314 (1:20, cell:*Candida* ratio).

Timecourse Assay

MyPH8-B6 derived neutrophils were washed in reaction medium (POM), counted and resuspended at 10^6 /ml in reaction medium containing APF (5 μ M). After APF loading, the cells were placed in a 37 °C water bath until needed, then a 1 ml aliquot of cells was put in a FACS tube. The typical amount of stimulant, as described above, was added and mixed by pipetting up and down, then the tube was placed immediately on the flow cytometer and acquisition begun. The cells were collected at a constant rate of approximately 100 events per second for 15 minutes.

MyPH8-B6 Derived Neutrophil Assay

MyPH8-B6 cells were differentiated over 4 days in differentiation medium. The cells were washed, counted and then resuspended at 10^5 cells per 60 μ l of reaction medium containing APF (5 μ M) and placed in a v-bottomed 96 well plate (10^5 cells per well). After loading with APF as described above, the cells were then stimulated. During these incubations the plate was covered with a sterile plate cover. The cells were then immediately put on ice and 100 μ l of '2X staining solution' containing both 7/4-Alexa647 (125 ng/ml), diluted in reaction medium, and left on ice in the dark for at least 20 minutes. The cells were then analysed by flow cytometry.

Inflammatory Cells Assay

Mice were injected i.p. with 1 ml 2% (w/v) BIOgel and 16-18 hours later a peritoneal lavage was performed using 5 ml of reaction medium (see above) and the cells immediately placed on ice. The cells were then filtered using a 40 μ m cell strainer to remove BIOgel particles

and then the cells were pooled. The cells were washed 3 times with reaction medium (350 x g, 5 minutes, 4 °C) and were suspended at 10^5 cells per 60 μ l of reaction medium containing APF (5 μ M) and placed in a v-bottomed 96 well plate (10^5 cells per well). After loading with APF as described above, the cells were then stimulated. During these incubations the plate was covered with a sterile plate cover. The cells were then immediately put on ice and 100 μ l of '2X staining solution' containing both ly-6G-PE (0.5 μ g/ml) and 7/4-Alexa647 (125 ng/ml) diluted in reaction medium, and left on ice in the dark for at least 20 minutes. The cells were then analysed by flow cytometry as described above.

When using anti-dectin-2 (D2.11E4 (Taylor, Reid et al. 2005)) or its rat IgG2a control (AbD Serotec) in blocking studies, the antibodies were included at 10ug/ml in the APF preincubation step and the assays were otherwise conducted as described above.

Whole Blood Assay

Blood was collected in heparin (5 μ l/ml of blood; working concentration of 50 U/ml; Sigma) or in 0.8% (w/v) sodium citrate (1 ml/ml of blood). Blood (20 μ l) was added to 100 μ l of reaction medium (OptiMem, 10% heat inactivated FCS, 1% penicillin/streptomycin, 1% L-glutamine and 30 μ M β -mercaptoethanol) in a v-bottomed 96 well plate (Greiner Bio-one). The blood was washed 3 times with 100 μ l of reaction medium (350 x g, 5 minutes, room temperature). Reaction medium (60 μ l) containing APF (5 μ M) was added to the cells, the plate covered with a sterile plate cover and the cells left at 37 °C, in a 5% CO₂ incubator, for 30 minutes. Stimulant diluted in reaction medium (40 μ l) was then added to the blood and the plate covered with a sterile plate cover. The plate was placed in a 37 °C water bath for 15 minutes. The cells were then immediately put on ice and 100 μ l of '2X staining solution' containing both ly-6G-PE (0.5 μ g/ml- diluted in reaction medium) and Draq5 (10 μ M, Bio

Status Limited) and left on ice in the dark for at least 20 minutes. The cells were then analysed directly by flow cytometry and without prior cell washing to prevent the effects of centrifugation on particle:cell interactions. Slower flow rates on flow cytometric acquisition ensure reduced background fluorescence from the unbound and unwashed antibody present during acquisition as this is diluted by the sheath fluid.

2.8. Protocols for Human Neutrophil Collection and Functional Assays

2.8.1. Collection of Salivary Neutrophils

Sterile PBS (5 x 10 ml) was used to thoroughly rinse the mouth of the donor. The resulting PBS was then spat out into a 50 ml falcon tube and the cells filtered using a 40 µm filter to remove any debris from the mouth and produce a single cell suspension. The presence of neutrophils was confirmed via cytopsin preparations and the cells inserted into the appropriate assay. Blocking receptors with antibodies were performed at 37°C for 30 minutes prior to stimulation of the cells. In the case of the APF assays, blocking was performed in conjunction with the APF loading stage. Human neutrophils were identified by their expression of CD66.

2.8.2. Collection of Human Blood Neutrophils

Blood was collected (10 ml) in a 50 ml syringe containing an equal amount of sterile PBS with 0.8% (w/v) sodium citrate (an alternative anti-coagulant to heparin (Bournazos, Rennie et al. 2008)). The cells were then lysed with ACK lysis buffer (0.15M NH₄Cl, 10mM KHCO₃ and 0.1mM EDTA, in ddH₂O, pH 7.2-7.4) counted and inserted into appropriate assay. Blocking receptors with antibodies were performed at 37°C 30 minutes prior to stimulation

of the cells. In the case of the APF assays, blocking was performed in conjunction with the APF loading stage. Human neutrophils were identified by CD66 expression.

2.8.3. Collection of Inflammatory Neutrophils via the Skin Window Technique

Inflammatory neutrophils were collected via a skin window as previously described (Marks, Radulovic et al. 2007). The forearm of the donor was cleaned with medical sterilising alcohol wipes then gently sanded down to the capillary bed (visible capillaries but prior to bleeding) with grade C sandpaper. A sterile piece of cellulose filter paper, that had been pre-soaked in sterile PBS, was placed over the wound and the arm wound wrapped in parafilm to prevent drying of the filter paper. The arm was then securely wrapped in a bandage to keep the filter paper in place. 18-24 hours later, the wound was unwrapped and the filter paper placed in a 50 ml falcon tube containing 10 ml sterile PBS and agitated (the tube was placed on a rotator and allowed to rotate for at least 5 minutes) to release the cells. The filter paper was discarded and the PBS filtered using a 40 µm filter. The presence of neutrophils was confirmed via cytopsin preparations and the cells used in the appropriate assay. Blocking receptors with antibodies were performed at 37°C 30 minutes prior to stimulation of the cells. In the case of the APF assays, blocking was performed in conjunction with the APF loading stage. Human neutrophils were identified using CD66 staining.

2.9 Statistical Tests

The T test was used when directly comparing two columns using only one parameter. This gives a p value that indicates significance. The Mann-Whitney U test is also used to compare two columns using only one parameter. This test was used in instances where the individual columns had large sample sizes, as it is less likely to indicate false significance generated by outliers. As with the T test, this gives a P value that indicates significance. The two way anova with Bonferri post-tests was used when comparing multiple columns using two or more parameters. It gives a p value for whether each parameter has an effect, a p value for whether the different parameters are interacting and individual p values comparing each column to every other column. All p values that were equal or less than 0.05 indicated significance. All analysis was conducted with GraphPad Prism (*=P<0.05; **=P<0.01; *** = P<0.001).

Chapter 3

Generation and Characterisation of

Conditionally-Immortalised Myeloid

Progenitor Cell Lines

3.1. Introduction

The use of animal models, whilst controversial, is an important tool in biomedical research. It enables the researcher to conduct experiments that would otherwise be ethically and morally unacceptable in humans and therefore increases the scope of research. However, in order to reduce animal suffering and loss of life, the guiding principles of animal based research are summarised in the three R's, which are outlined below:

1. **Replacement:** This refers to the use of non-animal models wherever possible.
2. **Reduction:** This refers to using methods that enable the researcher to obtain as much information from the fewest number of animals necessary.
3. **Refinement:** This refers to using methods that minimise and reduce animal suffering as well as increasing animal welfare.

The primary aims of this chapter were to characterise a model of *in vitro* neutrophil production for two reasons:

1. To simplify production of normal and genetically manipulated neutrophils to model innate immune responses.
2. To place the principles of the 3Rs at the forefront of the study of neutrophil biology.

A protocol for generating conditionally-immortalised myeloid progenitor cell lines has been developed by other researchers using retroviral infection of precursors with an oestrogen-dependent Hoxb8 fusion gene (Wang et al. 2006). In the presence of oestrogen and stem cell factor (SCF), the cells grow and remain as progenitors. Once oestrogen is removed the cells are then able to differentiate into myeloid cells, and when the lines were established in the presence of SCF, this is predominantly into neutrophils. These conditionally-

immortalised progenitor derived neutrophils were reportedly more mature than other cell line models, possibly due to the fact that the immortalisation factors are not maintained during differentiation, although to date no functional characterisation has been reported and details of differentiation potential are sparse. Hence, the objectives of this chapter were:

1. Reproduce the studies of Wang et al., generating conditionally-immortalised neutrophil precursors from mouse bone marrow.
2. Determine the differentiation potential of conditionally-immortalised neutrophil precursors to produce a pure population of neutrophils under a number of relevant *in vitro* conditions.
3. Assess the biological activity of the precursor-derived neutrophils in a series of functional assays relevant to neutrophil biology, using fungal recognition as a model.
4. Evaluate the usefulness of the precursor-derived neutrophils as a genetically tractable system for the study of neutrophil function *in vitro*.

3.2. Generation of Retrovirally Transduced Cell Lines from Mouse Bone Marrow

Femurs taken from mice were flushed and lineage marker negative stem cells enriched using the MACS murine lineage depletion kit (Miltenyi Biotec) (figure 3.1). The enriched lineage negative (lin^{-}) bone marrow (BM) stem cells (Figure 3.1.C) were expanded for 3 days in IL-3, IL-6 and SCF and then retrovirally transduced with viral supernatant containing a DNA construct encoding a Hoxb8:oestrogen receptor binding domain fusion protein and a puromycin resistance cassette (Figure 3.2) (Methods 2.5).

Forty-eight hours after transduction, the cells were cultured in the presence of oestrogen and those which had taken up the virus were selected with 1.5 $\mu\text{g}/\text{ml}$ of puromycin for 7-10 days. This led to the production of puromycin resistant proliferating cell lines, which after puromycin withdrawal, continued to proliferate in oestrogen containing media and hence were suitable for further characterisation.

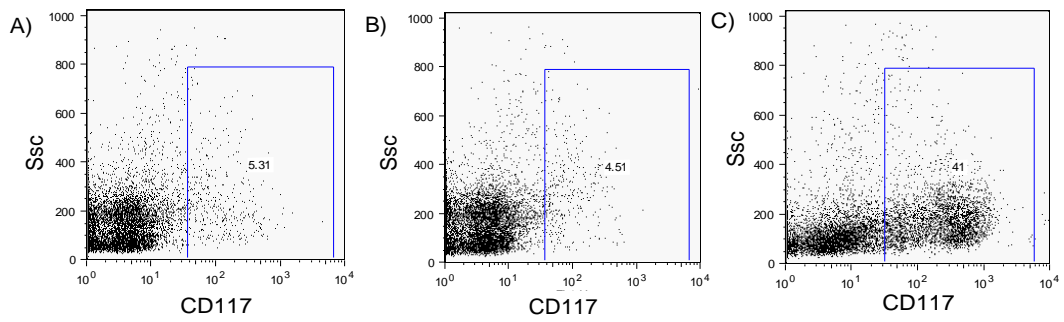


Figure 3.1: Flow cytometric analysis of lineage (lin) depletion of mouse BM cells: A) plot showing BM cells straight from the femur stained with CD117-APC ; B) plot showing BM cells that were removed by the MACS lineage depletion protocol; C) plot showing BM cells that have been depleted of lin⁺ cells and hence enriched in CD117⁺ cells. Numbers within the gate denote percentage of cells that are CD117⁺ and results are typical of the enrichments performed during this thesis.

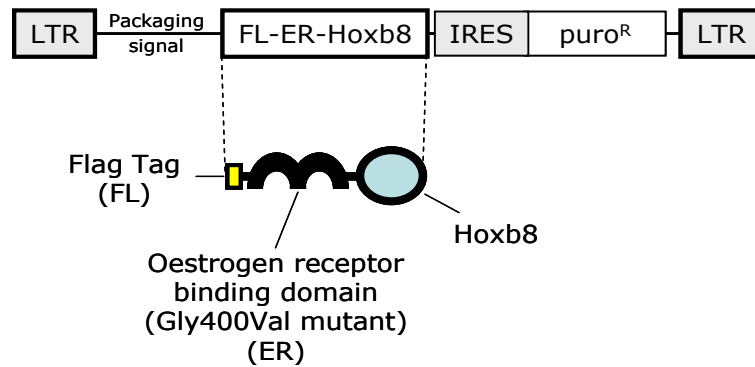


Figure 3.2: Viral DNA construct used to transduce lin^{-} BM cells for the production of a neutrophil progenitor cell line. The viral construct (top), expresses its transgene as part of a bicistronic transcript with puromycin resistance ($puro^R$) cassette driven from an internal ribosomal entry site (IRES). This enables expression of both coding sequences off of the single transcript. The transgene, a FLAG (FL)-tagged oestrogen receptor binding domain (ER)-Hoxb8 fusion gene, should hence be expressed in all cells which are puromycin resistant. The Gly400Val mutation reduces oestrogen binding, reducing background activation by ensuring that the gene is only activated in environments with artificially high concentrations of oestrogen which is used in this thesis. This vector has previously been used to make macrophage precursor cell lines (Rosas, Liddiard et al. 2008; Rosas, Osorio et al. 2010) and expression of the transgene was shown using an anti-FLAG antibody and western blotting (Rosas, unpublished). This vector was made by Dr. Phil Taylor and is described in detail elsewhere (Rosas, Liddiard et al. 2008).

3.3. Generation of Cell Lines from 129S6/SvEv Mice

Since *Clec7a*-deficient (*Clec7a*^{-/-}) mice were generated on the isogenic 129S6/SvEv genetic background (Taylor, Tsoni et al. 2007) and it was planned to use these animals to make cell lines and model the role of *Clec7a* in the innate immune responses of neutrophils (Chapter 4), initial attempts were made to generate neutrophil precursors from 129S6/SvEv mice. Attempts at generating this cell line using femurs from 129S6/SvEv mice were unsuccessful and resulted in the production of a cell line that was highly granular and ‘mast cell-like’ in appearance (figure 3.3. A). These cells expressed high levels of the mast cell markers CD117 (SCF Receptor) and FcεR1 (IgE Receptor 1) both in the presence and absence of oestrogen (figure 3.4) This supports the conclusion that the cells are of the mast cell lineage and that they do not differentiate after removal of oestrogen and are therefore not conditionally-immortalised. Similar results were obtained with 2 prior attempts made to generate these cells by other group members and empty vector controls produced similar results, indicating that this was a natural outgrowth of mast cell-like cells, which can be grown from BM with SCF, and not related to *Hoxb8* activity.

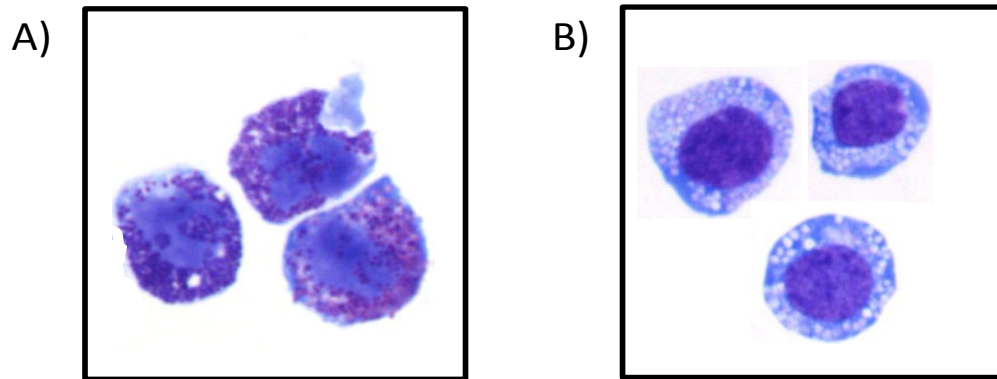


Figure 3.3: Cytospin preparations of lin^- cells transduced with viral construct containing *Hoxb8*. **A)** Typical appearance of ‘mast cell-like’ cell line produced using lin^- cells from 129S6/SvEv mice with viral DNA containing the oestrogen receptor binding domain-*Hoxb8* fusion gene and cultured in the presence of oestrogen and SCF. Note the heavy staining granules characteristic of mast cells. Similar results were obtained with an empty vector. Picture is representative of 3 individual experiments. **B)** MyPH8 cells derived from lin^- BM cells of C57BL/6 mice (MyPH8-B6), with a typical myeloid appearance of a large central nucleus and no visible granules. All cells were stained with eosin and methylene blue (Methods 2.4.4). Picture is representative of many experiments with >5 cell lines.

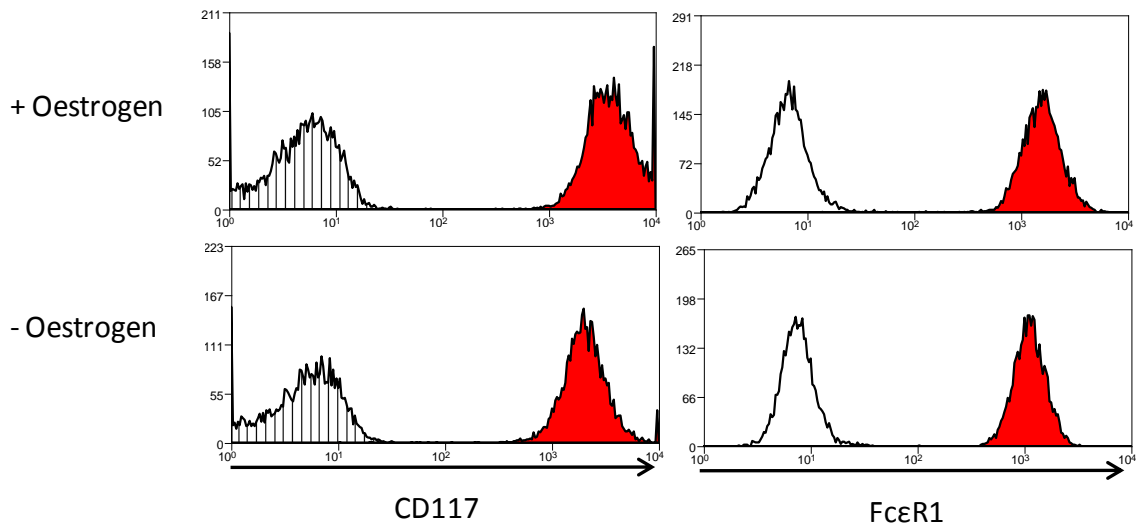


Figure 3.4: Cell surface expression of CD117 and FcεR1 on 'mast cell-like' progenitors. Histograms showing the cell surface expression, determined by flow-cytometry, of mast cell associated markers by the mast cell-like cell line produced from lin^- cells from 129S6/SvEv. Grown in the presence of oestrogen and SCF, the cells express high level of CD117 and FcεR1. On removal of oestrogen these cells still express high levels of both receptors. Data representative of 2 experiments, shaded histograms represent receptor-specific staining and bold lines denote isotype control staining.

3.4. Generation of Cell Lines from C57BL/6 and Balb/c mice

The procedure for the production of neutrophil precursors had been published as successful in relation to other mouse strains, specifically C57BL/6 and Balb/c (Wang, Calvo et al. 2006). Given the bias in 129 mice for the production of mast cells (Yamashita, Charles et al. 2007), subsequent attempts were made to generate cell lines from C57BL/6 and Balb/c mice. The Clec7a-deficient mice in Cardiff are also available backcrossed onto the C57BL/6 background (for 8 generations; >99.6% C57BL/6). Repeating the protocol with femurs from both C57BL/6 and Balb/c mice resulted in cells with a typical myeloid appearance of a large mostly circular or slightly irregular nucleus and largish cytoplasmic area and a distinct lack of the granules seen in cells derived from 129S6/SvEv mice (Figure 3.3.B). The cells were named Myeloid Progenitor Hoxb8 (MyPH8). BM cells, from these strains, that were transduced with an empty vector control did not continue to grow in the presence of oestrogen, due to a lack of Hoxb8 immortalisation and BM cells that were mock transduced did not continue to grow or survive puromycin selection.

3.5. Differentiation Studies on Myeloid Progenitor Cell Line

To verify whether the MyPH8 cell lines produced in these studies were, as published, a neutrophil progenitor cell line, differentiation studies using several different cytokine combinations (Table 3.1) were carried out on the C57BL/6 variant (MyPH8-B6). The cells were washed to remove oestrogen and resuspended in differentiation medium containing the appropriate cytokines. The cells were fed every two days and differentiation was followed for up to 5 days.

Best results at optimal time point			
Cytokines (20ng/ml)	Expansion (Fold increase)	Neutrophil purity (%)	Neutrophil total number
SCF	21.42	80.38	5,355,000
SCF + G-CSF	61.11	89.6	15,277,500
G-CSF	4.2	97.06	1,050,000
SCF + IL-5	100.8	23.52	25,200,000
IL-5	4.62	25.9	1,155,000
SCF + GM-CSF	408.24	57.5	102,060,000
GM-CSF	137.34	58.17	34,335,000

Table 3.1: Summary of differentiated MyPH8-B6 cells. Table showing the cytokine combinations used to differentiate MyPH8-B6 cells, the fold increase of cell counts, the percentage of cells that are neutrophils and the total neutrophil number. Cultures were started with 250,000 cells. “Best results at Optimal time point” refers to day number at which neutrophil purity is at its highest, cell numbers are at their highest and just before the culture begins to die. Data is derived from the experiments shown in Figure 3.7.

Differentiation of the cell line resulted in the generation of neutrophils as assessed by cytopsin preparations (Figure 3.5) and cell surface expression of murine neutrophil markers such as Ly-6B.2 (Figure 3.6). The cells were counted daily (Figure 3.7A) and neutrophil percentage determined using flow-cytometry (Figure 3.7B). Cells differentiated without SCF in the differentiation medium (with one exception) showed an immediate detrimental effect as a large proportion of these cells died (Figure 3.7A). These cells were able to recover from the large scale death; however, they produced much lower numbers of cells than those differentiated with SCF. However, this effect was not seen in cells differentiated with GM-CSF. In the cells differentiated with SCF in the medium there was a steady increase in cell number with the greatest increase seen in cells differentiated with SCF + GM-CSF.

Neutrophil purity was determined by looking at the percentage of cells that expressed Ly-6B.2. As shown in Figure 3.7B, cells differentiated with G-CSF in the medium produced the greatest neutrophil purity, with cells differentiated in G-CSF alone reaching a neutrophil percentage of over 97%; however, this was accompanied by extensive cell death and hence poor yields. Cells differentiated in SCF+G-CSF reached a percentage of approximately 90% purity, with substantially improved yields. Cells differentiated with GM-CSF in the medium, whilst providing high yields, only reached limited purity levels of approximately 50-60%. Cells differentiated in the presence of IL-5 only managed to produce purity levels of approximately 20-30%. Cells that were differentiated in medium containing only SCF managed to reach purity levels of over 80% (Figure 3.7B).

A further experiment looking at improving neutrophil purity, without a large death event, by growing in SCF+G-CSF for 2 days then removing the SCF and growing until day 5, prevented

the cell death, but showed no significant increase in neutrophil purity or yield compared to cells grown in SCF+GCSF for the full 5 days (data not shown).

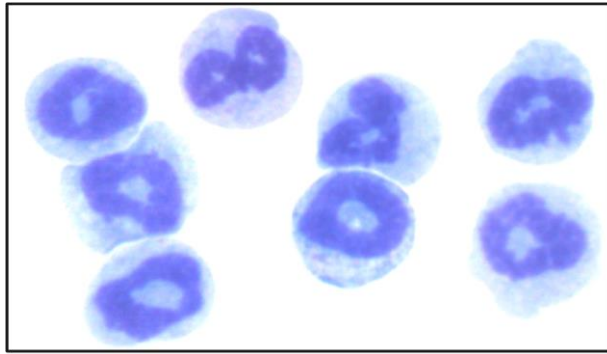


Figure 3.5: Cytospin preparation of differentiated MyPH8-B6 cells. Examples of cells that were differentiated in SCF+G-CSF for 4 days and then stained with eosin and methylene blue. The nuclear morphology is typical of mouse neutrophils. Selected examples are shown and are representative of many experiments with at least 5 different cell lines.

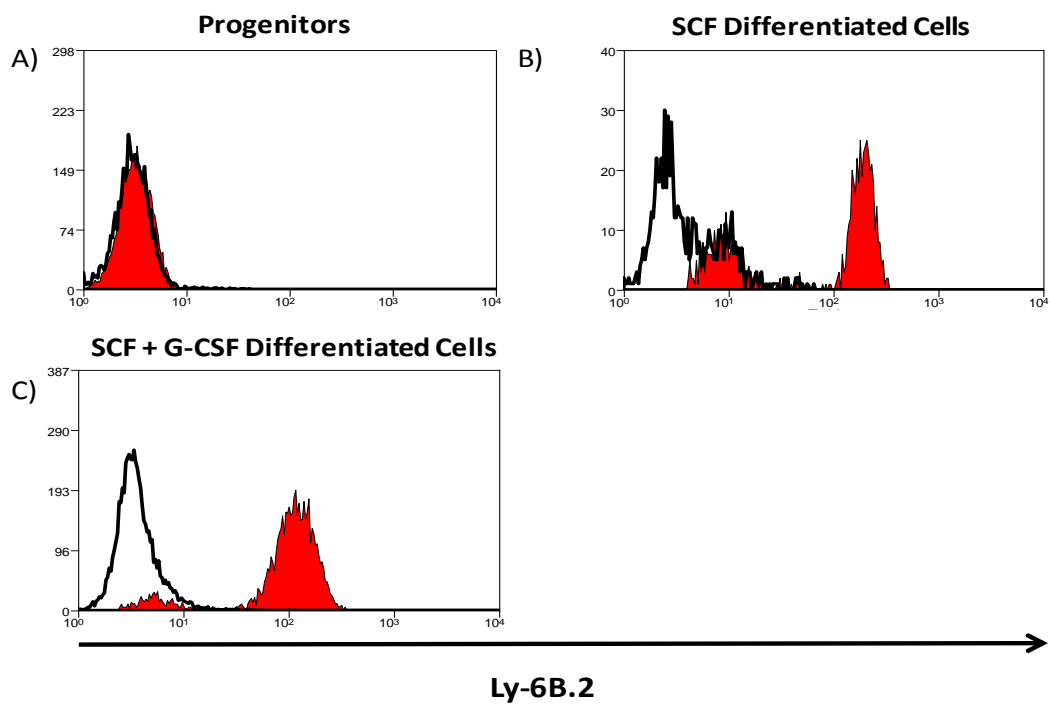


Figure 3.6: Cell surface expression of Ly-6B.2, a murine neutrophil marker. A) Undifferentiated progenitor cells grown in oestrogen and SCF do not show any Ly-6B.2 expression. B&C) The majority of cells differentiated in SCF (B) or SCF+G-CSF (C) for 5 days, show Ly-6B.2 expression. Red histogram shows cells labelled with Ly-6B.2 antibody, white histogram shows cells labelled with an isotype control antibody. Data is representative of 3 independent experiments.

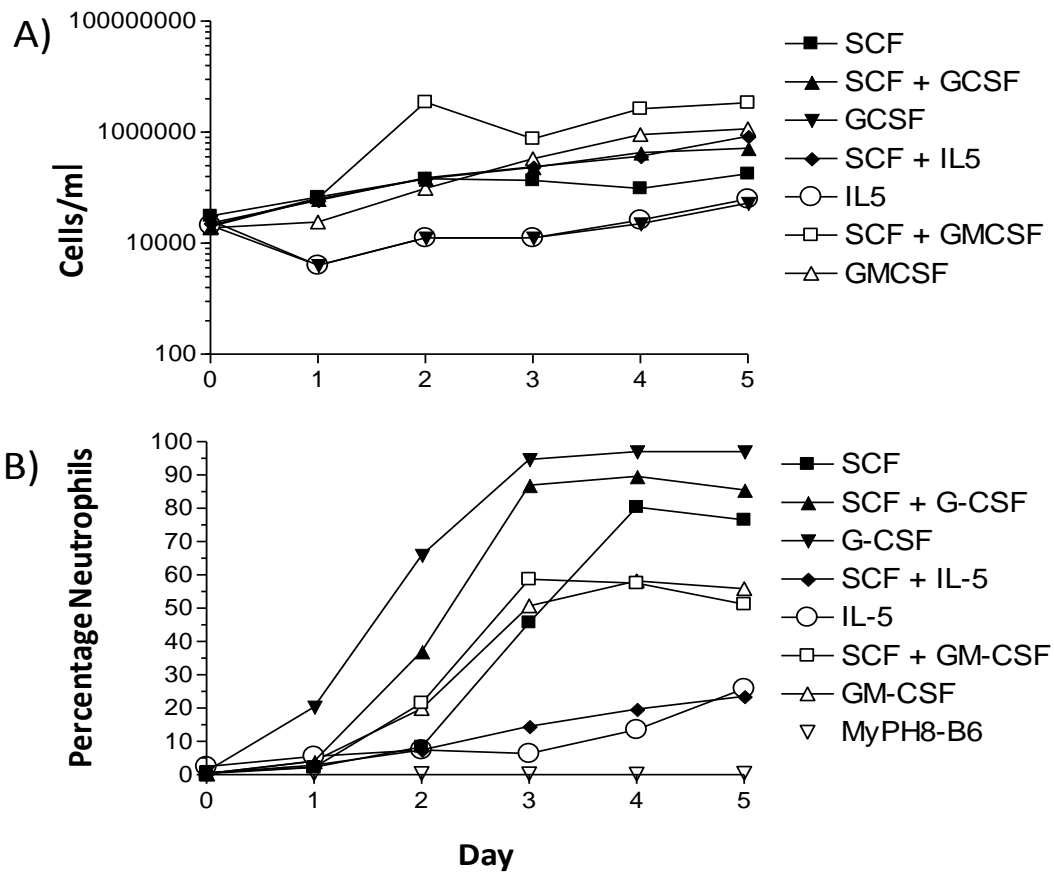


Figure 3.7: Differentiation of MyPH8-B6 cells over 5 days with 7 different cytokine combinations. A) The growth curve of MyPH8-B6 cells treated with different cytokines over 5 days. The fall in growth seen in day 3 of cells grown in SCF+GM-CSF is an artefact in this experiment due to overgrowth. B) Graph showing the percentage of cells in the culture that are Ly-6B.2⁺ neutrophils. The results from this preliminary experiment were used to inform future experiments.

Cell cycle (Figure 3.8 and 3.9) analysis was performed to determine whether the neutrophils derived from the conditionally-immortalised cell line were 'terminally-differentiated', which is characterised by cessation of cell division. The cells, differentiated as above, were stained with NIM-DAPI (Beckman-Coulter) and their nuclear DNA content was analysed on a 'Quanta SC MPL' flow cytometer, which has a mercury lamp for UV excitation of the DNA binding DAPI. After 5 days of differentiation, all cytokine combinations, except those containing IL-5, had noticeably decreased the proportion of their cells in S and G2/M phases, consistent with terminal-differentiation (Figure 3.8). IL-5 seems to slow down terminal-differentiation as supported by the low percentage of neutrophils and the higher percentage of cells in S and G2/M phases in comparison to all cytokine combinations. This could be due to the outgrowth of a percentage of cells (see Figure 3.7A), whose potential to differentiate later was not investigated.

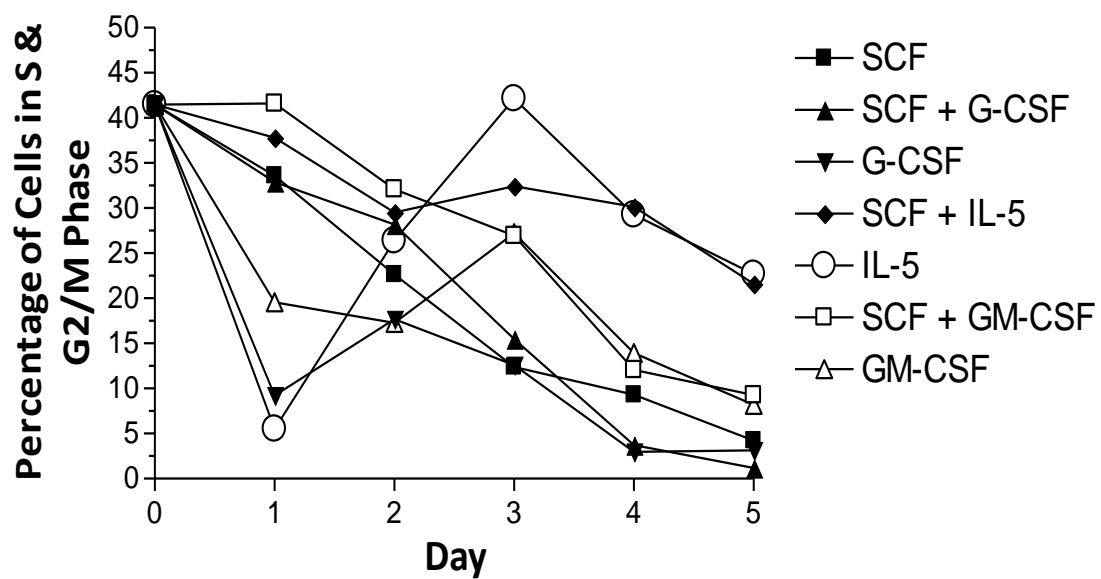


Figure 3.8: Percentage of differentiated cells in S and G2/M phases of the cell cycle over 5 days of differentiation. Data was compiled from the analyses shown in Figures 3.9B, C & D (below). The percentage of cells in the S and G2/M phase gradually decreases over time. This is an indicator of terminal differentiation, as cells stop dividing. Data is representative of 2 independent experiments.

