Modulation of Galectin Expression and Glycosylation Profile of Immune Cells during Inflammation

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ABSTRACT

Galectins-1, -3 and -9, are endowed with many immune-regulatory properties, with galectins-1 and -9 largely regarded as anti-inflammatory and galectin-3 as pro-inflammatory. Expression levels increase in activated adaptive immune cells, with peak expression often correlating with peak inflammation. Galectin actions are not only determined by their expression levels but also target tissue permissibility to galectin binding, which is in turn determined by the profile of specific carbohydrate residues, namely *N*-acetyllactosamine, recognised by these lectins. How expression levels and actions are modulated in innate immune cells during inflammation has not been systematically characterised. This study therefore set out to delineate the effects of inflammation on neutrophil glycophenotype, as well as elucidate the temporal and spatial modulation of galectins during resolving inflammation.

The neutrophil glycophenotype was modulated during trafficking with decreased levels of all terminal glycan residues assessed. However, this did not correlate with galectin binding permissibility suggesting this is not a useful indicator in this model. The overall change in glycosylation may theoretically be a consequence of rapid modulation of cell surface glycoproteins by activated neutrophils (i.e. CD62L shedding) rather than the actions of specific glycosylation enzymes as demonstrated in T- and endothelial cells.

Assessment of galectin levels in leukocytes over a 96h zymosan-induced resolving peritonitis demonstrated alterations both spatially and temporally with increased galectin-3 expression in neutrophils at the inflammatory site compared to the periphery and a peak expression at 24h adding supporting evidence that modulation of galectin expression allows delineation of galectin responses by neutrophils. This study also demonstrated a novel pro-resolution effect of galectin-3 with defective resolution observed in galectin-3 null mice.

In conclusion this work demonstrated that neutrophil permissibility for galectins-1, -3 and -9 binding is more likely a consequence of the exposure to galectins at specific time points in the resolving inflammatory response rather than due to a modulation of the glycophenotype upon activation. This study also

demonstrated that as well as an important role in the induction of an inflammatory response galectin-3 is involved in resolution, a novel finding which may lead to a better understanding of the resolution process.

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LIST OF ABBREVIATIONS

Ala	Alanine
ANOVA	Analysis of Variance
AnxA1	Annexin A1
APC	Allophycocyanin
Asn	Asparagine
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
C2GlcNAcT	Core 2 N-acetylglucosaminyltransferase
C3GlcNAcT	Core 3 N-acetylglucosaminyltransferase
C4GlcNAcT	Core 4 N-acetylglucosaminyltransferase
cDNA	Complimentary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
CGRP	Calcitonin gene-related peptide
CRD	Carbohydrate recognition domain
DAMP	Danger-associated moleular pattern
DAPI	4',6-diamidino-2-phenylindole
DMJ	Deoxymannojirimycin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triophosphate
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESAM	Endothelial cell-selectin adhesion molecule
ESL	E-selectin ligand
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FcγR1	FCγ receptor 1
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FSC	Forward Scatter
Fuc	Fucose
FucT	Fucosyltransferase
GAG	Glycosaminoglycan

Gal	Galactose
GalNAc	N-acetylgalactosamine
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
Glc	Glucose
GlcNac	N-acetylglucosamine
Glu	Glutamine
Gly	Glycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein coupled receptor
HAEC	Human aortic endothelial cell
HBSS	Hanks buffered saline solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMVEC	Human microvascular endothelial cells
HRP	Horseradish peroxidase
hrTNF	human recombinant tumour necrosis factor-α
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
Id	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Ip	Intraperitoneal
Iv	Intravascular
JAM	Junctional adhesion molecule
kDa	Kilodalton
LacNAc	N-acetyllactosamine
LAD	Leukocyte adhesion deficiency
LBG	Ligand binding groove
LEL	*Lycopersicon esculentum* lectin
LFA	Lymphocyte function-associated antigen
Lgals	Lectins, galactoside-binding
LPS	Lipopolysaccharide
Mac	Macrophage-1 antigen
MAL II	*Maackia amurensis* lectin II
Man	Mannose

MAPK	Mitogen-activated protein kinase
MCP	Monocyte-chemotactic protein
MerTK	Mer receptor tyrosine kinase
MFI	Median fluorescence intensity
MGAT	N-acetylglucosaminyltransferase
MHC	Major histocompatibility complex II
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuNAc	N-acetylneuraminic acid
NOD	Non-obese diabetic
OSGE	O-sialogycoprotein endopeptidase
PAF	Platelet-activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECAM	Platelet-endothelial cell adhesion molecule
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PHA-L	*Phaseolus vulgaris* leucoagglutinin
PMN	Polymorphonuclear cell
PNA	Peanut agglutinin
Poly(I:C)	Polyinosinic-polycytidylic acid
ppGalNAcT	Polypeptide N-acetylgalactosaminyltransferase
Pro	Proline
PS	Phosphatidylserine
PSGL	P-selectin glycoprotein ligand
RCA120	*Ricinus communis* Agglutinin 120
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
Sc	subcutaneous
SEM	Standard error mean
Ser	serine
SNA	*Sambucus nigra* agglutinin

SNAP	Soluble NSF attachment protein
SNARE	Soluble NSF attachment protein receptor
SSC	Side scatter
ST3GalT	α2,3-sialyltransferase-IV
ST6Gal	α2,6-sialyltransferase-1
TAE	Tris-acetate EDTA
TGF	Transforming growth factor
Thr	Threonine
Tim	T cell immunoglobulin domain and mucin domain
TLR	Toll-like receptor
TMB	3,3,5,5-tetramethylbenzidine
TNF	Tumour necrosis factor-α
TRPV1	Transient receptor potential cation channel subfamily 5 member 1
Tyr	Tyrosine
UDP	Uridine diphosphate
UEA	*Ulex europaeus* agglutinin I
VAMP	Vesicle-associated membrane protein
VCAM	Vascular cell adhesion molecule
WGA	Wheat Germ Agglutinin

CHAPTER 1:

INTRODUCTION

1 Introduction

1.1 Glycobiology

Glycosylation is one of the most important post-translational modifications of proteins with more than 50% of all mammalian proteins being glycosylated (van Kooyk et al., 2013). Approximately 10% of the expressed mammalian genome encodes enzymes that play a role in the glycosylation process; these include glycosyltransferases and glycosidases that add and remove monosaccharides from glycoconjugates respectively (Haslam et al., 2008). It was initially believed that glycosylation was restricted to eukaryotes however in 1976 Mescher and Strominger discovered a glycoprotein on the cell surface of *Halobacterium salinarum* and this provided the first evidence for glycoproteins in other kingdoms of life (Mescher and Strominger, 1976). Glycosylation has since been identified in ancient organisms including archaea indicating that it is an evolutionarily conserved process and thus is of importance for many facets of life (Jarrell et al., 2010).

The expression of glycosylation enzymes is regulated by the genome however the glycophenotype (the total expression of glycoconjugates on the surface of a cell) is considered a secondary gene product as glycan chains are modified post-translationally and can be affected by availability of monosaccharides (Grigorian et al., 2007, Lis and Sharon, 1993).

Glycan chains are frequently attached to cell surface proteins or lipids where they form glycoproteins and glycolipids respectively; their production is post-translational allowing cells of the same type to express different glycophenotypes dependent on the cellular environment. Thus unlike protein production, the glycoprotein status of a cell cannot be predicted by mRNA expression. This project focuses on the expression and regulation of glycoproteins on cells of the immune system with particular focus on those targeted by specific galectins, a family of proteins with known immunomodulatory functions.

Glycoproteins can be subdivided into two groups based on the amino acid in the protein chain to which they are bound; N-glycans are attached via an asparagine (Asn) residue while O-glycans are attached via a serine or threonine (Ser/Thr) residue. It is important to distinguish between the two as each has different binding permissibility for lectins.

1.1.1 N-Glycan Biosynthesis

N-glycan formation begins in the rough endoplasmic reticulum where, on the cytoplasmic side, two N-acetylglucosamine (GlcNAc) residues are added to a dolichol molecule via a uridine diphosphate (UDP)-GlcNAc donor by N-acetylglucosaminyl phosphate transferase (GlcNAc-1-phosphotransferase). Five mannose (Man) residues are added to the GlcNAc residues by mannosyltransferase enzymes via a guanosine diphosphate (GDP)-Man donor. The molecule is then translocated to the lumen of the endoplasmic reticulum where a further 4 mannose residues are added by mannosyltransferase enzymes via a lipid intermediate, dolichol-P-Man. Finally glucosyltransferases add three glucose (Glc) resid**ues** to the end of the structure (Burda and Aebi, 1999, Kornfeld and Kornfeld, 1985) Figure 1.1.

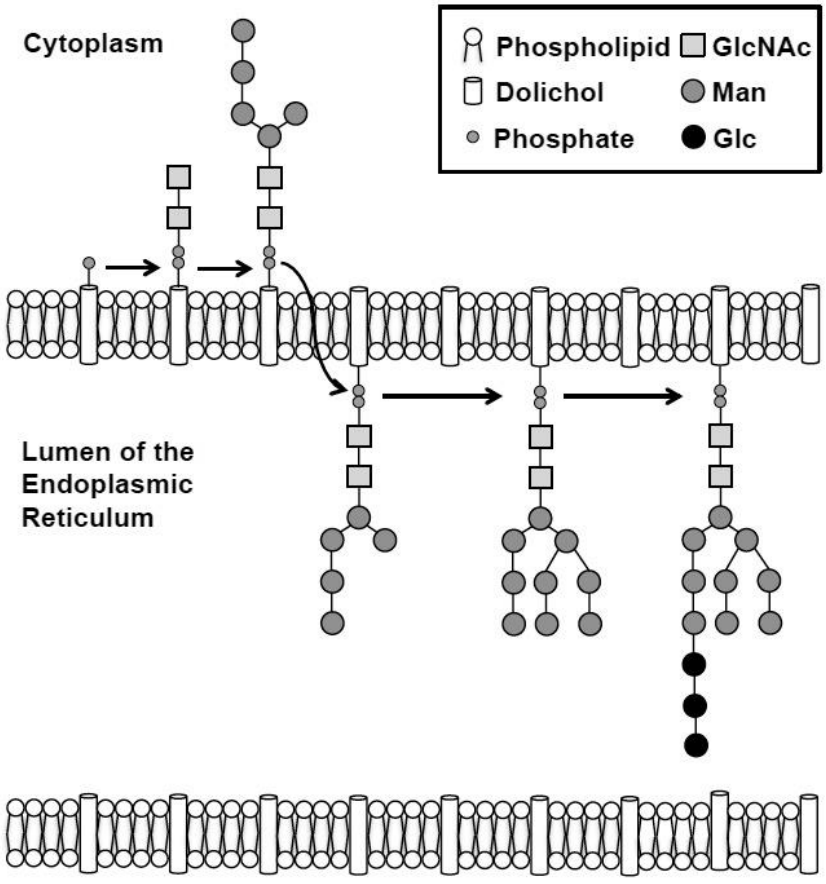


Figure 1.1: Schematic for the formation of a dolichol-linked precursor molecule in N-glycan biosynthesis. On the cytoplasmic side of the endoplasmic reticulum two N-acetylglucosamine residues are added followed by five mannose residues. The molecule is then translocated to the lumen where four more mannose residues and three glucose residues are added. Adapted from (Taylor and Drickamer, 2003).

The dolichol precursor molecule is transferred to an Asn residue on a polypeptide by an oligosaccharyltransferase enzyme. The Asn molecule is selected based on certain criteria: the amino acid sequence must follow the sequence Asn-X-Ser/Thr where X can be any amino acid except proline and the asparagine residue must lie in the correct position of the three-dimensional protein structure (Kornfeld and Kornfeld, 1985).

The first stage of processing is the removal of the three glucose residues and this is performed by glucosidases I and II, followed by up to four of the Man residues as shown in **Figure 1.2**.

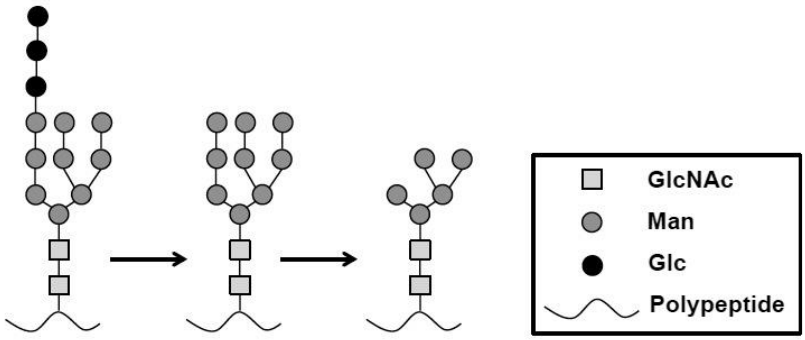


Figure 1.2: Processing of N-glycans following transfer to an asparagine residue on a polypeptide. Glucose residues are removed followed by up to 4 mannose residues depending on the type of N-glycan being generated. Adapted from (Taylor and Drickamer, 2003).

The glycoprotein is then translocated to the Golgi apparatus for the final processing to take place at which stage the N-glycan produced can be high mannose, hybrid or complex type depending on the extent of processing. High mannose N-glycans are those that have between five and nine Man residues and no further extensions. Complex N-glycans have all but three Man residues removed and each branch is extended with GlcNAc via N-acetylglucosaminyltransferase I (MGAT1). These can be subdivided into bi-, tri-, or tetra-antennary depending on the number of branches on the core structure and these branches can all be extended by N-acetyllactosamine chains (galactose bound to GlcNAc – LacNAc); extension usually continues until the chain is capped with a sialic acid residue that prevents further elongation. Fucosyltransferases also modify complex N-glycans and are important for the generation of sialyl Lewis x motifs. Hybrid N-glycans are formed when a GlcNAc is added onto the core structure containing three mannose residues, which can be extended but no further processing takes place as shown in **Figure 1.3** (Kornfeld and Kornfeld, 1985).

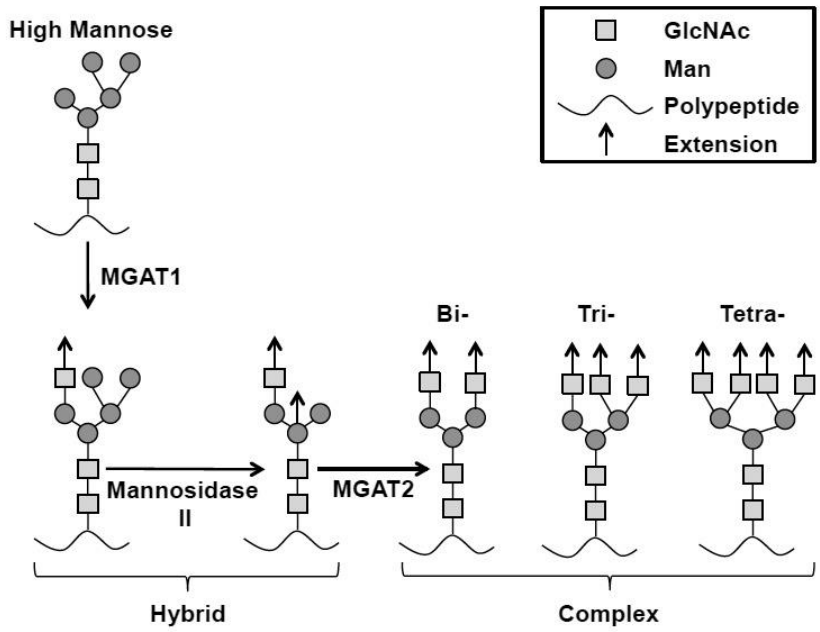


Figure 1.3: Schematic of the three types of N-glycans: high mannose, hybrid and complex. High mannose structures can be modified by N-acetylglucosaminyltransferase 1 to form hybrid structures and these can be further modified by mannosidase II and N-acetylglucosaminyltransferase 2 enzymes to form bi, -tri-, and tetra-antennary complex type N-glycans. Hybrid and complex N-glycans can be extended with LacNAc chains. Adapted from (Taylor and Drickamer, 2003).