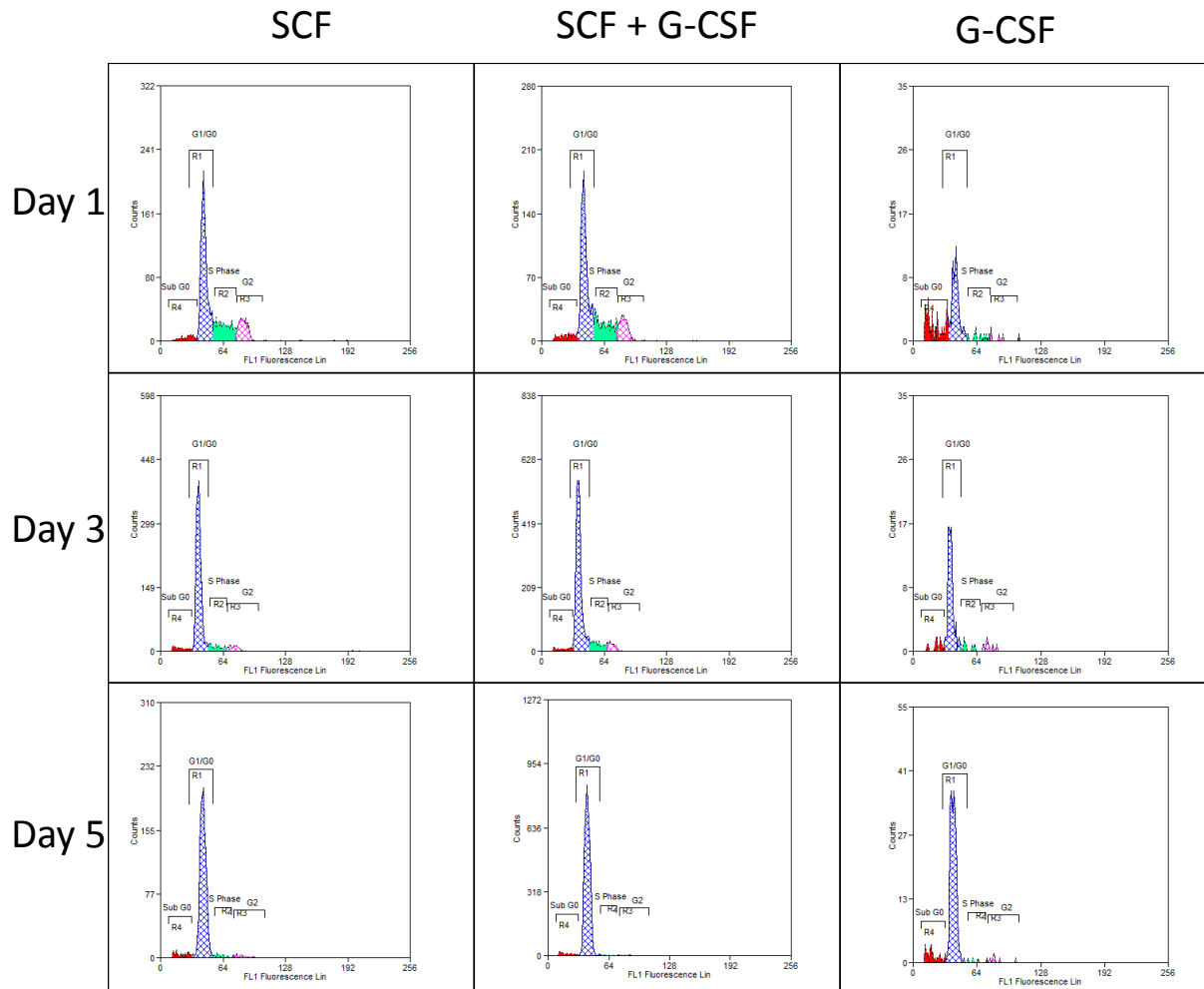


Figure 3.9A: Nuclear DNA analysis of SCF, SCF+ G-CSF and G-CSF differentiated cells. Nuclei were isolated from differentiated cells and analysed with a NIM-DAPI stain. Sub G0 cells refer to dead or dying cells; G0 are those that are resting and not in the process of division; G1 cells are in the cell cycle but it not possible to distinguish between G0 and G1 in this assay; S phase cells are preparing for division and are therefore increasing their DNA content; and G2/M cells have double the DNA content of G0/G1 cells and are ready to divide. Data are representative of 2 independent experiments.

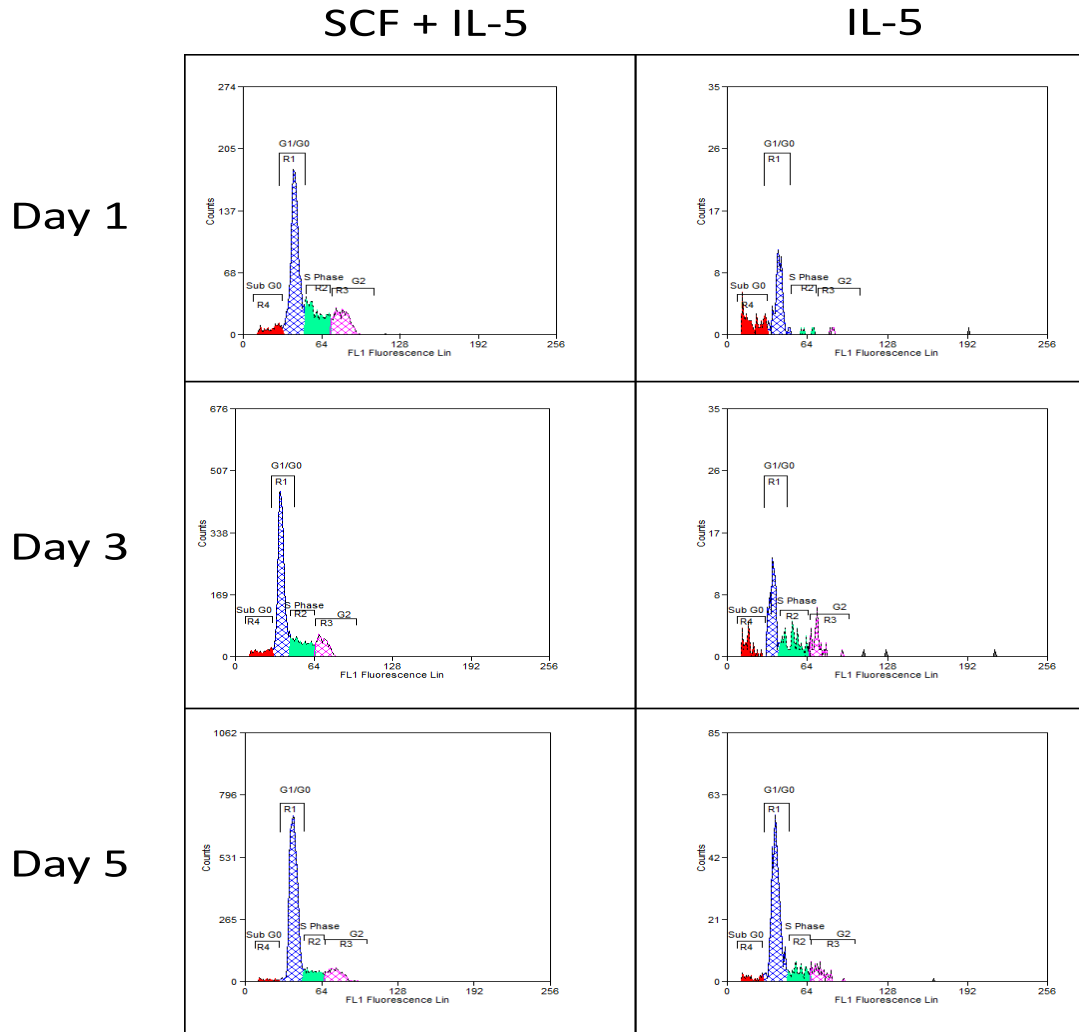


Figure 3.9B: Nuclear DNA analysis of SCF + IL-5 and IL-5 differentiated cells. Differentiated cells had their nuclei isolated and analysed with a NIM-DAPI stain. Sub G0 cells refer to dead or dying cells; G0 are those that are resting and not in the process of division; G1 cells are in the cell cycle but it not possible to distinguish between G0 and G1 in this assay; S phase cells are preparing for division and are therefore increasing their DNA content; and G2/M cells have double the DNA content of G0/G1 cells and are ready to divide. Data are representative of 2 independent experiments.

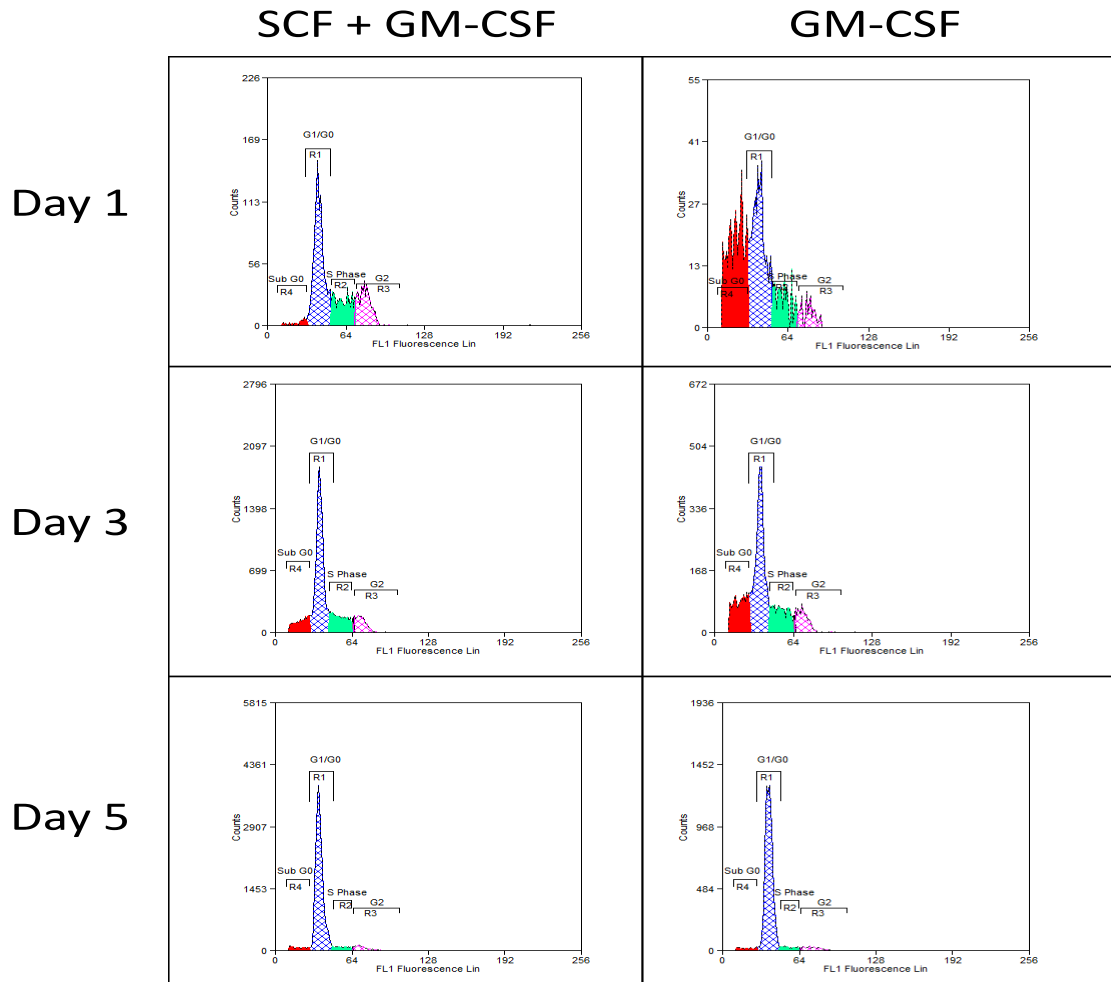


Figure 3.9.C: Nuclear DNA analysis of SCF + GM-CSF and GM-CSF differentiated cells. Differentiated cells had their nuclei isolated and analysed with a NIM-DAPI stain. Sub G0 cells refer to dead or dying cells; G0 are those that are resting and not in the process of division; G1 cells are in the cell cycle but it not possible to distinguish between G0 and G1 in this assay; S phase cells are preparing for division and are therefore increasing their DNA content; and G2/M cells have double the DNA content of G0/G1 cells and are ready to divide. Data are representative of 2 independent experiments.

After analysis of the different cytokine combinations and their effectiveness at generating neutrophils, the use of G-CSF or IL-5 without SCF was discontinued as inefficient due to the large scale death event on day 1 resulting in low cell yields. SCF + IL-5, SCF + GM-CSF and GM-CSF alone were also discontinued as they produced poor neutrophil purity, regardless of the very large yields, as they would require a further purification step to produce cells that are pure enough for most functional assays. Cells differentiated in SCF alone and SCF+G-CSF produced reasonable yields of at least 80% purity and these were selected for further study. These cells were differentiated over 5 days and labelled with different antibodies to look at neutrophil generation and maturity (Figure 3.10 and 3.11).

As expected, MyPH8-B6 progenitors all express CD117 (stem cell factor receptor, SCFR) (Figure 3.10). However, differentiation with SCF or SCF+G-CSF resulted in two populations of cells, one that does not express CD117 and a smaller subset that does. Cells that do not express CD117 are Ly-6B.2⁺ consistent with their differentiation into neutrophils. The second subset of cells that are CD117⁺ are also Ly-6B.2⁻, which is indicative of a small residual population of undifferentiated progenitor cells. This correlates with microscopic analysis of cytopsin preparations. As well as looking at CD117 expression a range of other antibodies were also looked at to assess normal neutrophil expression and maturation (Figure 3.11). Residual progenitor populations in the differentiated cell preparations were screened out by gating on Ly-6B.2⁺ cells. Differentiated neutrophils show clear positive expression of neutrophil markers such as Ly-6G and Gr-1 (which binds to both Ly-6G and Ly-6C), with no distinct difference between cells differentiated in SCF alone or SCF+G-CSF (Figure 3.11A). The differentiated cells were also positive for CD11b, which is a subunit of CR3, CD24, which is known to mediate myeloid cell binding to P-selectin (Aigner, Ruppert et

al. 1995) and CD80 a co-stimulatory molecule known to be involved in regulation of T cell activation (Mencacci, Montagnoli et al. 2002) (Figure 3.11B) with no differences between the two cytokine combinations (Figure 3.11A). The expression of CD88 (the C5a receptor) Clec7a (the β -glucan receptor) and CXCR2 (receptor for the chemokine macrophage inflammatory protein 2-alpha (MIP-2 α /CXCL2)) was seen to be different with the two cytokine combinations. Cells differentiated in SCF alone showed no, or only minimal, surface expression of the above markers, however those differentiated in SCF+G-CSF showed some low surface expression of the same markers. CD88 expression on these SCF+G-CSF differentiated neutrophils has since been repeated and shown to be greater than seen here (Karsten, Pandey et al. 2012).

3.5. DNA/RNA Profiling

To provide further evidence of terminal differentiation, cells differentiated in SCF+G-CSF for 4 days were stained with NIM-DAPI, to which had been added *Syto* RNASelectTM (Methods 2.5.8). The cells were then analysed in the flow cytometer (Figure 3.12). The nuclei of differentiated cells contained many more cells with a DNA content consistent with the G0/G1 stage of cell cycle than undifferentiated cells as shown previously, but they also contained much less RNA. This reduction of nuclear RNA is consistent with that seen in terminally-differentiated and quiescent cells (Figure 3.12).

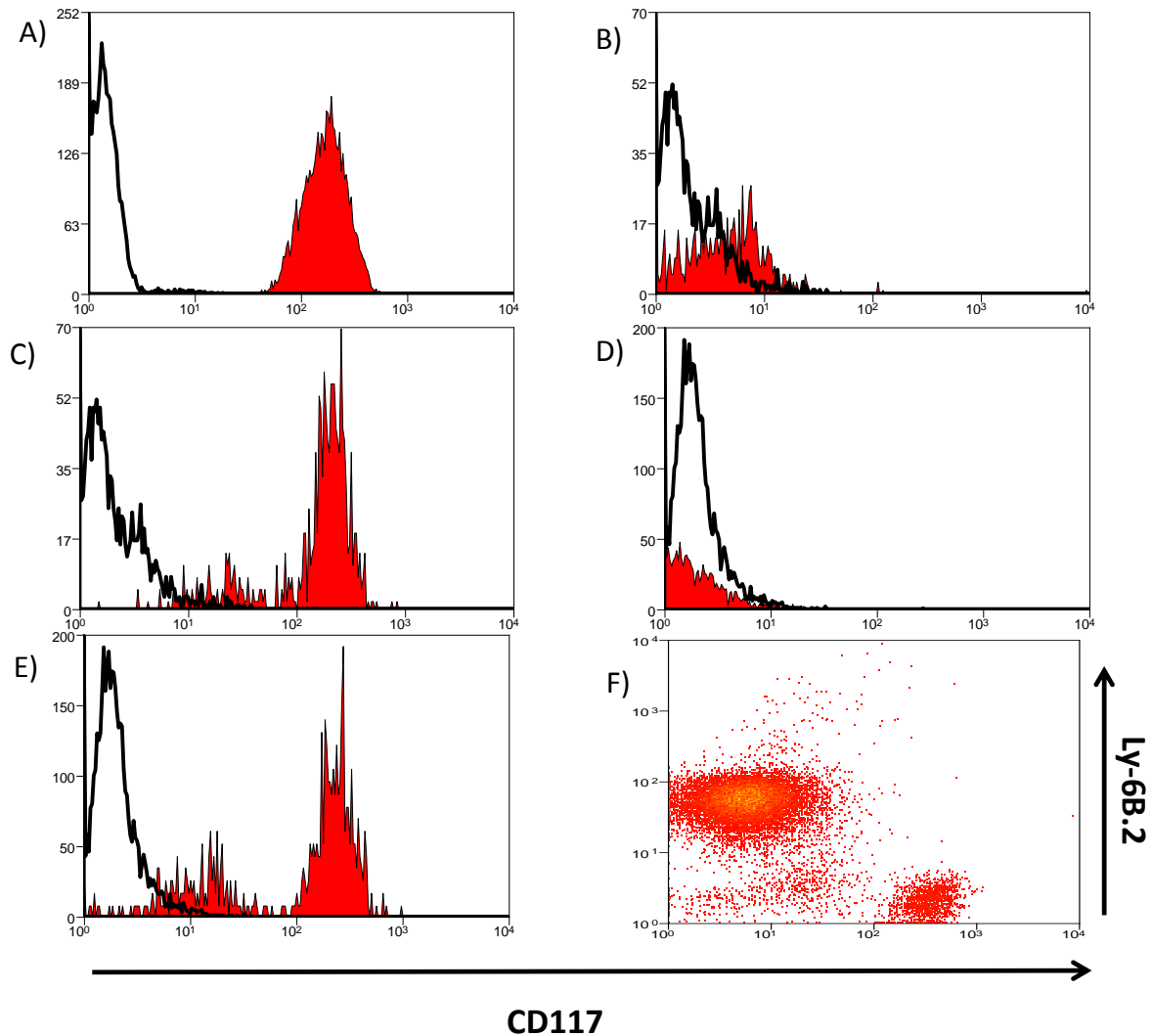


Figure 3.10: Expression of CD117 (SCFR). A) Progenitor cells show high CD117 expression as expected. B) Ly-6B.2⁺ neutrophils differentiated in SCF alone show greatly reduced CD117 expression, however C) a small population of Ly-6B.2⁻ cells show CD117 expression levels comparable to undifferentiated progenitors. This population is also present in cells differentiated in SCF+G-CSF (E). D) Ly-6B.2⁺ neutrophils differentiated in SCF+G-CSF show almost no expression of CD117. F) Dot plot of SCF+G-CSF differentiated cells showing the two distinct CD117⁺ and Ly-6B.2⁺ populations. Red histogram shows cells labelled with Ly-6B.2 antibody, white histogram shows cells labelled with an isotype control antibody. Data is representative of at least 3 independent experiments.

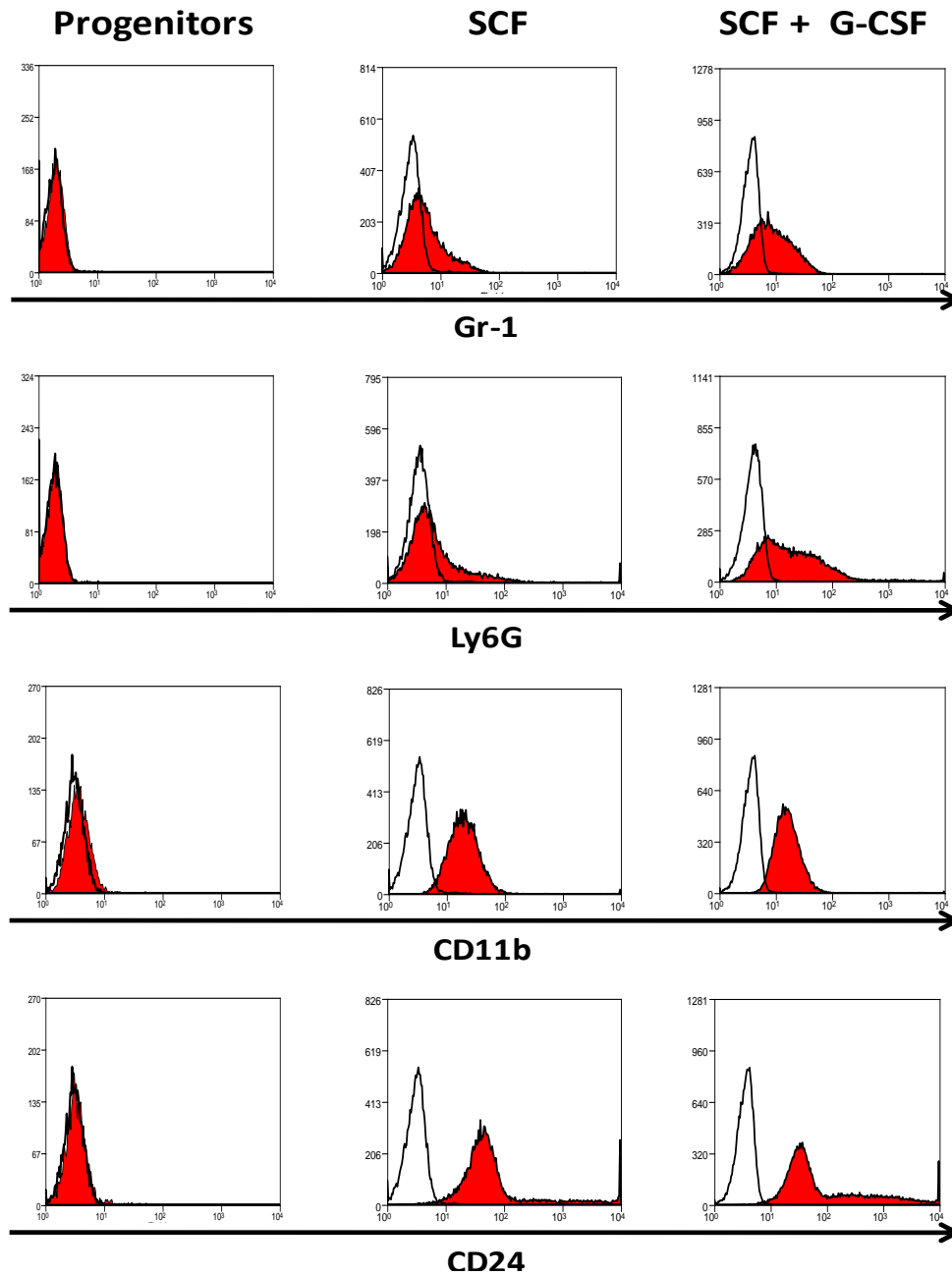


Figure 3.11A: Expression profile of MyPH8-B6 progenitors, cells differentiated in SCF and cells differentiated in SCF+G-CSF. Data for individual histograms is representative of at least 3 independent experiments and the plots of *in vitro*-differentiated cells are pre-gated on Ly-6B.2⁺ cells to restrict analysis to neutrophils. Data is representative of at least 2 individual experiments.

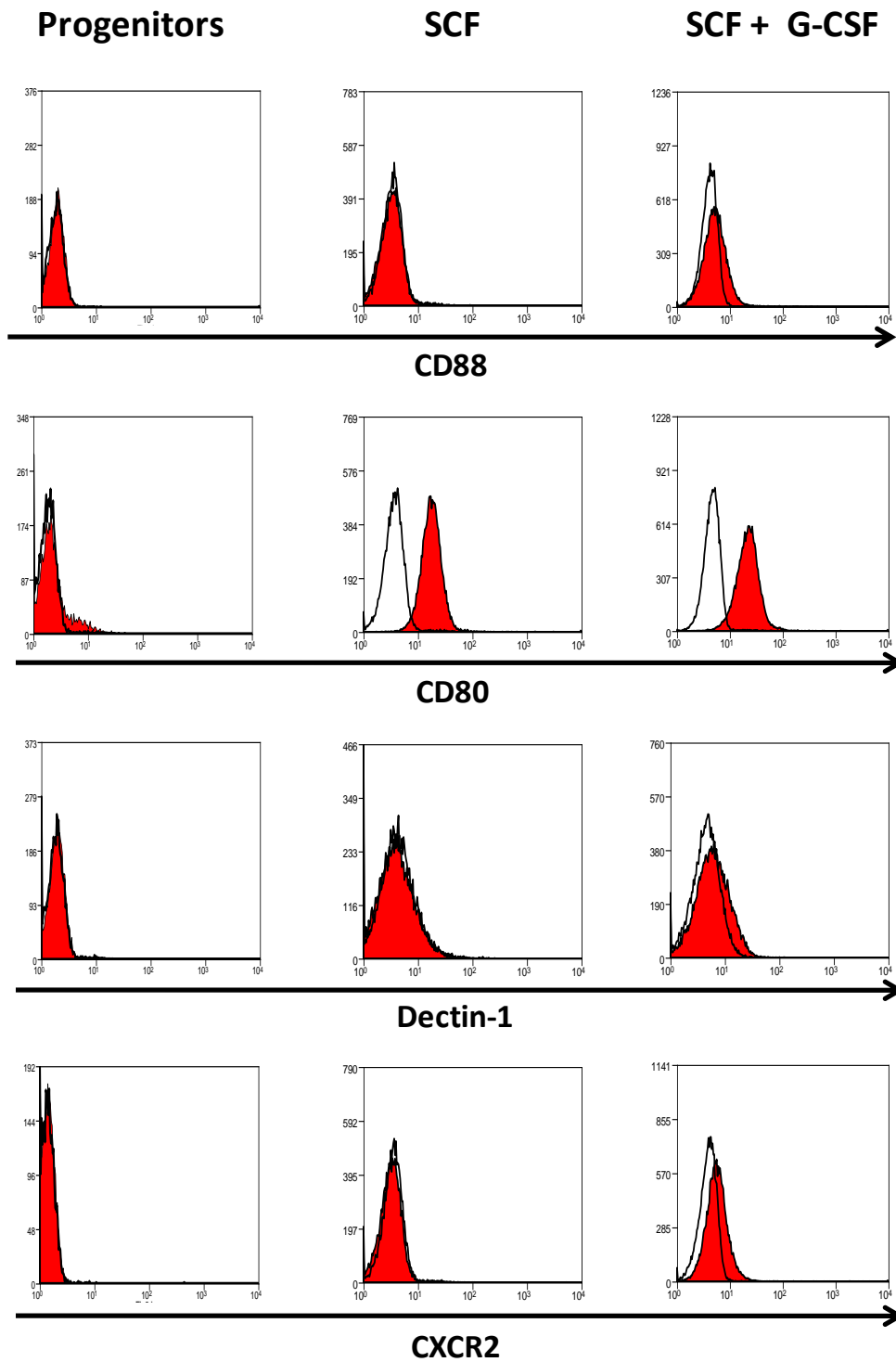


Figure 3.11B: Expression profile of MyPH8-B6 progenitors, cells differentiated in SCF and cells differentiated in SCF+G-CSF. Data for individual histograms is representative of at least 3 independent experiments and the plots of *in vitro*-differentiated cells are pre-gated on Ly-6B.2⁺ cells to restrict analysis to neutrophils. Data is representative of at least 2 individual experiments.

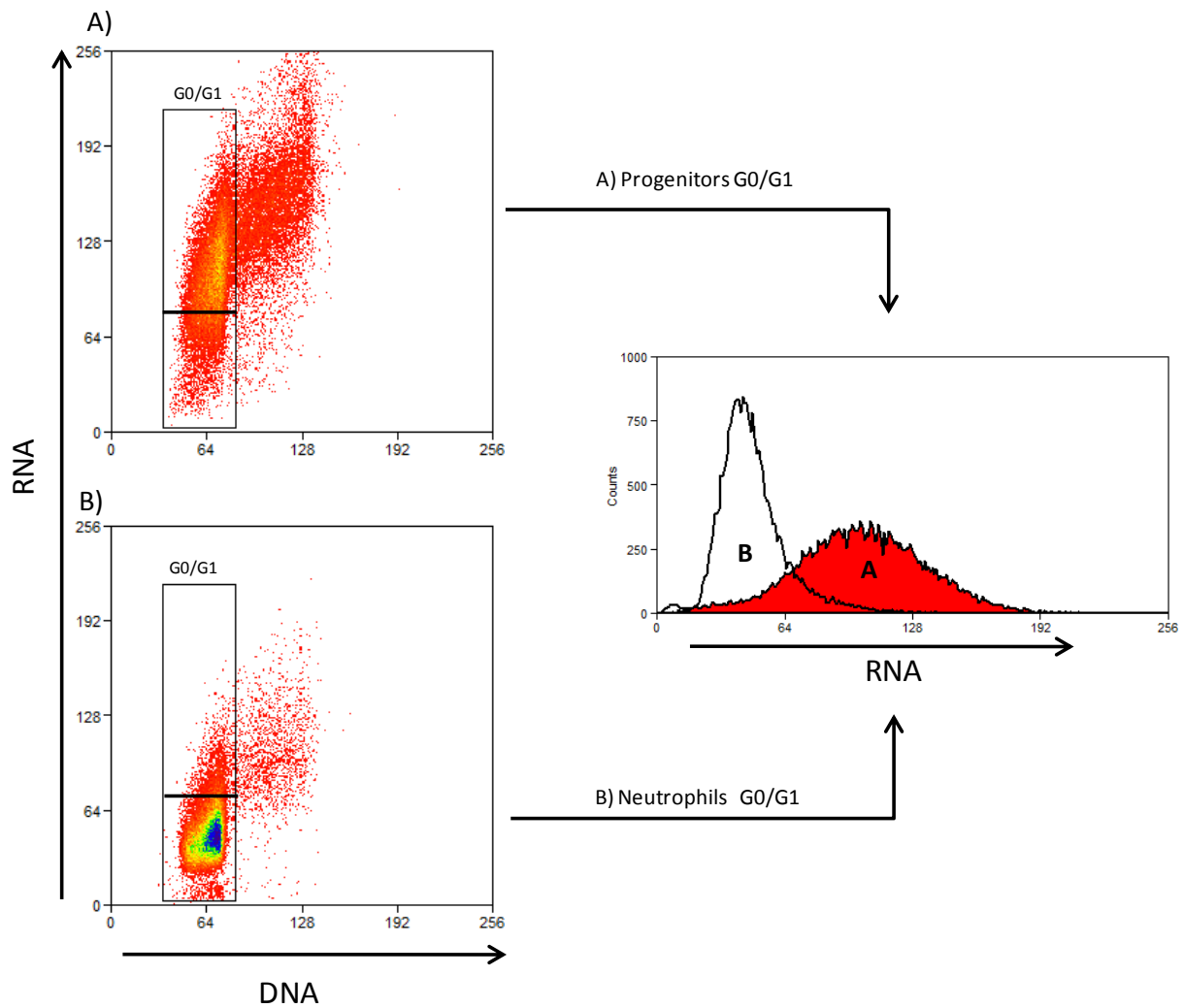


Figure 3.12: DNA/RNA Profile of MyPH8-B6 derived cells. A) Progenitor cells show a larger proportion of cells in with greater quantities of DNA and RNA which is an indication of cells that are still dividing. B) Neutrophils show less DNA and RNA than the progenitors. The plots in (A) and (B) are gated based on DNA content to identify the cells in G0/G1 and these events are shown in a histogram of RNA content (left panel). The arbitrary horizontal line within the gates used highlights the difference in RNA content of cells within these gates and is consistent with quiescence of the differentiated neutrophils. Data is representative of 2 individual experiments.

3.6. Purification of Viable MyPH8-B6-derived Neutrophils

During the differentiation process, cell death is common due to the short lived nature of neutrophils and occasional sub-optimal cell culture conditions (such as exhaustion of cytokine/growth factor). There is also a residual population of progenitors, possibly still dividing, after differentiating for 4 days. Peak neutrophil purity was achieved after 4 days in the presence of G-CSF and peak yields achieved when SCF was also present. To consistently achieve high purities of live neutrophils differentiated in SCF+G-CSF for 4 days, a protocol for dead cell removal, using a Miltenyi Biotec Dead Cell Removal Kit, was combined with a progenitor depletion step. To achieve this, anti-CD117 labelled with biotin was also added to the cells to aid in the removal of residual progenitors at the same time as the dead cell removal and the cells magnetically separated (Figure 3.13). Anti-CD117 negative selection was decided on as opposed to Ly-6B.2 positive selection because anti-CD117 does not bind to differentiated neutrophils but Ly-6B.2 does. Ly-6B.2 binding could lead to premature neutrophil activation, which could render the cells invalid for certain assays. In the extreme example shown for illustration purposes (Figure 3.13), the percentage of dead cells was significantly reduced from approximately 40% to less than 10% after magnetic separation. The percentage of neutrophils, as determined via Ly6B.2 expression and flow cytometric analysis, was further enriched from 90% to over 95% (Figure 3.13). Although this system was successful in the removal of dead cells and in improving neutrophil purity, it was never used for assays, as optimal cell culture conditions, where the cells were frequently fed and not allowed to overgrow or exhaust the growth medium, were subsequently established and purities of >90% and viabilities of >80% were routinely achieved.

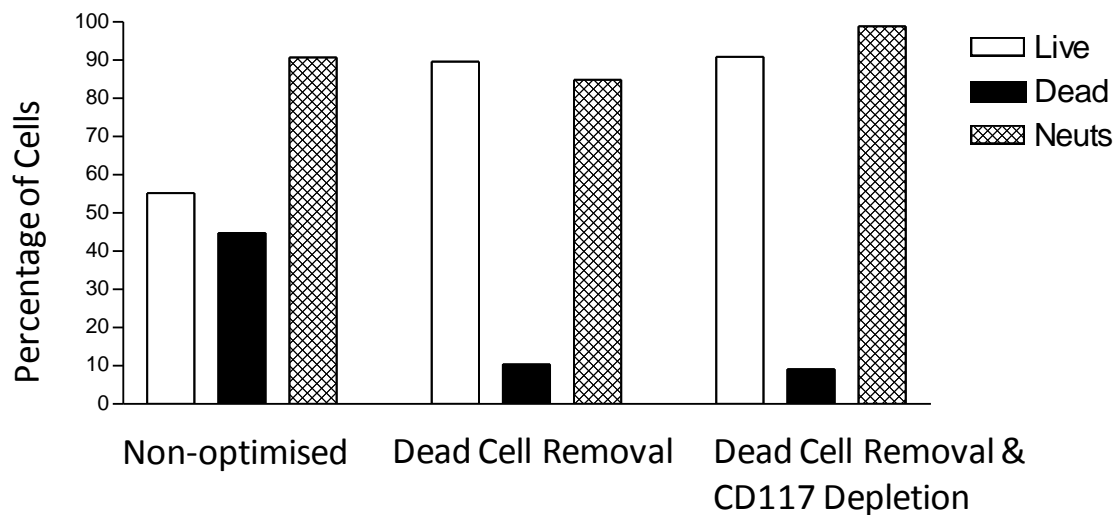


Figure 3.13: Percentage of cells that are either live or dead (determined by trypan blue exclusion) and neutrophils (Neuts) (determined by Ly-6B.2 staining) before and after purification with the dead cell removal kit and anti-CD117 depletion. Cells that were not purified from suboptimal culture conditions (non-optimised) were 40% dead with 90% of the cells being neutrophils. After purification and anti-CD117 depletion, only 10% of the cells were dead with over 95% of the cells being neutrophils. Typically, with good culture conditions >90% neutrophil purity and >80% viability were achieved without the need for dead cell and progenitor removal. Data representative of 2 independent experiments.

3.7. Recognition and Response of *in vitro* Generated Neutrophils to the Yeast

Particle Zymosan

A preliminary experiment in which MyPH8-B6 derived neutrophils were stimulated with either serum-opsonised or non-opsonised, fluorescently labelled, zymosan particles at 37°C, or 4°C was performed. The cells and zymosan particles were either centrifuged at 350 x *g* for 5 minutes to form a pellet and to some extent force an interaction or left in suspension for 5 minutes prior to the assay. After 30 minutes at the appropriate temperature, the interaction was analysed using flow-cytometry. MyPH8-B6-derived neutrophils were able to bind both opsonised and non-opsonised zymosan particles, but the extent to which the cells were able to bind was determined by the temperature and whether the particles were opsonised or not (Figure 3.14 and 3.15)

When the interaction was forced by centrifugation, there was greater zymosan binding than when left in suspension. At both temperatures, interactions with zymosan were much greater when the particles were opsonised as opposed to non-opsonised particles (Figure 3.14). The percentage binding seen in the suspension assay is poor compared to that which was observed in later experiments, which is probably due to poor opsonisation of the zymosan, as a result of the serum used for opsonisation being very old with diminished complement activity. Subsequent assays requiring serum opsonisation used fresh serum or serum that had been stored in aliquots at -80°C for a maximum of one week.

In order to determine the rate at which neutrophils can bind and respond to zymosan, Alexa Fluor 405 (A⁴⁰⁵) labelled zymosan particles were added to a suspension of MyPH8-B6 derived neutrophils that had been preloaded with 3'-(*p*-aminophenyl) fluorescein (APF) (methods Table 2.1 and methods 2.12) and immediately acquired on a flow cytometer until

a stable plateau in the association was reached. The cells were kept at 37°C immediately prior to addition of zymosan and acquisition. Opsonised zymosan was able to bind to approximately 60% of cells within the 30 seconds, after which point, the binding plateau was reached (Figure 3.16A, left). Non-opsonised zymosan remained at baseline level for the full length of collection (Figure 3.16A, right). Production of ROS, as measured by APF fluorescence, for cells that had bound zymosan became evident after about 1 minute 20 seconds (Figure 3.16B), approximately 1 minute after the initial association was evident. The speed of both binding and response was unexpectedly fast and due to this speed all subsequent binding and APF experiments were only 15 minutes long (Figure 3.16C).

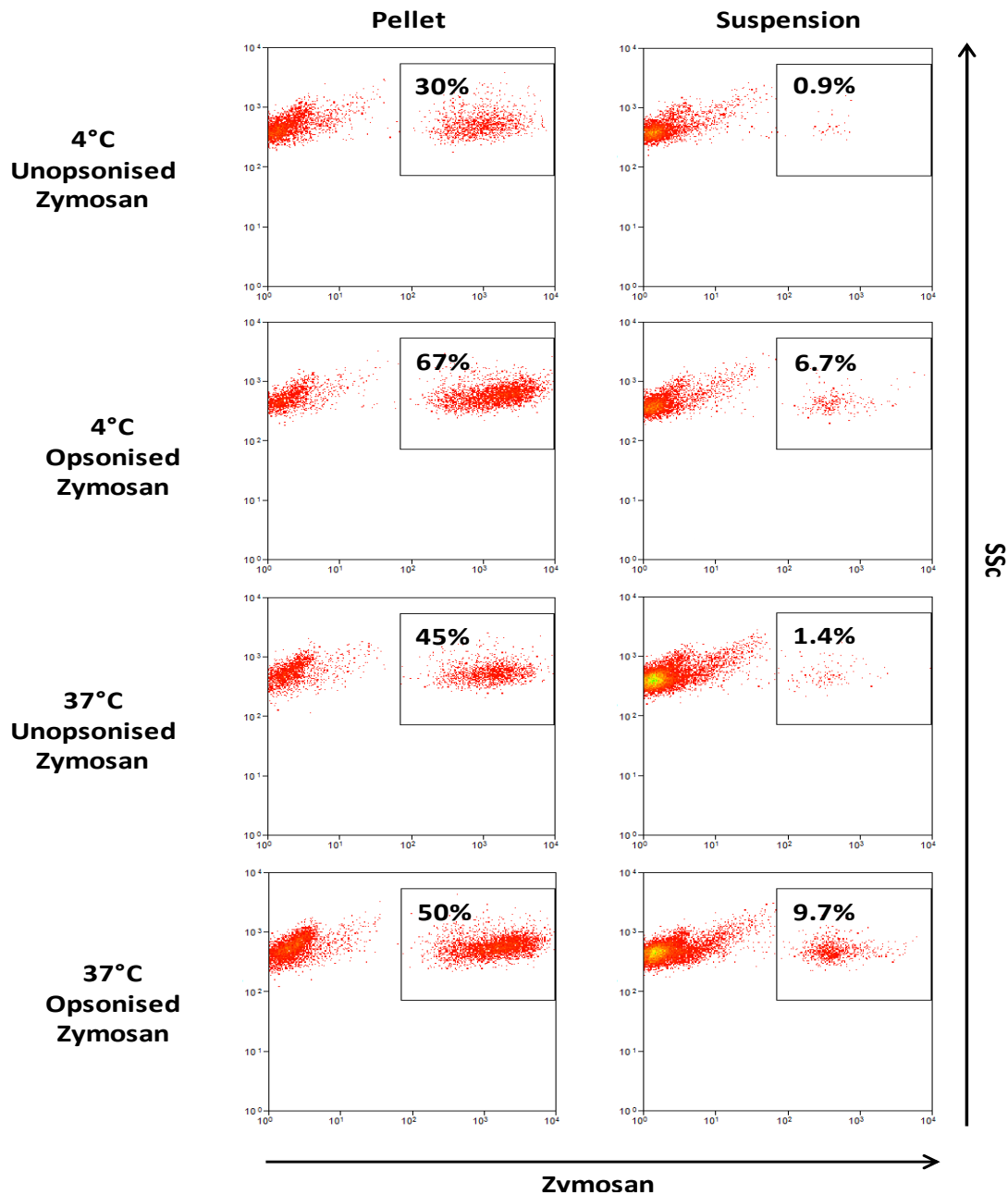


Figure 3.14: Flow cytometric plots showing the percentage of MyPH8-B6 neutrophils that have associated with either non-opsonised or serum opsonised zymosan, at either 4°C or 37°C. After 30 minutes incubation, cells in suspension showed much less binding than cells that had been pelleted and opsonised zymosan showed a greater ability to bind cells than non-opsonised zymosan. Data from a single preliminary experiment to indicate neutrophil ability to bind to zymosan.

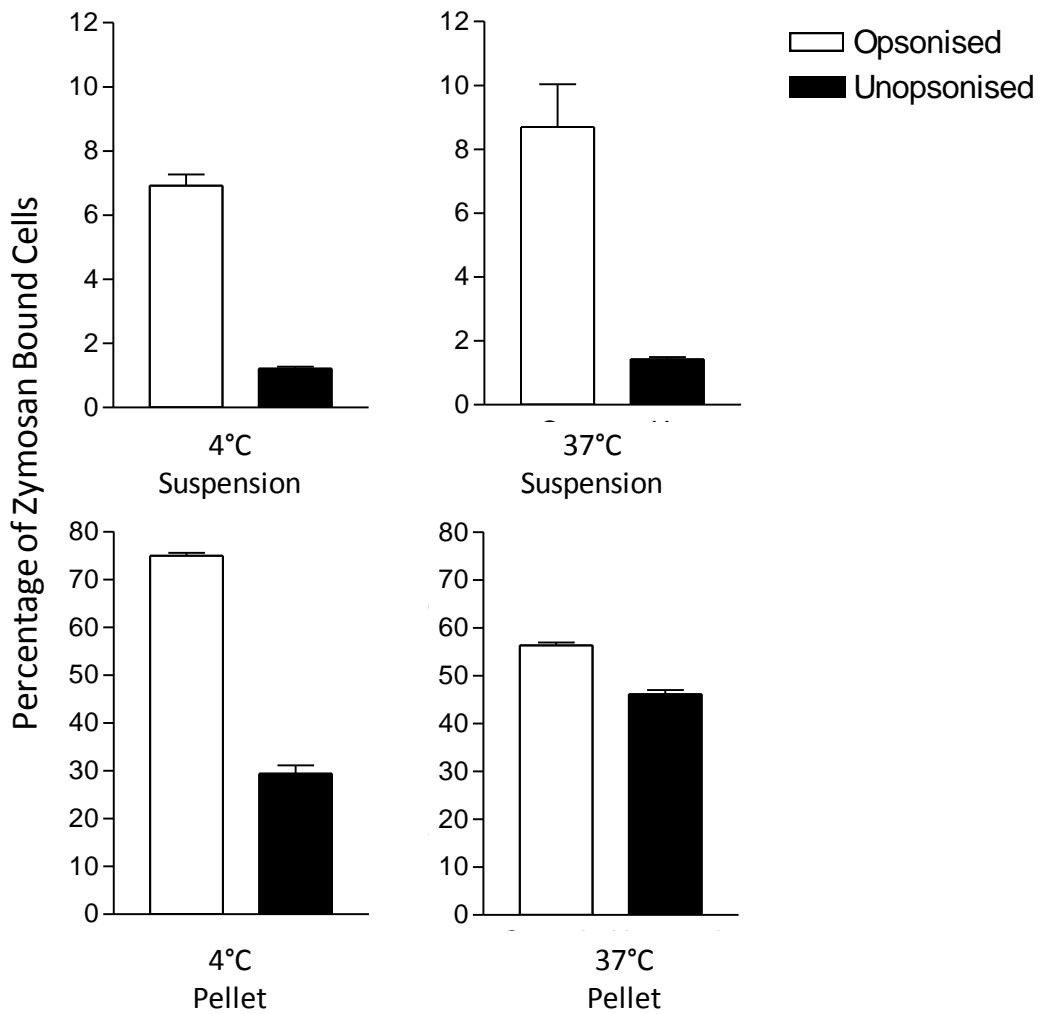


Figure 3.15: Graphical analysis of the data shown in Figure 3.14 showing the percentage of MyPH8-B6 neutrophils that have associated with either non-opsonised or serum opsonised zymosan, at either 4°C or 37°C. After 30 minutes incubation, cells in suspension showed much less binding than cells that had been pelleted and opsonised zymosan showed a greater ability to bind cells than non-opsonised zymosan. Data represents mean \pm SEM of triplicates.

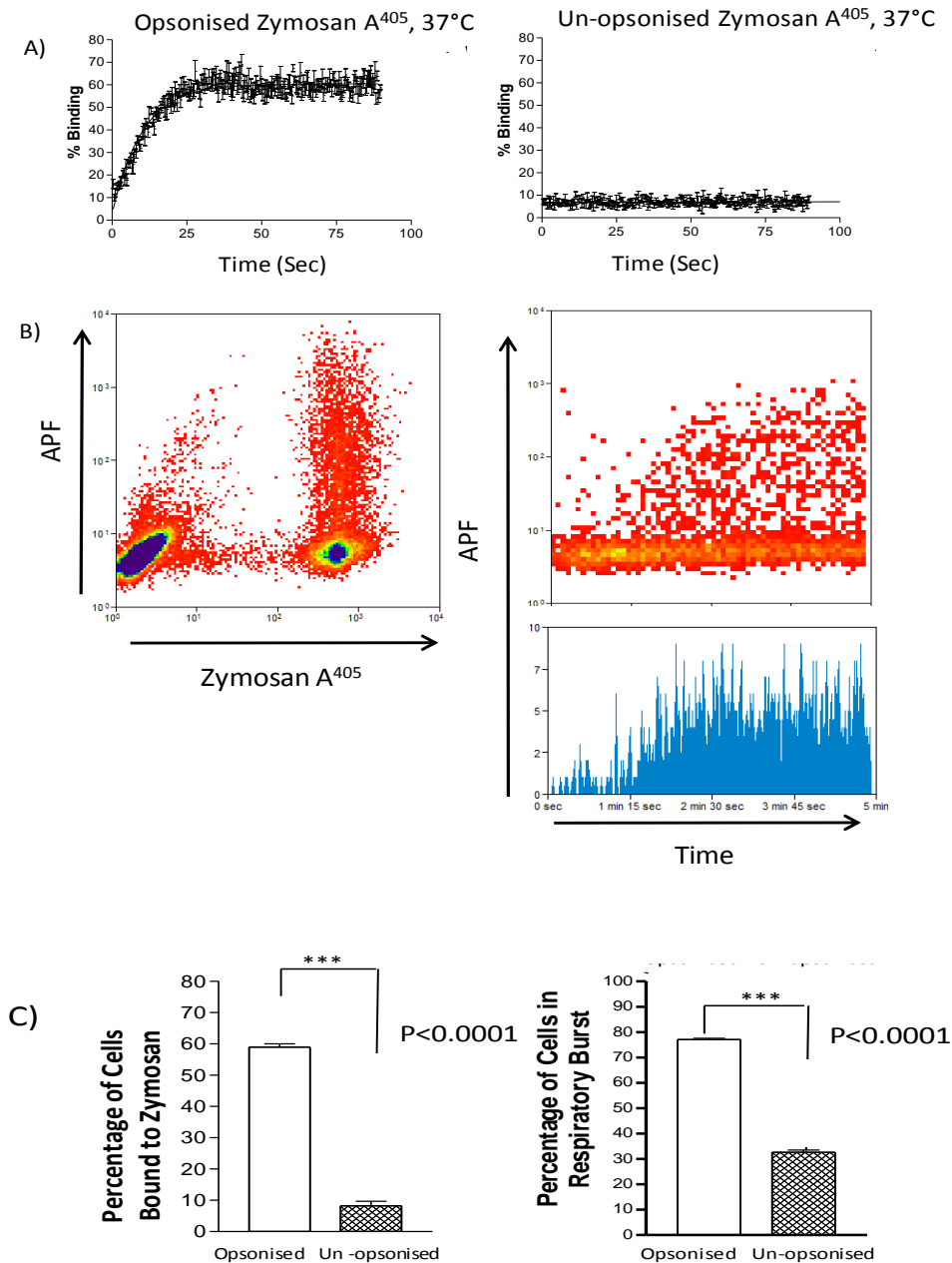


Figure 3.16: Zymosan binding and ROS production of MyPH8-B6 differentiated neutrophils. A) Percentage of MyPH8-B6 derived neutrophils binding to zymosan over time. Data represents mean \pm SEM of 3 replicates and is representative of 4 independent experiments. B) Flow cytometric plots showing that conversion of APF to its fluorescent form is restricted to cells that are interacting with zymosan and that this increases with time of incubation. C) Graphs showing the difference in zymosan binding and percentage of cells that have bound zymosan and are in respiratory burst, between cells stimulated with opsonised or non-opsonised zymosan. Data shown is calculated from the endpoint of a kinetic assay. Data represents mean \pm SEM of 3 replicates and is representative of 3 independent experiments. Statistical analysis: Two tailed T test.

3.8. Measurement of Intracellular Calcium Ions

Neutrophils are known to respond with an immediate increase in intracellular calcium ions (Ca^{2+}) in response to certain stimuli (Dewitt and Hallett 2002). In order to determine the ability of MyPH8-B6 to show this rapid increase in Ca^{2+} , MyPH8-B6 derived neutrophils were loaded with fluo-4 and fura-red for 30 minutes, at 37°C. The cells were left unstimulated or were stimulated with ionomycin (an ionophore that allows the transport of Ca^{2+} through the plasma membrane) as a positive control or fluorescently labelled zymosan. On addition of stimulant, the cells were immediately acquired on the flow cytometer and samples were collected over at least 5 minutes. Ca^{2+} measurements were obtained by taking the ratio of fluo-4/fura-red, since the fluorescence of Fluo-4 increases and Fura-red decreases in the presence of Ca^{2+} (Figure 3.17).

Unstimulated cells showed a low level baseline of intracellular calcium as expected (Figure 3.17A). On addition of 1µg/ml ionomycin, the levels of intracellular calcium immediately increased by a significant amount and remained at that level for the duration of the acquisition (Figure 3.17B). Neutrophils that were mixed with opsonised zymosan showed two distinct populations; cells that were not associated with zymosan particles and cells that were. Neutrophils that were not associated with zymosan particles showed a baseline level of intracellular Ca^{2+} , as seen with unstimulated cells (Figure 3.17C), while those that were associated with zymosan particles showed increased levels of intracellular Ca^{2+} (Figure 3.17D). This data shows that MyPH8-B6 derived neutrophils are capable of Ca^{2+} signalling in response to zymosan association.

These results were further explored by looking at intracellular calcium changes, on a cell by cell basis as opposed to whole populations, via microscopy. MyPH8-B6 derived neutrophils

were loaded with fura-2AM for 30 minutes, at 37°C. The cells were then allowed to stick to a glass slide and opsonised and non-opsonised zymosan particles were introduced to individual cells using a micropipette. Calcium changes were recorded in real time as phagocytosis progressed (Figure 3.18). Cells introduced to opsonised zymosan showed a rapid global increase in intracellular calcium ions and efficient phagocytosis of the zymosan particle (Figure 3.18A). The pattern of intracellular calcium changes seen by these cells was similar to that seen previously when human cells were introduced to iC3b-opsonised zymosan (Dewitt and Hallett 2002). Cells introduced to non-opsonised zymosan showed a much more erratic and unpredictable calcium signal (Figure 3.18B) as previously reported (Dewitt and Hallett 2002).

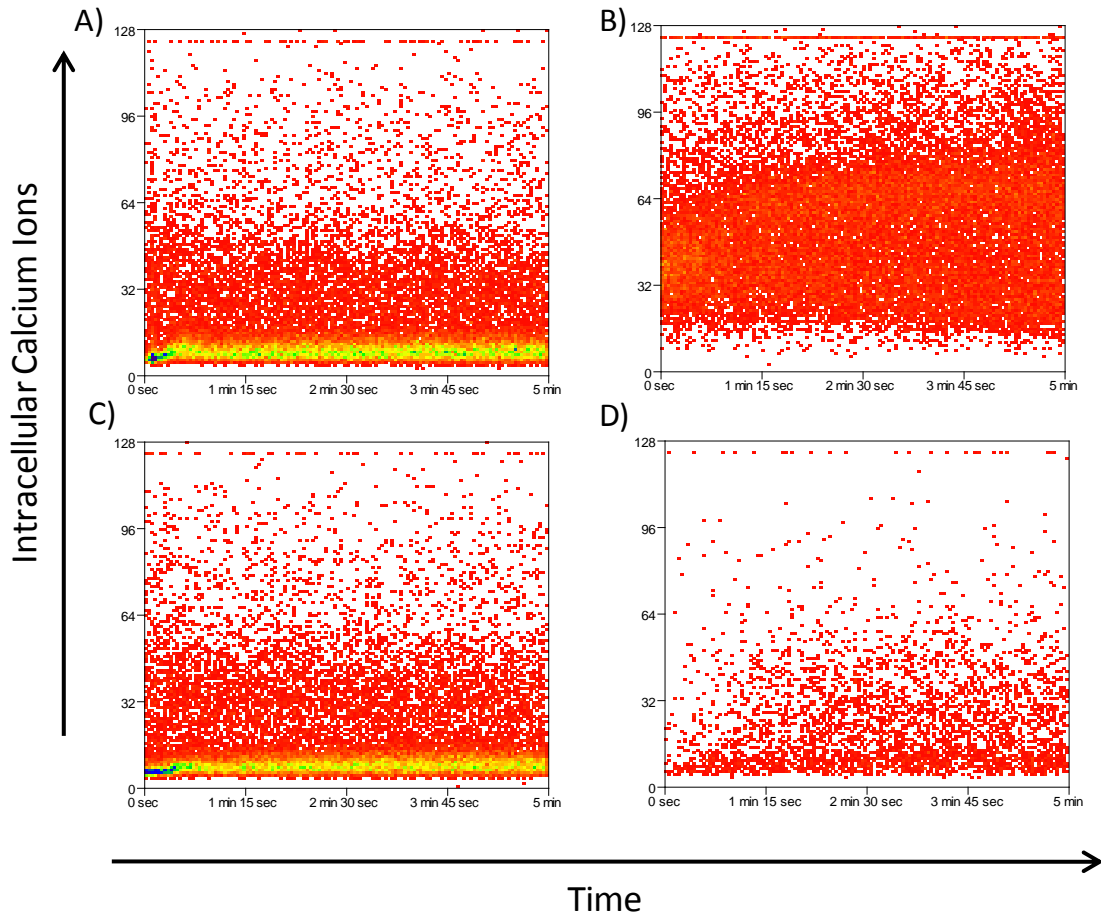


Figure 3.17: Intracellular calcium ion measurements (ratio of fluo-4/Fura-Red) of MyPH8-B6 derived neutrophils. A) Unstimulated neutrophils show a baseline low level of intracellular calcium ions, which increases on addition of $1\mu\text{g/ml}$ ionomycin (B). C) Cells that have been mixed with opsonised fluorescently labelled zymosan but that have not associated with zymosan show a baseline level of intracellular calcium ions, while cells from the same sample that have associated with zymosan particles show an increased level of intracellular calcium ions (D). Blue/turquoise lines denote the median of the calcium dye ratio. Data is representative of 2 individual experiments.

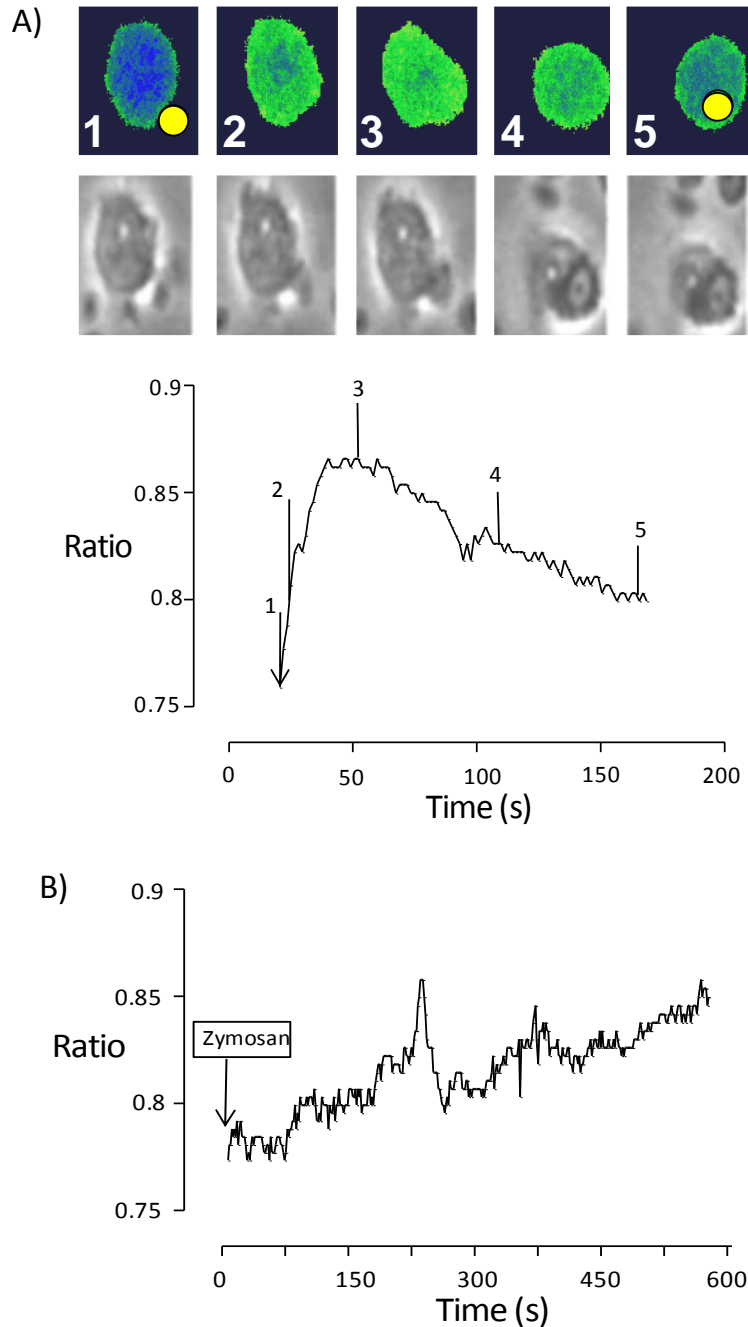


Figure 3.18: Global intracellular calcium changes within a single cell after introduction to a single zymosan particle with a micropipette. Calcium changes are measured by taking the ratio of fluorescence of fura-2 at two different excitation wavelengths (340nm/380nm). A) Phagocytosis of opsonised zymosan takes place in concert with the rapid increase of intracellular calcium within the cells and its slower decrease. A blue cell shows the calcium levels of the cell at rest. The yellow circle represents the zymosan particle. The green indicates elevated calcium levels. This data is representative of 6 cells. B) Non-opsonised zymosan produced an erratic and unpredictable pattern of intracellular calcium. This data is representative of 4 cells.

3.9. Transduction of MyPH8-B6 Progenitors to Generate Cells with Uniform

Transgene Expression

In an attempt to establish how easily the neutrophils could be genetically-modified, MMLV-derived retroviruses were used to transduce the precursor cells. MyPH8-B6 cells were transduced with a viral vector containing the calpain 1-GFP fusion gene (Nuzzi, Senetar et al. 2007). This work was done in conjunction with Kimberley Lewis, a PhD student of Prof. M Hallett who was studying the localisation of calpain-1 in neutrophils. The objective was to exploit her GFP tagged construct to examine the feasibility of genetic modification with a readily detectable transgene whilst hopefully generating a useful reagent for her studies. MyPH8-B6 cells were spin-infected with viral supernatant containing the calpain-1-GFP construct (Figure 3.19) in the presence of SCF and oestrogen. The GFP domain on the construct allowed for the transduced cells to be selected and sorted using a MoFlo Legacy flow cytometric cell sorter within Central Biotechnology Services, School of Medicine. The process of sorting the cells improved the percentage of GFP positive cells from 4% to over 98% (Figure 3.20). The cells were left in culture and percentage of GFP positive cells was assessed over the course of a few days. These cells were confirmed to be reasonably stable as approximately only 0.5% of cells lost GFP expression every 2 days (data not shown).

After sorting, GFP⁺ MyPH8-B6 progenitors were differentiated for 4 days according to normal protocols. Over the 4 days GFP expression decreased over time as Ly-6B.2 expression increased (Figure 3.21). By day 4 the expression of GFP was low and only a small number of neutrophils were detectable via microscopy. However, GFP could be readily detected for all 4 days via flow-cytometric analysis. This demonstrates that although the expression of the transgene decreases, it is still at detectable levels.

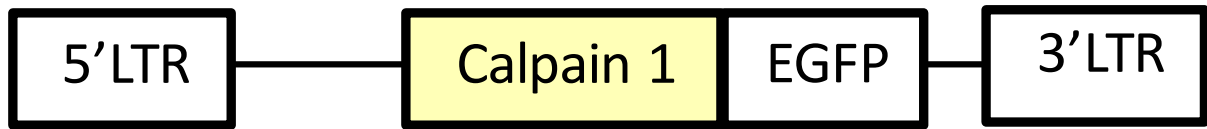


Figure 3.19: Viral DNA construct containing a calpain-1-EGFP fusion domain used to transduce MyPH8-B6 progenitors, to produce a cell line which can be differentiated into transgenic neutrophils (Nuzzi, Senetar et al. 2007).

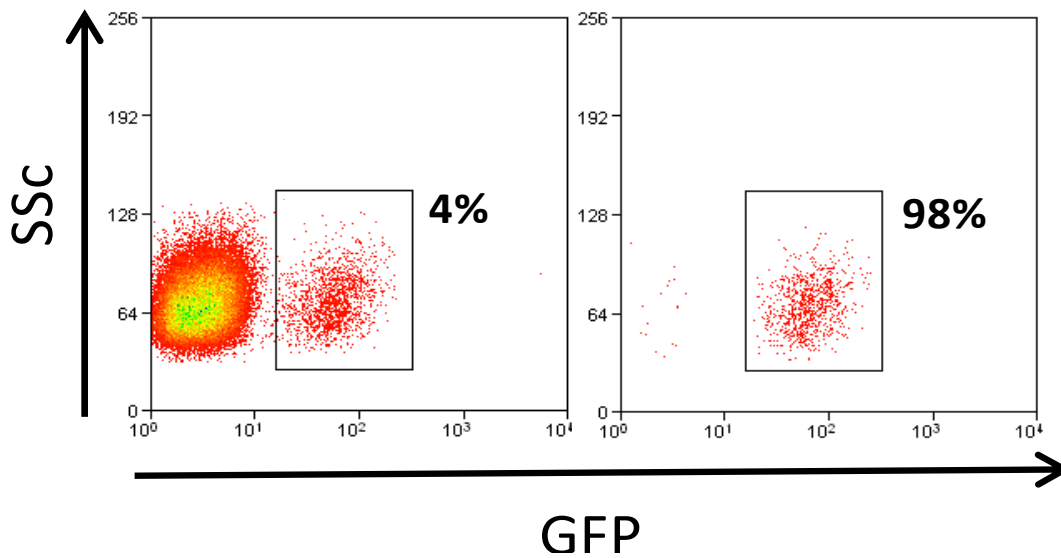


Figure 3.20: Flow-cytometric plots showing the GFP expression of MyPH8-B6 progenitors. Before cell sorting, 4% of cells expressed the transgene (left) but after sorting 98% of cells expressed the transgene (right).

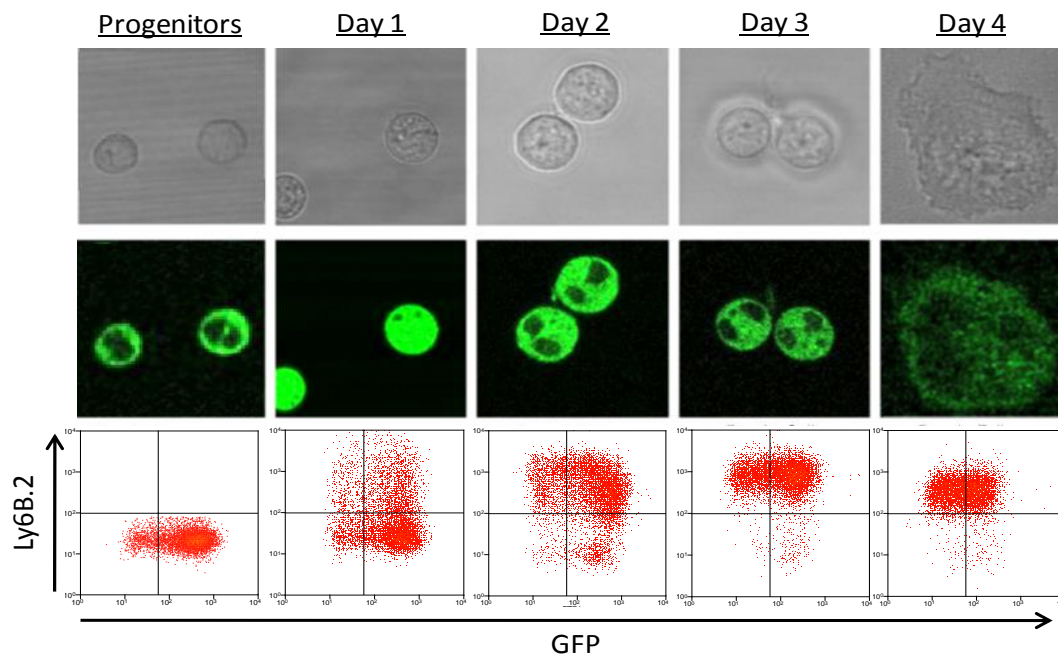


Figure 3.21: Differentiation time-course of MyPH8-B6 cells transduced to express GFP. Expression decreases over the 4 days of differentiation and although difficult to detectable via microscopy by day 4, detection is still possible via flow cytometry. Data is representative of one of two experiments. The confocal microscopy analysis was performed by Kimberley Lewis as part of her PhD studies of Calpain-1 localisation (note that the day 4 differentiated neutrophil has adhered and spread on the glass).

3.10. Adoptive Transfer of Neutrophils into Live Animals

In order to investigate whether it would be possible to adoptively transfer *in vitro* generated neutrophils into a live animal for *in vivo* study, *in vitro* generated neutrophils were fluorescently labelled and injected intravenously prior to induction of a model of acute peritoneal inflammation. Neutrophils generated from MyPH8-B6 were labelled with CellTrace™ DDAO-SE a fluorescent far red cell label and neutrophils generated from MyPH8-B6.*Itgam*^{-/-} cells (see Chapter 5 for characterisation) were labelled with both DDAO-SE and CellTrace™ calcein violet AM (the experiment was subsequently repeated with a ‘dye-swap’). The two neutrophil populations were premixed and 5x10⁶ of each cell type were i.v. injected into wild type C57BL/6 mice. Immediately after the i.v. injection the mice received 100 µg (~2x10⁷ particles) of FITC-labelled zymosan, i.p, or PBS as a control for the inflammatory response. The peritoneal cavity was then lavaged after 3 hours and the cellular composition analysed via flow cytometry.

Animals injected i.p. with PBS showed very limited recruitment of neutrophils into the peritoneal cavity while those injected with zymosan did (Figure 3.22). *In vitro* generated neutrophils from both cell lines could be identified via DDAO-SE and calcein violet labelling (Figure 3.22A). *Itgam*^{-/-} neutrophils (DDAO⁺, calcein violet⁺) showed a decreased ability to bind zymosan than the wild type neutrophils (DDAO⁺, calcein violet⁻) (Figure 3.22A). Endogenous neutrophils were identified using Ly-6G and Ly-6B.2 antibody staining (Figure 3.22B).

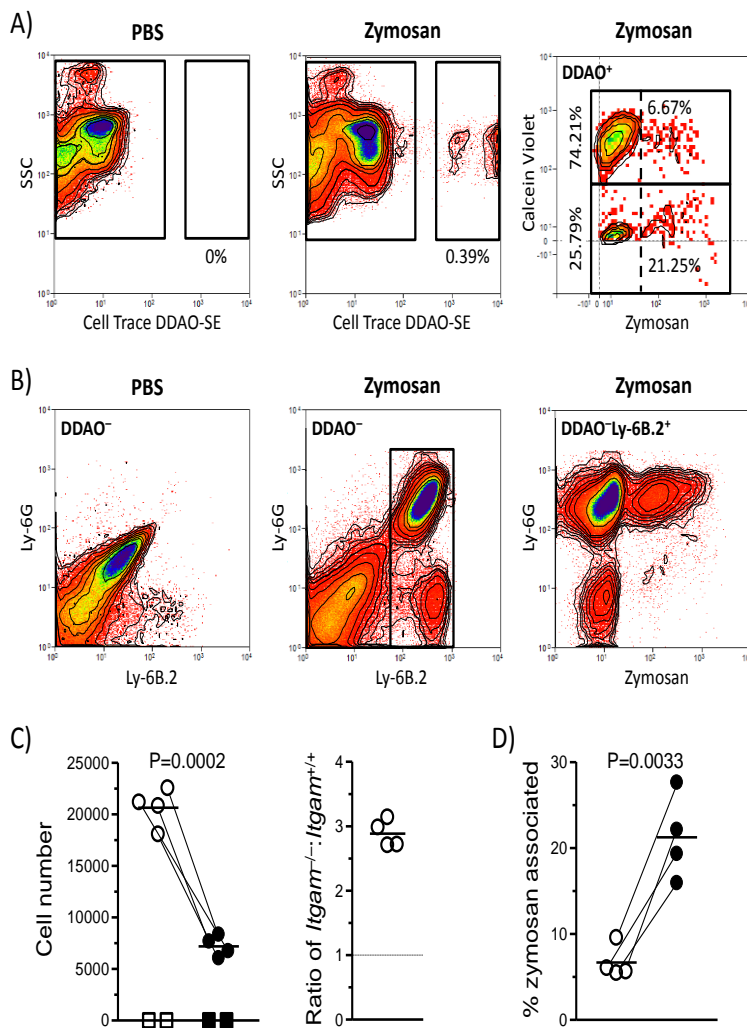


Figure 3.22: Adoptive transfer of *in vitro* generated neutrophils into C57BL/6 animals. A) Flow cytometric plots of *in vitro* generated neutrophils that were identified from peritoneal lavages 3 hours after zymosan induced recruitment into the peritoneal cavity. The left panel shows the absence of DDAO⁺ adoptively-transferred cells when control PBS i.p. injections were performed and the middle panel shows their recruitment when zymosan was administered i.p. The right panel indicates the typical division within the recruited DDAO⁺ cells for calcein violet labelling

(indicated by percentages outside of the gates) and the percentage of these two cell types that were zymosan associated (indicated by percentages within the gates). B) Identification of DDAO⁻ native neutrophils from C57BL/6 mice, shows the substantial recruitment of Ly-6G⁺Ly-6B.2⁺ neutrophils and Ly-6G⁻Ly-6B.2⁺ inflammatory monocytes after zymosan administration (middle panel), but not after control PBS injection (left panel). The right panel shows notable association of zymosan with the recruited endogenous neutrophils at this time point. C) Graphs showing the quantification of the *in vitro* generated neutrophils that were recruited into the peritoneal cavity of live mice. Almost three times more *Itgam*^{-/-} neutrophils (DDAO⁺, calcein violet⁺) were recruited into the peritoneal cavity than wild type neutrophils (DDAO⁺, calcein violet⁻). D) Graph showing the percentage of *in vitro* generated neutrophils that were associated with zymosan particles. *Itgam*^{-/-} neutrophils showed a decreased ability to bind zymosan than the wild type neutrophils. Exact P values are shown and were obtained by 2-tailed paired *t*-test. Data represent one of two identical experiments with FITC-zymosan in which dye swaps were performed.