1.1.2 O-Glycan Biosynthesis

O-glycans are generated in a different manner to the N-glycans with each monosaccharide added to a glycan chain individually without the production of a precursor molecule. Initially an N-acetylgalactosamine (GalNAc) residue is added to the protein at a serine or threonine residue by polypeptide N-acetylglucosaminyltransferase (ppGalNAcT) using UDP-GalNAc as a donor. The glycoprotein is then elongated using a variety of enzymes to form one of four core structures which each have different binding preferences.

Core 1 O-glycans are generated by addition of a single galactose (Gal) residue to the basal structure by core 1 β1,3-galactosyltransferase (T-synthase) and this can be further modified by core 2 β1,6-N-acetylglucosaminyltransferases (C2GlcNAcT) which adds a GlcNAc molecule to the basal structure of the core 1 O-glycan to form a core 2 structure. Core 3 O-glycans are generated from the initial basal structure but via the addition of a GlcNAc by C3GlcNAcT and this can be further modified by C4GlcNAcT, which adds a second GlcNAc residue to the core 3 O-glycan as shown in **Figure 1.4**. Each of the core structures can be elongated with LacNAc chains and these are

commonly terminated by the addition of sialyl Lewis x motifs by the actions of sialyltransferases and fucosyltransferases (Van den Steen et al., 1998).

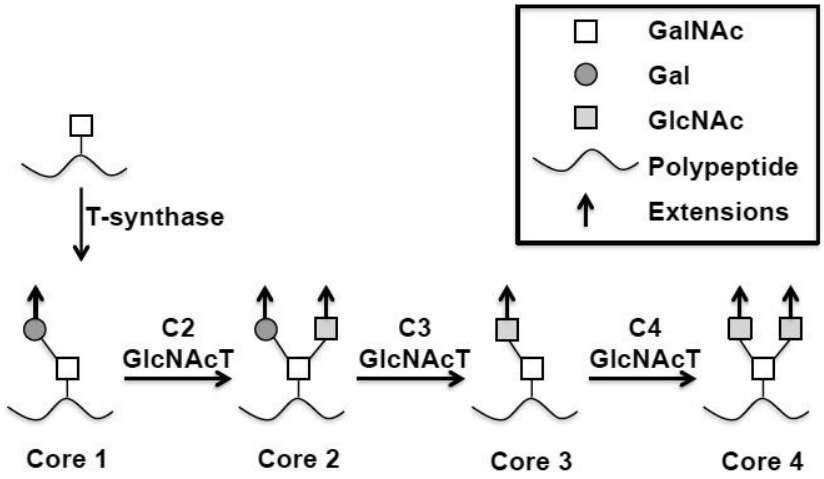


Figure 1.4: Core 1-4 O-glycan structures and the enzymes used to produce them. Core 1 O-glycans are generated by addition of galactose to the core structure, core 2 O-glycans are built onto the core 1 O-glycans, core 3 O-glycans are generated by the addition of N-acetylglucosamine to the core structure and core 4 O-glycans are built onto core 3 O-glycans. Adapted from (Taylor and Drickamer, 2003).

1.2 Inflammation

Inflammation is a physiological process designed to protect against infection or trauma by recruiting leukocytes from the circulation with the aim of destroying invading pathogens, repairing damaged tissue and restoring homeostasis to the area. This is accomplished via the sequential recruitment of leukocytes known as the leukocyte adhesion cascade, a term coined to explain the main stages of trafficking: capture and rolling, firm adhesion and transmigration (Butcher, 1991).

Initially neutrophils are captured from the bloodstream and begin to roll on the endothelium via interactions of selectins with their glycoconjugate counter-receptors. Selectins are a family of calcium-dependent lectins that interact with terminal sialyl Lewis x motifs (NeuNAc-α2,3-Gal-β1,4-(Fuc-α1,3)-GlcNAc) expressed on leukocyte glycoconjugates (Walz et al., 1990, Lowe et al., 1990). P-selectin and E-selectin are up-regulated on the endothelium following activation with pro-inflammatory cytokines released from resident macrophages and dendritic cells at the inflammatory site in response to injury or infection while L-selectin is constitutively expressed on leukocytes (Jung and Ley, 1999, Yang et al., 1999, Patel and McEver, 1997). The selectin-sialyl Lewis x interaction, along with secondary stimulation by chemokines released from the

inflammatory site and presented at the endothelium, leads to activation of integrins on the neutrophil surface, which interact with their respective ligands on the endothelium to promote firm adhesion (von Andrian et al., 1991, Laudanna and Bolomini-Vittori, 2009, Chesnutt et al., 2006). Once adherent neutrophils utilise a range of cell adhesion molecules, including platelet-endothelial cell adhesion molecule (PECAM)-1, endothelial cell-selectin adhesion molecule (ESAM)-1 and junctional adhesion molecule (JAM)-A to traverse the endothelial barrier and travel to the site of inflammation (Williams et al., 2011, Woodfin et al., 2009).

Once at the site of inflammation neutrophils play an important role in pathogen killing via phagocytosis, bacteria are detected by pattern recognition receptors such as toll-like receptors (TLRs) expressed on the neutrophil cell surface and endocytosed into phagosomes inside the neutrophil (van Kessel et al., 2014). Following this endocytotic process neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase becomes activated resulting in increased superoxide generation within the phagosome, due to the limited space within the phagosome the concentration of superoxide reaches approximately 20μM. Primary granule release into the phagosome also occurs leading to concentrations of myeloperoxidase (MPO) in the millimolar range; this MPO catalyses the conversion of hydrogen peroxide to hypochlorous acid and the highly acidic environment inside the granule is responsible for pathogen killing (Winterbourn and Kettle, 2013).

Aside from the well-organised mechanism of killing pathogens in phagosomes neutrophils also release their primary granule contents, including defensins, neutrophil elastase and MPO, into the extracellular environment to destroy invading pathogens; these mediators are not specific to microbial cells and thus cause damage to the host tissue if left unimpeded (Borregaard et al., 1993). To prevent damage to the host a second set of phagocytic leukocytes are recruited, the monocytes, which can be subdivided into two groups. Classical monocytes release cytokines to destroy any pathogens not removed by the neutrophils and are involved in cellular immunity; these are considered pro-inflammatory. Non-classical monocytes differentiate into macrophages at the inflammatory site and these phagocytose damaged tissue and apoptotic neutrophils to allow the tissue to return to homeostasis and are thus considered to facilitate/drive resolution (Gordon, 2003, Gordon and Taylor, 2005). Recent studies have shown that after a mild inflammatory stimulus the resolution phase is followed by population of the inflammatory site with a second wave of monocytes composed of monocyte-derived macrophages and tissue resident

macrophages; these cells act as a bridge between the innate and adaptive immune system before homeostasis can be restored (Newson et al., 2014).

1.2.1 Mechanisms of Neutrophil Degranulation

As described in the previous chapter neutrophil degranulation is essential to the inflammatory response and consequently is a tightly controlled process. Secretory vesicles, tertiary and secondary granules are required for trafficking to an inflammatory site and thus require release from the cell via exocytosis while primary granules are predominantly released into phagosomes for pathogen killing therefore a mechanism needs to be in place to control the release of granules.

One of the mechanisms in place for controlling sequential release of granules is their sensitivity to intracellular calcium flux; secretory vesicles are the most sensitive while primary granules are least sensitive however the downstream pathways following this activation have yet to be elucidated (Borregaard and Cowland, 1997, Kanaho et al., 2013).

Another mechanism by which granule release is controlled is via the binding of vesicle-associated membrane proteins (VAMPs) expressed on the granule surface to SNARE (soluble NSF attachment protein receptor) complexes expressed on the cell membrane. Granules contain different VAMPs to allow sequential release; secretory vesicles express VAMP-2, tertiary granules express both VAMP-2 and VAMP-7, while secondary and primary granules express VAMP-7 only. These proteins bind to SNARE complexes composed of soluble NSF associated proteins (SNAPs) and syntaxin-4 resulting in pore formation and release of the granule either from the cell or into a phagosome (Lacy, 2006).

1.3 Glycosylation in Inflammation

Glycosylation is of particular importance to the inflammatory process as the glycophenotype of an immune cell is crucial to its recruitment, activation and removal from inflammatory sites. The effect of glycosylation on leukocyte trafficking has been extensively studied using genetically manipulated mice and this has provided a framework for the identification of the roles of glycans on immune cells during inflammation.

1.3.1 Capture and Rolling

Glycosylation is important at all stages of leukocyte trafficking, especially during capture and rolling as these stages are dependent on interactions between selectins and sialyl Lewis x motifs expressed on cell surface glycoproteins (**Figure 1.5**).

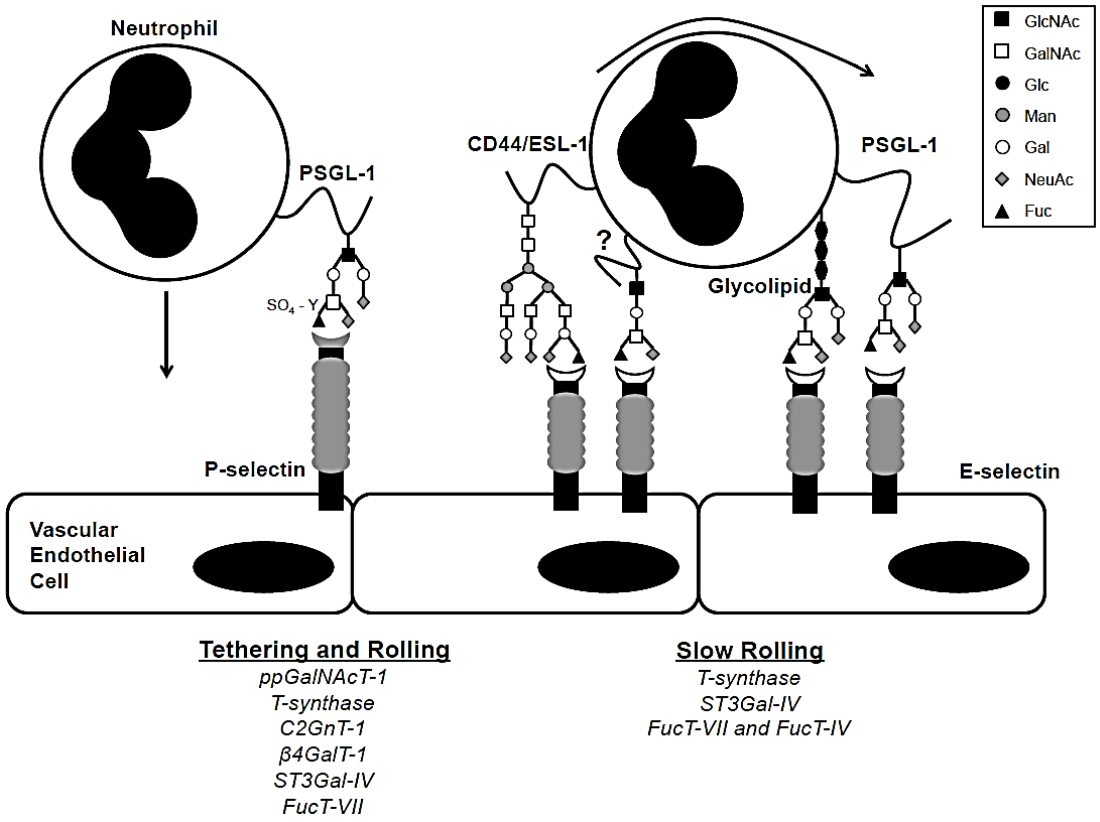


Figure 1.5: The role of glycosylation enzymes in neutrophil capture and slow rolling. Neutrophil tethering and rolling is mediated by interactions between P-selectin expressed on the endothelium in response to inflammatory stimulation and tyrosine sulphated Core 2 O-glycans on PSGL-1 constitutively expressed on the neutrophil. The expression of the core 2 O-glycans is dependent on the actions of ppGalNAcT-1, T-synthase, C2GnT-1, ST3Gal-IV and FucT-VII. Neutrophil slow rolling is mediated by interactions between E-selectin expressed on the endothelium in response to inflammatory stimulation and N-glycans expressed on CD44/ESL-1, Core 1 O-glycans expressed on unknown glycoproteins and Core 2 O-glycans expressed on glycolipids and PSGL-1. Slow rolling is dependent on the actions of T-synthase, ST3Gal-IV, FucT-VII and FucT-IV. Loss of the enzymes involved at either stage lead to a partial or complete loss of neutrophil trafficking. (Wright and Cooper, 2014).

The initial capture and rolling of leukocytes from the circulation relies on the interaction between P-selectin on activated endothelial cells and sialyl Lewis x motifs on constitutively expressed P-selectin glycoprotein ligand (PSGL)-1 on leukocytes. Mice lacking ppGalNAcT, the enzyme responsible for initiating O-glycan synthesis, demonstrate a significantly lower level of capture and rolling compared to wild type controls indicating that sialyl Lewis x expressed by PSGL-1 is displayed on core 2 O-

glycans (Block et al., 2012, Tenno et al., 2007). Similarly mice deficient in C2GlcNAcT-1 (responsible for core 2 extension) have severely reduced P-selectin-dependent leukocyte rolling (Sperandio et al., 2001).

The importance of β4GalT-1 (which is essential for extending core 2 O-glycans) is highlighted by null mice, which have reduced neutrophil recruitment in models of zymosan-induced dermatitis and skin wound healing due to defective selectin ligand biosynthesis (Asano et al., 2003, Mori et al., 2004).