3.11. Conclusion

This chapter demonstrates the successful reproduction of the conditionally-immortalised neutrophil precursor cell line originally generated by Wang et al. 2006. The first cell lines were attempted using 129S6/SvEv mice. However this proved unsuccessful as it generated highly granulated mast-cell like cells. The reason for this is unclear but studies have shown that mice from the 129 genetic background show significant differences in their mast cell biology. 129S6/SvEv mice have significantly increased serum IgE levels, increased responsiveness to anaphylactic challenge and increased levels of FcεR1 than C57BL/6 mice (Yamashita, Charles et al. 2007). This suggests a significant bias towards mast cell production that could be responsible for the highly granular cells produced (Krishnaswamy and Chi 2005).

The progenitor cell lines were generated using a viral DNA construct containing an oestrogen receptor binding domain-Hoxb8 fusion gene. The potential for this method to produce other myeloid cells has been reported (Wang, Calvo et al. 2006). In our laboratory, macrophage precursors are routinely generated that are differentiated into mature macrophages with GM-CSF or M-CSF, by changing the growth medium used to establish the cells from Opti-MEM containing SCF to RPMI 1640 containing GM-CSF (Rosas, Liddiard et al. 2008; Rosas, Osorio et al. 2010).

I was able to differentiate the neutrophil precursors into neutrophils by growing them in SCF+G-CSF containing medium for 4 days. Although it has been reported that these cell lines should be able to produce other myeloid cells, by changing the cytokine combinations (Wang, Calvo et al. 2006) used for differentiation, I found that this cell line was primarily a

neutrophil producing cell line and scope for generating other cell lines was limited in the time frames and conditions studied, although this was not extensively pursued.

Progenitors grown in SCF+G-CSF for 4 days produced high neutrophil purities and resulted in a large number of cells produced. Although other cytokine combinations gave greater neutrophil purity (G-CSF) and others gave greater cell expansion (SCF + GM-CSF), none gave the same combination of high purity and cell numbers that was seen with SCF+G-CSF. With a little optimisation (ensuring cell numbers were not so high as to deplete media or that growth factors were added in excess during the differentiation period), this protocol generates the high purities and viabilities that are required for a viable *replacement* for animals in research to be adopted by other laboratories.

Neutrophils are considered terminally differentiated cells. The *in vitro* generated neutrophils derived from the conditionally immortalised cell lines had DNA and RNA contents that were consistent at the very least with cessation of cell cycle and a quiescent like phenotype.

Neutrophils from conditionally-immortalised precursor cells were able to bind and phagocytose zymosan and exhibited calcium mobilisation (both confirmed by both flow-cytometry and live microscopy) and respiratory burst. These are key functions of primary neutrophils that these *in vitro* neutrophils can perform.

Neutrophils generated *in vitro* were successfully adoptively transferred into live animals and were successfully identified after culling. In addition, functional differences could be recorded in these *in vivo* studies when CD11b-deficient (*Itgam*^{-/-}) cells were used.

Specifically it was demonstrated that there was almost three times as many CD11b deficient

in vitro generated neutrophils located in the peritoneal cavity than wild type neutrophils.

This is consistent with the published research that CD11b deficient neutrophils accumulate in the peritoneal cavity in response to a challenge due to reduced apoptosis of the cells (Coxon, Rieu et al. 1996), and adds further information by confirming an *in vivo* defect in fungal particle recognition in the absence of CD11b.

In addition to these studies reported here, collaborators in Prof. Marina Botto's laboratory in London demonstrated the usefulness of these cell lines in a chemotactic assay across an endothelial barrier towards MIP-2 (McDonald, Cortini et al. 2011). The receptor for MIP-2 is CXCR2, which was found selectively on the SCF+G-CSF differentiated cells (Figure 3.11B).

Additionally, they demonstrated a reduced propensity for the *Itgam*^{-/-} cells to die after migration, when compared to wild type cells, which reflects the phenotype of primary *Itgam*^{-/-} neutrophils (Coxon, Rieu et al. 1996). In a currently unpublished collaborative study conducted with Prof. Jörg Köhl (Lübeck, Germany) these cells have been similarly shown to migrate towards recombinant C5a (Karsten, Pandey et al. 2012), consistent with their expression of CD88/C5a receptor.

Taken together these studies indicate that the cells can be effectively used in carefully controlled studies and can readily identify phenotypes in genetically-deficient *in vitro* generated neutrophils that have been previously reported with primary cells.

In conclusion, I have validated this method of producing conditionally immortalised neutrophil progenitors and shown that it produces a reasonable model of a mouse neutrophil that exhibits many of the properties of a primary mouse neutrophil. A further advantage of these cells is that the progenitors can be frozen and a cell stock kept and thawed for future use. However, they also exhibit some alterations, such as low Ly-6G and

Clec7a expression that indicate that they may not represent a fully mature mouse neutrophil. In spite of this, they are currently one of the best *in vitro* models of mouse neutrophils and have the further advantage that they can be readily genetically-modified for functional studies, which is a significant step forward as there are no comparable methods of producing genetically modified neutrophils without the need to generate new mouse colonies (see also chapter 5).

**Chapter 4: Analysis of the Role of Clec7a
(Dectin-1) in Fungal Particle Recognition
on Primary Neutrophils**

4.1. Introduction

The incidence of human fungal infections is on the increase especially those caused by normally non-pathogenic species, e.g. *Candida albicans*. This is due to the increasing numbers of people that have a compromised immune system due to HIV, the use of immunosuppressants and other medical interventions (Romani 2004¹).

Fungal cell walls are predominantly composed of carbohydrates, including β -glucans, but also glycoproteins, lipids and inorganic salts. Study of β -glucan interactions has traditionally used zymosan, which is a particle made from the cell walls of *Saccharomyces cerevisiae*, which contains β -glucans as well as other polysaccharides, proteins and lipids (Di Carlo and Fiore 1958). As well as using zymosan to model β -glucan recognition, this chapter also uses curdlan microparticles, which are a selective clec7a agonist (Rosas, Liddiard et al. 2008).

Clec7a is a cell surface receptor that recognises β -glucans (Brown and Gordon 2001; Brown, Taylor et al. 2002). It is a type II transmembrane protein with a C-type lectin-like domain and it contains an immunoreceptor tyrosine-based activation-like motif on its cytoplasmic tail (Ariizumi, Shen et al. 2000). Clec7a is broadly expressed on myeloid cells, predominantly macrophages, monocytes and neutrophils (Taylor, Brown et al. 2002; Taylor, Tsoni et al. 2007). Opsonic recognition of β -glucans and fungal particles is known to proceed via complement receptor 3 (CR3: CD18/CD11b) (Brown 2006). Until relatively recently, CR3 was thought to be the primary non-opsonic β -glucan receptor (Xia, Vetvicka et al. 1999). More recent studies demonstrated that Clec7a is a major non-opsonic β -glucan receptor on macrophages (Brown, Taylor et al. 2002). However, additional β -glucan receptors that have been proposed are SCARF-1 and CD36 (Means, Mylonakis et al. 2009).

The main aims of this chapter are as follows:

1. To develop a model for studying the relative contributions of Clec7a and CR3 (including via complement opsonisation) in β -glucan recognition by neutrophils *in vivo* and *ex vivo*.
2. To explore the contribution of Clec4n (dectin-2) in neutrophil recognition and response to fungal particles.
3. To explore the function of Clec7a on distinct populations of human neutrophils.

4.2. Establishing Parameters for *In Vivo* Exploration

A series of experiments were performed to determine the parameters required for neutrophils to interact with fungal particles *in vivo* and hence for the contribution of Clec7a to these inflammatory and recognition processes to be assessed. 129S6/SvEv mice were injected intraperitoneally (i.p.) with 2×10^7 FITC-labelled zymosan. The peritoneal cells were then harvested at different time points and analysed. In lower dose models ($\leq 2 \times 10^6$), inflammatory cell recruitment and cytokine production in this model has a partial dependency on Clec7a (Taylor, Tsoni et al. 2007), which I found to be reproducible in an experiment where mice were injected with 10^5 particles of FITC-labelled zymosan (data not shown). Cells taken from the peritoneal lavage of an animal that had not been injected i.p. with zymosan showed a prominent population of tissue resident macrophages as confirmed by high expression of F4/80 (Austyn and Gordon 1981; Taylor, Martinez-Pomares et al. 2005) and almost no neutrophils (Figure 4.1.A). After injection with zymosan, neutrophils were identified by Ly-6G and Ly-6B.2 expression and 4 hours after injection neutrophils proved to be the predominant cell present (Figure 4.1.B). After 18 hours, the predominant cell type was still the neutrophil, however, number had started to decrease while monocyte (Ly-6B.2⁺ and Ly-6G⁻) numbers had started to rise (Figures 4.1. C). By days 3 and 7 post-injection, cells of the monocyte/macrophage lineage had become predominant, with very low numbers of neutrophils (Figure 4.1.D & E).

Quantification of cell numbers showed that of the time points examined, neutrophil numbers peaked at the 4 hour time point (Figure 4.2.A) but quickly decreased down to baseline levels by day 3. The percentage of neutrophils associated with zymosan followed this same pattern with the peak at 4 hours (Figure 4.2.B). All cells of the

monocyte/macrophage lineages showed a sharp decline at 4 hours but then a steady increase, with the peak at day 3. This sharp decline is probably a result of the macrophage disappearance reaction due to acute inflammation that has been previously reported (Barth, Hendrzak et al. 1995). Monocyte/macrophage numbers then seem to settle back to baseline by day 7, although this population almost certainly represents a mixed population of cells (Davies, Rosas et al. 2011). The percentage of monocyte/macrophages that are associated with zymosan show a steady increase, until they peak at day 3 (Figure 4.2.B). This late association of zymosan with macrophages is likely explained by one of two, not mutually exclusive, mechanisms: the phagocytic clearance of dying neutrophils that have previously phagocytosed zymosan or the re-appearance of tissue resident macrophages, which still contain zymosan (Davies, Rosas et al. 2011).

It is clear from this data that the greatest association between zymosan and neutrophils is occurring at the 4 hour time point. As that is the interaction of interest, all further experiments looking at the role of Clec7a on neutrophils was performed with cells taken 4 hours after i.p. injection.

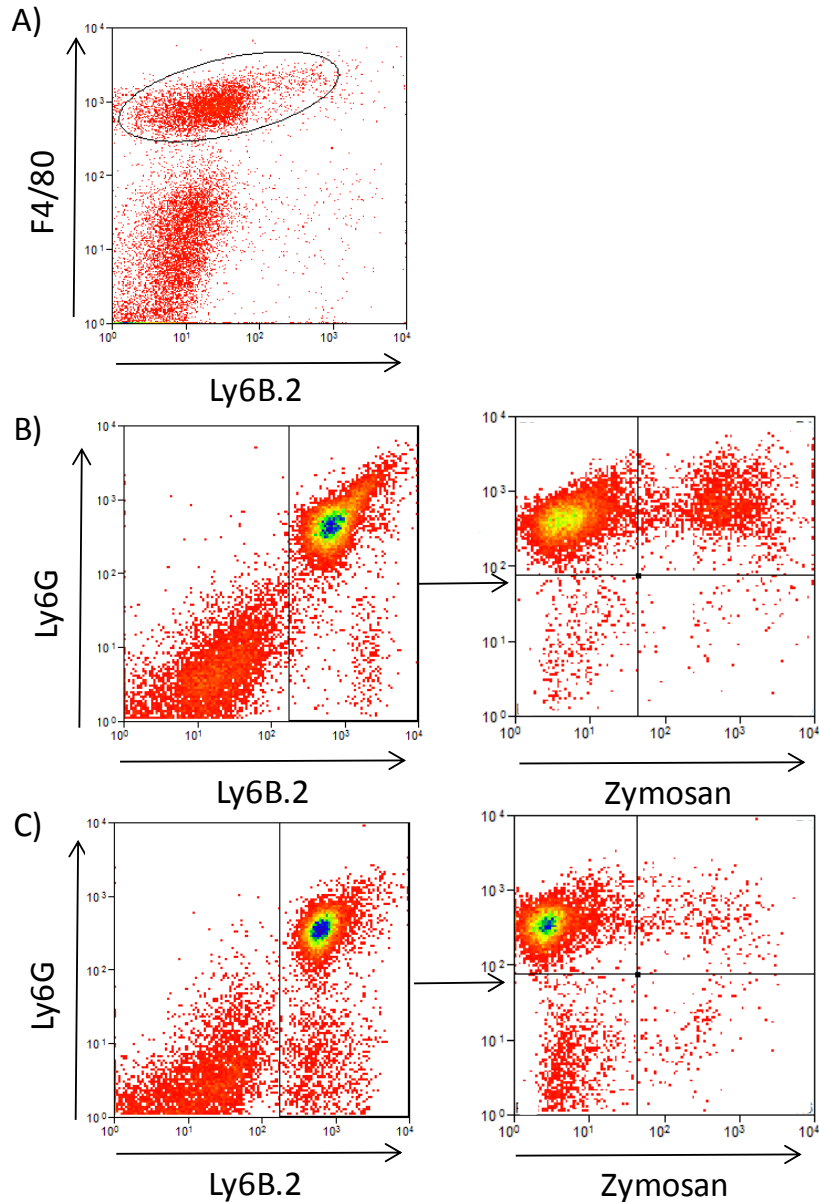


Figure 4.1: Flow-cytometric plots showing peritoneal cells from 129S6/SvEv mice. A) Resident peritoneal cells from an animal that has not been injected with zymosan. The gated region shows resident macrophages as denoted by high F4/80 staining. There is no distinct population of neutrophils as shown by the lack of a distinct population showing high Ly-6B.2 and no F4/80 staining. B) Recruited peritoneal cells 4 hours after i.p. injection with FITC-zymosan. The Ly-6G⁺Ly-6B.2⁺ cells denote a large population of infiltrating neutrophils while the Ly-6G⁻Ly-6B.2⁺ show a smaller population of infiltrating monocytes. At this time point the majority of the zymosan particles are associated with neutrophils. C) Recruited peritoneal cells 18 hours after i.p. injection with FITC-zymosan. Neutrophil numbers are lower than at 4 hours while monocytes numbers have increased (See Figure 4.2 below). Plots are representative of at least 2 mice per time point.

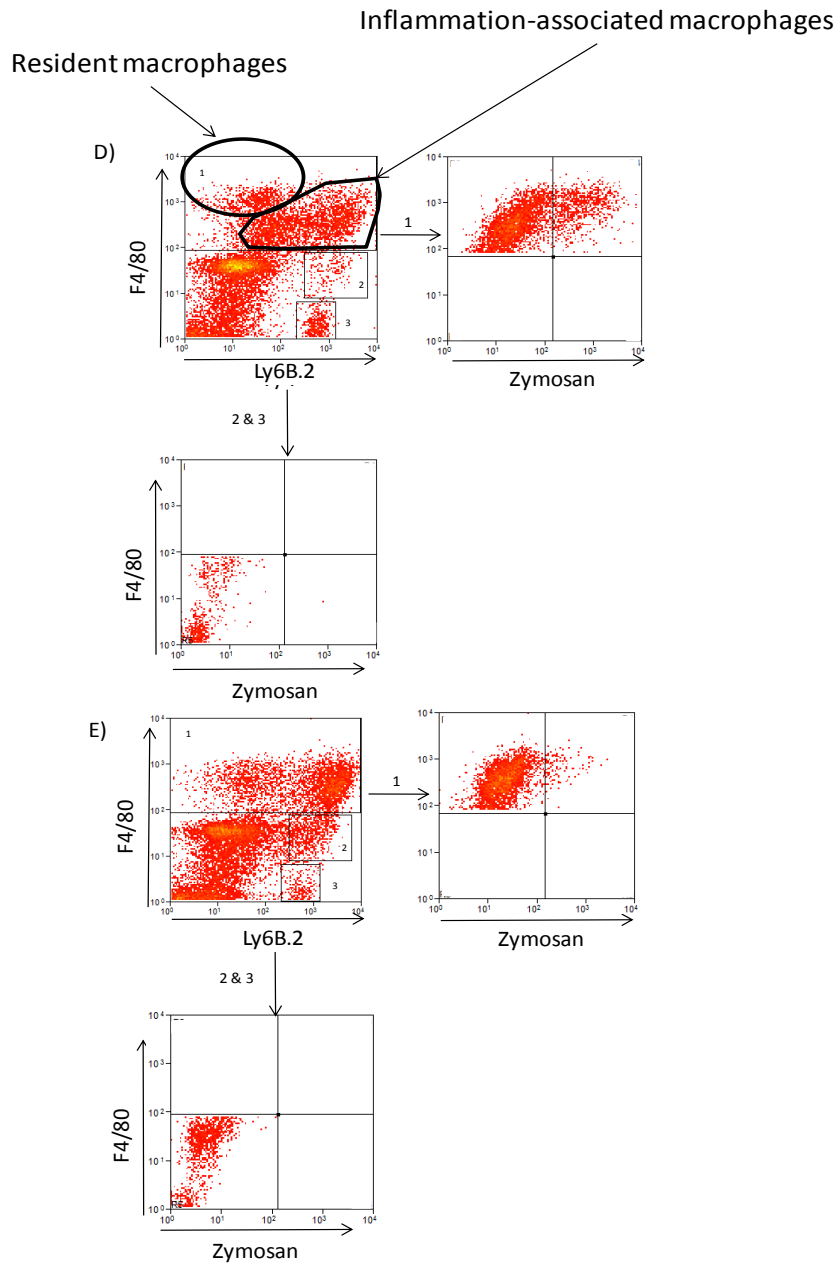


Figure 4.1: Flow-cytometric plots showing peritoneal cells from 129S6/SvEv mice. D) Recruited peritoneal cells 3 days (72 hours) after i.p. injection with FITC-zymosan. Present are: F4/80 high macrophages (1), composed of residual F4/80^{high}Ly-6B.2^{low-high} population of resident macrophages as well as an abundant F4/80^{high}Ly-6B.2^{low-high}; a much smaller F4/80^{low}Ly-6B.2^{high} population of monocytes(2); and a F4/80^{high}Ly-6B.2^{high} population of neutrophils. The majority of zymosan association is now with the F4/80^{high} macrophage populations. Almost no zymosan is associated with the residual neutrophils and F4/80^{low} monocytes. These results are also seen 7 days (168 hours) after i.p. injection with zymosan, although there are fewer cells associated with FITC-zymosan at this time point (E). Plots are representative of at least 2 mice per time point.

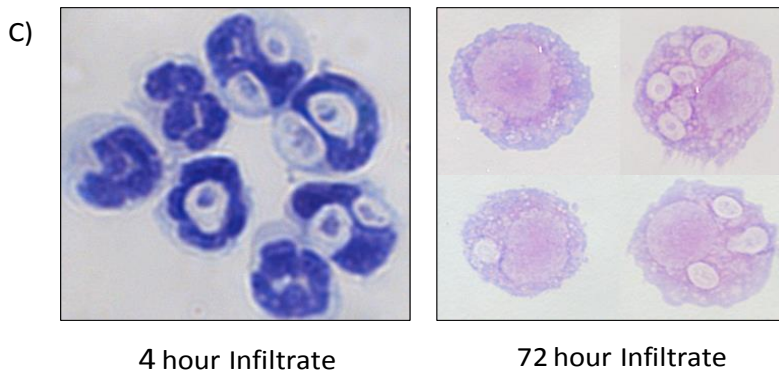
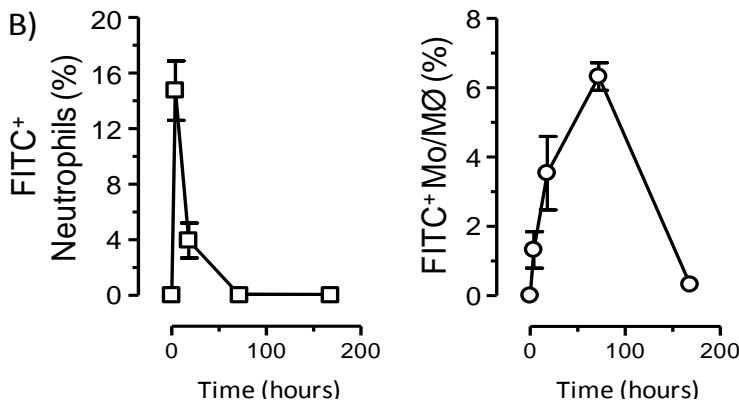
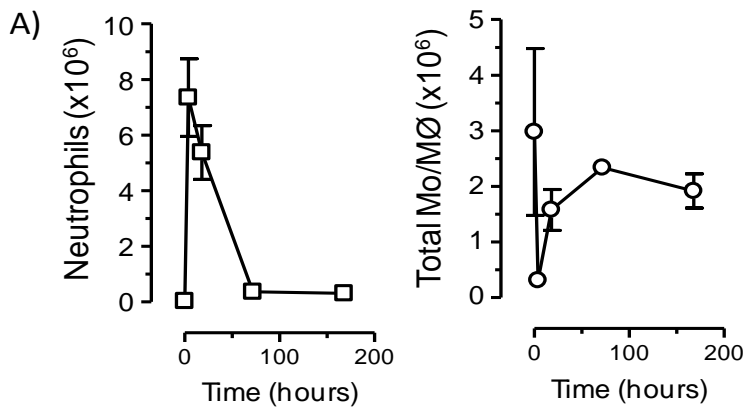


Figure 4.2: A) Graphical representation of **neutrophil and monocyte/macrophage numbers in the peritoneal cavity before and after acute zymosan peritonitis**. n=3 129S6/SvEv mice per group and is representative of 2 independent experiments. Data is shown as mean±SEM. There is a rapid influx of neutrophil on i.p. injection with FITC-zymosan, which peaks at 4 hours after injection. Neutrophil numbers then decrease until approaching baseline at 72 hours. After i.p. injection with FITC-zymosan, monocyte/macrophage numbers rapidly decrease, with the trough at 4 hours after injection. After

this point numbers slowly increased until approaching baseline levels at 72 hours, albeit that these macrophages represent a mixture of macrophage phenotypes at this time (Davies, Rosas et al. 2011). B) Graphical representation of the percentage of neutrophils and monocytes/macrophages present that were associated with zymosan. Zymosan association with neutrophils was greatest 4 hours after injection and had all disappeared by day 7. Zymosan association with the monocyte/macrophage populations gradually increased to peak at 72 hours before returning to baseline by day 7. C) Representative picture of neutrophils in a 4 hour inflammatory infiltrate showing zymosan associated and unassociated cells (left panel) and MoFlo purified day 3 macrophages (right panel). The macrophages in the lower panel show examples of association with no, one or multiple zymosan particles, although, the percentage of macrophages associated with zymosan at this time point is low (see B).

4.3. A Model for the *In Vivo* Study of the Function of Clec7a on Neutrophils During Peritonitis Caused by Fungal Infection

129S6/SvEv and 129S6/SvEv.*Clec7a*^{-/-} mice were injected i.p. with 2×10^7 FITC-labelled zymosan. After 4 hours, the peritoneal cavity was lavaged and the recruited cells analysed. Supernatants from this lavage were kept and analysed for IL-6 production as a marker of cellular activation and inflammation.

Resident macrophages were identified prior to zymosan injection by high expression of CD11b and lack of Ly-6B.2 (Figure 4.3A). After zymosan injection, the number of resident macrophages recovered from the peritoneal cavity was very low (<5% of the numbers in naive mice)(Figure 4.3A and 4.4A). Of the remaining resident macrophages, there were more cells from *Clec7a*^{-/-} mice associated with zymosan particles compared to wild type cells (Figure 4.3A and 4.4B). Neutrophils and monocytes were identified by expression of Ly-6G and Ly-6B.2. Neutrophils were predominantly present while a small population of the cells were monocytes. Both cells type showed noticeable association with zymosan particles (Figure 4.3B).

The numbers of neutrophils present in the lavage 4 hours post injection were significantly less in *Clec7a*^{-/-} mice than in wild type mice. However, this was reversed for monocytes, with *Clec7a*^{-/-} mice showing a significantly greater number of monocytes than wild type mice. The numbers of neutrophils associated with zymosan particles was significantly lower in *Clec7a*^{-/-} mice than in wild type mice. There was no significant difference between the monocytes. IL-6 production was also significantly decreased in *Clec7a*^{-/-} mice (Figure 4.4.C). This data suggests that Clec7a has some role in neutrophil interaction with zymosan, but

indicates that the inflammatory response is not normal in the *Clec7a*^{-/-} mice as previously reported (Taylor, Tsoni et al. 2007).

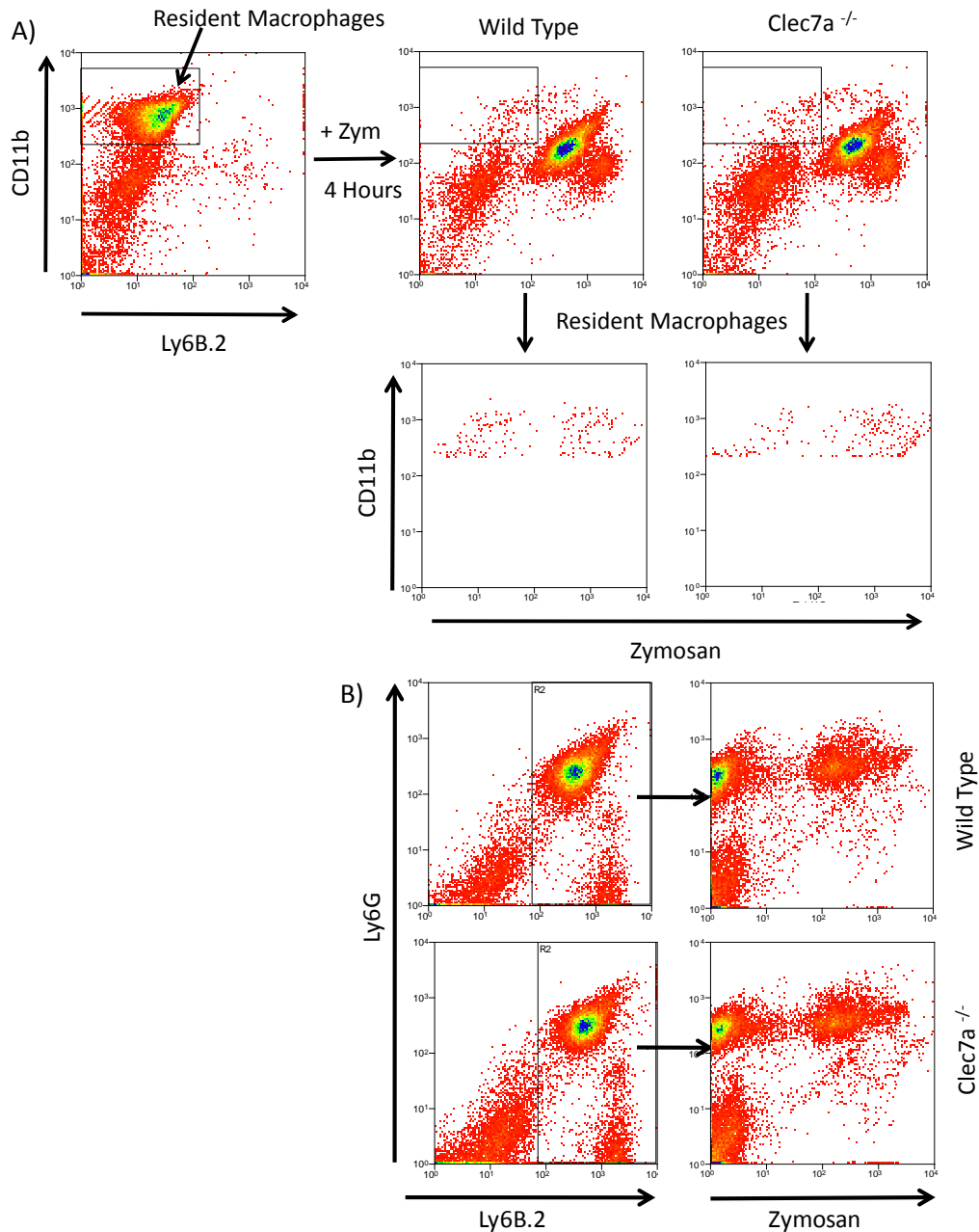


Figure 4.3: Flow-cytometric plots showing peritoneal cells from 129S6/SvEv mice and 129S6/SvEv.*Clec7a*^{-/-} mice before and after acute zymosan peritonitis. A) Mice injected i.p. with FITC-zymosan show a rapid disappearance of the majority of the resident macrophages in both wild type and *Clec7a*^{-/-} animals. Residual resident macrophages show marked association with zymosan particles. B) Both wild type and *Clec7a*^{-/-} animals show neutrophil and monocyte recruitment into the peritoneum, 4 hours after i.p. injection with FITC-zymosan. The majority of zymosan particles are associated with neutrophils, however some monocytes also show association with zymosan. Data shown is from representative mice with the full dataset shown below (Figure 4.4).

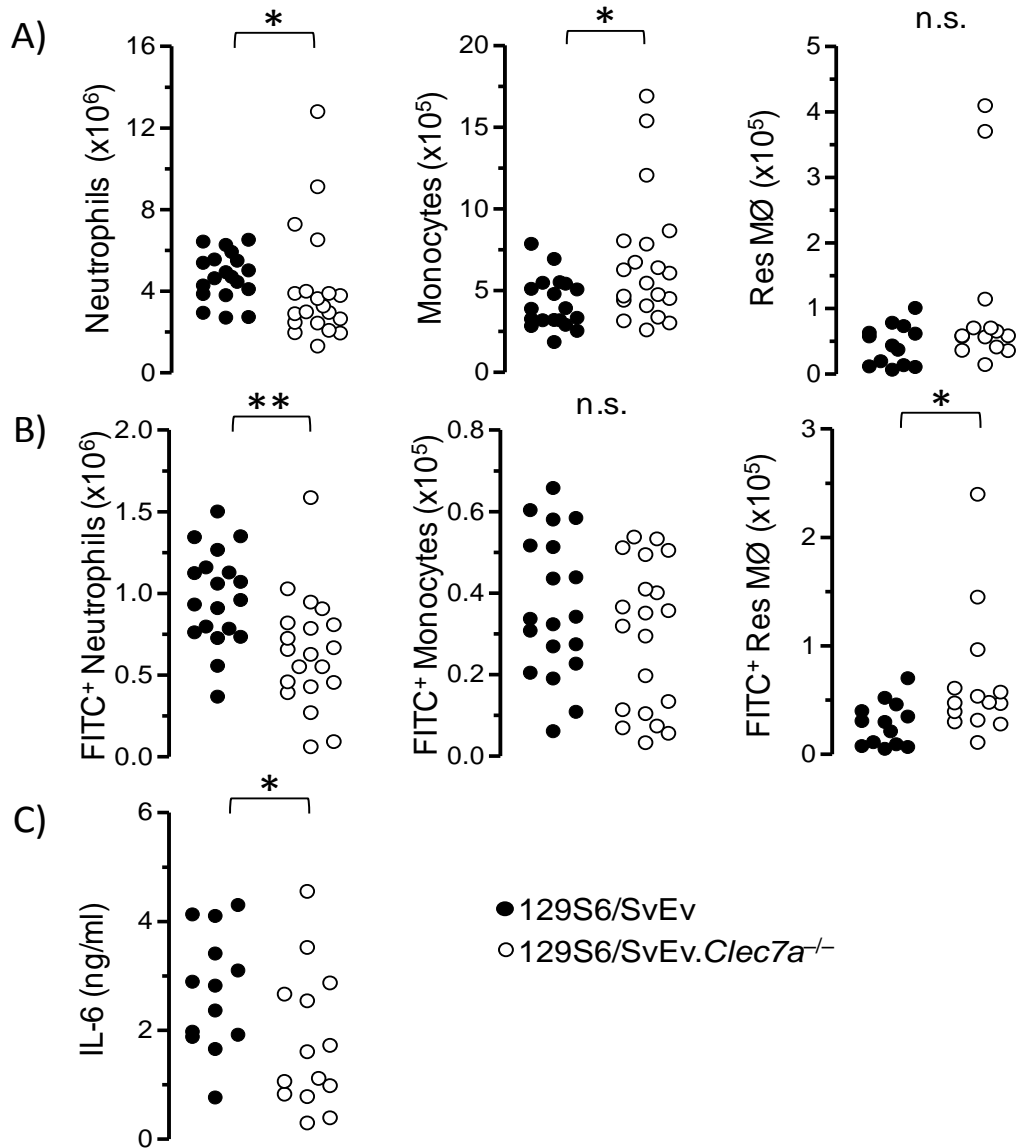


Figure 4.4: Characterisation of high-dose zymosan peritonitis in *Clec7a*-deficient mice. Wild type (closed circles) and *Clec7a*-deficient (*Clec7a*^{-/-}; open circles) 129S6/SvEv mice were injected intraperitoneally with 2×10^7 zymosan particles and inflammatory infiltrates examined 4 hours later. A) Graphs showing the number of neutrophils, monocytes and residual tissue resident macrophages (Res MØ) in the peritoneal cavity at this time. B) Graphs showing the numbers of those cells indicated in (A) above that are associated with FITC-zymosan. C) Quantification of IL-6 in the peritoneal lavage fluid of zymosan injected mice. For A, B and C data were normalised and pooled from 2 (res MØ and IL-6 analysis) and 3 (Neutrophil and monocyte analysis) independent experiments. Each symbol represents an individual mouse. Data were analysed by non-parametric Mann-Whitney U-test. P values: *=P<0.05; **=P<0.01.