Finally sialyl Lewis x motifs are capped with sialic acid and fucose, generated by the actions of α2,3-sialyltransferase-IV (ST3GalT-IV) and α1,3-fucosyltransferase-VII (FucT-VII) and deletion of either of these enzymes results in mice which have significantly reduced leukocyte capture and rolling compared to wild types controls in inflamed cremaster models using intravital microscopy (Ellies et al., 2002, Malý et al., 1996). As glycosylation is imperative for leukocytes to elicit their roles in inflammation a large amount of biological redundancy is innate in the system with many enzymes capable of compensating in case of loss of one, thus complete inhibition of the recruitment pathway usually requires the knock down of multiple enzymes. For example although FucT-IV mice have no deficiency in capture and rolling compared to wild type mice the remaining response seen in FucT-VII null mice is completely abolished in double null mutants (FucT-VII/FucT-IV) suggesting that the FucT-IV enzyme can compensate for FucT-VII loss but does not act constitutively (Homeister et al., 2001). Fucosylation is of particular importance to leukocyte trafficking as demonstrated in patients suffering from leukocyte adhesion deficiency (LAD)-II; these patients have a genetic defect in the gene encoding the GDP-fucose transporter and thus do not generate selectin ligands. The disease manifests with recurrent bacterial infections, persistent leukocytosis and mental and growth retardation (Etzioni et al., 1992, Frydman et al., 1992, Price et al., 1994).

Once leukocytes have begun to roll on the endothelium in a P-selectin-dependent manner they need to decrease their velocity in order for adhesion and transmigration to begin and this is mediated by the interactions of E-selectin with its counter-receptors. E-selectin binds PSGL-1 in much the same manner as P-selectin but knock-down of PSGL-1 is unable to completely abolish E-selectin-dependent rolling suggesting the lectin is able to bind other receptors (Yang et al., 1999). E-selectin also binds to fucosylated N-glycans on CD44 and E-selectin ligand (ESL)-1 as removal of O-glycans

from myeloid cells using O-sialoglycoprotein endopeptidase (OSGE) does not abolish E-selectin-dependent rolling (Katayama et al., 2005, Levinovitz et al., 1993).

Recent evidence has also demonstrated a role for core 1 O-glycans as E-selectin ligands as mice lacking T-synthase, the key enzyme responsible for the formation of core 1 O-glycans, in haematopoietic cells show significant reductions in E-selectin-dependent neutrophil recruitment to the peritoneum in response to thioglycollate injection. These mice had a significantly diminished response when compared to the C2GlcNAcT-1 null mice suggesting core 1 O-glycans are also E-selectin ligands (Yago et al., 2010).

1.3.2 Firm Adhesion

E-selectin-dependent slow rolling induces the intermediate activation of integrins on the leukocyte surface including Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18), full activation of the integrins is stimulated by the interactions between chemokine receptors on the leukocyte surface and chemokines that are released from activated endothelial cells or presented on the surface of endothelial cells following their release from other cellular sources (von Andrian et al., 1991, Rot, 1992, Alon and Ley, 2008). Chemokines are presented to leukocytes by the endothelium on glycosaminoglycans (GAGs) such as heparan sulphate; GAGs are also found in the extracellular matrix and this allows formation of an immobilised chemokine gradient that can be followed by the leukocytes to the site of inflammation (Patel et al., 2001).

Chemokine receptors themselves are glycosylated and this increases the binding affinity of the chemokine for the receptor, for example α2,6-linked sialic acid is required for chemokine binding to CCR5 and CXCR4 (Bannert et al., 2001, Zhou and Tai, 1999). The role of sialylation has been further investigated using ST3GalT-IV null mice, which have decreased CXCR2-mediated firm adhesion as assessed by intravital microscopy of the cremasteric microcirculation in mice (Frommhold et al., 2008). The ST3GalT-IV null mice also have decreased recruitment of both neutrophils and monocytes to the peritoneal cavity in response to CCL2 when compared to wild type mice (Döring et al., 2014). As well as altering chemokine affinity, glycosylation of chemokine receptors also protects them from proteolytic removal from the cell thus prolonging the effect of the chemokine (Ludwig et al., 2000).

Activated integrins on the PMN surface bind to members of the Ig superfamily on the endothelial cell surface including ICAM-1 and VCAM-1. These receptors are

themselves glycosylated and N-glycans play important roles in the process of firm adhesion. Human ICAM-1 has eight sites that are permissible for N-glycosylation and importantly the extent of processing affects the binding of integrins. For example, treatment of human umbilical vein endothelial cells (HUVECs) with deoxymannojirimycin (DMJ), an inhibitor of mannosidase I that blocks N-glycans from being processed past high mannose type, results in increased neutrophil adhesion (Bloom et al., 1996). This effect is due to Mac-1 binding preferentially to ICAM-1 with simple high mannose N-glycans; it has also been demonstrated that LFA-1 binds to ICAM-1 with large complex N-glycans (Diamond et al., 1991). VCAM-1 contains seven potential N-glycosylation sites and is decorated with α2,6-linked sialic acid however the sialylation of VCAM-1 does not affect VCAM-1-dependent leukocyte adhesion under flow conditions and the role of this glycosylation has yet to be determined (Hanasaki et al., 1994, Abe et al., 1999) **Figure 1.6**.

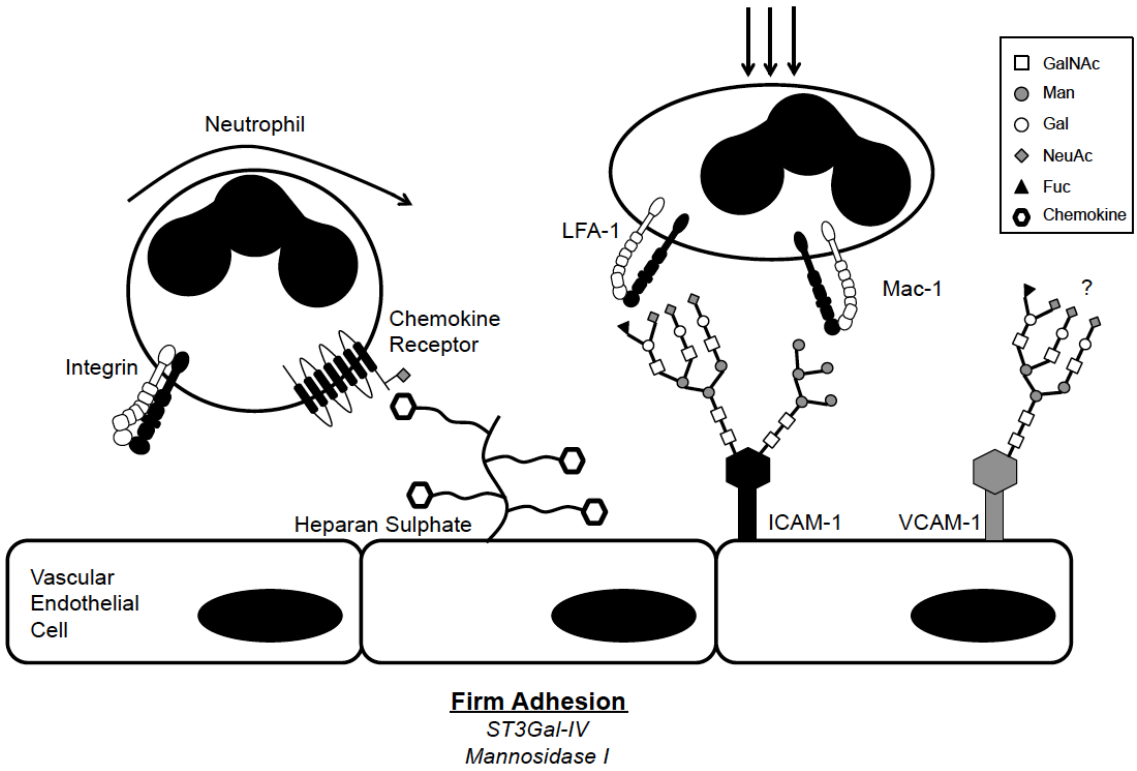


Figure 1.6: The role of glycosylation enzymes in neutrophil firm adhesion. Neutrophil firm adhesion is mediated by interactions between integrins expressed by leukocytes and their ligands on the activated endothelium. Slow rolling on E-selectin promotes the intermediate activation of the integrin molecules and these are fully activated by interactions between chemokines, which are immobilised on endothelial glycosaminoglycans, and their GPCR receptors. GPCR receptors require modification by ST6GalT-IV to bind chemokines. Once fully active the integrins bind to ICAM-1 and VCAM-1 that are N-glycosylated. Loss of enzymes at either stage leads to a partial loss of neutrophil trafficking. (Wright and Cooper, 2014).

1.3.3 Transmigration

Transmigration is mediated by a number of adhesion molecules including ESAM-1, JAM-A, PECAM-1 and vascular endothelial (VE)-cadherin and although studies have shown that many of these are glycosylated it is not yet clear whether this directly affects leukocyte trafficking (**Figure 1.7**).

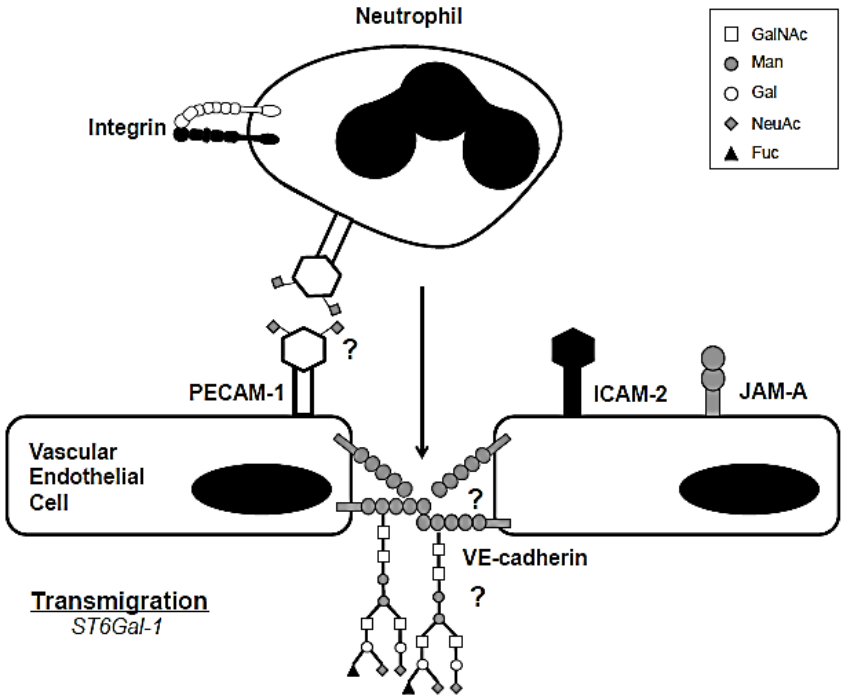


Figure 1.7: The role of glycosylation enzymes in neutrophil transmigration. Neutrophil transmigration is mediated by interactions between cell adhesion molecules expressed on the leukocyte and the endothelium including PECAM-1, ESAM-1 and JAM-A. Of these PECAM-1 requires modification by ST6Gal-1 to be retained at the cell surface. VE-cadherin is required to disassemble in order for trafficking to take place and is known to be glycosylated however it is unclear how this affects trafficking. (Wright and Cooper, 2014).

PECAM-1 is expressed by both the endothelium and leukocytes and forms homophilic interactions to initiate the transmigration stage of trafficking (Piali et al., 1995). The expression of PECAM-1 on endothelial cells in ST6Gal-1 null mice is reduced when compared to wild type cells suggesting sialylation is important for surface retention of the adhesion molecule and thus may play an indirect role in trafficking (Kitazume et al., 2010).

VE-cadherin is expressed on endothelial cells and forms zipper-like cell-cell interactions that maintain vascular integrity during homeostasis and thus prevent trafficking; VE-cadherin is required to disassemble in order for leukocytes to pass

through the endothelial cells during inflammation (Shapiro et al., 1995). There are seven potential N-glycosylation sites on VE-cadherin, all of which are in the extracellular domain; these are predominantly sialylated and fucosylated biantennary complex N-glycans with some expression of sialylated hybrid-type N-glycans (Suzuki et al., 1991, Breviario et al., 1995, Geyer et al., 1999). Studies using bacterially-produced VE-cadherin suggested the protein forms trimers at the cell surface however this was recently demonstrated to be an artefact caused by a lack of glycosylation and it is now believed that the high level of sialylation provides the protein with a negative charge thus preventing association with molecules on the same cell and promoting association with molecules on opposing cells to maintain the “zipper-like” structure (Legrand et al., 2001, Bibert et al., 2002, Brasch et al., 2011).

1.3.4 Modulation of the Glycophenotype during Inflammation

It is known that the glycophenotype of the endothelium is fluid during inflammation and is modulated upon pro-inflammatory stimulation, such as tumour necrosis factor (TNF)-α and disturbed flow, and this leads to an increase in the expression of hypoglycosylated N-glycans that are predominantly high-mannose structures (Chacko et al., 2011). This modulation has been shown to promote an increase in the local adhesion of monocytes under flow and thus the glycophenotype of the endothelium has been proposed to act as a “zip code” for directing specific recruitment of leukocytes in different vascular beds in response to inflammatory stimuli (Renkonen et al., 2002).

The modulation of the endothelial glycophenotype also impacts the binding of lectins such as galectins that have a role in leukocyte trafficking. Treatment of the endothelium with immunosuppressive cytokines (such as interleukin (IL)-10 and transforming growth factor (TGF)-β) leads to increased expression of complex tri- and tetra-antennary N-glycans with poly-LacNAc chains and decreased expression of α2,6-linked sialic acid residues; this glycophenotype is permissive for the binding of galectin-1, a lectin that is known to decrease leukocyte trafficking through the endothelium (Croci et al., 2014, Norling et al., 2008).

There is some evidence to suggest that the glycophenotype of leukocytes is also modulated during inflammation. Analysis of neutrophils from healthy volunteers by mass spectrometry found the majority of N-glycans were complex bi-, tri-, or tetra-antennary structures with LacNAc extensions and sialylated and fucosylated branches.

The O-glycans were predominantly highly sialylated and fucosylated core 2 structures (Babu et al., 2009). The glycophenotype was is also modulated by pro-inflammatory stimuli as activation with Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) promoted rapid and transient neuraminidase release from secondary and tertiary granules, leading to removal of sialic acids from glycoproteins, exposing the underlying glycans and allowing binding of lectins (Cross and Wright, 1991, Cross et al., 2003). It is not yet clear how the glycophenotype is modulated during the process of neutrophil trafficking through the endothelium.

1.4 Galectins

Galectins are a family of lectins that are defined as having "affinity for β-galactosides and significant sequence similarity in their carbohydrate binding sites, the relevant amino acids of which have been determined by X-ray crystallography" (Barondes et al., 1994). Studies have identified 15 mammalian galectins, which are numbered sequentially based on date of discovery, however one of these (galectin-11) has since been designated 'galectin-like' as it does not bind β-galactoside (Cooper et al., 2012, Leffler et al., 2004). Galectin-10 is also potentially galectin-like as it preferentially binds mannose and not β-galactoside (Swaminathan et al., 1999). Galectins have been found in all animal kingdoms as well as plants and fungi and are therefore evolutionarily conserved suggesting many important roles for this family of proteins (Leffler et al., 2004).