4.4. Ex Vivo Study of the Function of Clec7a on Primary Neutrophils

In order to determine whether the apparent defects in neutrophil zymosan interactions seen in *Clec7a*^{-/-} mice described above were due to the intrinsic role of Clec7a on neutrophils or defects in recruitment and activation of the neutrophils caused by impaired cellular activation and induction of inflammation, an *ex vivo* assay was developed. Inflammatory cells were recruited into the peritoneum in a Clec7a independent manner using BIOgel polyacrylamide beads. The neutrophils (Ly-6G⁺ Ly-6B.2⁺) and monocytes (Ly-6G⁻ Ly-6B.2⁺) were tested for their expression of both Clec7a and CD11b. Wild type neutrophils and monocytes were both positive for Clec7a and CD11b while *Clec7a*^{-/-} cells were positive for CD11b but not Clec7a (figure 4.5). The cells were preloaded with APF, an indicator of ROS, then stimulated with zymosan (2x10⁶ particles), serum-opsonised zymosan, *C. albicans* (2x10⁶ live cells) or serum-opsonised *C. albicans*. PBS was used as a negative control. As well as analysing peritoneal neutrophils (figure 4.6), recruited Ly-6B⁺ monocytes were also analysed (figure 4.7).

Only cells that had interacted with fungal particles produced ROS as a result of the respiratory burst (figure 4.8). Clec7a deficiency significantly (P<0.0001) impairs the ability of neutrophils and monocytes to bind both opsonised and non-opsonised zymosan, and their ability to respond via production of reactive oxygen species (figure 4.8.A). However, the effect of Clec7a is not significant in regards to the ability of neutrophils to respond to opsonised zymosan. In all cases opsonisation increases binding of zymosan and in the case of neutrophils, opsonisation increases the ability of cells to respond to zymosan. This supports the established important role for CR3 as well as Clec7a mediated binding.

When looking at binding and response to live *C. albicans*, Clec7a deficiency significantly impairs the ability of neutrophils and monocytes to associate with live *C. albicans* .

Response to *C. albicans*, by production of ROS, is impaired in neutrophils when associated with both opsonised and unopsonised *C.albicans*. However in monocytes response is only significantly impaired when associated with unopsonised *C.albicans* (figure 4.8.B). This broadly mirrors the results seen with zymosan particles but shows less dependence on Clec7a to initiate respiratory burst in interacting neutrophils.

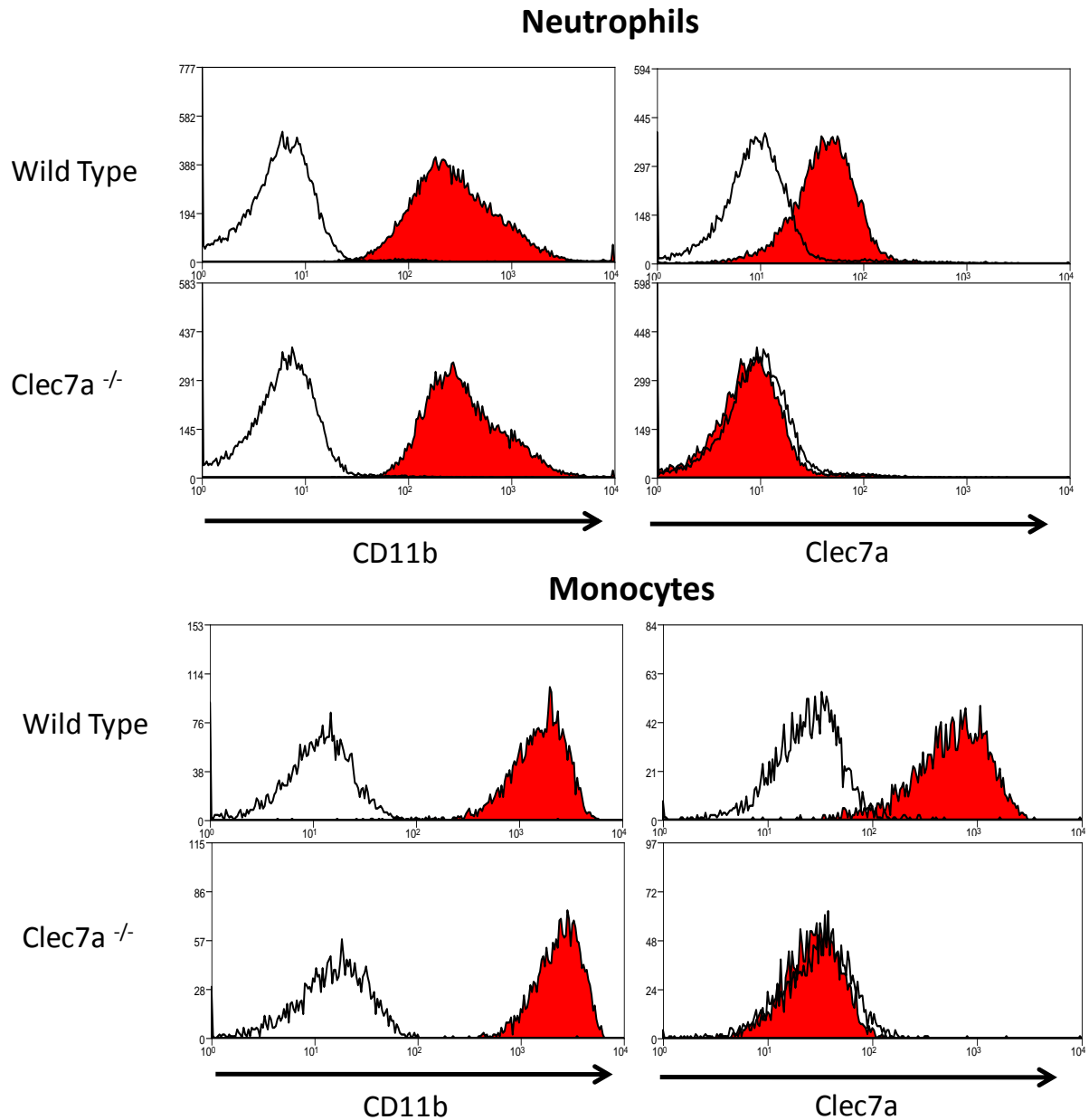


Figure 4.5: Expression of Clec7a and CD11b on the neutrophils of wild type and *Clec7a*^{-/-} mice. Both cell types express comparable levels of CD11b. Only wild type cells show clec7a expression. White histograms show isotype control staining. Red histograms show anti-CD11b or anti-Clec7a expression. Data is representative of 3 independent experiments.

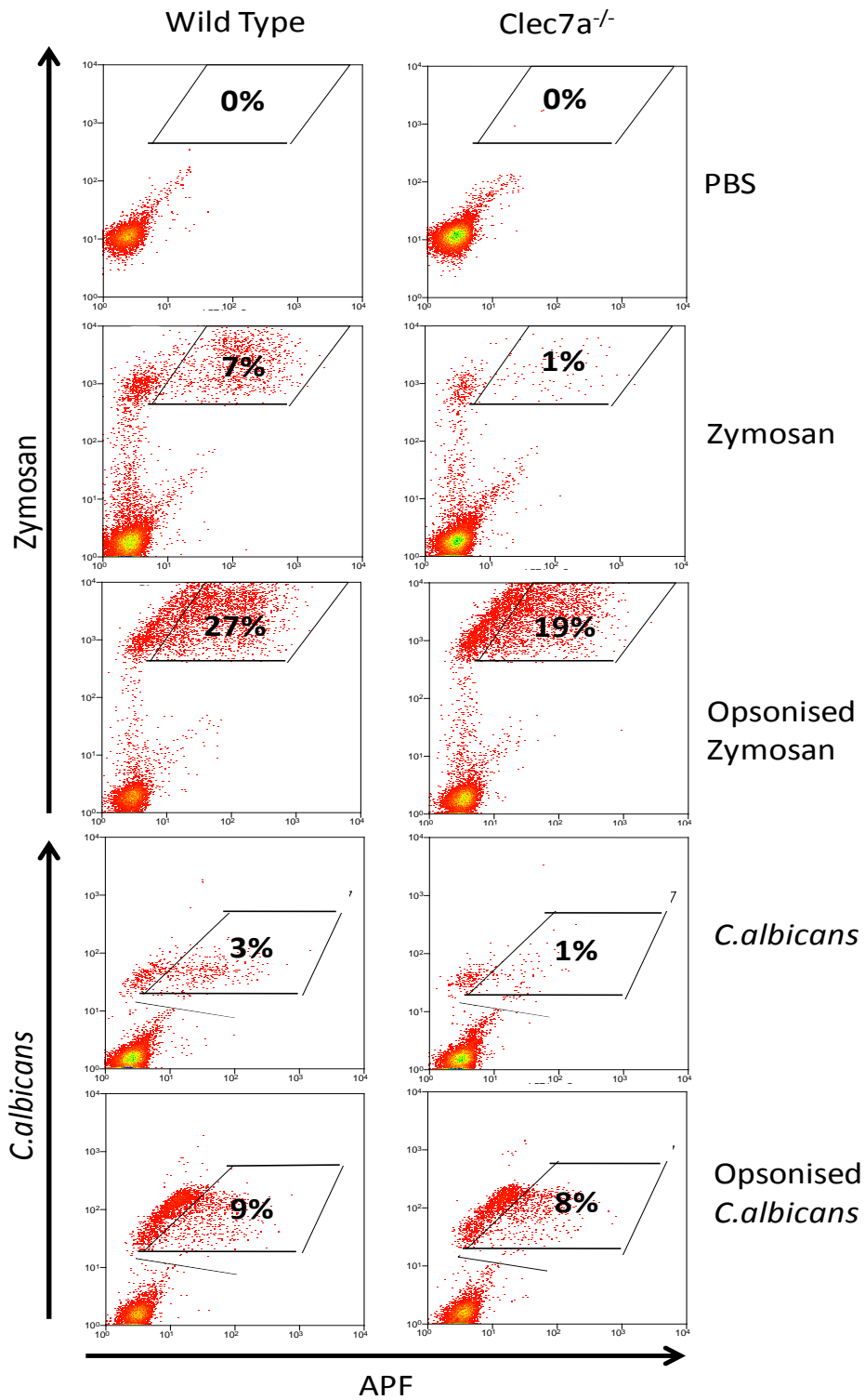


Figure 4.6: Flow-cytometric plots showing the association of neutrophils with labelled zymosan or live *C. albicans* and the resulting production of ROS as measured via APF fluorescence. Data representative of 3 independent experiments.

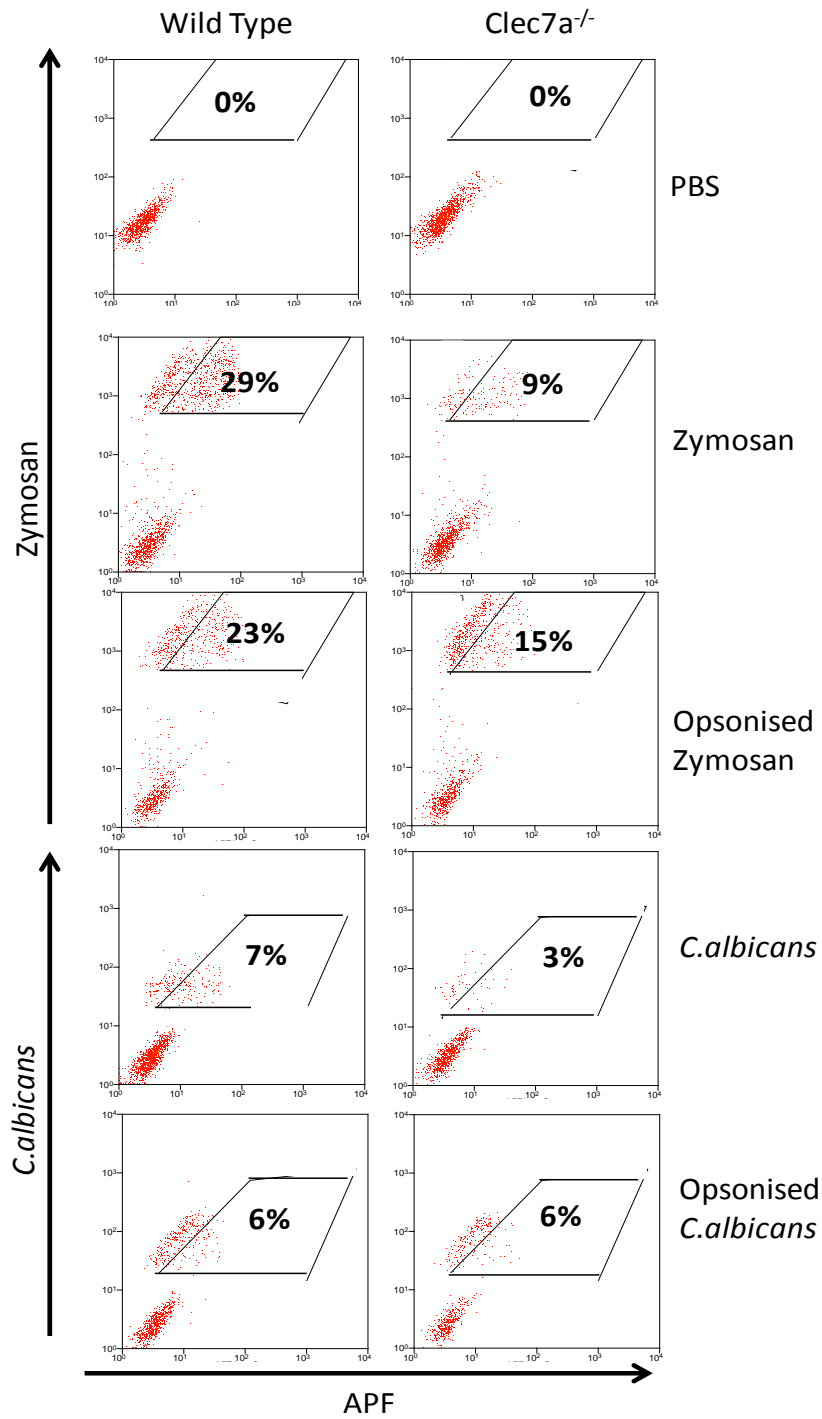


Figure 4.7: Flow-cytometric plots showing the association of inflammatory monocytes with labelled zymosan or live *C.albicans* and the resulting production of ROS as measured via APF fluorescence. Data representative of 3 independent experiments.

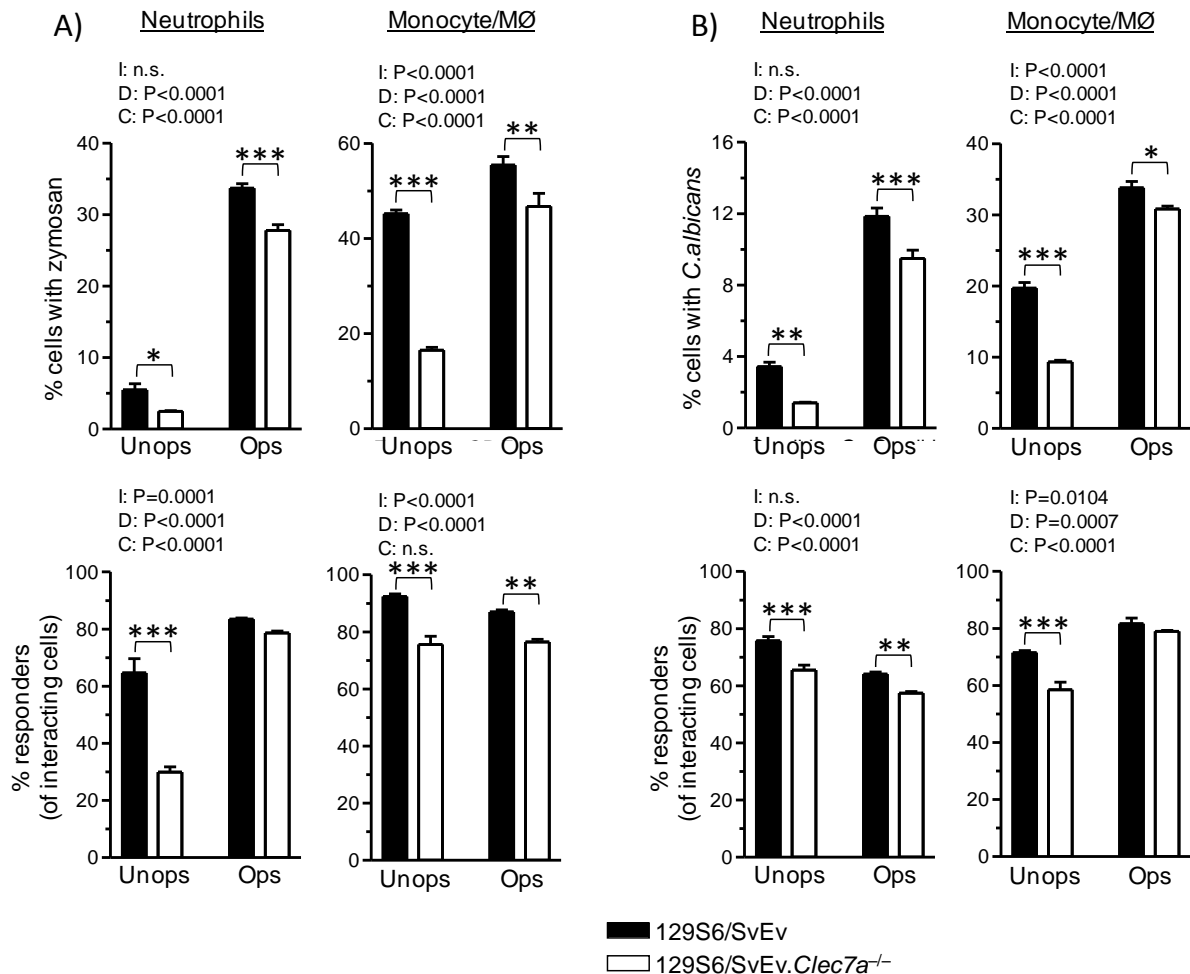


Figure 4.8: Graphical analysis of the recognition and response to zymosan (2×10^6 particles) and *C. albicans* (2×10^6 live cells) of primary neutrophils and monocytes. A) Inflammatory cells were loaded with APF and then incubated with serum-opsonised or non-opsonised zymosan for 15 minutes. After this time the association of the inflammatory cells with zymosan was measured by flow-cytometry (upper panels) and in those cells that were interacting with zymosan the evidence for fluorescent conversion of APF was also quantified (lower panels). B) The experiments shown in (A) were repeated with live pacific orange-labelled *C. albicans*. Neutrophils and monocytes stimulated with either serum opsonised or unopsonised live *C. albicans*. Unops = non-opsonised; Ops = opsonised. Both (A) and (B) are representative experiments from 3 independent experiments and data shown represented the mean \pm SEM of triplicates. Both (A) and (B) were analysed by two-way ANOVA with Bonferroni post-tests. I = Interaction; D = significance of the effect of Clec7a; C = significance of effect of complement opsonisation. P values derived from individual Bonferroni post-tests are indicated with bracketed pairs of samples. P values: *= $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$.

4.5. Role of Clec7a and Complement Opsonisation in β -glucan Recognition by Peripheral Blood Neutrophils

Whole blood was collected from 129S6/SvEv and 129S6/SvEv.*Clec7a*^{-/-} mice in heparin. The cells were washed, preloaded with APF and stimulated with zymosan (2×10^6 particles), opsonised zymosan, *C. albicans* (2×10^6 live cells) or opsonised *C. albicans*. PBS was used as a negative control. Neutrophils were identified via draq5 staining of DNA and ly-6G positive staining (figure 4.9). The neutrophils were collected via flow cytometry and analysed for association and production of reactive oxygen species (figure 4.10).

Clec7a deficiency had no effect on the ability of these cells to associate with or respond to zymosan or *C. albicans* (figure 4.11). Opsonisation of zymosan increased the ability of the neutrophils to associate with and respond to the zymosan however, although association with *C. albicans* was increased by opsonisation of the yeast, response was not affected (figure 4.11). This suggests that *Clec7a* is not essential for binding or response in peripheral blood neutrophils, but that in order for significant binding to occur, opsonisation of the particle is required.

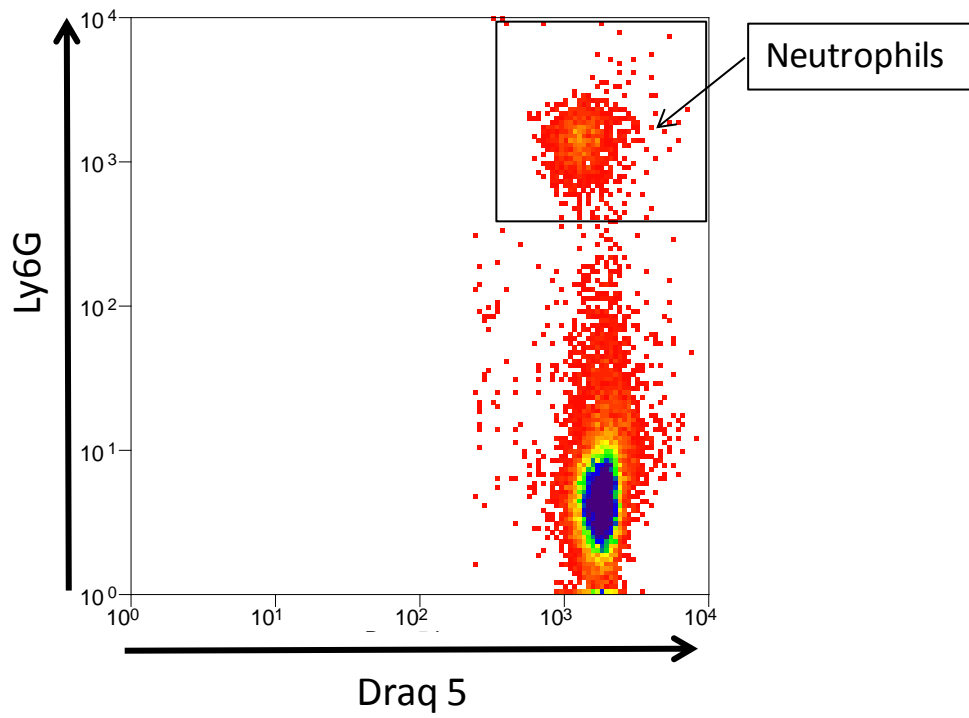


Figure 4.9: FACS plot showing the identification of neutrophils from heparinised whole blood using draq 5 (DNA dye) and Ly6G staining. Non-leukocytes (Draq5⁻) are excluded by a Draq5 threshold gate.

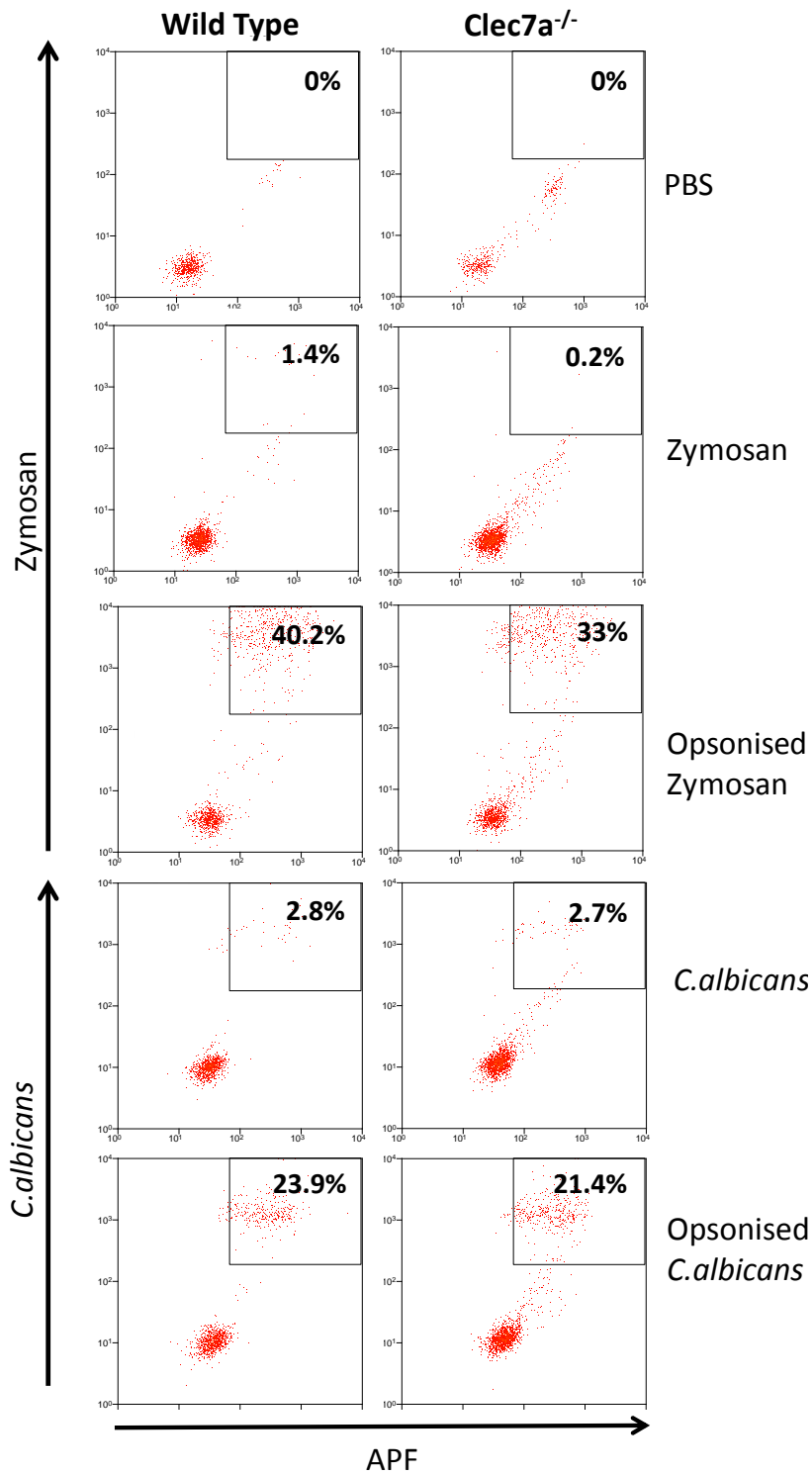


Figure 4.10: FACS plots showing individual samples of whole blood neutrophil association with labelled zymosan or live *C.albicans* and the resulting production of ROS as measured via APF fluorescence. Data representative of 3 independent experiments.

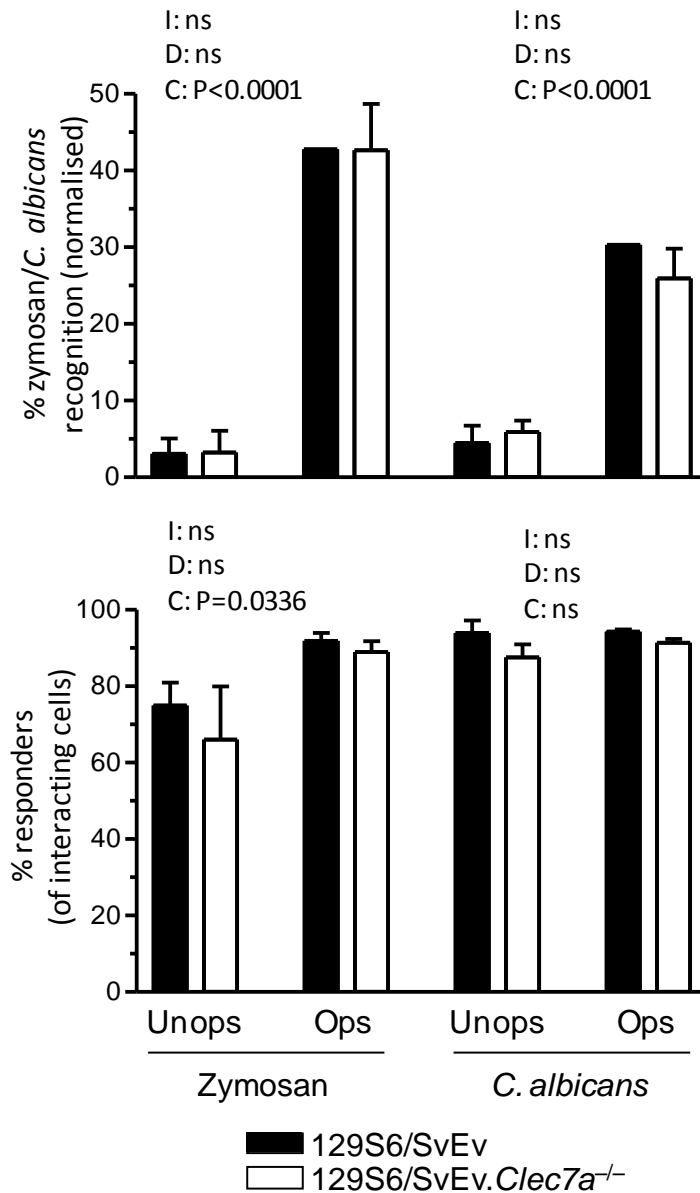


Figure 4.11: Graphical analysis of the recognition and response to zymosan and *C. albicans* of neutrophils in whole blood. *Clec7a* deficiency had no effect on the ability of neutrophils to bind or respond to zymosan and *C.albicans*. No opsonisation of the stimulant resulted in very low levels of recognition, which was restored with serum opsonisation. Unops = non-opsonised; Ops = opsonised. Data is representative experiments from 3 independent experiments and data shown represents the mean±SEM of triplicates. Data was analysed by two-way ANOVA with Bonferroni post-tests. I = Interaction; D = significance of the effect of *Clec7a*; C = significance of effect of complement opsonisation.

4.6. Investigations into the Role of Clec7a on Primary Inflammatory and Whole Blood Neutrophils Using a Clec7a Specific Agonist

Both zymosan and *C. albicans* are recognised by several different receptors on immune cells so to look more specifically at Clec7a, curdlan microparticles were used. Curdlan microparticles are particulate β -glucans which are approximately 2.5-3 μ m in size, can be phagocytosed, and are selective Clec7a agonists (Rosas, Liddiard et al. 2008).

Inflammatory neutrophils and whole blood neutrophils were stimulated with different concentrations of curdlan microparticles and the ROS response measured. Clec7a deficient cells, both inflammatory and whole blood, showed a consistent level of baseline ROS response up to concentrations of 1 mg/ml (figure 4.12.A). In contrast, wild type inflammatory neutrophils were able to respond at all concentrations, from approximately 25% of cells responding at 30 μ g/ml, up to over 60% of cells responding at 1 mg/ml (figure 4.12.A). Wild type whole blood neutrophils were much poorer in their ability to respond to curdlan microparticles. These cells showed a baseline response for the lower concentrations and at 1 mg/ml, less than 20% of the cells gave an ROS response (figure 4.12.A).

To determine the extent to which the use of whole blood and the anticoagulant affect the outcome of the assay, inflammatory neutrophils were mixed with whole blood neutrophils. Whole blood was taken from CD45.2⁺ mice, while inflammatory neutrophils obtained from CD45.1⁺ mice. Using the CD45.1 antibody allowed for the differentiation between the two blood and inflammatory neutrophils. This introduced red blood cells into the inflammatory assay and therefore any differences seen between the two cell types would be due to the

pre-activation of the inflammatory cells. This also exposed the inflammatory cells to heparin.

Inflammatory neutrophils that had been mixed with whole blood neutrophils in a 1:1 ratio, did not show the same level of response as those that had not been mixed with whole blood (figure 4.12.B). They showed the same level of response as whole blood neutrophils. Whole blood cells were collected using heparin as an anticoagulant. Due to its well reported anti-inflammatory effects (Young 2008), inflammatory neutrophils that had been collected in the absence of heparin, were exposed to the same concentration of heparin as the whole blood cells for 10 minutes, then washed, to determine whether heparin has an effect on the ability of neutrophils to respond to the curdlan microparticles. These cells showed a significant decrease (t -test: $P = 0.0091$) in the percentage of cells that responded (figure 4.12.B).

To address some of these problems the experiment was repeated with sodium citrate as an alternative anticoagulant and the blood cells lysed with ACK lysis buffer, after which the inflammatory cells were mixed with the blood cells, all prior to loading with APF. There were no differences seen between inflammatory neutrophils and blood neutrophils in this assay (figure 4.12.C).

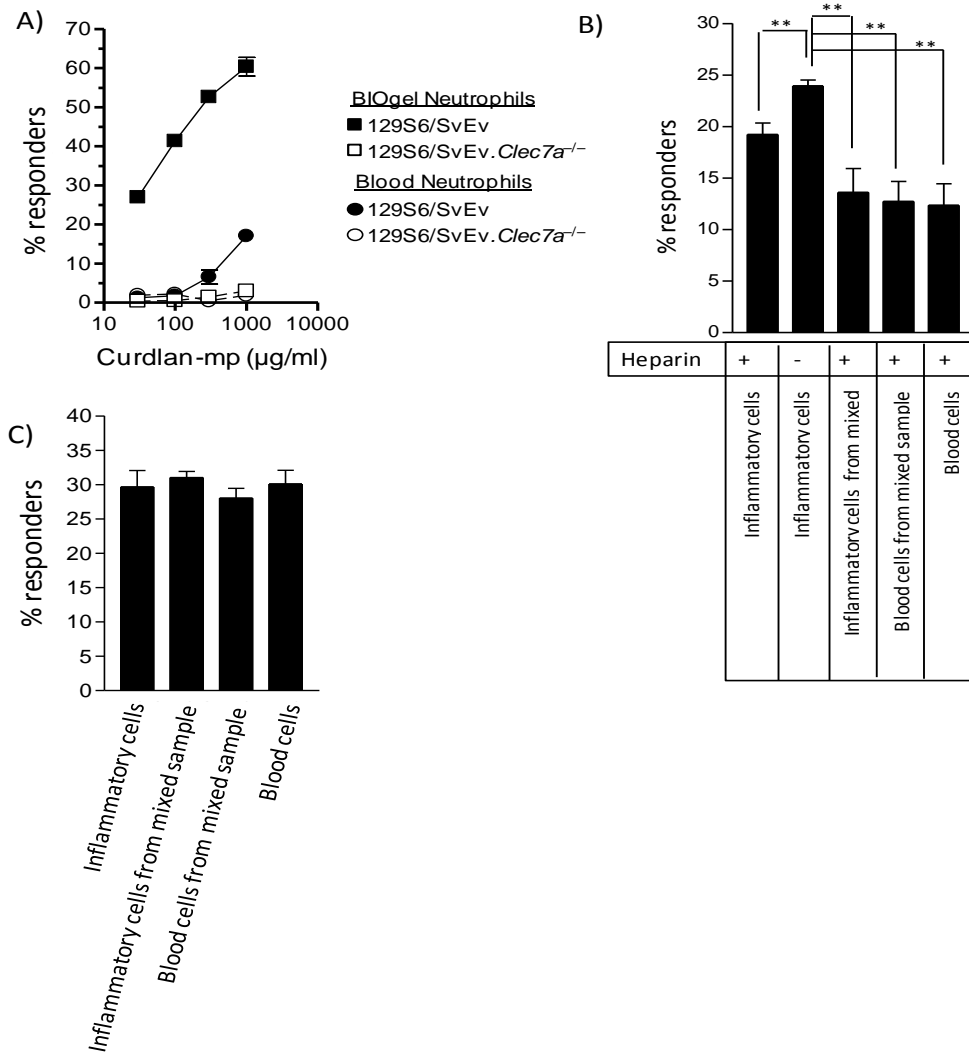


Figure 4.12: Blood and inflammatory neutrophil responses to curdlan mp. A) Graph showing the percentage of cells responding to curdlan mp by producing ROS in both inflammatory and whole blood neutrophils from wild type and Clec7a deficient mice. Wild type inflammatory neutrophils show a much greater ability to respond than whole blood and clec7a deficient neutrophils. Data represents mean \pm SEM and is representative of 3 independent experiments B) Graph showing the percentage of cells responding to curdlan mp (300 μ g/ml) by producing ROS in whole blood assay that had been spiked with inflammatory neutrophils in an approximate 1:1 ratio. Data represents mean \pm SEM and is representative of 2 individual experiments. C) Graph showing the percentage of cells responding to curdlan mp by producing ROS in an assay with whole blood cells that had been collected with sodium citrate, lysed with ACK lysis buffer and mixed with inflammatory neutrophils in a 1:1 ratio prior to stimulation. Data represents mean \pm SEM and is representative of 3 independent experiments. Data was analysed using T Test; P values: **=P<0.01.