Of all the galectins, three have been identified to play important roles in innate immunity; these are galectin-1, galectin-3 and galectin-9 and thus will be the focus of this project (Dias-Baruffi et al., 2010, Cherayil et al., 1989, Kim et al., 2007, Wada and Kanwar, 1997, Wada et al., 1997).

1.4.1 Structure

Galectin-1 was initially discovered in the electric eel where it was termed electrolectin, since then many galectins have been identified in various species, tissues, and cell types, all of which were differently named depending on the source and the investigator (Teichberg et al., 1975). This led to confusion and an inability to link different reports of structure and function; therefore in 1993 the galectins were characterised into three subtypes depending on their structural characteristics in an attempt to provide some clarity to the various members of the family as shown in **Figure 1.8** (Hirabayashi and Kasai, 1993).

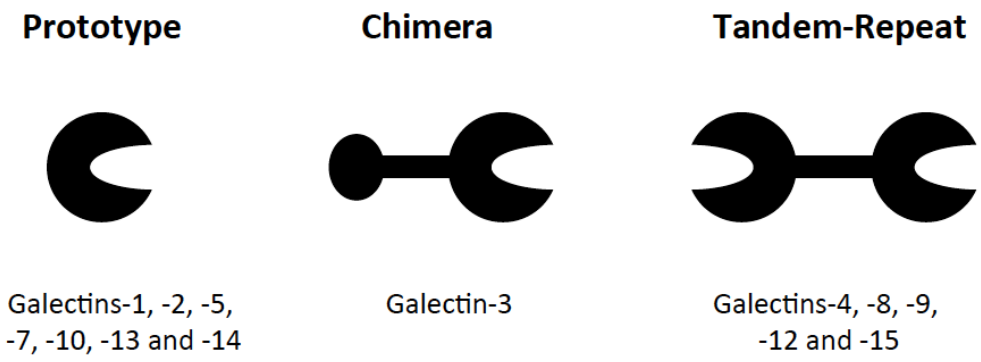


Figure 1.8: Schematic of the structure of the three subtypes of galectins. Prototype galectins have a single CRD; chimeric galectins have a single CRD with an extended N-terminus and tandem repeat galectins have two distinct CRDs joined by a short linker peptide.

Prototype galectins have a single carbohydrate recognition domain (CRD) and can homodimerise physiologically to exert their effects; these include galectins-1, -2, -5, -7, -10, -13 and -14 (it has since been shown that galectin-5, -7 and -10 can exist as monomers under physiological conditions) (Hirabayashi and Kasai, 1993). Galectin-3 is the only known chimeric galectin, it has a single CRD linked to an extended non-lectin region (Hirabayashi and Kasai, 1993). Tandem-repeat galectins have two distinct CRDs with different binding properties that are joined by a short-linker peptide; it was initially postulated that proteolytic cleavage of the linker peptide enabled each CRD to elicit its effects however it is now known that both CRDs as well as the linker peptide are essential for the effects of the protein through increasing signalling potency (Earl et al., 2011). Tandem-repeat galectins include galectins-4, -8, -9, -12 and -15 (Hirabayashi and Kasai, 1993).

The X-ray crystallography structure of the CRD was determined in 1993 by Lobsanov and colleagues who determined that each monomer is composed of eleven strands arranged in a β-sandwich with five strands in one sheet and six strands in the other (Lobsanov et al., 1993). The CRD consists of 135-140 amino acids however only four are conserved between the entire galectin family, these are a glycine at position 25, a tryptophan at position 80, a glutamic acid at position 83 and an arginine at position 125. These conserved amino acids reside in a structure known as the ligand binding groove (LBG), the part of the CRD where the β-galactoside residue binds (Di Lella et al., 2011). Although only these four amino acids residues are conserved there is high homology between the other residues in the CRD especially between galectins of the same subtype (Guardia et al., 2011).

1.4.2 Expression

Galectins are soluble proteins found throughout all cellular compartments however it is also well documented that they are found in the extracellular environment where they act on cell surface receptors to elicit their effects however extensive analysis of the genes confirms the proteins do not express a secretion signal sequence (Hughes, 1999), thus they join proteins such as Annexin A1 (AnxA1) in being exported from the cell by an as yet unknown mechanism (March et al., 1985, Perretti and D'Acquisto, 2009). Until 2001 IL-1β was included in this category however it was determined that it is secreted via microvesicles that are released from monocytes in response to an increase in cytosolic calcium, this mechanism has yet to be assessed for galectin secretion (MacKenzie et al., 2001).

Of the fifteen galectins described in the literature eleven are expressed in humans. Galectins-1, -3 and -9, known to elicit immunomodulatory functions, are as would be expected, expressed in cells pertinent to the inflammatory response with expression levels modulated in response to inflammatory mediators.

1.4.2.1 Endothelial Cells

Vascular endothelial cells are known to express low levels of galectins-1, -3 and -9 under basal conditions but this is modulated in response to activation of the cells (Thijssen et al., 2008). Galectin-1 expression is increased upon activation of HUVECs with human serum or lipopolysaccharide and in human aortic endothelial cells (HAECs) following stimulation with minimally oxidised low-density lipoprotein (Baum et al., 1995). The increased expression of galectin-1 seen following activation correlates with a localisation of the protein to the cell surface (Thijssen et al., 2008). Galectin-3 in HUVECs is up-regulated upon activation with IL-1β (50ng/mL) for 4 hours (Rao et al., 2007). However *in vivo* studies have shown a decrease in the expression of galectin-3 in rat endothelial cells following 4-hour carrageenan-induced peritonitis (Gil et al., 2006b)(Gil et al., 2006b). Galectin-9 is also increased in HUVECs following stimulation with interferon-γ (10ng/mL) or polyinosinic-polycytidylic acid (poly-IC), a double-stranded RNA (Alam et al., 2011, Ishikawa et al., 2004, Imaizumi et al., 2002).

1.4.2.2 Monocytes and Macrophages

Galectin-1 is detected on the surface of isolated human monocytes under basal conditions, levels are unaltered upon classical activation of the cells to an M1 phenotype but surface levels increase upon alternative activation to M2 monocytes

(Novak et al., 2012). Galectin-1 is also detected in macrophages from sputum samples and expression is significantly decreased in asthmatic patients (Sanchez-Cuellar et al., 2012). Galectin-3 is highly expressed by human and murine monocytes and macrophages and levels increase in correlation with the differentiation of the cell; murine alternative macrophages express significantly more galectin-3 than classical macrophages (Ho and Springer, 1982, Sato and Hughes, 1994, MacKinnon et al., 2008, Novak et al., 2012). Upon activation with calcium ionophore (A23187) the expression of galectin-3 significantly decreases and this coincides with an increase in the extracellular environment confirming the lectin is being released from the cell (Liu et al., 1995). Resident peritoneal macrophages express low levels of galectin-3, these cells are a distinct population of macrophages that are derived from the yolk sac during embryogenesis and these differentiate to repopulate the peritoneal cavity following depletion (Yona et al., 2013). Galectin-9 is expressed in human macrophages collected from sputum samples in healthy volunteers and, similar to galectin-1, levels are significantly decreased in asthmatic patients (Sanchez-Cuellar et al., 2012).

1.4.2.3 Neutrophils

Galectin expression in both human and murine neutrophils is negligible, although there are reports of modification during inflammation. Galectin-1 expression is significantly decreased upon human neutrophil adhesion to an endothelial monolayer *in vitro* (Gil et al., 2006b)(Gil et al., 2006b). The expression of galectin-3 in neutrophils is somewhat controversial and appears to be species-dependent as a report by Gil et al showed an increase in the expression of the protein following carrageenan-induced peritonitis in recruited neutrophils in a rat model while Sato and colleagues showed no expression of the protein in murine neutrophils that were recruited to the air pouch in response to lipopolysaccharide (LPS) (Gil et al., 2006b, Truong et al., 1993, Farnworth et al., 2008, Sato et al., 2002b). This discrepancy may be a result of the differences in detection methods; Gil and colleagues used immunofluorescence to assess the expression of galectin-3 *in situ* while Sato et al plated exudate from the air pouch onto plastic and collected the non-adherent neutrophils before analysis by western blot, a method which may have lacked the sensitivity to detect low levels (Gil et al., 2006b, Sato et al., 2002b). Human neutrophils express low levels of galectin-3 and this is not modulated upon transendothelial migration (Gil et al., 2006b)(Gil et al., 2006b), the lectin could also be detected in neutrophils from sputum samples collected from healthy and asthmatic patients and no difference was seen between the two groups (Sanchez-Cuellar et al., 2012).

1.4.3 Function

Although galectins bind specifically to LacNAc residues they do so with relatively low affinity, this is increased by up to 100-fold by arrangement of the LacNAc residues into repeating chains (Rabinovich and Toscano, 2009, Ahmad et al., 2002). They bind residues expressed on cell surface glycoproteins and form galectin-glycoprotein lattices as shown in **Figure 1.9**.

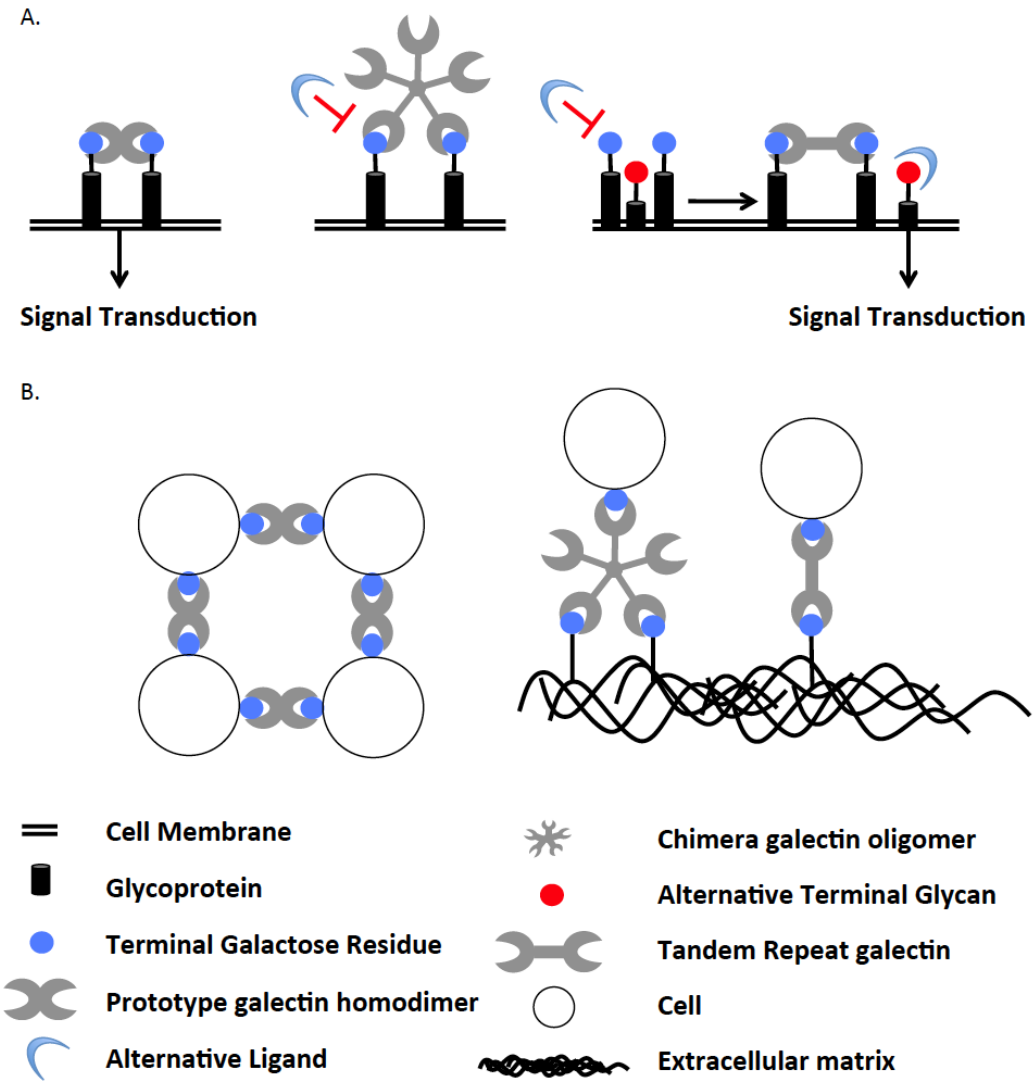


Figure 1.9: Galectin functions on a single cell and between cells. A. Galectins can cross-link receptors on a cell surface to induce signal transduction, they can bind receptors to inhibit the binding of alternative ligands and they can cluster receptors to allow other ligands access to their receptors. B. Galectins can cross-link cells to promote cell-cell adhesion or cells to extracellular matrix to promote cell-matrix adhesion.

Galectins can cross-link receptors on the same cell leading to activation of signalling pathways, inhibition of other ligands binding to the receptors or even increase binding by clustering inappropriate receptors out of the way allowing ligands to reach their

receptors (Rabinovich et al., 2007). The galectins can also cross-link receptors between cells or between cells and extracellular matrix proteins to promote adhesion (Rabinovich et al., 2007). The functions of galectins-1, -3 and -9 upon binding to their ligands will be discussed individually.

1.4.4 Galectin-1

Galectin-1 is a 14 kilodalton (kDa) prototype galectin that homodimerises under reducing conditions via specific amino acids in its N-terminus; the monomer is also bioactive but has a lower affinity for ligands than the dimeric form; this is important as both forms can be detected in solution (Cho and Cummings, 1995, Hirabayashi and Kasai, 1984, Leppänen et al., 2005, Giudicelli et al., 1997). Galectin-1 can be oxidised or reduced reversibly, which is important as reduced galectin-1 has immunoregulatory and pro-apoptotic properties while oxidised galectin-1 has no effect on immune cells (Guardia et al., 2014). The highly oxidative environment at inflammatory sites facilitates the oxidation of galectin-1 allowing leukocytes to be recruited and elicit their effects without being impeded by the anti-inflammatory properties of the lectin. As the leukocytes migrate away from the inflammatory site into healthy tissue the environment normalises and the reduced galectin-1 homodimerises to elicit its effects and protect healthy tissue (Stowell et al., 2009a).

Galectin-1 binds preferentially to LacNAc monomers expressed on core 2 and core 4 O-glycans or bi-antennary complex N-glycans and the binding is inhibited by the presence of an α2,6-linked, but not by α2,3-linked, sialic acid residues as the lectin binds to the terminal motif (Stowell et al., 2004, Stowell et al., 2008a, Leppänen et al., 2005).

Many of the studies assessing galectin-1 functions have been performed on T cells as the lectin plays an important role in chronic inflammatory diseases where it has been designated anti-inflammatory. Galectin-1 promotes the apoptosis of human and murine Th1 and Th17 cells while having no effect on Th2 cells due to differential glycosylation of the cells; Th1 and Th17 cells express high levels of LacNAc on their surfaces which are permissive for galectin-1 binding while on Th2 cells these residues are capped with α2,6-linked sialic acid that blocks galectin-1 binding (Toscano et al., 2007). The LacNAc residues on Th1 and Th17 cells are expressed on core 2 O-glycans of CD7, CD43 and CD45, upon galectin-1 binding CD43 and CD45 are sequestered on the cell surface leaving CD7 isolated and this initiates the apoptotic caspase pathway (Pace et al., 1999, Brandt et al., 2008, Galvan et al., 2000).