4.7. The Effect of Clec4n (Dectin-2) Blockade on Yeast Particle Recognition by Inflammatory Neutrophils

A role for dectin-2 in neutrophil fungal recognition has not been previously reported, perhaps because it was thought to be minimally expressed by these cells, with most expression confined to inflammatory monocytes and tissue resident macrophages (Taylor, Reid et al. 2005). I found that inflammatory neutrophils like monocytes express dectin-2, though not at comparable levels, with inflammatory monocytes showing greater dectin-2 expression (figure 4.13).

Inflammatory neutrophils and monocytes were tested for their ability to respond via ROS production to opsonised and non-opsonised zymosan particles. *Clec7a*^{-/-} cells were used to unmask any redundancy in the system and blocking of dectin-2 was achieved by pre-incubation of the cells with anti-dectin-2 (D2.11E4) and Rat IgG2a was used as an isotype control. Although blocking dectin-2 on neutrophils shows no significant impairment to zymosan binding it does significantly impair the ability of neutrophils to respond to non-opsonised zymosan particles. This impairment is not seen in response to opsonised particles (figure 4.14). Blocking dectin-2 has a greater effect on inflammatory monocytes by impairing their ability to bind non-opsonised zymosan and their ability to respond to both opsonised and non-opsonised zymosan (figure 4.14).

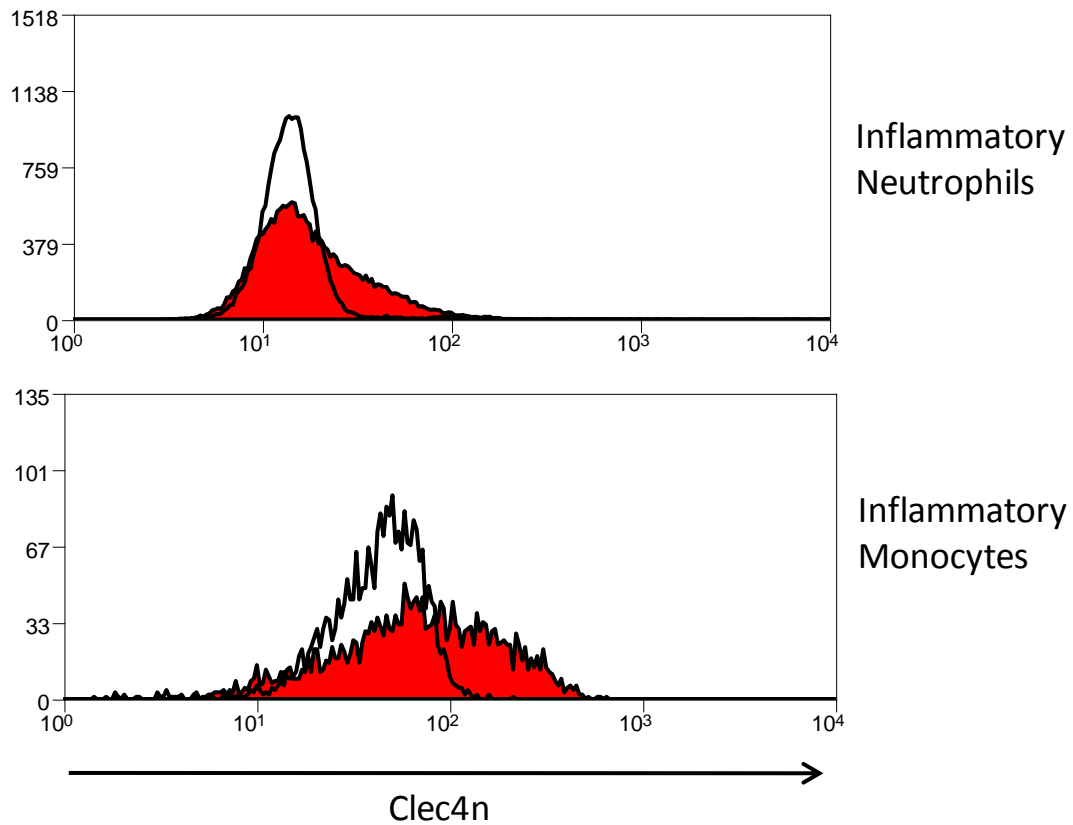


Figure 4.13: Expression of dectin-2 on the inflammatory neutrophils and monocytes of wild type mice. Both cell types show some expression of dectin-2 but not at comparable levels. Neutrophils show less expression of dectin-2 than monocytes. White histograms show isotype control staining. Red histograms show anti-Clec4n (D2.11E4) expression. Data representative of 2 independent experiments.

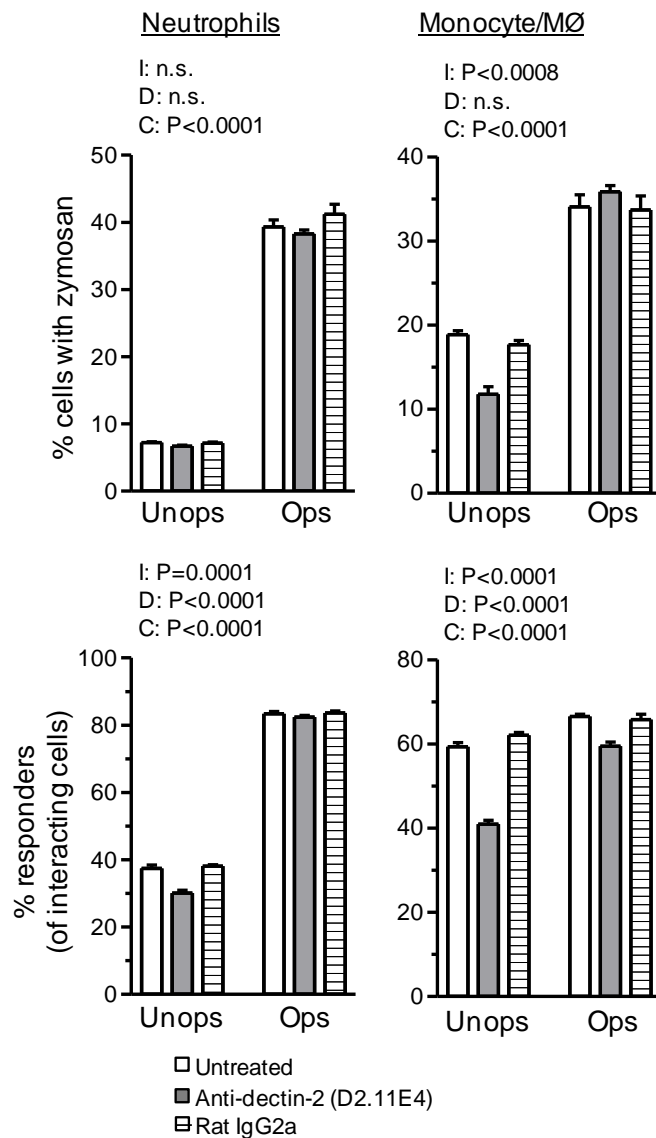


Figure 4.14: Graphical analysis of the recognition and response to zymosan of primary neutrophils and monocytes from Clec7a deficient mice. The cells were left untreated (white bar), pre-treated with anti-dectin-2 (grey bar) or pre-treated with a rat IgG2a control antibody (horizontal lines bar). Unops = non-opsonised; Ops = opsonised. Data is representative experiments from 3 independent experiments and data shown represented the mean±SEM of triplicates. Data was analysed by two-way ANOVA. I = Interaction; D = significance of the effect of Clec4n; C = significance of effect of complement opsonisation.

4.8. Application of *In Vitro* APF Model to Human Neutrophils

i) Extravasated salivary neutrophils

The model of looking at the role of Clec7a in fungal interactions was applied to primary human neutrophils. Human saliva is a good source of inflammatory neutrophils (al-Essa, Niwa et al. 1994), with between 1.1×10^7 – 2×10^7 cells harvested. The cells were loaded with APF and stimulated with zymosan and response analysed. Two different Clec7a-specific antibodies (GE2 (Willment, Marshall et al. 2005) and anti-human Clec7a (mAb 259931) by R&D Systems) were added to some samples to attempt to block Clec7a. Laminarin, a soluble β -glucan, was also added as a blocking agent. Neutrophils were identified by flow-cytometry with a CD66-specific staining and confirmed via cytopsin preparations (figure 4.15). It was immediately apparent that these inflammatory neutrophils would not be suitable for the APF assay as they showed an extraordinarily high background level of ROS production that would mask any genuine results due to zymosan stimulation (figure 4.16). The level of zymosan binding was assessed to determine the success of the blocking antibodies. Blocking of Clec7a appeared unsuccessful in reducing zymosan binding with neither antibody nor laminarin having a detectable effect in this assay (figure 4.17). The cytopsin preparations confirmed the presence of neutrophils, as identified by their highly stained, multi-lobed nuclei (figure 4.15B). Epithelial cells were also present but were excluded from the flow-cytometric assay by their lack of CD66 staining.

ii) Peripheral blood neutrophils

Human whole blood was used as a source of sterile neutrophils. The blood was collected in sodium citrate to avoid any possible negative effects of heparin and a whole blood APF assay was performed. The assay was successful in that there was no background ROS

production and the response seen was clearly as a result of particulate stimulation (figure 4.18A). Curdlan microparticles as well as zymosan were used to stimulate these cells. The zymosan was able to elicit a greater APF response than the curdlan microparticles; however, no evidence of Clec7a blocking by the antibodies was seen (figure 4.18B). Blocking also did not affect the binding of zymosan (figure 4.18C).

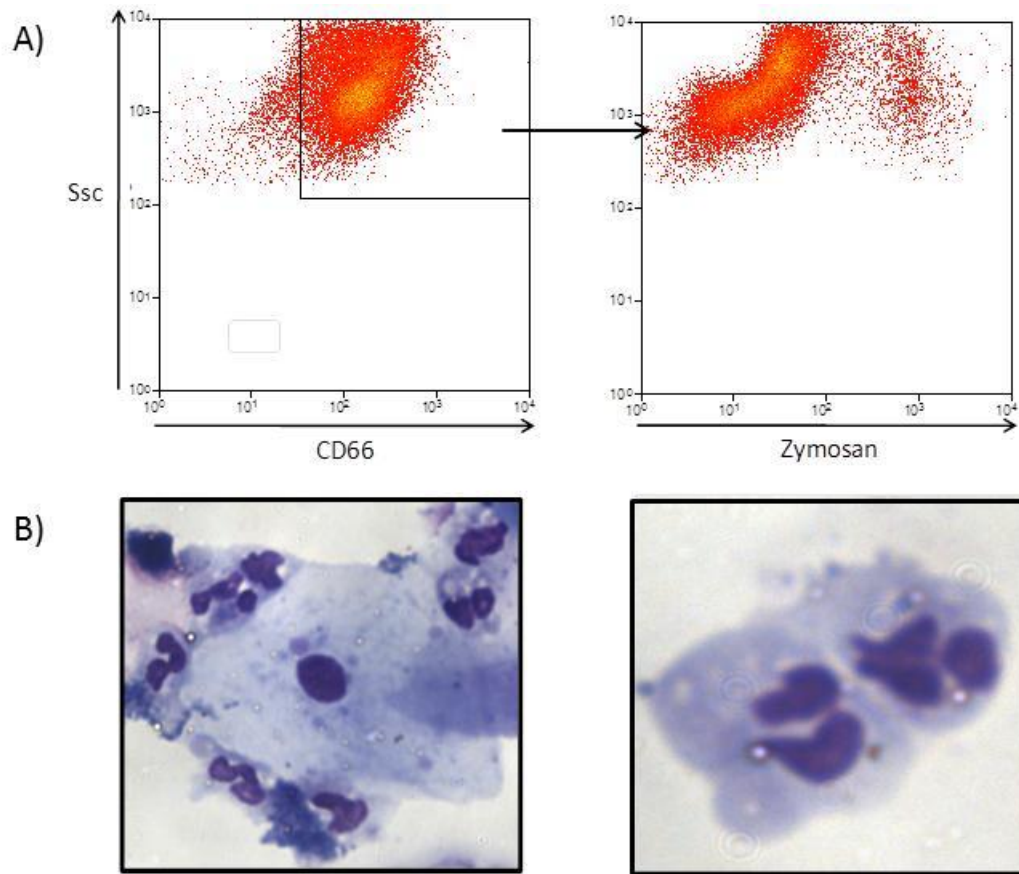


Figure 4.15: Identification of human salivary neutrophils. A) Flow-cytometric plots showing CD66 identification of salivary neutrophils and their association with zymosan. B) Cytospin slides showing cells from human saliva. The picture to the left shows an epithelial cell surrounded by numerous neutrophils, while the picture to the right shows close up of two neutrophils with their distinct highly stained, multi-lobed nuclei. All cells were stained with eosin and methylene blue. Data representative of 3 independent experiments.

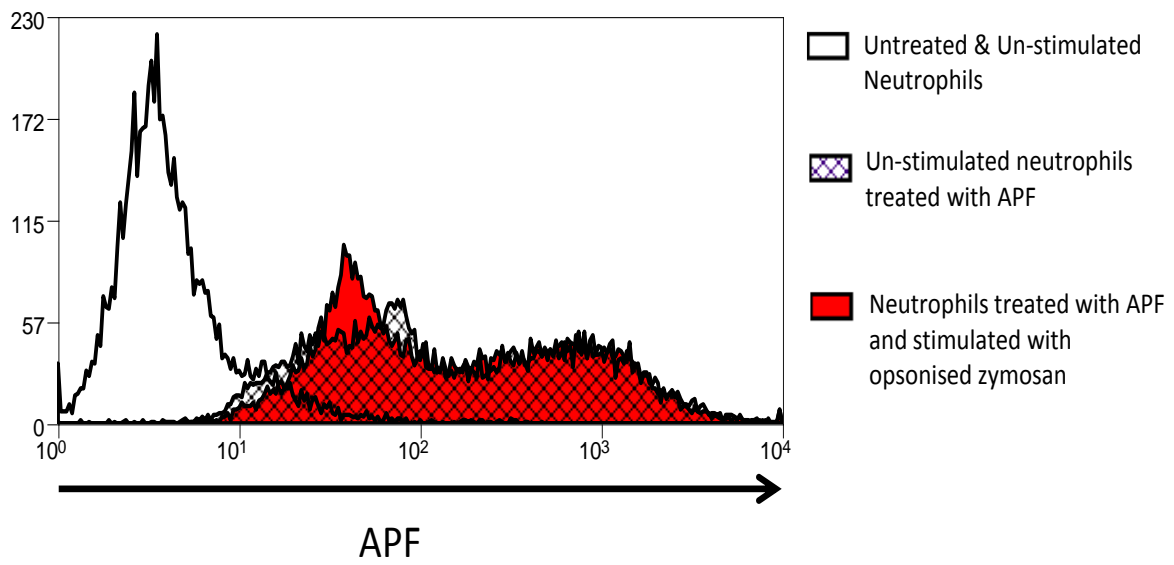


Figure 4.16: Histograms showing the production of ROS via APF fluorescence in salivary neutrophils. The white histogram shows neutrophils that have been not been treated with APF or stimulated with zymosan. The purple crosshatched histogram shows neutrophils treated with APF and not stimulated with zymosan and the red histogram shows neutrophils that have been treated with APF and have bound zymosan particles. Cells not incubated with APF and not stimulated with zymosan showed limited autofluorescence. Both zymosan stimulated and unstimulated APF treated neutrophils showed similarly high amounts of APF conversion into the fluorescent reporter indicating a high level of basal ROS production in extravasated salivary neutrophils that could mask any zymosan induced ROS production. Data is representative of 2 independent preliminary experiments to determine background APF fluorescence levels.

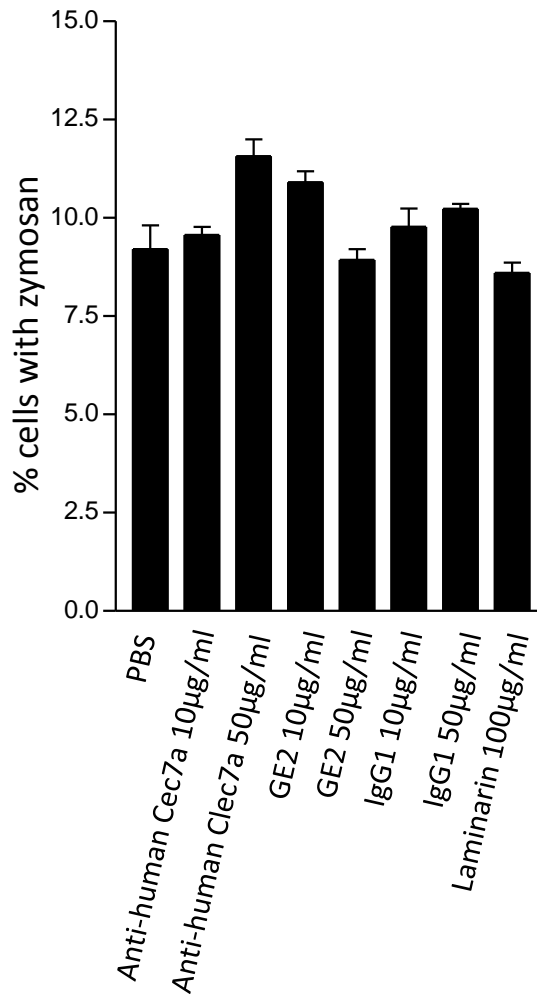


Figure 4.17: Graph showing the percentage of salivary neutrophils that have associated with non-opsonised zymosan. Only a small percentage of neutrophils interacted with the zymosan particles and neither of the monoclonal antibodies specific to dectin-1 nor laminarin were able to block this zymosan binding. Data representative of 2 independent experiments from 2 different donors. Data shown represent the mean±SEM of triplicates.

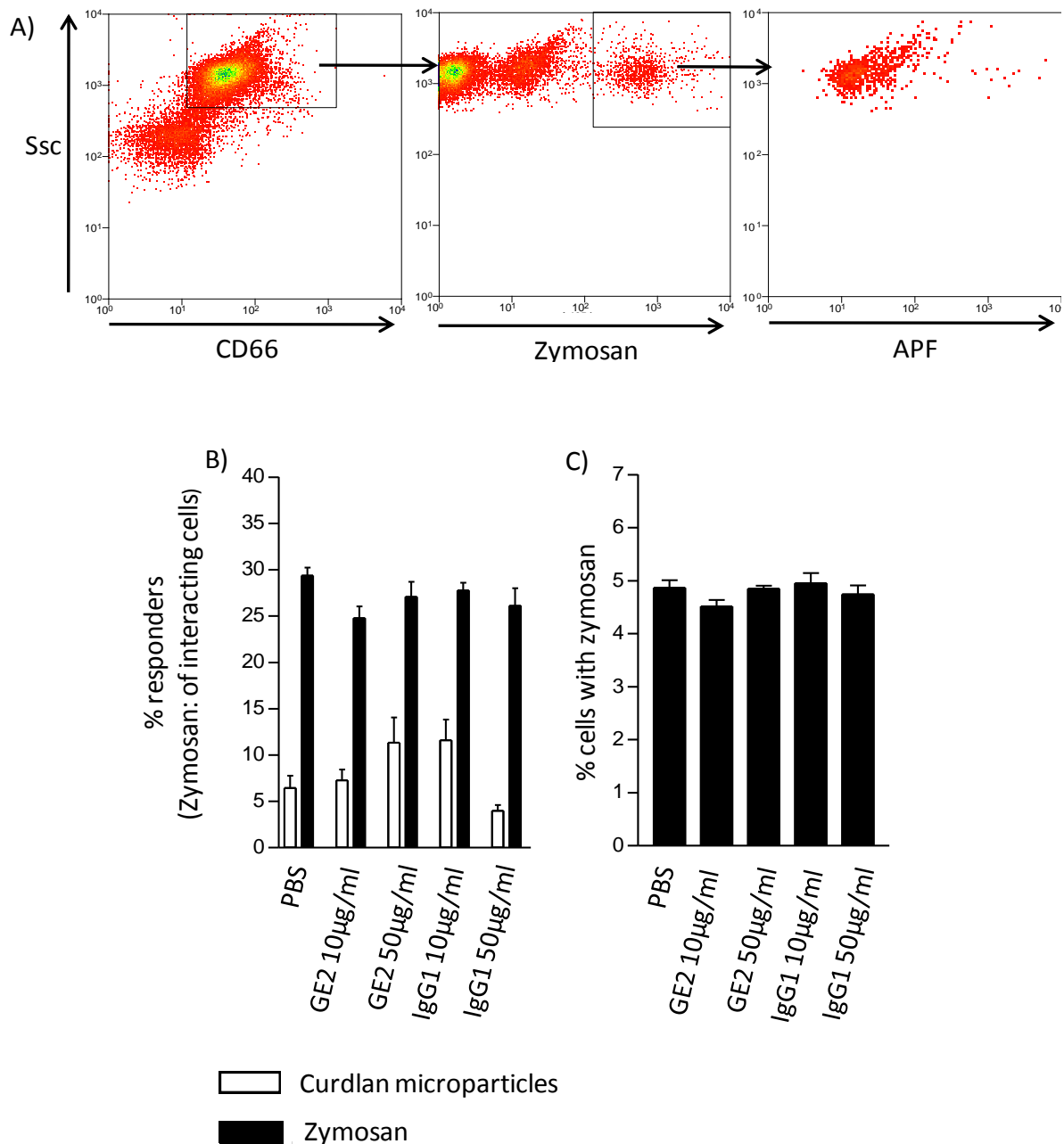


Figure 4.18: Identification of human peripheral blood neutrophils and their recognition and response to curdlan microparticles and zymosan. A) Flow-cytometric plots showing CD66 identification of blood neutrophils, their association with zymosan and subsequent APF response. B) Graph showing the percentage of curdlan microparticle stimulated cells that have responded by ROS production and the percentage of zymosan associated cells that have responded with ROS production. GE2 shows no blocking action with zymosan or curdlan microparticles. Data from single preliminary experiment from a single donor. C) Graph showing the percentage of whole blood neutrophils that have associated with zymosan. Data representative of 2 independent experiments from 2 different donors. Data shown represented the mean±SEM of triplicates.

iii) Inflammatory 'skin window' neutrophils

A process of obtaining sterile inflammatory neutrophils, via recruitment through a skin window (Marks, Radulovic et al. 2007), produced clearly identifiable neutrophils (figure 4.20A). However, this technique produced far fewer neutrophils than saliva extraction, with each 6 x 3 cm window producing less than 10^6 cells. Like the salivary neutrophils, these cells showed a significant background production of ROS (figure 4.19). This is consistent with priming effects associated with their extravasation to an inflammatory site and with possible further activation in the wound. Binding of these cells to zymosan was extremely low and Clec7a blocking antibodies had no effect on the level of binding (figure 4.20B).

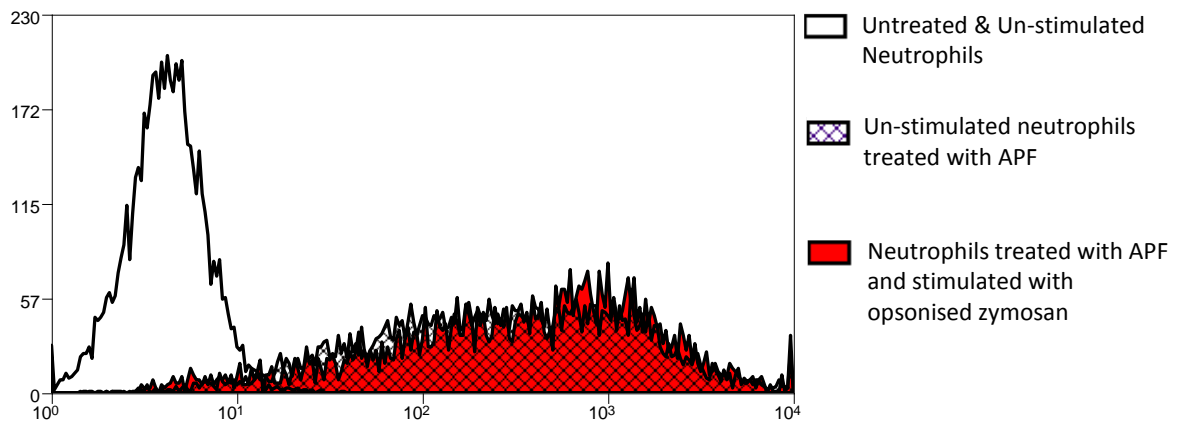


Figure 4.19: Histograms showing the production of ROS via APF fluorescence in neutrophils derived from the skin window technique. Cells not incubated with APF and not stimulated with zymosan showed background autofluorescence (white histogram). There was no difference observed between the cells incubated with APF but not stimulated with zymosan (purple cross-hatched histogram) and the cells incubated with APF and bound to zymosan (red histogram). Both groups of APF loaded cells show a high level of APF expression meaning that the background APF expression is too high as to be indistinguishable from APF fluorescence induced by zymosan interactions. Data shows the result of a preliminary experiment to determine background APF fluorescence levels.

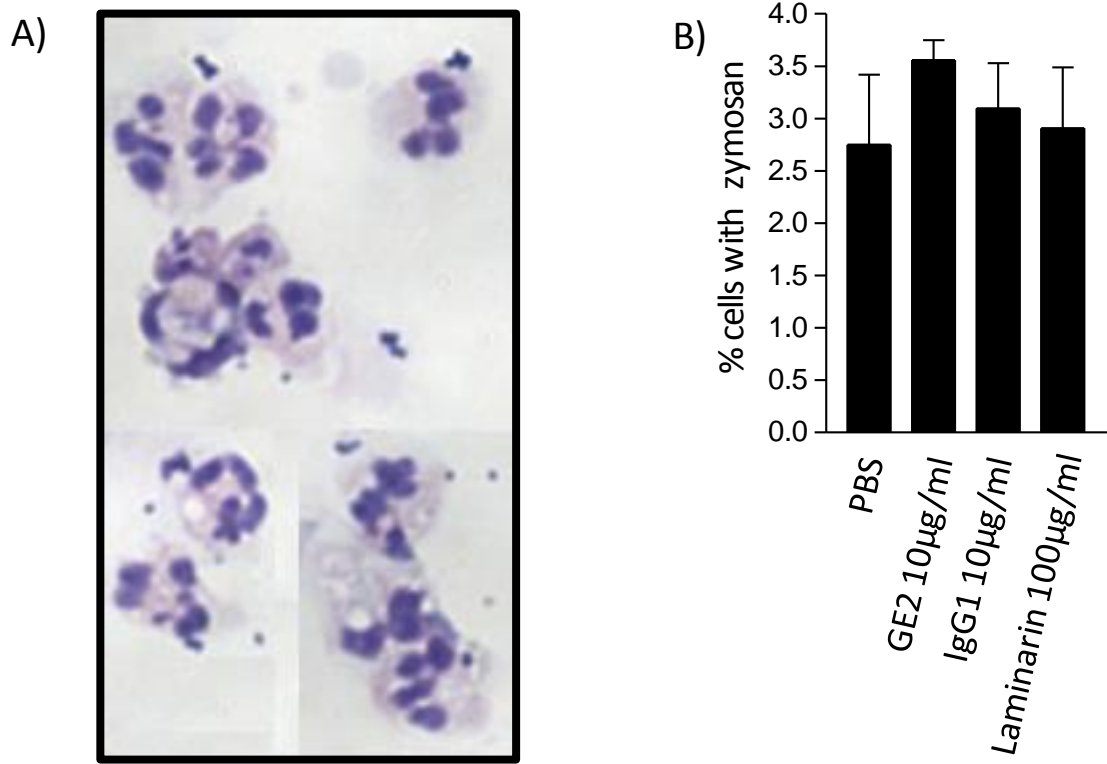


Figure 4.20: Cytospin preparation of 'skin window' neutrophils and their association with zymosan. A)

Cytospin slide showing human inflammatory neutrophils recruited through the skin via the skin window. Cells

were stained with eosin and methylene blue. B) Graph showing the percentage of neutrophils that are

associated with zymosan. The addition of GE2 and laminarin had no blocking effect on the association of

zymosan with neutrophils. IgG1 was used as a negative control for the GE2 antibody. Data is representative of

2 independent experiments from a single donor. Data shown represented the mean ± SEM of triplicates.

4.9. Conclusion

Zymosan induced peritonitis is typically studied using zymosan doses of at between 2×10^7 - 2×10^8 particles. Previous studies with *Clec7a*^{-/-} mice looked at doses $\leq 2 \times 10^6$ particles (Taylor, Tsoni et al. 2007), where the particles were sufficiently cleared by tissue resident macrophages. An increased dose of zymosan would 'overwhelm' the resident macrophages and result in the activation of alternative inflammatory responses helping to drive the recruitment of inflammatory cells to the peritoneal cavity. Previous studies with low doses of zymosan have shown that *Clec7a* is involved in the activation of tissue resident phagocytes in response to fungal challenges (Taylor, Tsoni et al. 2007; Rosas, Liddiard et al. 2008). A higher dose of zymosan would allow for the direct study of the role of *Clec7a* on inflammatory neutrophils and monocytes in response to fungal challenges by permitting direct interaction of the cells with zymosan after their recruitment to the peritoneal cavity. The inflammatory parameters of a high dose model of zymosan peritonitis were established. After i.p. injection of 2×10^7 particles of zymosan, an acute inflammatory response was observed typified by a large drop in recoverable resident macrophage numbers and a large influx of inflammatory neutrophils and some monocytes. Neutrophil numbers and association with zymosan peaked 4 hours after zymosan injection so this was considered a useful time point for the study of neutrophil interactions. Macrophage interactions with zymosan peaked after 3 days and this could either be attributed to the apoptosis of dead neutrophils containing zymosan particles or the persistence of tissue resident macrophages late into the inflammatory process (Davies, Rosas et al. 2011).

The role of *Clec7a* on primary neutrophils was studied with the aid of *Clec7a* deficient mice. *Clec7a* deficient mice were impaired in their ability to recruit neutrophils to the site of

infection in response to zymosan. These neutrophils were also impaired in their ability to bind to zymosan particles. This could suggest a direct causal link between Clec7a deficiency on neutrophils and impaired ability to recognise fungal particles. However, the significantly reduced levels of IL-6 observed in conjunction with lower numbers of neutrophils recruited suggest an impaired inflammatory response in general. This may occur via a lack of Clec7a on tissue resident cells, resulting in a reduced inflammatory response to zymosan and therefore decreased recruitment and cellular activation of neutrophils.

In order to specifically look at Clec7a on neutrophils an *ex vivo* assay with primary neutrophils was established. This *ex vivo* assay showed that Clec7a deficiency does directly impair the ability of inflammatory neutrophils to recognise and respond to fungal particles and live *C. albicans*. However, *in vivo*, there are a variety of recognition mechanisms that neutrophils utilise to help phagocytose fungal pathogens. Complement opsonisation is a crucial recognition system and the abundance of complement in the *in vivo* environment should ensure effective recognition of an invading organism. Serum opsonisation of both zymosan and *C. albicans*, *in vitro*, prior to exposure to the neutrophils significantly increased their binding potential. These data show that although Clec7a does contribute to the recognition and response to fungal particles, its role is partially redundant as complement opsonisation has a larger effect on particle recognition. Very recently it was shown that Clec7a activates CR3 in neutrophils and that both receptors work together to enhance neutrophil response to fungal pathogens. The ability of Mg^{2+} , which activates CR3, to restore phagocytosis of zymosan suggests that the reduction of recognition and phagocytosis of fungal particles in Clec7a deficient neutrophils is down to a lack of CR3 activation by Clec7a (Li, Utomo et al. 2011). My data supports the suggestion that *in vivo*

Clec7a is not essential to clearance of fungal particles and this suggests that the activation of CR3 by Clec7a *in vivo* is also a redundant process. Further evidence for a partial redundancy in the system can be seen in the observation that Clec7a deficient human neutrophils are able to phagocytose yeast particles under adherent conditions and in the presence of serum (Ferwerda, Ferwerda et al. 2009).

The method for the whole blood assay produced several challenges that made it difficult to draw any conclusions about the differences seen between inflammatory peritoneal neutrophils and blood neutrophils. The use of heparin as an anticoagulant is common; however, it has been shown to have negative side effects that could skew the result of any assay looking at cellular immunity (Young 2008). This was overcome by the use of sodium citrate as an alternative anticoagulant. Also the presence of red blood cells in the whole blood assay may have blocked interactions between neutrophils and the particulate stimulants and led to the diminished responses seen in blood neutrophils in the whole blood assay. This was addressed by first introducing inflammatory peritoneal cells into the whole blood assay. The results suggested that the red blood cells were interfering with cell:particle interactions. In order to remove this interference, the red blood cells were lysed. This seemed to increase the percentage of blood neutrophils that were responding, up to the same level of the inflammatory cells. This could be indicative of two things:

1. The removal of the red blood cells removes the interference and the natural ability of the cells to respond to particulate challenge is restored.
2. The lysing of the red blood cells resulted in priming of the cells making them more able to respond than would otherwise be the case.

The attempt to apply this model to primary human neutrophils using similar assay conditions was largely unsuccessful due to a mixture of assay specific factors. Neutrophils obtained from the saliva were already responding to challenges from bacteria present in the mouth and this made exploring specific cellular responses of any interactions very difficult. This same limitation was present with skin window produced neutrophils. Only peripheral blood neutrophils exhibited a largely ROS⁻ phenotype after isolation. In order to specifically look at the role of Clec7a on human neutrophils, the blocking of Clec7a was required. The binding of zymosan particles to human neutrophils (isolated from either of the 3 protocols) was consistently low and neither antibody blocking (with two distinct monoclonal antibodies) or the use of a soluble β -glucan were able to successfully show any blocking of Clec7a and this made the study of the receptor on human neutrophils very difficult. Ideally, these studies could be conducted using neutrophils from the blood of naturally Clec7a-deficient humans (Ferwerda, Ferwerda et al. 2009; Plantinga, van der Velden et al. 2009), but these were not available for these studies. Studies using naturally Clec7a-deficient humans have shown that neutrophils are not impaired in their ability to phagocytose and kill live *C.albicans* even in the absence of Clec7a, further highlighting the redundancy Clec7a in fungal recognition (Ferwerda, Ferwerda et al. 2009). This is consistent with the lack of inhibitory activity of the anti-dectin-1 antibodies and soluble glucans observed in this thesis.

Chapter 5: Modelling Innate Immunity

Using Neutrophils Derived From conditionally-Immortalised Cell Lines

5.1. Introduction

In chapter 3, the generation of conditionally-immortalised neutrophil precursor cell lines as a means to minimise animal suffering and the loss of animal life in scientific research and put the three R's (replacement, reduction and refinement) at the forefront of all neutrophil research was discussed. The cell line was found to be morphologically and functionally similar to primary mouse neutrophils. To determine whether these cells are viable substitutes for the use of primary murine neutrophils in basic research, these cells need to be inserted into established models of neutrophil research and their behaviour compared to that of primary neutrophils.