1.4.4.1 Galectin-1 in Animal Models of Inflammation

Galectin-1 null mice were generated and exhibit no phenotypic abnormalities under standard conditions except for a deformity in olfactory nerve development; these mice do have deficiencies when challenged as discussed below (Poirier and Robertson, 1993, Puche et al., 1996). Galectin-1 null mice have been subjected to various inflammatory pathologies in order to assess the phenotype when challenged as shown in **Table 1-1**.

Pathology	Outcome
Experimental Autoimmune Encephalomyelitis	Increased susceptibility to disease (Toscano et al., 2007)
Collagen-induced Arthritis	Increased severity of disease (Iqbal et al., 2013)
Graft vs Host Disease	Earlier rejection of skin grafts (Moreau et al., 2012)
Interleukin-1β Intravital Microscopy	Decreased leukocyte adhesion and emigration (Cooper et al., 2008)
Experimental Acute Myocardial Infarction	Increased inflammation and damage (Seropian et al., 2013)
Carrageenan-induced Paw Oedema	Reduced leukocyte recruitment in the second half of an inflammatory response (Iqbal et al., 2011)

Table 1-1: Overview of the phenotype of galectin-1 null mice subjected to various inflammatory pathologies.

Galectin-1 null mice have increased susceptibility to experimental autoimmune encephalomyelitis and significantly more pathological signs of collagen-induced arthritis compared to wild type mice and this correlates with an increase in interleukin-17 and interferon-γ levels suggesting an increased number of Th1 and Th17 cells compared to control (Toscano et al., 2007, Iqbal et al., 2013). Galectin-1 null mice have also been used to study the innate immune response using intravital microscopy to visualise the microvasculature following activation with IL-1β where increased leukocyte recruitment was observed compared to wild type mice (Cooper et al., 2008). Paw oedema was induced in galectin-1 null mice using carrageenan and this led to reduced leukocyte recruitment in the second half of the inflammatory response, as galectin-1 is an anti-inflammatory protein this was an unexpected finding however it was also demonstrated that galectin-9, a second anti-inflammatory member of the galectin family, was increased in these mice.

Through the induction of T cell apoptosis galectin-1 is able to attenuate many T cell-driven pathologies as demonstrated by several groups using recombinant galectin-1 in various models of inflammation as shown in **Table 1-2**.

Pathology	Species	Dose	Outcome
Experimental Autoimmune Myasthenia Gravis	Rabbit	100μg/intradermal (id) single dose with antigen or at onset of clinical symptoms	Preventative and therapeutic effects (Levi et al., 1983)
Collagen-induced Arthritis	Mice	100μg/intraperitoneal (ip) daily for 11 days beginning at onset of clinical symptoms	Skewing from Th1 to Th2 profile and attenuation of the disease (Rabinovich et al., 1999)
Concanavalin-A-induced Hepatitis	Mice	20μg/intravenous (iv) 30 minutes prior to Con-A administration	Decreased pro-inflammatory cytokine production and increased T cell apoptosis (Santucci et al., 2003)
Graft vs Host Disease	Mice	250μg/ip three times weekly from bone marrow transplant until death	Amelioration of weight loss, inflammatory destruction of target organs and mortality (Baum et al., 2003)
Trinitrobenzene Sulphonic Acid-induced Colitis	Mice	400μg/iv daily for 7 days following TNBS injection or starting 2 weeks after onset of clinical symptoms	Protective and therapeutic effects (Santucci et al., 2003)
Autoimmune Retinal Disease	Mice	50μg/ip every other day for 1 week either week 1 or week 3 after disease induction	Suppression of pathology (Toscano et al., 2006)
Experimental Acute Myocardial Infarction	Mice	100μg/ip single dose 3 minutes prior to reperfusion	Attenuation of pathology (Seropian et al., 2013)
Phospholipase A_2-induced Paw Oedema	Rats	5ng/subplantar (sp) single dose 30 minutes prior to phospholipase A_2	Decreased leukocyte recruitment (Rabinovich et al., 2000)
IL-1β-induced Peritonitis	Mice	0.3μg/ip single dose 1 hour prior to IL-1β administration	Decreased neutrophil recruitment to peritoneal cavity (La et al., 2003)

Table 1-2: Overview of the phenotype of mice following use of recombinant galectin-1 in animal models of inflammation.

The effects of exogenous galectin-1 have been studied by administration of recombinant protein to animals at different stages of the inflammatory process in different disease models and these studies provide evidence that galectin-1 is able to both prevent and treat many inflammatory conditions. The first evidence of an anti-

inflammatory role for galectin-1 came from its effect in a model of experimental autoimmune myasthenia gravis. Rabbits were administered 100µg of galectin-1 both prophylactically and therapeutically with both schedules leading to prevention and attenuation of clinical symptoms respectively (Levi et al., 1983). The majority of the literature has since focused on the effects of galectin-1 in T cell driven models such as collagen-induced arthritis, Concanavalin-A-induced hepatitis and Trinitrobenzene Sulphonic acid-induced colitis; these have been performed using doses of rGal-1 from 20µg - 400µg per mouse and these all promoted amelioration of disease due to induction of apoptosis in Th1 and Th17 cells (Rabinovich et al., 1999, Santucci et al., 2003, Baum et al., 2003, Toscano et al., 2006, Seropian et al., 2013).

The first study to investigate the role of galectin-1 in a model of innate immunity was performed by Rabinovich et al (1992) in a model of phospholipase A_2-induced paw oedema. Rats were administered 5ng of galectin-1 into the paw 30 minutes before phospholipase A_2 and decreased leukocyte recruitment was seen (Rabinovich et al., 2000). In another study mice were given 0.3µg rGal-1 in a model of neutrophil-driven inflammation, interleukin-1β-induced peritonitis; these mice showed decreased neutrophil recruitment to the peritoneal cavity (La et al., 2003). The mechanisms by which galectin-1 exerts its anti-inflammatory effects have yet to be fully elucidated, however it is known to bind innate immune cells and elicit various responses as discussed below.

1.4.4.2 Monocytes and Macrophages

Galectin-1 binds to an as yet unknown receptor on human monocytes and promotes increased expression of Fcγ receptor 1 (FCγR1) leading to increased phagocytosis and a decreased expression of major histocompatibility class II (MHC II) thus decreasing the ability of monocytes to act as antigen presenting cells (Barrionuevo et al., 2007). The lectin is chemotactic for human monocytes, but not human macrophages, via a p44/42 mitogen activated kinase (MAPK) pathway (Malik et al., 2009) and galectin-1 is able to convert murine macrophages to a pro-resolving phenotype, characterised by expression of 12/15-lipoxygenase and loss of phagocytic capacity, which is important for the resolution of the inflammatory response (Rostoker et al., 2013).

1.4.4.3 Neutrophils

Almkvist and colleagues demonstrated increased binding of galectin-1 to human neutrophils that had exudated compared to those in the peripheral circulation (Almkvist

et al., 2002). Once bound to its counter-receptors on neutrophils galectin-1 is able to induce a reversible exposure of phosphatidylserine (PS), which is not linked to an increase in apoptosis of the cells but acts as an 'eat-me' signal inducing preaparesis of the neutrophils by monocytes, an act that may serve to limit damage to healthy tissue (Stowell et al., 2009b, Dias-Baruffi et al., 2003). Galectin-1 also inhibits platelet-activating factor (PAF)-induced CD11b upregulation and decreased neutrophil-endothelial interactions and neutrophil chemotaxis (Cooper et al., 2008, La et al., 2003).

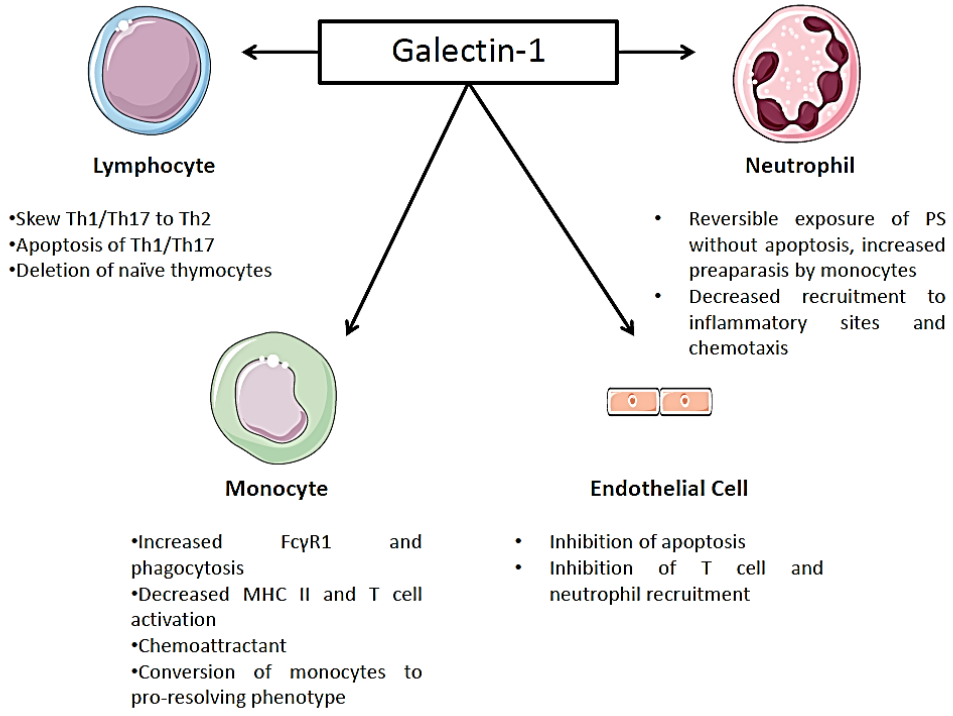


Figure 1.10: Summary of the effects of galectin-1 on immune cells. The effects of galectin-1 on lymphocytes, monocytes, neutrophils and endothelial cells as determined by studies on isolated human cells and *in vivo* using wild type and galectin-1 null mice.

1.4.5 Galectin-3

Galectin-3 is a 29kDa chimera type galectin that oligomerises in solution via its extended N-terminus to form multimers that can cross-link heterogeneous receptors (Ahmad et al., 2004). The non-lectin N-terminus is composed of repeats of the amino acid sequence (Tyr-Pro-Gly-Pro-Glu-Ala-Thr-Pro-Ala-Pro-Gly-Ala), which is repeated ten times in murine galectin-3 and five times in human galectin-3 (Albrandt et al., 1987, Cherayil et al., 1990). The N-terminus is a target for matrix metalloproteinases (MMP)-2 and -9 and this cleavage inhibits oligomerisation of galectin-3 and its ability to cross-

link receptors and thus many of the effects of galectin-3 on cells (Hsu et al., 1992, Yang et al., 1998, Ochieng et al., 1994, Yamaoka et al., 1995).

Galectin-3 binds preferentially to poly-LacNAc residues with at least 3 repeats of the sequence expressed on N-glycans elongated by N-acetylglucosaminyltransferase V (MGAT5). The lectin is able to bind in the presence or absence of sialic acid residues as it binds internal residues on a glycan chain and it's binding is improved in the presence of α-fucose (Rabinovich and Toscano, 2009, Henrick et al., 1998, Stowell et al., 2008a).

1.4.5.1 Galectin-3 in Animal Models of Inflammation

Like their galectin-1 null counterparts, galectin-3 null mice exhibit few phenotypic alterations when not challenged with the exception of reduced cellularity in their bone marrow when compared to littermate controls under standard conditions (Brand et al., 2011, Colnot et al., 1998a). It was initially postulated that galectin-1 and galectin-3 might compensate for each other however galectin-1/galectin-3 double null mutant mice have no overt phenotypic abnormalities confirming that these lectins have distinct actions (Colnot et al., 1998a).

Further studies have been performed using the galectin-3 null mice in models of inflammatory disease as summarised in Table 1-3.

Pathology	Outcome
Hepatic Fibrosis	Reduced fibrosis (Henderson et al., 2006)
Ovalbumin-induced Asthma	Decreased airway hypersensitivity with decreased Th2 responses but increased Th1 responses (Zuberi et al., 2004)
Experimental Autoimmune Encephalomyelitis	Milder disease and reduced leukocyte infiltrate (Jiang et al., 2009)
Antigen-induced Arthritis	Suppression of joint damage and decreased cytokine production (Forsman et al., 2011)
LPS-induced Septic Shock	Increased susceptibility (Li et al., 2009)
Thioglycollate-induced Peritonitis	Reduced neutrophil and monocyte recruitment not associated with increased apoptosis or phagocytosis (Colnot et al., 1998b, Hsu et al., 2000)
Wound Healing	Slower wound healing, could be rescued by galectin-7 administration (Cao et al., 2002)
Pneumococcal Pneumonia with *Streptococcal pneumoniae*	Increased bacteraemia and lung damage, decreased phagocytic capability of macrophages – can be rescued by administration of recombinant galectin-3 (Farnworth et al., 2008)
Respiratory Tularaemia	Decreased leukocyte infiltration and augmentation of sepsis development (Mishra et al., 2013)
Parasitic protozoan cutaneous infection with *Leishmania major*	Decreased neutrophil recruitment in initial 48 hours followed by normalisation to wild type levels, significantly increased parasitic burden – can be rescued by administration of recombinant galectin-3 (Bhaumik et al., 2013)

Table 1-3: Overview of the phenotype of animal models of inflammation in galectin-3 null mice.

Galectin-3 was initially designated a pro-inflammatory lectin due to its roles in T cell-driven inflammatory disease including hepatic fibrosis, ovalbumin-induced asthma, experimental autoimmune encephalomyelitis, all of which were attenuated in the galectin-3 null mice; this is most likely due to the ability of galectin-3 to skew the T cell profile from Th2 towards Th1 and Th17-driven pathology (Henderson et al., 2006, Zuberi et al., 2004, Jiang et al., 2009). Recent studies have demonstrated a role for galectin-3 in innate immunity protecting against specific infectious stimuli potentially by acting as a danger-associated molecular pattern (DAMP) (Bhaumik et al., 2013). Infection with *Streptococcus pneumoniae* or *Leishmania major* is considerably more severe in galectin-3 null mice compared with wild type animals but no differences were seen in the response to *Escherichia coli* infection; these protective effects were rescued by administration of recombinant galectin-3 (Farnworth et al., 2008, Bhaumik et al., 2013, Sato et al., 2002b). This may be linked to the ability of galectin-3 to promote the recruitment of leukocytes to inflammatory sites as demonstrated by a reduction of neutrophil and monocyte recruitment in galectin-3 null mice in models of

thioglycollate-induced peritonitis and wound healing (Colnot et al., 1998b, Hsu et al., 2000, Cao et al., 2002).

The ability of galectin-3 to act as a leukocyte chemoattractant was confirmed in the murine dorsal air pouch. Injection of galectin-3 into the air pouch resulted in recruitment of monocytes (Sano et al., 2000).

Pathology	Species	Dose	Outcome
Dorsal Air Pouch	Mouse	1μM/subcutaneous (sc) single dose 4 hours prior to exudate collection	Increased recruitment of monocytes than induced by monocyte chemoattractant protein (MCP)-1 (Sano et al., 2000)

Table 1-4: Overview of the phenotype of mice following use of recombinant galectin-3 in animal models of inflammation.

Mechanistic insights into the effect of galectin-3 on innate immunity have been provided by *in vitro* studies.

1.4.5.2 Endothelial Cells

High concentrations (1μg/mL, equivalent to 32nM) of galectin-3 promote the release of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 from HUVECs and human microvascular endothelial cells (HMVECs) *in vitro*, these cytokines act in an autocrine or paracrine manner to up-regulate E-selectin, ICAM-1 and VCAM-1 on endothelial cells (Chen et al., 2013) which may explain some of the effects of galectin-3 on leukocyte trafficking. As well as actions on the endothelium, galectin-3 also directly affects leukocytes.

1.4.5.3 Monocytes and Macrophages

It acts cooperatively with LPS to promote increased expression of IL-1β from human monocytes (Jeng et al., 1994); it also cross-links CD13 expressed on human monocyte cell surfaces to promote homotypic aggregation (Mina-Osorio et al., 2007). Studies using a Boyden chamber have demonstrated an ability of galectin-3 to act as a chemoattractant for both human monocytes and macrophages (Sano et al., 2000). Whilst murine galectin-3 null macrophages exhibit defective phagocytic capabilities compared to wild type cells confirming the role of the lectin as an opsonin (Sano et al., 2003, Farnworth et al., 2008, Karlsson et al., 2009). Presence of galectin-3 is able to increase both the proportion of macrophages that can phagocytose and the number of

cells phagocytosed per macrophage leading to a large increase in net phagocytosis (Karlsson et al., 2009); this may be due to the lectin "bridging" phagocytic prey to the phagocytes via the Mer receptor tyrosine kinase (MerTK) (Caberoy et al., 2012). Galectin-3-deficient macrophages have defective alternative activation; perhaps suggesting the resolution of inflammation would be impaired in the galectin-3 null mice (MacKinnon et al., 2008). This hypothesis is strengthened by a recent study which showed galectin-3 (60μg/mL) increases the production of IL-10 from human monocytes when co-cultured with a TLR1/TLR2 ligand (19-kD lipopeptide) and this inhibits the GM-CSF-induced differentiation into dendritic cells thus reducing inflammation (Chung et al., 2013).

1.4.5.4 Neutrophils

Resting human neutrophils express low levels of the receptors for galectin-3 (CD66a and CD66b) when compared to activated cells as they are released from secondary granules; thus providing an explanation for the lack of response to the lectin by peripheral blood neutrophils (Almkvist et al., 2001, Feuk-Lagerstedt et al., 1999). Upon binding to the activated neutrophil galectin-3 increases the phagocytic capability, degranulation and the production of reactive oxygen species (ROS); the lectin can also cross-link neutrophil receptors to laminin and promote adhesion of the cells (Fernández et al., 2005, Kuwabara and Liu, 1996, Farnworth et al., 2008, Yamaoka et al., 1995). Addition of exogenous galectin-3 to neutrophils promotes survival of the cells and this may be due to the ability of the lectin to activate the neutrophil (Farnworth et al., 2008, Hsu et al., 2000).

Like galectin-1, galectin-3 is able to induce the exposure of PS on the human neutrophil cell surface without a concurrent induction of apoptosis and this promotes the preaparesis of neutrophils by monocytes and macrophages (Stowell et al., 2008b). This suggests galectin-3 may play a role not just in the initiation of the neutrophil-driven inflammatory response as described above but may also be involved in the clearance of neutrophils once their function is completed, yet further work is required to assess this pathway.

Limited experimentation has been performed on the role of galectin-3 in murine neutrophils due to published reports suggesting murine neutrophils do not express the lectin (Sato et al., 2002b, Farnworth et al., 2008). However contrary to this hypothesis it is known that galectin-3 plays a role in neutrophil recruitment as galectin-3 null mice have decreased numbers of neutrophils recruited in models of thioglycollate-induced

peritonitis and pneumococcal pneumonia compared to wild type mice, a phenotype that can be rescued by the use of recombinant galectin-3 (Colnot et al., 1998b, Hsu et al., 2000, Henderson et al., 2006). This may be due to the ability of the lectin to cross-link cells to the extracellular matrix thus acting directly as an adhesion molecule, as demonstrated in β2-integrin-independent transmigration (Sato et al., 2002b).

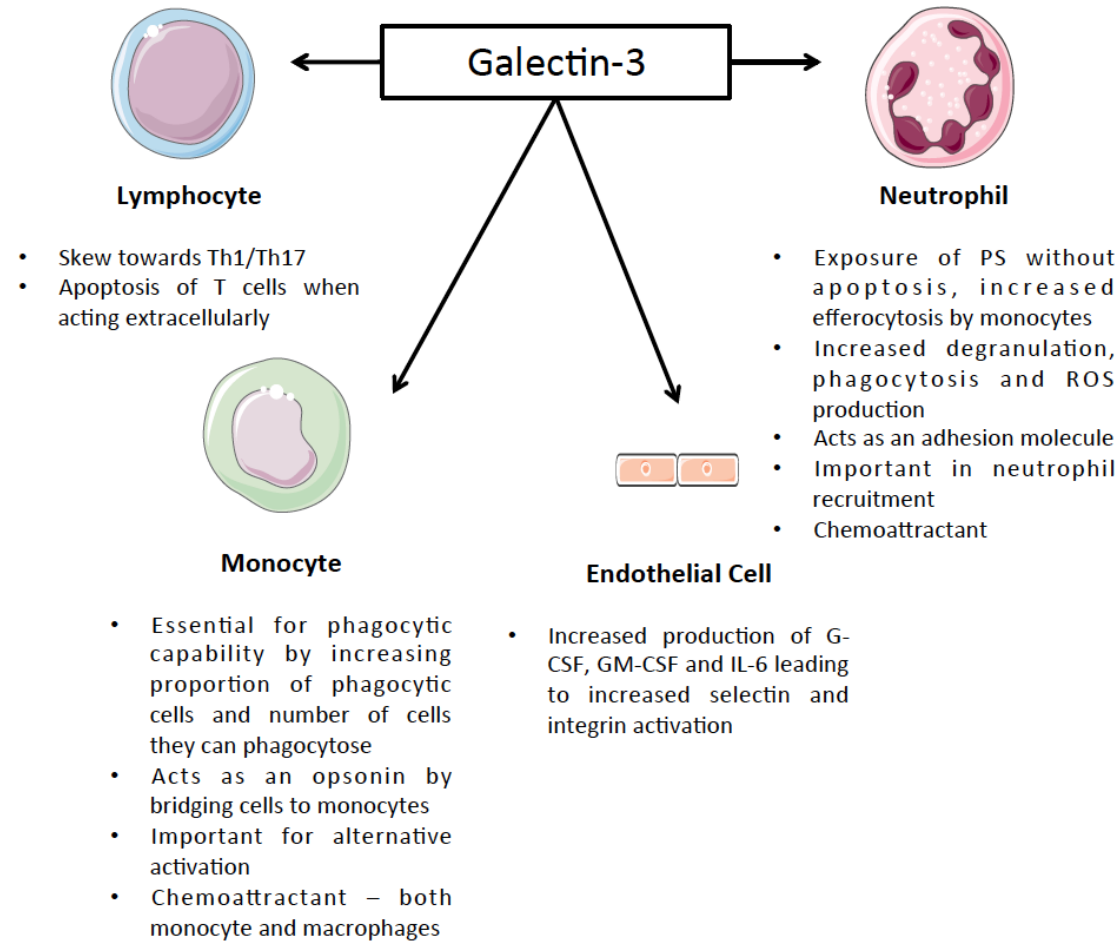


Figure 1.11: Summary of the effects of galectin-3 on immune cells. The effects of galectin-3 on lymphocytes, monocytes, neutrophils and endothelial cells as determined by studies on isolated human cells and *in vivo* using wild type and galectin-3 null mice.

1.4.6 Galectin-9

Galectin-9 is a 36kDa tandem-repeat lectin that has two functionally distinct CRDs; the murine C-terminal CRD is 70% homologous to rat galectin-5 while the N-terminal CRD is 40% homologous to murine galectin-3, the two CRDs are only 35% identical (Nagae et al., 2006). There are five known isoforms of galectin-9 that are produced by splice variation and three of these differ in length of the linker region, they are 35, 36 and 40kDa in size with the most common isoform being the medium (36kDa) isoform, the

fourth has a truncated C-terminal CRD while the final isoform has a posttranslational modification that results in restricted expression (Chabot et al., 2002, Heusschen et al., 2014). Studies have suggested the N-terminus of galectin-9 can homodimerise however this has not been shown *in* vivo (Nagae et al., 2006).

Galectin-9 binds to poly-LacNAc residues with at least 3 repeats of the sequence on complex tri- and tetra-antennary N-glycans following removal of the sialic acid capping structure (Rabinovich and Toscano, 2009, Sato et al., 2002b, Sato et al., 2002a).

Originally identified as a T cell-derived eosinophil chemoattractant galectin-9 has since been demonstrated to be produced by and to elicit effects on other inflammatory cells including T cells, monocytes and neutrophils (Hirashima, 2000).

Like galectins-1 and -3, galectin-9 also shows activity in T cell-driven pathologies, again through its ability to induce apoptosis of Th1 and Th17 cells (Wada et al., 1997); this occurs via binding to glycan chains on T-cell immunoglobulin domain and mucin domain (Tim)-3, which is highly expressed by both Th1 and Th17 cells and increases upon their activation (Zhu et al., 2005, Hastings et al., 2009). T cell apoptosis is achieved through a different pathway than galectin-1, thus lending evidence to the hypothesis that galectin-1 and galectin-9 can compensate for each other in the absence of the lectin or its ligands, a fact further supported by studies using galectin-1 null mice in which increased levels of galectin-9 were seen (Bi et al., 2008, Iqbal et al., 2011).

1.4.6.1 Galectin-9 in Animal Models of Inflammation

Numerous *in* vivo models have been used to demonstrate the immunomodulatory actions of galectin-9. It appears to have anti-inflammatory properties when administered during inflammation as in all of the models reported to date it attenuates many of the clinical symptoms of the disease, generally these effects can be assigned to the ability of galectin-9 to induce T cell apoptosis including the effects during experimental autoimmune encephalomyelitis, collagen-induced arthritis and graft vs host disease (Zhu et al., 2005, Seki et al., 2007, Seki et al., 2008, Sakai et al., 2011, He et al., 2009) **Table 1-5**.

Pathology	Species	Dose	Outcome
Experimental Autoimmune Encephalomyelitis	Mouse	100μg/ip daily from day 3 of disease induction to day 9	Reduced disease severity and mortality (Zhu et al., 2005)
Collagen-induced Arthritis	Mice	10μg/iv daily from date of booster vaccine (day 21)	Decreases Th1 and Th17 cytokines and induces differentiation of T_{regs} (Seki et al., 2007, Seki et al., 2008)
Concanavalin-A-induced Hepatitis	Mice	100μg/iv 30 minutes prior to Con-A injection	Amelioration of disease (Lv et al., 2012)
Graft vs Host Disease	Mice	100μg/ip daily for 14 days from onset of clinical symptoms	Decreased leukocyte infiltration and pro-inflammatory cytokine production (Sakai et al., 2011, He et al., 2009)
Ovalbumin-induced Asthma	Mice	1mg/ip 30 minutes prior to inhalation of Ova	Suppression of IgE-antigen complex formation and reduced allergic activity (Niki et al., 2009)
Shwartzman reaction	Mouse	7μg/ip with LPS injection	Recruitment of prostaglandin E_2-secreting neutrophils and suppression of the response (Tsuboi et al., 2007)

Table 1-5: Overview of the phenotype of mice following use of recombinant galectin-9 in animal models of inflammation.

Non-obese diabetic (NOD) mice were generated that over-expressed galectin-9 and were shown to have protection against development of autoimmune diabetes compared to littermate controls; suggesting a role for galectin-9 in protection against inflammatory disease (Chou et al., 2009).

Galectin-9 null mice are more susceptible to LPS-induced vasculitis, using the Shwartzman reaction, this appears to be due to the ability of galectin-9 to recruit a population of neutrophils that are pro-resolution and release prostaglandin E_2 (Tsuboi et al., 2007)

Several *in vitro* studies have been performed that assess the role of galectin-9 in myeloid leukocytes and these have demonstrated roles of the lectin in acute inflammation.

1.4.6.2 Monocytes and Macrophages

Galectin-9 induces apoptosis of monocytes in a CRD-dependent manner by promoting an influx of calcium into the cell leading to caspase activation, providing further evidence that galectin-9 is anti-inflammatory (Kashio et al., 2003).

1.4.6.3 Neutrophils

A recent study has shown that Tim-3 is expressed on human neutrophils and increases upon activation of these cells; binding of galectin-9 to this receptor induces granule release from the neutrophil. Galectin-9 also acts as an opsonin for gram-negative bacteria as shown by increased phagocytosis of Pseudomonas aeruginosa by neutrophils; this occurs via cross-linking receptors on the bacterial cell surface to Tim-3 expressed on human neutrophils (Vega-Carrascal et al., 2014). This data suggests that galectin-9 plays an important role in the clearance of bacteria; potentially enforcing the hypothesis that galectin-9 is a pro-resolution protein.

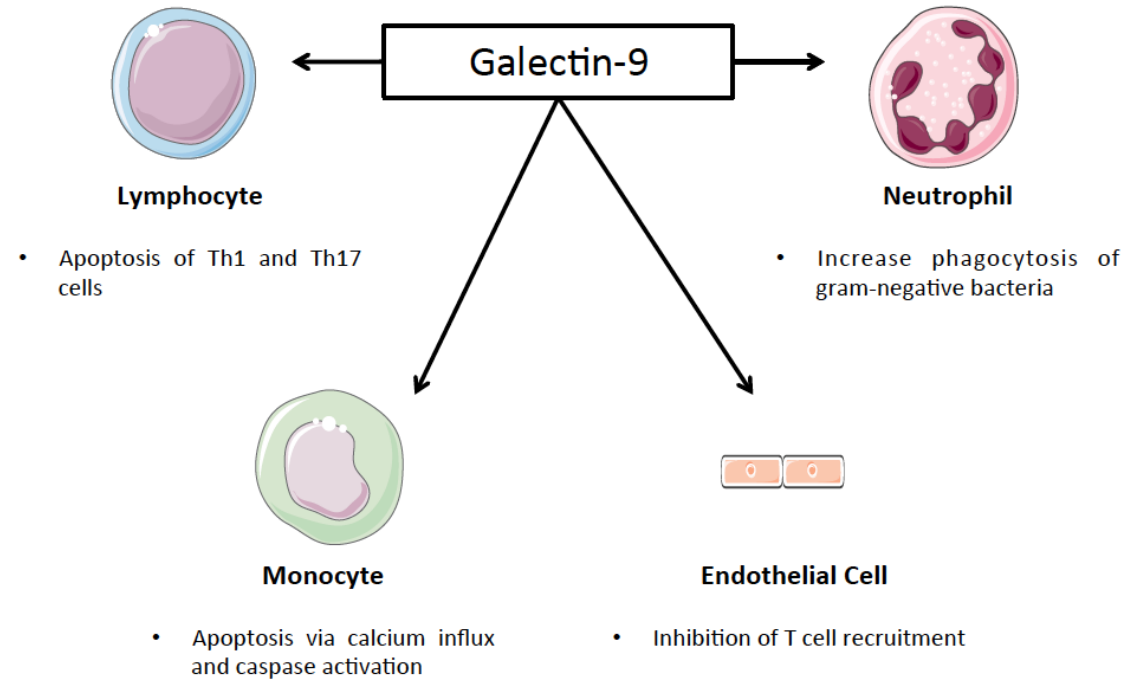


Figure 1.12: Summary of the effects of galectin-9 on immune cells. The effects of galectin-9 on lymphocytes, monocytes, neutrophils and endothelial cells as determined by studies on isolated human cells and *in vivo* using wild type mice.