The previous chapter looked at the relative contributions of Clec7a and complement opsonisation in the recognition and innate immune response to fungal pathogens. In this chapter, the aim was to use the *in vitro* generated neutrophils described in chapter 3 to explore the mechanisms described in chapter 4 as a means to demonstrate the usefulness of these cell lines as an effective replacement for animals in the study of immune response mechanisms.

Clec7a has been shown to exist in two distinct isoforms; Clec7a.A and Clec7a.B. Both isoforms are expressed in human and mouse cells (Willment, Marshall et al. 2005; Heinsbroek, Taylor et al. 2006). In order to study Clec7a, *in vitro*, conditionally-immortalised neutrophil progenitor cell lines were generated from *Clec7a*^{-/-} mice and reconstitutions performed using the two different isoforms and MMLV-derived retroviral vectors. This strategy was adopted because of the very low Clec7a expression observed on *in vitro* generated neutrophils (Chapter 3), which already identifies some limitation of *in vitro* neutrophil generation to model immune responses.

As further proof of concept, a conditionally-immortalised neutrophil progenitor cell line was generated from CD11b-deficient (*Itgam*^{-/-}) mice and reconstituted with two different human CD11b isoforms; G230 and A230 (Sachs et al. 2004 and Macpherson et al. 2010). At the time of this study, this polymorphism in *Itgam* has been linked to susceptibility to autoimmune disease (Sachs, Chavakis et al. 2004; Nath, Han et al. 2008; MacPherson, Lek et al. 2011), with no mechanistic explanation to explain this. The recognition and immune response to fungal pathogens of these cell lines was then explored to determine to what level CD11b function had been restored and whether there were any evident functional differences between the two isoforms.

5.2. Generation of *Clec7a*^{-/-} and *Itgam*^{-/-} Conditionally-Immortalised Neutrophil

Precursor Cell lines

Cell lines were generated using femurs from *Clec7a*^{-/-} and *Itgam*^{-/-} C57BL/6 mice as described in chapter 3 and methods 2.5. These knock out cell lines were confirmed not to express Clec7a or CD11b, respectively (Figure 5.1).

5.3. Transduction of Conditionally-Immortalised Neutrophil Precursors with Clec7a

The MyPH8-B6 cell line progenitors were transduced with viral DNA constructs containing *Clec7a.A*, *Clec7a.B* or an empty vector (pMXs-IZ) as described in chapter 3 and methods 2.5.5. The cells were then differentiated (methods chapter 2.5.6) and analysed for differentiation and expression of Clec7a (Figure 5.2). The empty vector progenitors expressed limited levels of endogenous Clec7a compared to the Clec7a transduced cells.

The transduced progenitors both express Clec7a, with the *Clec7a.A* transduced cell line showing greater expression than the *Clec7a.B* cell line (Figure 5.2).

In the *Clec7a* transduced cell lines, Clec7a expression decreased with neutrophil differentiation (Figure 5.2). However, day 4 neutrophils from these cells still showed greater Clec7a expression than the empty vector transduced neutrophils (Figure 5.2). The levels of Clec7a in day 4 neutrophils were noticeably different in cell lines transduced with the two different isoforms. Those differentiated with Clec7a.A showed a much greater cell surface expression of the receptor than those differentiated with Clec7a.B (Figure 5.2).

The *Clec7a*^{-/-} cell line was similarly reconstituted with Clec7a as described above, using the different isoforms. Expression of Clec7a on neutrophils derived from these cell lines was confirmed via RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR). The RT-PCR showed that MyPH8-B6 derived wild type neutrophils predominantly expressed the B isoform of Clec7a. The *Clec7a*^{-/-} cell line neutrophils did not show any Clec7a expression (Figure 5.3.A). Clec7a expression on the transduced cell lines was compared to that on primary neutrophils, and while neutrophils derived from the *Clec7a.A* cell line showed higher Clec7a expression than primary neutrophils, neutrophils derived from cell lines transduced with *Clec7a.B* showed comparable expression levels (Figure 5.3.B). These expression differences of the two isoforms were consistent in repeat transductions (see also Figure 5.2).

Due to the wild type cell line predominantly expressing the Clec7a.B isoform, and the comparable levels of expression produced by cell lines transduced with this isoform, it was decided that all further studies would be conducted using only this isoform. This decision was further supported by a functional assay, where zymosan binding was examined and this

showed that zymosan binding by the neutrophils derived from the *Clec7a.A* transduced cell line showed much higher binding than wild type cells (Figure 5.4.). Although this could be attributed to the isoform itself, it is likely that it is the increased expression of the receptor that produces this effect.

In order to examine the contribution of CD11b to neutrophil recognition of fungal particles in the context of normal *Clec7a* expression, the MyPH8-B6.*Itgam*^{-/-} cell line was transduced with the *Clec7a.B* isoform (or empty control vector) and *Clec7a* expression analysed via flow cytometry. Neutrophils derived from this cell line also showed levels of *Clec7a* expression comparable to primary neutrophils (Figure 5.3.B).

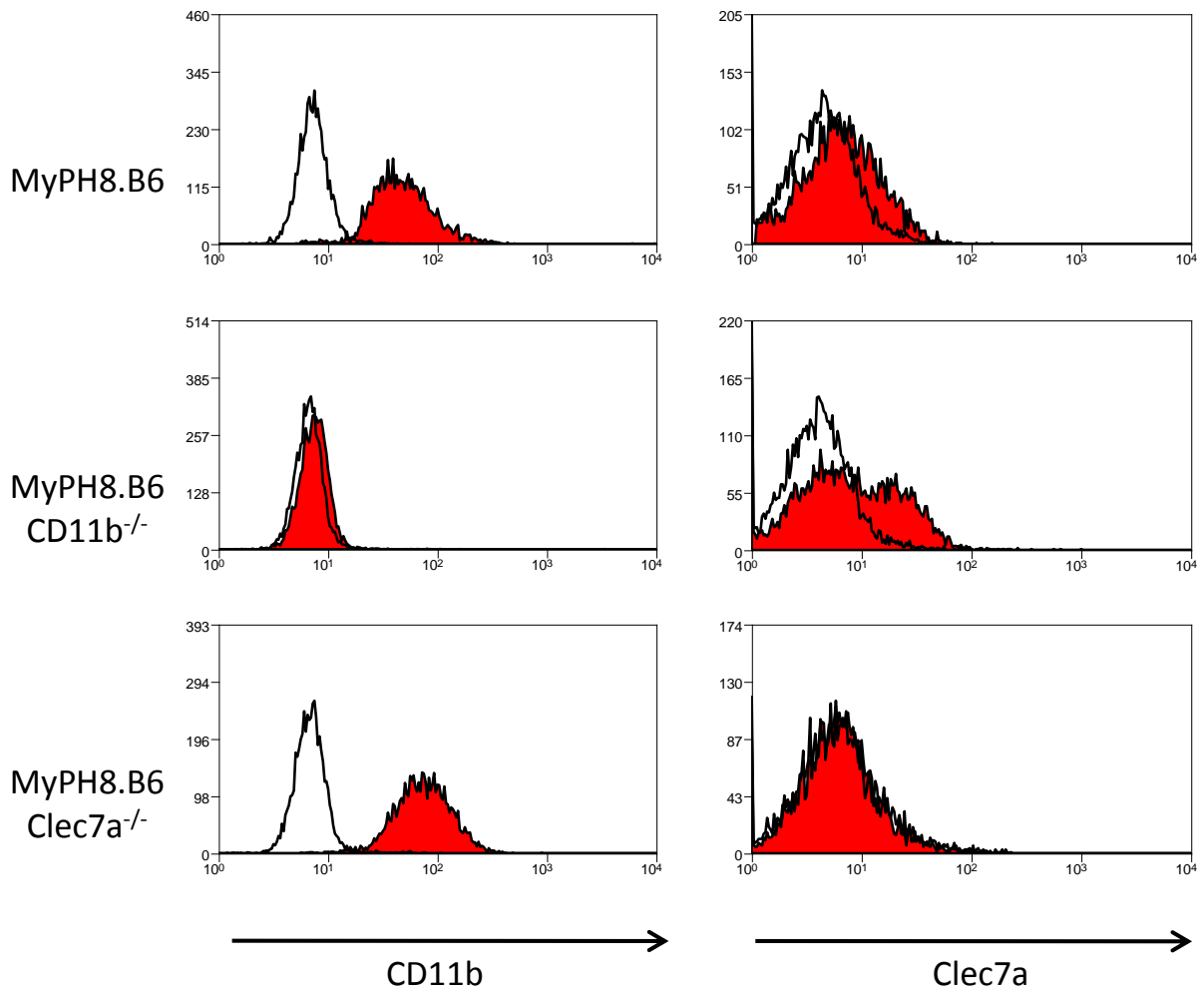


Figure 5.1: Cell surface expression of Clec7a and CD11b on 4 day *in vitro* generated neutrophils produced from conditionally-immortalised neutrophil progenitor cells line. Red histogram show cell surface expression of indicated receptor and white histogram shows isotype control. The cell lines from the *Itgam*^{-/-} and *Clec7a*^{-/-} also confirm the specificity of the antibodies. The wild type MyPH8-B6 cell line expresses CD11b and low levels of Clec7a. The CD11b knockout cell line expresses Clec7a but not CD11b and the Clec7a knockout cell line expresses CD11b but not Clec7a. Data representative of 2 independent experiments

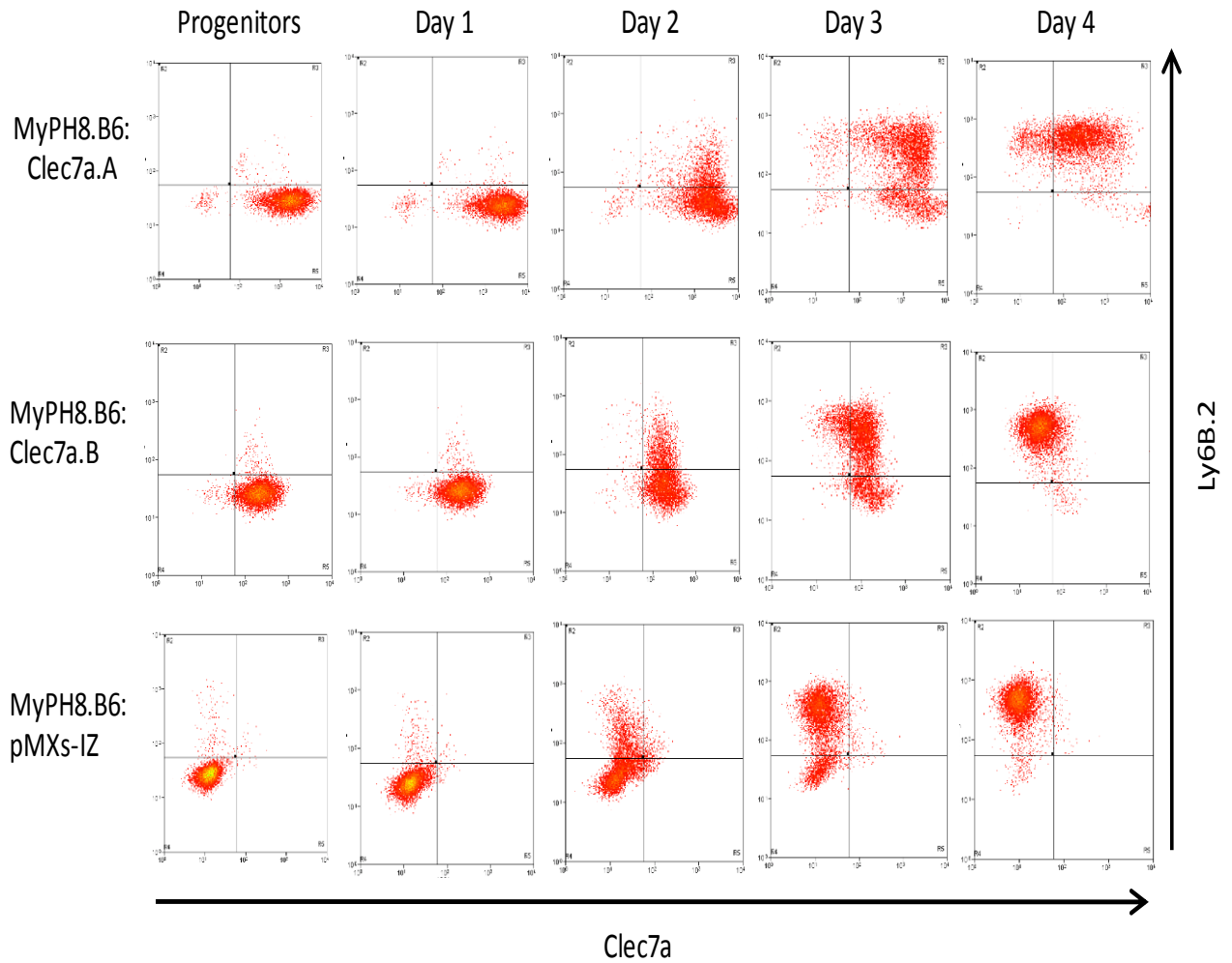


Figure 5.2: Four day differentiation flow cytometry plots of MyPH8-B6:*Clec7a.A*, MyPH8-B6:*Clec7a.B* and MyPH8-B6:*pMXs-IZ*. Ly6B.2 was used as a marker of neutrophil differentiation and Clec7a cell surface expression was determined at each time point. Ly6B.2 expression increases steadily and by day 4 all cell lines show expression of Ly6B.2. By day 4 the cell lines transduced with *Clec7a* show a marked increase in Clec7a than the empty vector control cell line. Although Clec7a expression decreased in the cell lines transduced with *Clec7a*, the expression of the cell surface receptor was still greater than seen in the empty vector control cell line. Note that the quadrants delineate Ly-6B.2 positivity and an arbitrary vertical reference line that marks the lower limit of expression of Clec7a by the *Clec7a.B* transduced cells (middle row). The *Clec7a.B* expression can be seen to cross this arbitrary vertical marker line after *in vitro* differentiation. Data representative of 3 independent experiments.

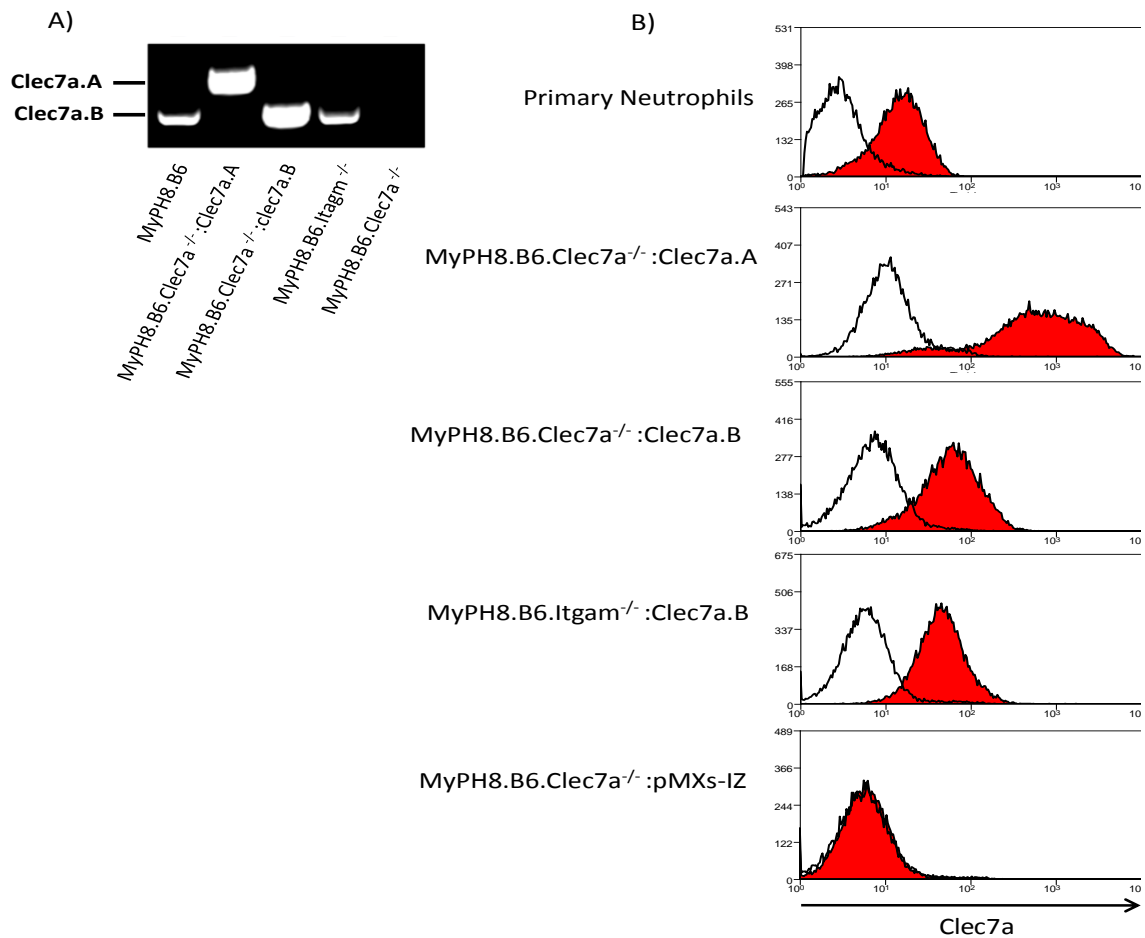


Figure 5.3: mRNA and expression of Clec7a on *in vitro* generated, Ly-6G⁺, neutrophils derived from conditionally-immortalised cell lines. A) mRNA expression of Clec7a splice-forms on neutrophils derived from conditionally-immortalised cell lines as determined via RNA extraction followed by RT-PCR (methods chapter 2.4.6). MyPH8-B6 wild type cells are shown to express predominantly the “B” isoform. The knockout cell line MyPH8-B6.Clec7a^{-/-} shows no expression and the knockout cell lines that were transduced show expression of the Clec7a isoform they had been transduced with. B) Histograms showing expression of Clec7a on *in vitro* generated, Ly-6G⁺, neutrophils derived from conditionally-immortalised cell lines. The red histogram denotes Clec7a expression and the white histogram shows the negative control. Primary neutrophils were extracted from the peritoneal cavity of mice 18 hours after an i.p. injection with 1ml 2% (w/v) BIOgel. Neutrophils from MyPH8-B6.Clec7a^{-/-} cells transduced with Clec7a.A shows a noticeably increased expression of Clec7a than primary neutrophils and neutrophils derived from cell lines transduced with Clec7a.B, which show comparable levels of Clec7a expression. As expected, neutrophils derived from the empty vector control cell line show no expression of Clec7a.

5.4. Assay Development: Zymosan Binding

A zymosan binding time course assay, using fluorescently labelled zymosan particles (100µg/ml), was performed with neutrophils derived from conditionally-immortalised cell lines (Figure 5.4). The percentage of cells bound to zymosan reached a stable plateau within the first 90 seconds of the assay. This was seen with all cell lines and demonstrated the relatively short amount of time required for such an assay.

Temperature dependency of this assay was also examined. There were no differences in the results obtained with non-opsonised particles when the assay was conducted at either 4°C or 37°C and only a slight increase in binding at 37°C with opsonised particles, compared to 4°C (Figure 5.4). The higher temperature was used for future assays partly as it showed an increase in binding but most importantly it allowed for the measurement of cellular response to the cell:particle interaction as it was physiologically relevant.

For both temperatures, opsonised particles showed much greater binding than non-opsonised particles. Neutrophils derived from the cell line transduced with Clec7a.A, were the only cells able to show a greater than background level of binding of non-opsonised zymosan. Neutrophils derived from the *Itgam*^{-/-} cell line, unsurprisingly, showed only background levels of binding for both non-opsonised and opsonised zymosan particles. For all subsequent studies fungal particles were both opsonised and non-opsonised.

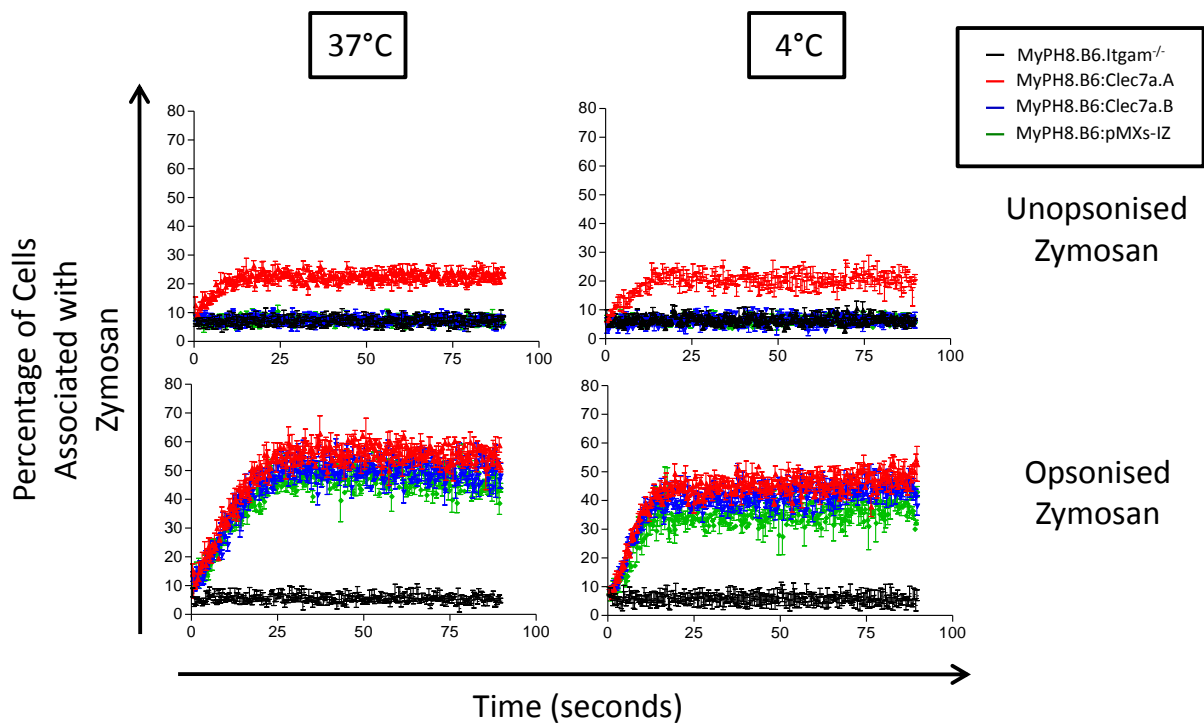


Figure 5.4: Zymosan binding time course of neutrophils derived from conditionally-immortalised cell lines.

Neutrophils were pre-warmed or pre-chilled to the appropriate temperature, then mixed with either non-opsonised or serum opsonised zymosan and binding examined with acquisition over time. Only MyPH8-B6:Clec7a.A neutrophils were able to bind non-opsonised zymosan above a background level at both temperatures. Neutrophils from all cell lines except the CD11b deficient cell line were able to bind opsonised zymosan at both temperatures. Data represents mean \pm SEM of 3 replicates and is representative of 2 independent experiments.

5.5. Assay Development: Respiratory Burst

In order to study the response of cells to fungal pathogens, a time course assay looking at the production of ROS in response to fluorescently labelled zymosan was performed (methods 2.7.2)(Figure 5.5). Neutrophils derived from the MyPH8-B6 cell line were pre-loaded with 3'-(*p*-aminophenyl) fluorescein (APF), a substance that becomes fluorescent in the presence of certain ROS (methods 2.1.4) produced during the respiratory burst. The cells were then stimulated with opsonised fluorescently labelled zymosan and ROS production measured over time. Cells that showed no association with zymosan also showed no increased production of ROS (Figure 5.5.A). However, some zymosan associated cells showed a marked increase in ROS production, which after a steep rise began to level out (Figure 5.5.B/C). These preliminary experiments showed that zymosan association had reached a plateau within 90 seconds and within 10 minutes, the ROS generation had reached a point where ROS production was being drastically limited, possibly by the physical ability of the cell to make ROS. An endpoint assay where the cells were stimulated for 15 minutes prior to flow cytometric analysis was proposed for future analysis as this was deemed sufficient to fully measure zymosan association and ROS production. It was also decided that the concentration of zymosan used (100 µg/ml) was sufficient to stimulate a measurable cellular response and that an internal control for APF production was possible using the neutrophils that had not associated with the fluorescently labelled particle.

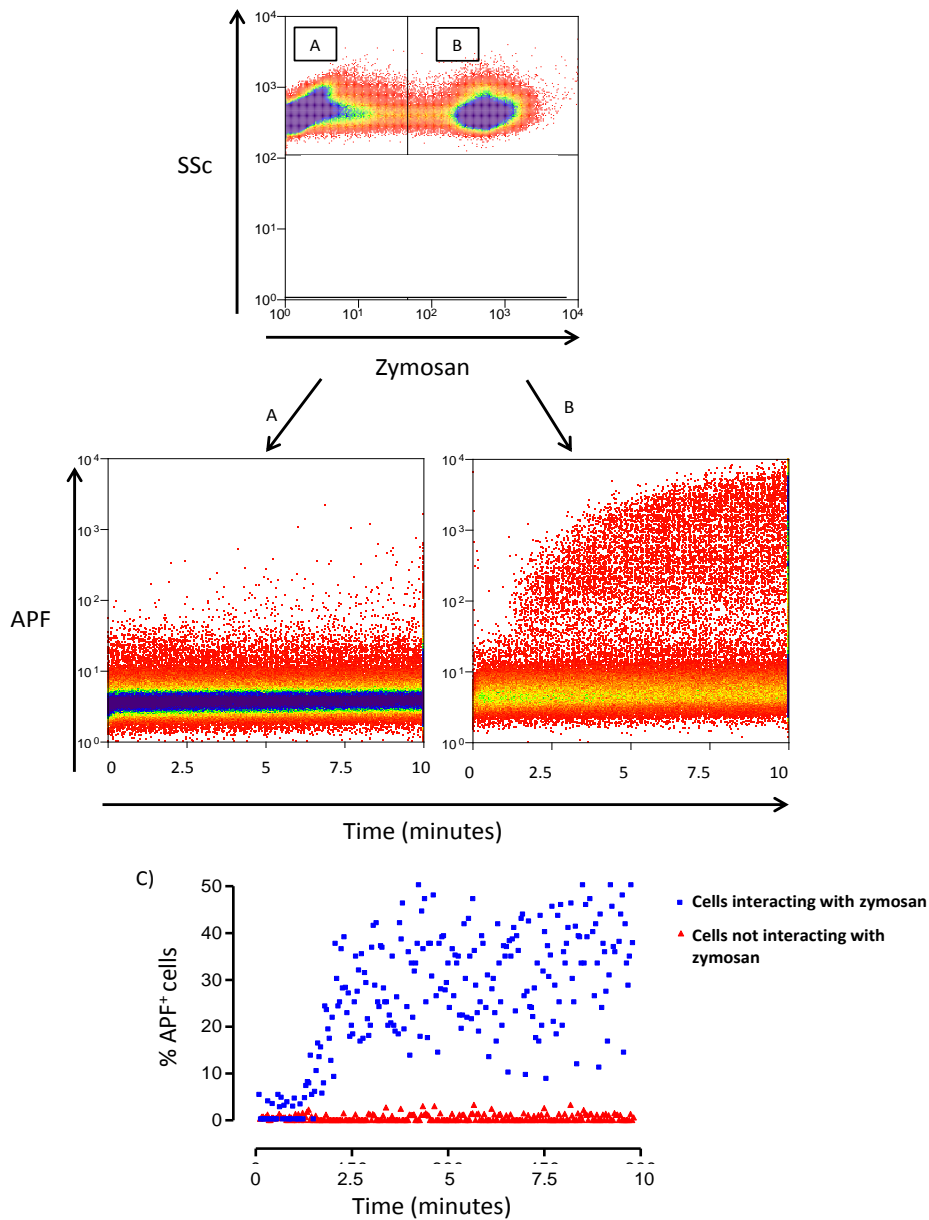


Figure 5: Neutrophils derived from MyPH8-B6 cell line were stimulated with fluorescently labelled opsonised zymosan particles. A) Flow cytometric representation of neutrophils that showed no association with zymosan and showed no increase in the production of ROS. B) Flow cytometric representation showing that some neutrophils that were associated with zymosan showed a steep increase in ROS production, which began to level out within 10 minutes. C) Graphical representation of the percentage of cells producing ROS over time. Data representative of 2 independent experiments.

5.6. *In Vitro* Study of the Function of Clec7a on Neutrophils Derived from Conditionally-immortalised Cell Lines

Neutrophils derived from conditionally-immortalised cell lines were loaded with APF then stimulated with fluorescently labelled zymosan or live *C. albicans* SC5314 for 15 minutes, at 37°C (methods 2.7.2). When mixed with non-opsonised zymosan, both wild type and Clec7a deficient neutrophils showed background levels of zymosan binding (Figure 5.6). On opsonisation of the particles, levels of zymosan binding significantly increased (Figure 5.6). However with live *C. albicans*, Clec7a deficiency resulted in a significant decrease in binding for both non-opsonised and opsonised *C. albicans* (Figure 5.6). Clec7a deficiency also impaired the ability of *in vitro* generated neutrophils to respond to non-opsonised and opsonised zymosan interactions by the production of ROS. This was not seen with *C. albicans* and instead a significant increase in ROS production was seen with Clec7a deficient cells as a response to non-opsonised *C. albicans* (Figure 5.6). However, this latter result was not reproduced in a second experiment.

Although, these results show a role for Clec7a on neutrophils in fungal particle recognition and cellular response, the expression of Clec7a on MyPH8-B6 wild type generated neutrophils is very low (Figure 5.1) and not comparable to that seen on primary neutrophils (Figure 5.3). Neutrophils from the Clec7a deficient cell line that had been reconstituted with *Clec7a.B* did show more comparable levels of the protein expression so this assay was repeated using neutrophils derived from this cell line, the *Clec7a*^{-/-} deficient cell line and a *Itgam*^{-/-} cell line that had been transduced with *Clec7a.B* (Figure 5.7).

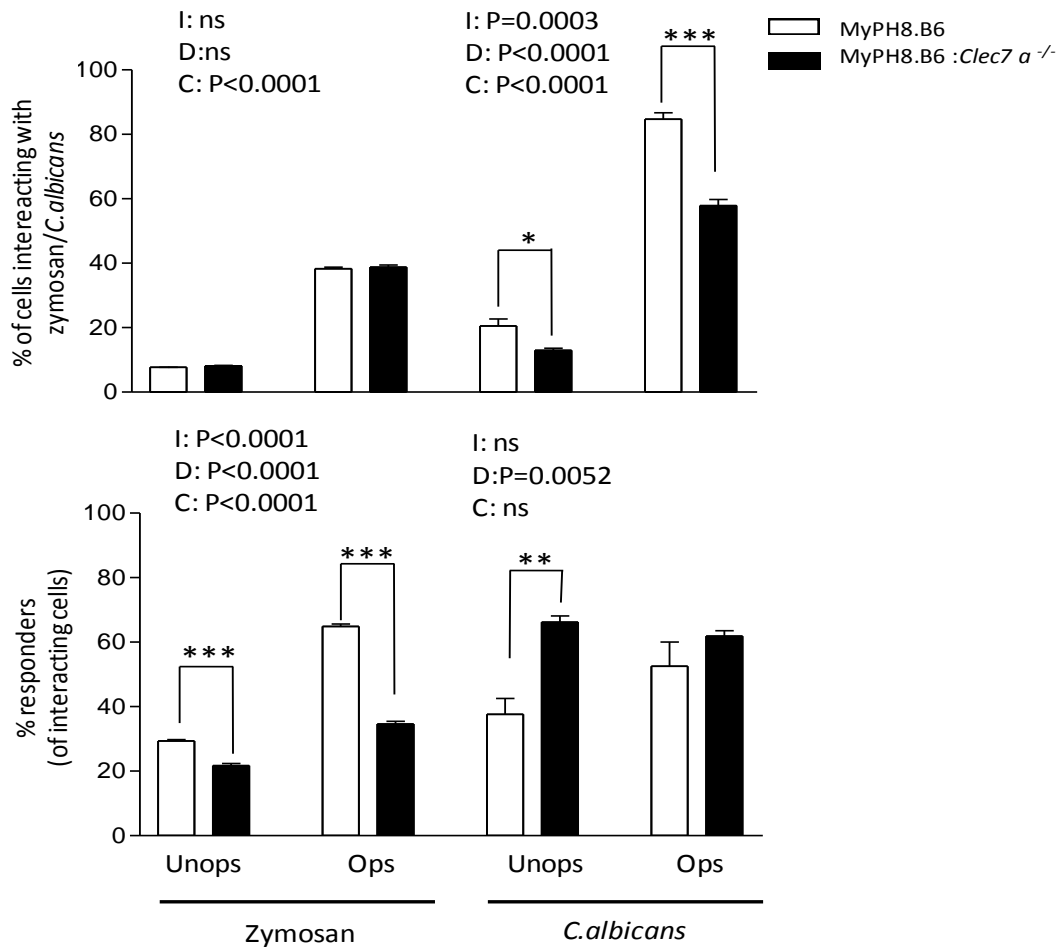


Figure 5.6: Graphical analysis of the recognition and response to zymosan and *C. albicans* of MyPH8-B6 and MyPH8-B6:*Clec7a*^{-/-} neutrophils. Neutrophils were stimulated with either serum opsonised or non-opsonised zymosan particles or serum opsonised or non-opsonised live *C. albicans*. *Clec7a* deficiency did not impair binding to non-opsonised and opsonised zymosan, however its deficiency did impair the response of these cells to the stimulant. Opsonisation of zymosan significantly increases the percentage of cells binding zymosan and the percentage of these interacting cells that respond by producing ROS. In cells stimulated with *C. albicans*, *Clec7a* deficiency impairs binding to both opsonised and non-opsonised *C. albicans*. Opsonisation of *C. albicans* significantly increases the percentage of neutrophils that bind *C. albicans*. Unops = non-opsonised; Ops = opsonised. Data shown is representative of 2 independent experiments and data shown represented the mean±SEM of triplicates of one experiment. Analysis by two-way ANOVA with Bonferroni post-tests. I = Interaction; D = significance of the effect of *Clec7a*; C = significance of effect of complement opsonisation. P values: * = P<0.05; ** = P<0.01; *** = P<0.001.

Curdlan microparticles (curdlan mp) are a selective Clec7a agonist (Rosas et al. 2008). Clec7a deficient neutrophils show no response when stimulated with curdlan mp, however, Clec7a deficient cells that were reconstituted with the B isoform were able to respond (Figure 5.7.A). Interestingly, CD11b deficient neutrophils that had been transduced with Clec7a.B showed a significantly greater response to curdlan mp than either of the previous neutrophil types (Figure 5.7.A). This could be due to the slightly higher clec7a expression on these cells due to the fact that they already expressed low levels of Clec7a prior to transduction.

When stimulated with non-opsonised and serum opsonised zymosan, MyPH8-B6.*Clec7a*^{-/-}:Clec7a.B neutrophils showed a significant increase in zymosan binding and ROS production, compared to both MyPH8-B6.*Itgam*^{-/-}.Clec7a.B neutrophils and the empty vector control neutrophils (Figure 5.7.B). Opsonisation of the zymosan particles significantly increased the percentage of neutrophils binding zymosan, however, opsonisation did not affect the ROS production of those cells that did bind the particle (Figure 5.7.B). When stimulated with *C. albicans*, MyPH8-B6.*Clec7a*^{-/-}:Clec7a.B neutrophils showed greater binding than the other two cell lines. Serum opsonisation of the yeast resulted in significantly increased binding in all cell lines except the CD11b deficient cells (Figure 5.7.B). Again serum opsonisation had no effect on the ROS production of neutrophils that had bound the *C. albicans*.