1.5 Scope of the Thesis

1.5.1 Hypothesis

Previous reports have demonstrated a clear role for glycosylation in the modulation of an inflammatory response; this is mediated by the effects of lectins binding to glycoconjugates that are differentially expressed dependent on the leukocytes maturation and activation status. This glycosylation determines the recruitment, activation and removal of leukocytes from the site of inflammation by regulating the lectin binding permissibility of the cell.

This thesis challenges the hypothesis that the glycophenotype of a myeloid cell is modulated over the course of an inflammatory response allowing permissibility for the binding, and therefore actions of galectins-1, -3 and -9 in order to promote resolution and restore homeostasis.

1.5.2 Aims

The hypothesis will be addressed using the following aims:

1. To characterise the modulation of the glycophenotype of human and murine neutrophils following an inflammatory stimulus and/or cell trafficking using a lectin binding assay with a validated panel of lectins.

2. To assess whether galectin binding to human neutrophils is altered by transmigration and if this correlates with the changes in the glycophenotype.

3. To investigate how the expression of galectins alters in human leukocytes following an inflammatory stimulus and/or cell trafficking.

4. To investigate how the expression of galectins-1, -3 and -9 changes over the course of a resolving inflammatory response using a murine model of zymosan-induced peritonitis.

5. To determine the role of galectin-3 on murine leukocytes during a resolving inflammatory response.

CHAPTER 2:

MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

2.1.1 Cell Culture

Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium and DPBS without calcium and magnesium, fetal bovine serum (FBS), fungizone, Hanks buffered saline solution (HBSS), human serum, Medium 199 with Earle's Salts with L-glutamine (M199), penicillin/streptomycin and Roswell Park Memorial Institute medium 1640 (RPMI) with L-glutamine were purchased from GE Healthcare, Buckinghamshire, UK. CellTrace™ carboxyfluorescein succinimidyl ester, Dynabeads® and trypsin/EDTA (0.025%/0.01%) were purchased from Invitrogen, Paisley, UK. Type II collagenase was purchased from Lorne Laboratories, Reading, UK. Accutase and fibronectin from human plasma were purchased from Millipore, Watford, UK. Acetic acid, capsaicin, cell dissociation solution, crystal violet, dextran (molecular weight 450,000-650,000), fMLP, gelatin type B from bovine skin, Hanks Balanced salt solution 10X, Histopaque 1077, phosphate buffered saline (PBS), sodium citrate, human recombinant tumour necrosis factor-α (hrTNF-α) and zymosan were purchased from Sigma-Aldrich, Poole, UK. 3µm transwell inserts for 6-well plates were purchased from Scientific Laboratory Supplies, Yorkshire, UK. An EasySep murine neutrophil negative selection kit was purchased from StemCell, Grenoble, France. Nunc™ LabTek™ II Chamber Slide Systems were purchased from Thermo Scientific, St-Leon Rot, Germany.

2.1.2 Flow Cytometry

Annexin V-FITC apoptosis detection kit and BD FACS lysing solution were purchased from BD Pharmingen, Oxford, UK. Intracellular fixation and permeabilisation buffers were purchased from eBioscience, Hatfield, UK. A secondary streptavidin antibody conjugated to phycoerythrin (PE) was purchased from Invitrogen, Paisley, UK. Bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) sodium salt, paraformaldehyde (PFA) and sodium chloride were purchased from Sigma-Aldrich, Poole, UK. Recombinant forms of human Galectins-1, -3 and -9 (stable) were kindly provided by GalPharma, Takamatsu, Kagawa, Japan.

2.1.3 Molecular Biology Reagents

Gel red DNA dye was purchased from Biotium, Cambridge, UK. Trizol reagent was purchased from Invitrogen, Paisley, UK. A 1-kilobase molecular weight DNA ladder was purchased from New England Biolabs, Hitchin, UK. Tris-Acetate EDTA (TAE) buffer was purchased from Promega, Southampton, UK. Deoxyribonucleotide triphosphate (dNTP), dithiothreitol (DTT), first strand Buffer, oligo$(DT)_{15}$, primers, Qiagen mini-kit, QiaShredder columns, RNAse out and SuperScript were purchased from Qiagen, Manchester, UK. 2-Propanolol, β-mercaptoethanol, chloroform, Galectin-3 genotyping primers, molecular biology grade 100% ethanol, and REDExtract-N-Amp 2 PCR kit were purchased from Sigma-Aldrich, Poole, UK. ReddyMix PCR master-mix was purchased from Thermo-Scientific, St-Leon Rot, Germany.

2.1.4 Other Reagents

Isoflurane was purchased from Abbott Laboratories, Maidenhead, UK. ProLong® gold antifade mountant with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Life Technologies, Paisley, UK. Mouse Galectin-1 and Galectin-3 duo set ELISA kits were purchased from R&D Systems, Abingdon, UK. Haematoxylin was purchased from Sigma-Aldrich, Poole, UK. Eosin was purchased from VWR, Leicestershire, UK.

2.1.5 Antibodies

Species/ Antigen	Host	Isotype	Fluorochrome	Clone	Supplier	Working Conc
Human Galectin-1	Goat	IgG	Purified	Poly	R&D Systems	8μg/mL
Human Galectin-3	Goat	IgG	Purified	Poly	R&D Systems	8μg/mL
Human Galectin-9	Goat	IgG	Purified	Poly	R&D systems	8μg/mL
Human CD11b	Mouse	IgG1 κ	APC	ICRF44	eBioscience	100ng/mL
Human CD62L	Mouse	IgG1 κ	PE-Cy5.5	DREG-56	eBioscience	100ng/mL
Human CD14	Mouse	IgG1 κ	APC	61D3	eBioscience	250ng/mL
Human CD3	Mouse	IgG1 κ	PE	UCHT1	eBioscience	300ng/mL
Human CD66b	Mouse	IgG1	FITC/PE	80H3	AbD Serotec	1μg/mL
Human CD35	Mouse	IgG1	PE	E11	AbD Serotec	20μg/mL
Human CD62E/P	Mouse	IgG1	FITC	1.2B6	AbD Serotec	100ng/mL
Human CD146	Mouse	IgG2a	Alexa-Fluor 647	SHM-57	BioLegend	250ng/mL
Mouse Galectin-1	Goat	IgG	Purified	Poly	R&D Systems	8μg/mL
Human and Mouse Galectin-3	Rat	IgG2a	PE	M3/38	eBioscience	1μg/mL
Mouse Galectin-9	Goat	IgG	Purified	Poly	R&D systems	8μg/mL
Mouse F4/80	Rat	IgG2a	Alexa-Fluor 488/PE/APC	BM8	eBioscience	250ng/mL Alexa-Fluor 488 and PE 100ng/mL APC
Mouse Ly6G	Rat	IgG2a	FITC/PE/ Purified	1A8	BD Pharmingen	500ng/mL FITC 200ng/mL PE 10μg/mL purified
Mouse Gr-1	Rat	IgG2b	APC	RB6-8C5	eBioscience	200ng/mL
Mouse CXCR4	Rat	IgG2b	Alexa-Fluor 647	2B11	eBioscience	200ng/mL
Mouse Ly6C	Rat	IgG2c	PerCP-Cy5.5	HK1.4	eBioscience	200ng/mL
Mouse Neutrophil	Rat	IgG2a	FITC	7/4	Abcam	250ng/mL
Mouse CCR2	Rat	IgG2b	Fluorescein	475301	R&D Systems	250ng/mL
Goat IgG	Chicken	Whole	Alexa-Fluor 488	Poly	Invitrogen	400ng/mL

Table 2.1: List of antibodies used for Flow Cytometry.

Host/Isotype	Fluorochrome	Clone	Supplier
Mouse IgG1 κ	APC	P3.6.2.1	eBioscience
Mouse IgG1 κ	Pe-Cy5.5	P3.6.2.8.1	eBioscience
Mouse IgG1	PE	P3.6.2.8.1	eBioscience
Mouse IgG1 κ	PE	P3.6.2.8.1	eBioscience
Mouse IgG1	FITC	P3.6.2.8.1	eBioscience
Rat IgG2a	PE	eBR2a	eBioscience
Rat IgG2a	Alexa Fluor-488	eBR2a	eBioscience
Rat IgG2a	APC	eBR2a	eBioscience
Rat IgG2a	FITC	eBR2a	eBioscience
Rat IgG2b	APC	eB149/10H5	eBioscience
Rat IgG2b	Alexa Fluor-647	eB149/10H5	eBioscience
Rat IgG2c	Pe-Cy5.5	R2C-23A3	eBioscience

Table 2.2: List of isotype controls used for Flow Cytometry.

2.1.6 Lectins

Biotinylated *Lycopersicon esculentum* Lectin (LEL), Biotinylated Peanut Agglutinin (PNA), Biotinylated *Phaseolus vulgaris* Leucoagglutinin (PHA-L), Biotinylated *Ricinus communis* Agglutinin$_{120}$ (RCA$_{120}$), Biotinylated *Sambucus nigra* Agglutinin (SNA) and Biotinylated *Ulex europaeus* Agglutinin I (UEA I) were purchased from Vector Laboratories, Peterborough, UK.

2.2 In Vitro Methods

2.2.1 Isolation and Culture of Primary Human Umbilical Vein Endothelial Cells (HUVEC)

Umbilical cords were kindly supplied by the midwifery staff at the Royal London Hospital (Ethics approval REC reference number: 06/Q0605/40). Cords were collected in HBSS containing penicillin (100U), streptomycin (10µg/mL) and fungizone (2.5µg/mL) and stored at 4°C until processing. HUVECs were isolated by collagenase digestion of the interior umbilical vein as described by Jaffe et al with some minor modifications as described below (Jaffe et al., 1973).

A butterfly needle was inserted into one end of the umbilical vein and clamped in place. Approximately 30mL PBS containing antibiotics (penicillin (100U), streptomycin (10µg/mL) and fungizone (2.5µg/mL)) was perfused through the vein using a 50mL sterile syringe to wash away residual blood and identify any perforations in the cord. The other end of the cord was clamped and approximately 20-25mL 0.1% type II collagenase in serum-free M199 (containing antibiotics) was added; the vein was then incubated for 14 minutes in a humidified chamber in 5% carbon dioxide at 37°C.

The collagenase solution was transferred to a 50mL falcon tube and the cord flushed with 30mL PBS (containing antibiotics), which was also added to the tube; the cord was then flushed through with air to remove any remaining cells. Cells were centrifuged at 300*g* for 10 minutes, the supernatant removed and the pellet re-suspended in 10mL complete medium (M199 containing antibiotics and 20% human serum) and transferred to a T75 flask (75cm^2), which had been pre-coated with 0.5% gelatin for 20 minutes.

The cells were incubated in a humidified chamber in 5% carbon dioxide at 37°C; the medium was changed after 24 hours to remove the erythrocytes and then every 48 hours until approximately 95% confluent. The yield from this procedure was typically 0.5-1.5 x 10^6 per cord.

Once at 95% confluence the cells were sub-cultured approximately one T75 flask into three. Cells were rinsed with PBS prior to addition of 0.025% Trypsin/0.01% EDTA solution to remove any serum; once the cells began to detach and round up the flask was tapped firmly to loosen all cells and complete medium was added to inactivate the trypsin. The cells were then split between the required gelatinised T75 flasks. Cells were used up to passage 3 where they still retained a good response to hrTNF-α as assessed by adhesion molecule up-regulation.

2.2.2 Isolation of Human PMN from Whole Blood

The local research committee approved experiments with healthy volunteers and informed consent was provided according to the declaration of Helsinki. Human blood was obtained from healthy volunteers using a 21-gauge needle and transferred to a 50mL falcon tube containing a 1/10 volume of 3.2% (w/v) sodium citrate to prevent clotting. Blood was initially centrifuged at 137*g* for 20 minutes to separate the platelet-rich plasma, which was then removed, before 10mL DPBS (without calcium or magnesium) and 8mL dextran (6% w/v) was gently layered on top of the blood. The tube was sealed and inverted gently until homogenously distributed and then left for erythrocytes to bind dextran and sediment for 30 minutes. The top layer containing leukocytes was then gently collected and layered onto 10mL Histopaque 1077 in a 50mL falcon tube and this was centrifuged at 483*g* for 30 minutes resulting in layer formation as shown in **Figure 2.1**.

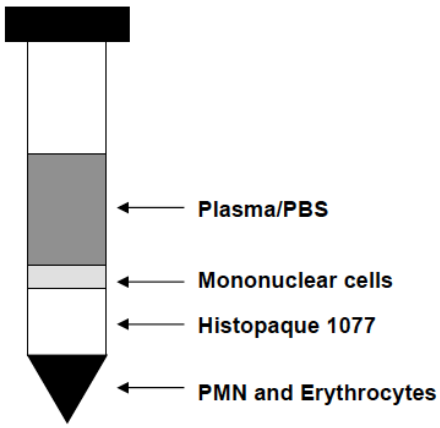


Figure 2.1: PMN isolation from human whole blood using dextran sedimentation. Following removal of platelet-rich plasma and sedimentation of erythrocytes the blood was layered gently onto Histopaque 1077 and centrifuged at 483*g* for 30 minutes resulting in layers of plasma/PBS, peripheral blood mononuclear cells, Histopaque 1077 and PMN/erythrocytes.

Peripheral blood mononuclear cells (PBMCs) were removed first to prevent contamination of the polymorphonuclear cells (PMN), following this most of the Histopaque and plasma layers were removed leaving only the PMN and erythrocytes. The erythrocytes were lysed using 9mL of ice-cold ultrapure water for approximately 10 seconds and once lysed the cells were rapidly mixed with 1mL 10X Hanks Balanced Salt Solution to return the solution to the correct isotonic balance. This was made up to 50mL with PBS and centrifuged at 215*g* for 10 minute to pellet the PMN.