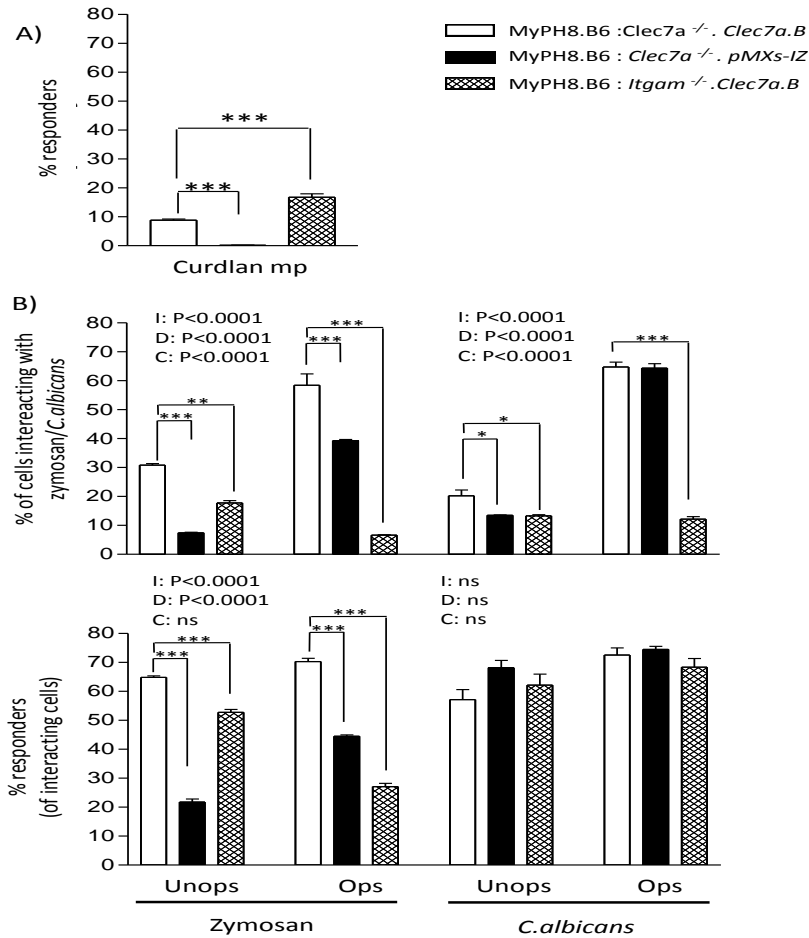


Figure 5.7: Graphical analysis of the recognition and response to curdlan mp, zymosan and *C. albicans* of *in vitro*-derived neutrophils. A) Percentage of cells stimulated with curdlan mp that respond by producing ROS. The *Clec7a* deficient neutrophils showed no response to curdlan mp but both cell lines with *Clec7a.B* were able to respond. B) Neutrophils were stimulated with either serum opsonised or non-opsonised zymosan or live *C. albicans*. *Clec7a* deficiency impairs binding and response to non-opsonised and opsonised zymosan in neutrophils. Opsonisation of zymosan increases the percentage of cells that bind zymosan in all cell line except the CD11b deficient cells. When stimulating with live *C. albicans*, *Clec7a* deficiency impaired only the binding of non-opsonised zymosan to the neutrophils. *Clec7a* deficiency and opsonisation of *C. albicans* had no effect on the response of the neutrophils to the live *C. albicans*. Unops = non-opsonised; Ops = opsonised. Data are representative of 3 independent experiments and data shown represented the mean±SEM of triplicates of one experiment. (A) Analysis by two tailed *t* test. (B) Analysis by two-way ANOVA with Bonferroni post-tests (asterisks). I = Interaction; D = significance of the genetic deficiencies; C = significance of effect of complement opsonisation. P values: *= $P < 0.05$; **= $P < 0.01$; *** = $P < 0.001$.

5.7. Reconstitution of *Itgam*^{-/-} Cell Line with Human CD11b

An *Itgam*^{-/-} neutrophil precursor cell line was reconstituted with two forms of the human *Itgam* gene as described in chapter 3 and chapter 2.5.5. The expression of CD11b on the *in vitro* generated neutrophils was compared to CD11b expression on primary neutrophils (Figure 5.8). Both of the transduced cell lines appeared to express CD11b and the empty vector control cell line showed no expression of CD11b (Figure 5.8). Although expression of human CD11b appeared to be lower than mouse CD11b on primary cells, this could not be concluded as both of the antibodies (either clone 5C6 or M1/70) used to determine CD11b expression were anti-mouse antibodies that were used purely because of their known cross-reaction with human CD11b.

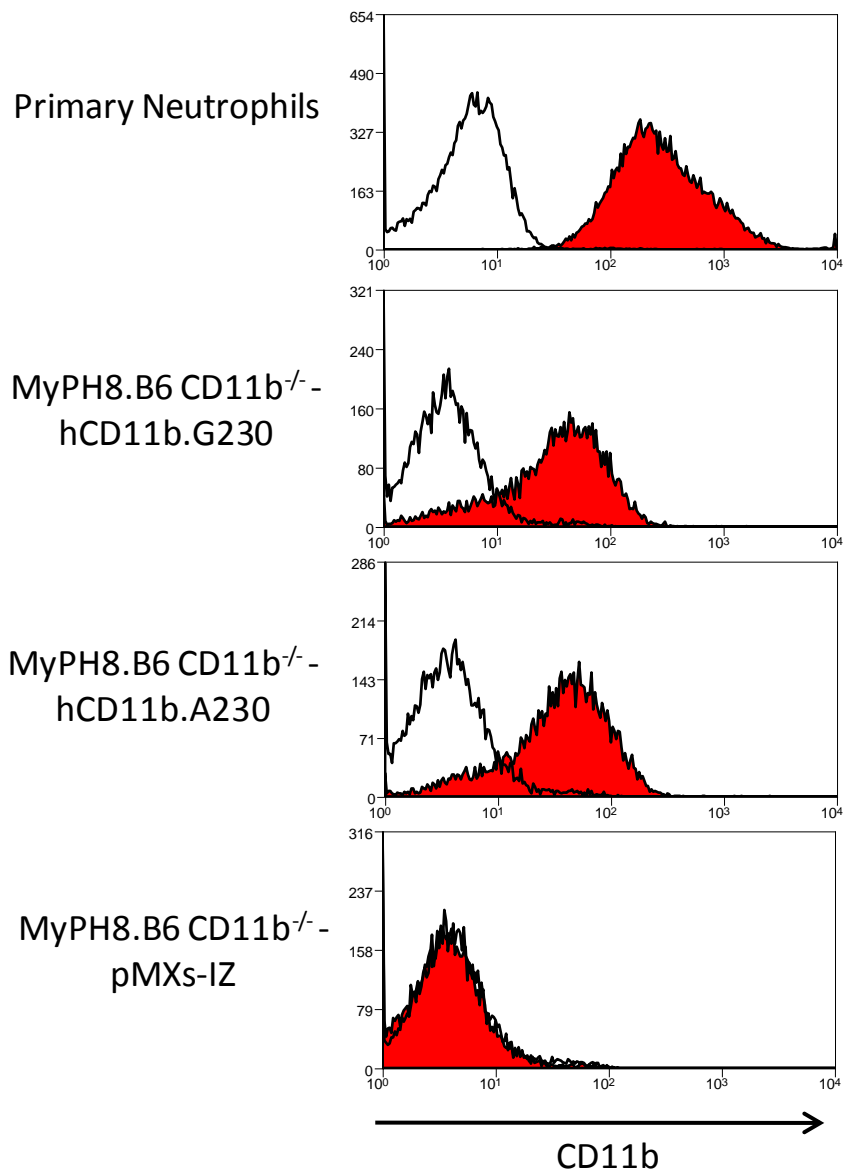


Figure 5.8: Expression of CD11b on primary cells and *in vitro* generated neutrophils derived from transduced *Itgam*^{-/-} cell lines. The cell lines transduced with human CD11b isoforms ('G230' and 'A230') both show similar expressions of CD11b which are 'lower' than that seen on primary neutrophils. The empty vector control cell line showed no expression of CD11b. The red histogram denotes CD11b expression and the white histogram shows the negative control. Data representative of 2 independent experiments.

5.8. Restored Function of CD11b on Reconstituted Cell Lines

Neutrophils derived from the MyPH8-B6.*Itgam*^{-/-}:G230, MyPH8-B6.*Itgam*^{-/-}:A230 and MyPH8-B6.*Itgam*^{-/-}:pMXs-IZ cell lines were preloaded with APF then stimulated with fluorescently labelled zymosan or live *C. albicans* for 15 minutes, at 37°C (methods chapter 2.7.2). All three cell lines showed poor binding of non-opsonised zymosan but the reconstituted CD11b cell lines were able to bind serum opsonised zymosan (Figure 5.9). Opsonisation was also able to significantly increase the ROS production of zymosan associated cells. The empty vector control cell line showed the low levels of binding and ROS production observed for non-opsonised particles (Figure 5.9).

When stimulated with non-opsonised *C. albicans*, neutrophils derived from the G230 and A230 cell lines showed significantly increased binding to *C. albicans* compared to neutrophils derived from the empty vector control cell line. The CD11b reconstituted cell lines, but not the empty vector control, showed a significant increase in *C. albicans* binding when the yeast was serum opsonised. ROS production in response to non-opsonised *C. albicans* is significantly increased in the A230 reconstituted cell line, but not in the G230, in comparison to the empty vector control (Figure 5.9), which contrasts previously published data (MacPherson, Lek et al. 2011). Serum opsonisation of *C. albicans* significantly increased ROS production in both reconstituted cell lines in comparison to the control cell line.

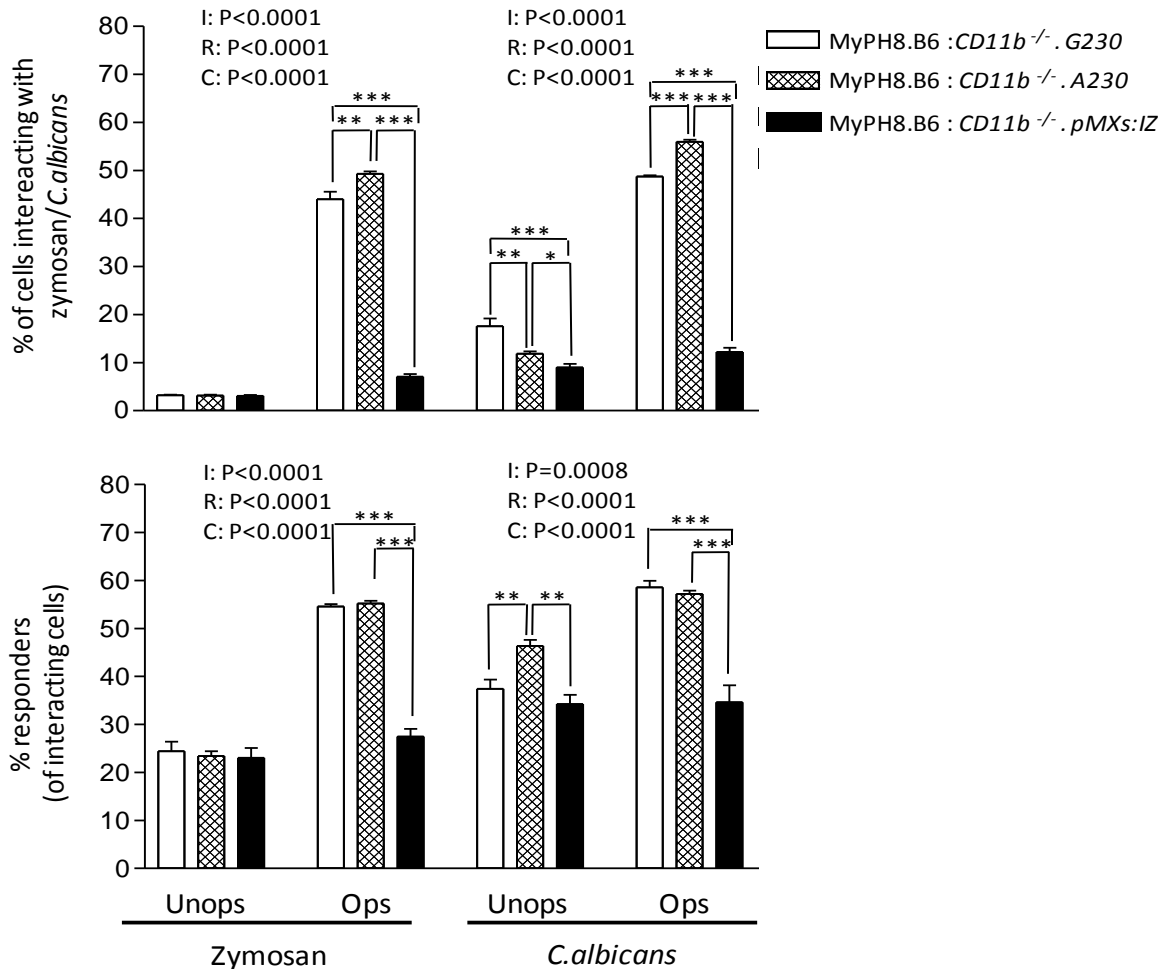


Figure 5.9: Graphical analysis of the recognition and response to zymosan and *C. albicans* of *in vitro* neutrophils.

Neutrophils were stimulated with non-ops or serum ops zymosan or live *C. albicans*. Very low binding of non-ops zymosan was observed with all cell lines. However, ops of zymosan resulted in a significantly increased level of binding in the MyPH8-B6:*Itgam*^{-/-}.G230 and MyPH8-B6:*Itgam*^{-/-}.A230 cells but not in the empty vector control cells. Response of these cells to zymosan was also significantly increased by ops in the cells reconstituted with CD11b. Levels of binding and response to *C. albicans* was increased by ops of the live yeast in the reconstituted cell lines but not in the empty vector control. Unops = non-ops; Ops = ops. Data representative of 3 independent experiments and data shown represented the mean±SEM of triplicates. Analysis by two-way ANOVA with Bonferroni post-tests (asterisks). I = Interaction; R = significance of the effect of the transduced receptor; C = significance of effect of complement ops. P values: * = P < 0.05; ** = P < 0.01; *** = P < 0.001.

5.9. Conclusion

In this chapter, neutrophils derived from conditionally-immortalised precursor cell lines were used to model innate immunity. The decision to use reconstituted *Clec7a*^{-/-} cells as opposed to wild type cells was predominantly based on the difference of Clec7a expression between the wild type MyPH8-B6 neutrophils and primary neutrophils. *In vitro* generated wild type neutrophils showed low expression of Clec7a, suggesting that they are not quite fully matured murine neutrophils but this was addressed in a controlled manner suitable for the model being explored. Neutrophils derived from the *Clec7a*^{-/-} cell line showed a similar impairment in the recognition and response to zymosan and *C. albicans* as that seen with primary neutrophils in chapter 4. Reconstitution of these deficient cells with Clec7a was able to restore the lost function. Also opsonisation of the stimulating particles, increased recognition of both zymosan and *C. albicans*, with the exception being the CD11b deficient cells, as CD11b is a subunit of complement receptor 3 (CR3), which recognises serum opsonised particles.

CD11b deficient cells were successfully reconstituted with two different isoforms of human CD11b. The reconstitution of CD11b restored the increased recognition and response to opsonised zymosan and *C. albicans*. Interestingly, human CD11b transduced cell lines did not exhibit CD11b surface expression until they were differentiated, suggesting that surface expression could not be achieved until differentiation induced expression of its partner molecule (CD18) had occurred. Additionally these experiments depended upon an association between human CD11b and mouse CD18 and these results provided circumstantial evidence that this occurs and that this heterodimer is functional. Contrary to previously published data (Macpherson et al. 2010), which suggests that cells homozygous

for the A230 allele are impaired in their immune response, the results seen here suggest the A230 allele has a slight advantage in its ability to recognise and respond to zymosan and *C.albicans*. There are several factors to be taken into account when looking at these results. Firstly, neither fungal particles nor serum-opsonised fungal particles are specific for CD11b and mouse serum was used to opsonise, which most likely has altered affinity for human CD11b. Also the cross-species mouse CD18:human CD11b heterodimer, whilst functional may not be truly represent the activity of the human heterodimer and the cell lines expression may differ as a consequence of *in vitro* passage and not solely as a consequence of CD11b polymorphism. Ideally these experiments need to be repeated with selective CD11b ligands, and verified with a separate set of cell lines. In the context of the 3Rs, this shows that this type of model is a useful, but experiments need to be carefully controlled. Since completion of these studies the MyPH8-B6.*Clec7a*^{-/-} parental cell line transduced with either Clec7a.B or the empty vector control has been used to model a novel immune regulatory function of Clec7a (Karsten, Pandey et al. 2012). These studies with the cell line derived neutrophils gave very similar results to primary cells, both validating this cell line approach and showing the advantage of being able to genetically alter neutrophils to tackle difficult scientific questions.

In summary, these studies demonstrate the ability of neutrophils derived from conditionally-immortalised cell lines to model innate immunity, but with some specific limitations and considerations that must be considered in experimental plans. The relative ease with which these cells can be successfully genetically modified increases the scope of neutrophil study and the study of human proteins in a mouse model.

Chapter 6: Discussion

The study of neutrophil biology is primarily achieved through the use of primary neutrophils obtained from human donor blood or tissues (Yamamoto, Saeki et al. 1991; Adams 2004; Marks, Radulovic et al. 2007; Nauseef 2007), or experimental animal models (Cowburn, Condliffe et al. 2008). Although directly relevant these primary cells are short lived, can be difficult to handle for experimental purposes and can lead to a large loss in animal life. To address some of these issues a myeloid progenitor cell line was developed (Wang, Calvo et al. 2006). However, extensive functional characterisation was not performed and this thesis aimed to address this deficiency and to determine if these cells could be a useful replacement for the animals in neutrophil research.

The cell line was generated by the conditional immortalisation of lin^- bone marrow cells by the expression of mouse Hoxb8 fused to an oestrogen-receptor binding domain. This gene prevents the progenitor cells from terminal differentiation and allows the cell line to be kept in culture indefinitely, provided a growth factor is present. However, the transcriptional activity of Hoxb8 is only active in the presence of oestrogen, hence it's conditional nature, and removal of the oestrogen allows for the cells to terminally differentiate producing neutrophils. This makes this cell line distinct from other cell lines, in that the immortalising factor is removed before differentiation possibly leading to more mature neutrophils being produced (Collins, Gallo et al. 1977; Collins, Ruscetti et al. 1978; Guchhait, Tosi et al. 2003; Fleck, Romero-Steiner et al. 2005; Wang, Calvo et al. 2006).

The removal of the immortalising factor also makes these cells particularly suitable for adoptive transfer into live animals as once in the animal they won't persist leading to possible problems with tumour formation. The successful i.v injection of these cells into live mice and their recruitment from the peritoneal cavity, show that in an *in vivo* system, these

cells are able to behave like endogenous cells and can pass from the blood stream and extravasate into other tissues of the body. Differences in the functional behaviour of different cells were still noticeable, as CD11b deficient cells were less able to bind zymosan particles used to recruit the cells in the peritoneal cavity and were present in higher numbers as previously reported in the knockout animals (Coxon, Rieu et al. 1996).

Wang et al. reported that conditional-immortalisation with Hoxb8 in the presence of SCF resulted in a precursor cell line could be manipulated to produce other myeloid cells, i.e. eosinophils and macrophages (Wang, Calvo et al. 2006) by the manipulating of differentiation cytokines. I specifically examined IL-5 treated cultures for the presence of eosinophils, as IL-5 is a known growth factor for eosinophils (Lopez, Begley et al. 1986; Sanderson 1990; Wang, Calvo et al. 2006), but did not observe cells with the morphology of eosinophils (as compared to the peritoneal lavages from naive mice) after eosin, methylene blue staining (data not shown). Over the time (6 days) and culture conditions used there was little evidence of macrophage differentiation, with differentiated cells being non-adherent and predominantly F4/80⁻. This data suggests that the progenitors produced are already committed to the neutrophil lineage. The differences seen with the cells produced by Wang et al. could be due to a less homogenous progenitor population, where a few progenitors are not yet committed to the neutrophil lineage so are still able to produce the eosinophils and macrophages seen.

MyPH8-B6 neutrophils that had been differentiated in SCF alone, as original performed by Wang and colleagues (Wang, Calvo et al. 2006) and in SCF+G-CSF were chosen for further study due to the observation that they produced large numbers of cells with a high (>80%) neutrophil purity. Their expression of different cell surface markers was examined and they

were shown to express several different myeloid, and specifically, neutrophil markers. However, the expression patterns seen suggest that although SCF alone and the SCF+G-CSF differentiated cells are neutrophils, they have slightly different phenotypes. Primary neutrophils express Clec7a and CD88 (Chenoweth and Hugli 1978; Taylor, Brown et al. 2002). Cells differentiated in SCF alone do not express these markers and this suggests they are perhaps not as mature as primary neutrophils. However, cells that were differentiated in SCF+G-CSF show a low level of expression of both these markers, relative to primary cells. Additionally, compared to primary cells, these *in vitro* generated cells exhibited low expression of Ly-6G (and hence Gr-1). Overall, this meant that in studies of Clec7a where these *in vitro* generated neutrophils were used, in order to achieve physiologically relevant levels of Clec7a expression, Clec7a knockout cell lines were reconstituted with Clec7a. The reason for choosing to reconstitute Clec7a knockout cells rather than simply overexpress Clec7a in wild type cells is that this allowed direct comparison of the knockout cells with the same parental cell line reconstituted with Clec7a without the risk of artefacts created by differences between distinct cell lines.

The functional behaviour of MyPH8-B6-derived neutrophils was assessed. These neutrophils were able to bind and phagocytose zymosan particles and respond with respiratory burst. A flow cytometric assay showed that neutrophils stimulated with ionomycin exhibited a strong increase in intracellular calcium ion levels. Similarly, zymosan was also able to induce an increase in intracellular calcium ions. A limitation to this approach is that it only provides a snapshot of cell activity as the cells are excited and detected by the flow cytometer. An alternative way to examine the kinetics of calcium mobilisation would be to continuously monitor individual cells over a period of time. A

single live-cell microscopy assay allowed for individual cells to be identified and introduced to a zymosan particle by micromanipulation techniques (Dewitt and Hallett 2002), and for the subsequent calcium ion changes and phagocytosis of the particle to be monitored. This assay also allows for a direct correlation between calcium ion changes and the progress of phagocytosis to be observed. The *in vitro* generated neutrophils were found to produce a calcium ion signal similar to that seen by human primary neutrophils (Dewitt and Hallett 2002; Dewitt, Laffafian et al. 2002), when introduced to a zymosan particle.

Primary neutrophils have a very short life span and are terminally differentiated (Cowburn, Condliffe et al. 2008). This makes them almost impossible to genetically modify. This method of producing neutrophil precursors allows for the generation of genetically modified precursor cells that can be used to generate neutrophils. I have shown that the progenitor cells can be transduced with a viral construct containing the gene of interest and differentiated into neutrophils that also contain the gene. This has been similarly seen with conditionally-immortalised macrophage precursors (Wang, Calvo et al. 2006; Rosas, Osorio et al. 2010) as well as SCF differentiated neutrophil precursors (Koedel, Frankenberg et al. 2009). There is a reduction of transgene expression, which can perhaps be attributed to terminal differentiation and reduction in RNA production and new protein synthesis, as differentiation occurs, but by the end of the differentiation process, expression of the gene is still detectable at levels which confer functional alterations upon the cells. This is one of the key advantages of this method of neutrophil production as it expands the scope of studying neutrophil biology immensely.

The contribution of Clec7a to neutrophil recognition and response to fungal infection has been recently explored. Clec7a deficient murine neutrophils show decreased zymosan

binding and an attenuated respiratory burst in response to zymosan (Taylor, Tsoni et al. 2007). On human neutrophils, Clec7a has been shown to promote fungicidal activity towards *C.albicans* (Kennedy, Willment et al. 2007). However, this is complicated as blocking Clec7a activity on human neutrophils has no effect on *C. parapsilosis* phagocytosis even though it reportedly shows more β -glucans on its surface than *C.albicans* (Linden, Maccani et al. 2010). If a model to demonstrate the role of Clec7a on neutrophils could be developed, it would be a useful model in which to test the efficacy of *in vitro* generated neutrophils in modelling innate immune responses and to analyse the contribution of Clec7a to neutrophil activation. A time course experiment in wild type 129S6/SvEv mice using zymosan peritonitis generated a useful model for the response of the immune system to zymosan. Zymosan particles (2×10^7) injected i.p. encounter tissue resident cells first, including macrophages that were able to phagocytose some of the particles. In this model, inflammation is triggered by a number of systems including macrophage activation (Brown and Gordon 2001; Underhill 2003), complement activation (Pillemer, Blum et al. 1954; Mizuno, Ito et al. 2009) and mast cell activation (Kolaczowska, Seljelid et al. 2001). The chemokines and other inflammatory mediators, such as C5a, that are produced recruit neutrophils. In these studies, this manifested itself in a rapid increase in neutrophil presence in the peritoneum, which coincided with the 'disappearance' of tissue resident macrophages, and the high dose of zymosan ensures that enough zymosan persists for the neutrophils to phagocytose. Neutrophil numbers decreased over a matter of hours and numbers of infiltrating monocytes and macrophages began to gradually increase. These macrophages and monocytes, particularly those monocytes that arrive earlier in the inflammatory cascade would be able to phagocytose any residual zymosan particles as well as apoptotic neutrophils and 7 days after initial injection, the absolute numbers of

macrophages had almost recovered completely. Since neutrophil presence in the peritoneal cavity was notable and increasing at 4 hours and this was deemed a suitable time point at which to explore the effect of Clec7a deficiency on neutrophil interactions with zymosan *in vivo*.

Deficiency in Clec7a diminishes the recruitment of neutrophils and their ability to associate with zymosan. It was unclear as to whether this is due to an intrinsic role of Clec7a on the neutrophils or perhaps just a defect in inflammation in response to zymosan (a ligand of Clec7a). A defect in cellular activation in general in the absence of Clec7a is suggested by the reduction in IL-6 observed in the peritoneal washouts of Clec7a-deficient mice when compared to the wild type controls and this is consistent with Clec7a being a major receptor for the non-opsonic recognition of β -glucan containing particles such as zymosan (Brown, Taylor et al. 2002). Tissue resident macrophages express alternate receptors for zymosan, including the DC-SIGN homologue SIGNR1 (Taylor, Brown et al. 2002; Taylor, Brown et al. 2004) as well as alternate scavenger receptors and lectins that may play, perhaps lesser, but important potential roles in fungal recognition. To explore whether Clec7a is a major receptor for the non-opsonic recognition of β -glucan, an *ex vivo* assay looking at primary neutrophil recognition of zymosan was developed. When conducted with inflammatory neutrophils, it was found that although Clec7a deficiency does impair the ability of neutrophils to bind and respond to zymosan particles; however serum-opsonisation of the zymosan particles was much more important for neutrophil-mediated recognition. This suggests that Clec7a does have a role to play in recognition of fungal particles but opsonisation and CD11b have a larger role, making the role of Clec7a appear partially redundant. Live *C. albicans* interactions with neutrophils were also explored and similar

observations and conclusions were made. In contrast, peripheral blood neutrophils showed no dependency on Clec7a for the recognition of fungal particles. This obvious difference between inflammatory neutrophils and whole blood neutrophils could be down to the nature of the cells. Inflammatory neutrophils have been recruited through the endothelial barrier in response to different cytokine signals and are primed and activated to respond, which could enhance the receptor specific responses seen. Whole blood neutrophils have not been through this priming process and this could contribute to some of the differences seen. One possible limitation of this assay is the presence of large numbers of red blood cells. The red blood cells are not lysed prior to the assay to avoid priming of the blood neutrophils; however, the red blood cells could result in steric inhibition of neutrophil activity. This limitation is not present in the inflammatory neutrophil assay and would allow for a greater response. Another possible limitation is the use of heparin in this assay. Heparin, an anticoagulant used to stop the blood from clotting, is reported to have anti-inflammatory properties (Young 2007).

To test the limitations of this assay, curdlan microparticles, specific Clec7a agonists, were used to stimulate the cells and whole blood samples were spiked with inflammatory BIOgel neutrophils, which could be distinguished with an allotypic marker. This resulted in a lowering of the response of the inflammatory neutrophils and supports the idea that red blood cells are interfering with the curdlan microparticle and neutrophil interaction and lowering the ROS response. However, heparin may also be having an anti-inflammatory effect on these neutrophils (Young 2008). Testing whether the use of heparin affected our assay, showed that inflammatory cells that had been exposed to heparin had a decrease in ROS production compared to those that had not been exposed. A different anti-coagulant,

sodium citrate, and lysis of the red blood cells was used to address these issues, however, this resulted in no difference being seen between inflammatory neutrophils and quiescent blood neutrophils, as the blood neutrophils had increased the percentage of cells responding 3-fold. There are two possible reasons for the results seen. Firstly, lysis of the red blood cells removes any interference between the neutrophils and curdlan microparticles, thereby allowing more blood neutrophils to interact and respond. Also the process of lysing the red blood cells could activate the previously quiescent blood neutrophils, increasing their ability to interact and respond to curdlan microparticles. Distinguishing between these two reasons would be extremely technically difficult and highly purified quiescent blood neutrophils would need to be isolated before this limitation could be addressed. There is a study that purports to produce such highly purified quiescent blood neutrophils (Cotter, Norman et al. 2001). My results suggest that although heparin may affect the response of cells to curdlan microparticles, the interference of red blood cells is a much bigger limitation in this assay.

This ROS response assay was applied to human neutrophils to determine if there was a functional difference between the murine model and human model of response. Obtaining suitable inflammatory human neutrophils proved to be a significant challenge as the methods we used each had their own limitations. The saliva contains large amounts of neutrophils that have extravasated from the blood and are mixed with potential microbial material and are therefore primed. Collection of these neutrophils was simple and large amounts could be obtained, however, the background ROS response was prohibitively high and would make subtle differences in response very difficult to see. This can probably be attributed to bacteria and other microbial material that is found in the mouth that the

neutrophils have already been activated by. Another method of obtaining inflammatory human neutrophils involved using a “skin window” by lightly removing the top layer of skin using sand paper and collecting the infiltrating cells on filter paper soaked with PBS (Marks et al. 2007). This method produced far less neutrophils than the saliva and obtaining the cells requires injury to the donor, which is not a desirable side effect. These cells also showed a high background ROS response which would cause significant problems when examining the low level of non-opsonic recognition of yeast and glucan particles by human neutrophils. Human peripheral blood neutrophils were easily obtainable in sufficient numbers and the ROS response background levels were minimal. The main problem when attempting to apply this model to human neutrophils was the lack of Clec7a-deficient patients in Cardiff. Methods of blocking Clec7a activity were investigated and two different blocking antibodies were looked at but neither had any noticeable effect on the neutrophil binding to zymosan, in contrast to the Clec7a-dependency seen with murine neutrophils. Also, laminarin, a soluble β -glucan, which has been used previously to inhibit Clec7a activity (Brown, Taylor et al. 2002), had no effect on the non-opsonic zymosan binding to neutrophils. Increasing neutrophil: zymosan interaction through the centrifugation (Voyich and DeLeo 2002; Kennedy, Willment et al. 2007) could have helped remove these limitations, however, the questionable physiological relevance of a forced interaction was the deciding factor in forgoing this step.

A murine model looking at the role of Clec7a on neutrophils was developed and results indicate that the role of Clec7a on neutrophils is complicated. Clec7a deficiency does impair fungal particle recognition and ROS production, which shows an important role for Clec7a on neutrophils, however, opsonisation of the fungal particles is able to largely restore

recognition and ROS production showing that there is a level of redundancy in the system and that the role of Clec7a in response to fungal infection is part of a large framework of responses, rather than a protein with one distinct function. The ubiquitous nature of complement in the body perhaps gives it a more significant role in fungal recognition than Clec7a. This is consistent with the observation that although Clec7a deficient humans may have higher recurrent instances of fungal infections, they are not highly susceptible to invasive fungal disease and show no defect in fungal killing (Ferwerda, Ferwerda et al. 2009). Recent studies have shown that Clec7a activates CR3 but that this is likely to also be redundant as the immune system has other mechanisms, such as other PRR, that can compensate for the lack of Clec7a (Li, Utomo et al. 2011).

Neutrophils derived from conditionally immortalised cell lines were used to replicate the studies performed on the role of Clec7a on primary neutrophils, as a proof of concept study. Clec7a knockout cells were used and reconstituted with both the Clec7a isoforms (Clec7a-A and Clec7a-B); because it was felt that the expression of Clec7a by the wild type cells was lower than that seen on primary neutrophils. The reconstitution was successful and after flow cytometric studies comparing Clec7a expression on these cells to primary neutrophils, it became evident that the Clec7a-A isoform reconstituted cell line expressed noticeably more Clec7a expression than the Clec7a-B reconstituted cell line, which had expression levels comparable to primary neutrophils. RNA extraction and RT-PCR analysis showed that MyPH8-B6 wild type cells primarily express the B isoform, similar to the primary macrophages isolated from the same parental strain of mice (C57BL/6) (Heinsbroek, Taylor et al. 2006). Some initial zymosan binding studies were performed using wild type cell line-derived neutrophils that had been transduced with the different Clec7a isoforms. Taking

into consideration that MyPH8-B6-derived neutrophils primarily express the Clec7a-B isoform and that the Clec7a^{-/-} cell line that had been reconstituted with Clec7a-B showed comparable levels of expression to the primary neutrophils, all further studies were performed only with this cell line and not the Clec7a-A reconstituted cell line whose activities would have to be considered physiologically irrelevant, due to the fact that it is overexpressed on the cell line neutrophils, in comparison to primary wild type neutrophils. Attempts to flow-cytometrically cell sort low expressers from the Clec7a-A expressing cells failed and the population stabilised with high expressing soon after. In conjunction with the Clec7a-B reconstituted cell line empty vector containing Clec7a^{-/-} cells were also made and for direct comparison the same two vectors were also transduced into the Itgam^{-/-} cell line. These studies were able to replicate the results seen with primary mouse neutrophils in that although Clec7a deficiency does impair binding and response to both zymosan and *C. albicans*, the effect is limited and serum opsonisation seems to play a much bigger role in recognition and response.

Further proof of concept studies were performed using *Itgam*^{-/-} *in vitro* derived neutrophils. These cells were reconstituted with 2 polymorphic variants of human CD11b. These neutrophils were assessed for their ability to bind and respond to opsonised zymosan and *C. albicans*. The reconstitution of CD11b on these cells was able to restore binding to opsonised particles, confirming that human CD11b could effectively partner with mouse CD18. Also these studies demonstrate the versatility of these cells in that human as well as mouse genes may be studied and manipulated on these cells.

To summarise, neutrophils derived from conditionally immortalised are morphologically and functionally similar to primary neutrophils. They can be genetically modified and provide a

biologically relevant model for the study of neutrophil biology. However, extreme caution should be taken not to over-interpret data that could also be explained, by defects in maturation of *in vitro*-derived neutrophils or by non-physiological expression levels of transgenes. Maturation defects have also been seen in *ex vivo* neutrophils generated from CD34⁺ human stem cells which impaired their ability to kill *E.coli* and *S. pneumoniae* (Dick, Prince et al. 2008). Although, those cells were functional similar to peripheral blood neutrophils, in that they were able to recognise and phagocytose pathogens, they contained immature granules which impaired their killing ability. This technique of producing *in vitro* generated neutrophils is novel and increases the scope of the study of neutrophil biology.

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