The PMN pellet was re-suspended in 5-10mL PBS (depending on the size of the pellet) and a 10µL aliquot was mixed with 990µL Turks Stain (dH_2O containing 3% acetic acid and 0.01% crystal violet) and counted using a haemocytometer as seen in **Figure 2.2.**

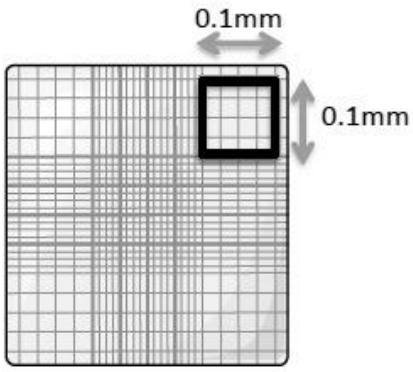


Figure 2.2: Counting grid on a Neubauer Haemocytometer. Cells are counted in each of the 4 corners as indicated by the red square.

The calculation below in **Figure 2.3** was used to determine the total number of PMN in the solution and then following a further wash step in PBS the cells were re-suspended in RPMI (+ 10% FBS) at a concentration of 3 x 10^6 per mL ready for use.

$$\frac{\text{Total cells counted in chamber}}{4} \quad (\text{x } 10^4) \text{ x dilution factor} = \text{cells per mL}$$

Figure 2.3: Calculation used to determine total number of cells after counting with a haemocytometer.

2.2.3 Collection of Exudated Human PMN

Capsaicin is a stimulus that promotes neurogenic inflammation (through activation of dorsal root ganglion) by activation of Transient Receptor Potential Cation Channel subfamily V member 1 ($TRPV_1$) receptors. Activation results in the release of calcitonin-related gene product (CGRP) and substance P, these neuropeptides act on the local vasculature to promote inflammation (Helme and McKernan, 1985). Tobasco sauce (purchased from the McIlhenny Co, Avery Island, Los Angeles, USA) contains high levels of capsaicin (0.33mg capsaicinoids/mL) as determined by high pressure liquid chromatography (Gonzalez et al., 1998) and thus induces this effect when administered orally.

The local research committee approved experiments with healthy volunteers and informed consent was provided according to the declaration of Helsinki (Ethics approval REC reference number: QMREC2010/17). Volunteers were asked to rinse the buccal cavity three times with 20mL of a 0.9% saline mouthwash (made with Maldon

sea salt as it contains low levels of impurities compared to other table salts and Evian water) for 30 seconds and then a 10% Tobasco solution in saline (20mL for 30 seconds). These were discarded and the volunteer asked not to eat or drink anything for the following hour. The volunteers were then asked to rinse again three times with saline and these samples were collected and centrifuged at 300*g* for 10 minutes to pellet the cells. The cells were pooled and re-suspended in 5mL PBS (+0.1% BSA) and an aliquot (10µL) was taken and diluted 1/5 in Turks solution for counting using a light microscope. Approximately 5-10 x 10^6 cells were collected per donor using this protocol and 80-90% of these were PMN with the remainder of cells collected being buccal epithelial cells.

2.2.4 PMN Adhesion to a HUVEC Monolayer in a 6-well Plate

HUVECs were seeded onto 0.5% gelatin coated wells of a 6-well plate and left to become fully confluent in a humidified incubator at 5% carbon dioxide at 37°C, all subsequent incubation steps were performed in a humidified incubator. Once confluent endothelial monolayers were treated with 10ng/mL hrTNF-α for 4 hours to promote activation and up-regulation of adhesion molecules, particularly E-selectin and ICAM-1.

Following this incubation HUVECs were washed with PBS and PMN were added (1mL complete RPMI containing 3 x 10^6 PMN per well) for 30 minutes.

After the incubation period the RPMI was collected and HUVECs were washed gently with PBS to remove any non-adherent PMN, these were collected into a falcon tube. Cell Dissociation Solution was used to remove PMN that were adherent to the underlying HUVEC monolayer and the cells were then washed gently with PBS to collect all adherent PMN. The HUVECs were then incubated with Accutase for 10 minutes and the well was washed with PBS to ensure all remaining cells were recovered. The three populations of cells, non-adherent, adherent and transmigrated were centrifuged at 300*g* for 10 minutes to pellet the cells. An aliquot was taken and assayed for the expression of neutrophil activation markers as described later and the remaining pellet was then re-suspended in PBS (+0.1% BSA) with 1% PFA to fix the cells. These were stored overnight at 4°C overnight before lectin binding assays were performed.

2.2.5 PMN Transmigration through a HUVEC Monolayer on a Transwell Insert

A previous study demonstrated that although the 6-well transmigration assay allows easy assessment of adherent leukocytes it cannot distinguish between those bound to the apical surface and those that have transmigrated (Muller and Luscinskas, 2008); therefore a second model of transmigration was utilised using a transwell insert as performed by Krankel et al in 2011 and modified for neutrophil, rather than monocyte, transmigration (Kränkel et al., 2011).

A 3μm transwell insert was placed inside a well of a 6-well plate and coated with 10ng/mL fibronectin in 0.5% gelatin; this was left to incubate for a minimum of 2 hours. The inserts were then washed with PBS before HUVECs were seeded onto the membrane and grown to confluence; this was confirmed using staining with Wheat Germ Agglutinin (WGA). Briefly HUVECs on the insert were fixed with 1% PFA overnight and then stained with WGA conjugated to Alexa-Fluor 488 in PBS at a concentration of 5μg/mL for 20 minutes. Cells were visualised using an EVOS Digital Inverted Microscope as shown in **Figure 2.4**.

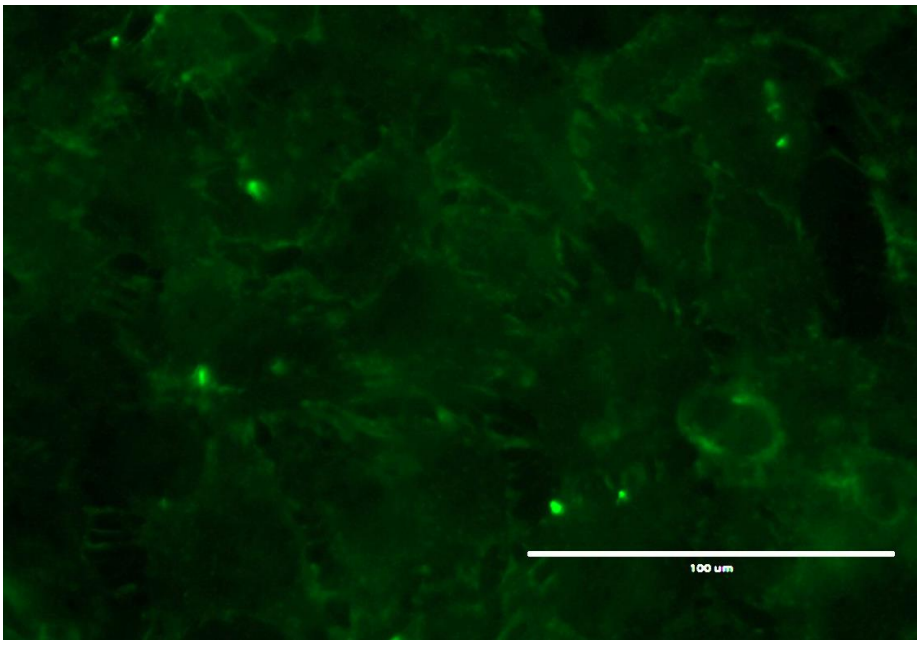


Figure 2.4: Wheat germ agglutinin staining of a confluent monolayer of HUVECs on a transwell insert. HUVECs were plated on a 3μm transwell insert that had been pre-coated with 10ng/mL fibronectin in 0.5% gelatin; cells were grown to confluence and fixed in 1% PFA overnight. HUVECs were incubated with WGA (5μg/mL) for 20 minutes and visualised at 40X magnification.

For transmigration assays the HUVECs were treated with 10ng/mL hrTNF-α for 4 hours, at which point the medium was removed and the transwell insert gently washed with PBS. 1mL complete HUVEC medium was placed at the bottom of the well and 1mL RPMI (containing 3 x 10^6 PMN) was placed on top of the transwell insert and incubated for 90 minutes.

Non-adherent cells were collected from the top of the transwell insert by gently washing with PBS, the transwell insert was transferred to a new 6-well plate and incubated for 10 minutes with Accutase to remove all adherent PMN and the transmigrated PMN were collected from the lower well and the underside of the transwell insert.

The three populations of cells, non-adherent, adherent and transmigrated were centrifuged at 300*g* for 10 minutes to pellet the cells. The pellet was then either re-suspended in PBS (+0.1% BSA) and 1% PFA to fix the cells for lectin binding, or used immediately in galectin expression, galectin binding assays or to assess the expression of neutrophil activation markers as described below. Fixed cells were stored at 4°C overnight before lectin binding was performed.

2.2.6 Secreted Factor Diffusion Assay

HUVECs were grown to confluence on a 3μm transwell insert as in the transwell transmigration assay however isolated PMN (3 x 10^6 per mL in RPMI + 10% FCS) were placed in the bottom of the well with 1mL RPMI (+10% FCS) on top of the transwell. The plate was incubated at 37°C in 5% carbon dioxide for 90 minutes and the PMN collected from under the transwell.

2.2.7 Flow Cytometric Analysis

Flow Cytometry is a technique used to determine the properties of individual cells in a solution using their size and density characteristics and can also be used to quantify the expression levels of specific antigens on the cell surface using fluorescently labelled antibodies. The sample is passed through a central channel on the Flow Cytometer, which is surrounded by an outer sheath through which sheath fluid (PBS) passes at a speed greater than that of the sample. This creates a phenomenon known as hydrodynamic focusing in which a drag force is exerted on the central chamber and the sample is narrowed to form a single stream of cells as shown in **Figure 2.5.**

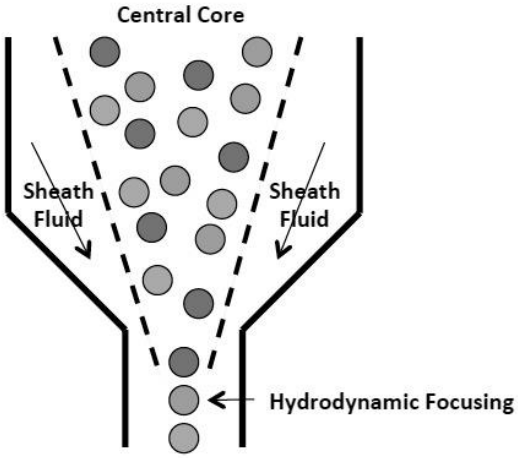


Figure 2.5: Schematic of the flow cell of a Flow Cytometer. The faster stream of sheath fluid creates a single stream of cells that can be analysed individually.

Cells are individually analysed for size, shape and fluorescence using specific lasers. As they pass through the laser each cell scatters the light in different directions, light scattered in a straight line is detected by the forward scatter lens (FSC) and this is a measure of the cell size. Light scattered approximately 90° to the side is detected by the side scatter lens (SSC) and is a measure of the density of the cell and therefore its granularity. This allows cells with different properties to be distinguished from each other, for example human whole blood can be differentiated into lymphocytes, monocytes and neutrophils as seen in **Figure 2.6**.

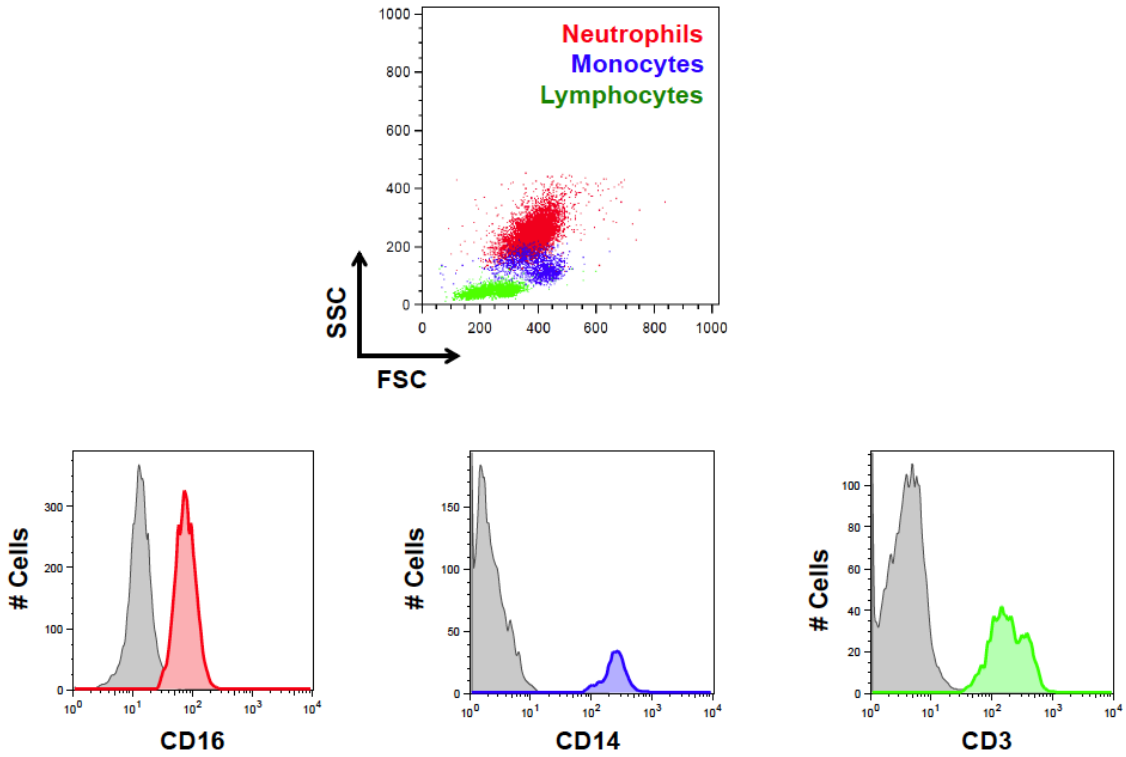


Figure 2.6: Forward/Side scatter plot for human whole blood following red blood cell lysis. Different properties allow cells to be differentiated in a heterogeneous population. Human blood was collected from healthy volunteers and the red cells lysed using BD FACS Lysing solution, leukocytes were then labelled with antibodies for CD16 (neutrophils), CD14 (monocytes), and CD3 (lymphocytes) and analysed by Flow Cytometry.

Cells can be labelled with antibodies directed to specific antigens on the cell surface or intracellularly, these antibodies can be directly conjugated to a fluorochrome, or a secondary antibody conjugated to a fluorochrome can be used. Fluorochromes absorb light of specific wavelengths released by lasers in the system and emit light of a longer wavelength; the light emitted is passed through a series of filters set to specific wavelengths, light of the specific wavelength is refracted to the lens for detection while light of a different wavelength passes straight through to the next laser as shown diagrammatically in **Figure 2.7**.

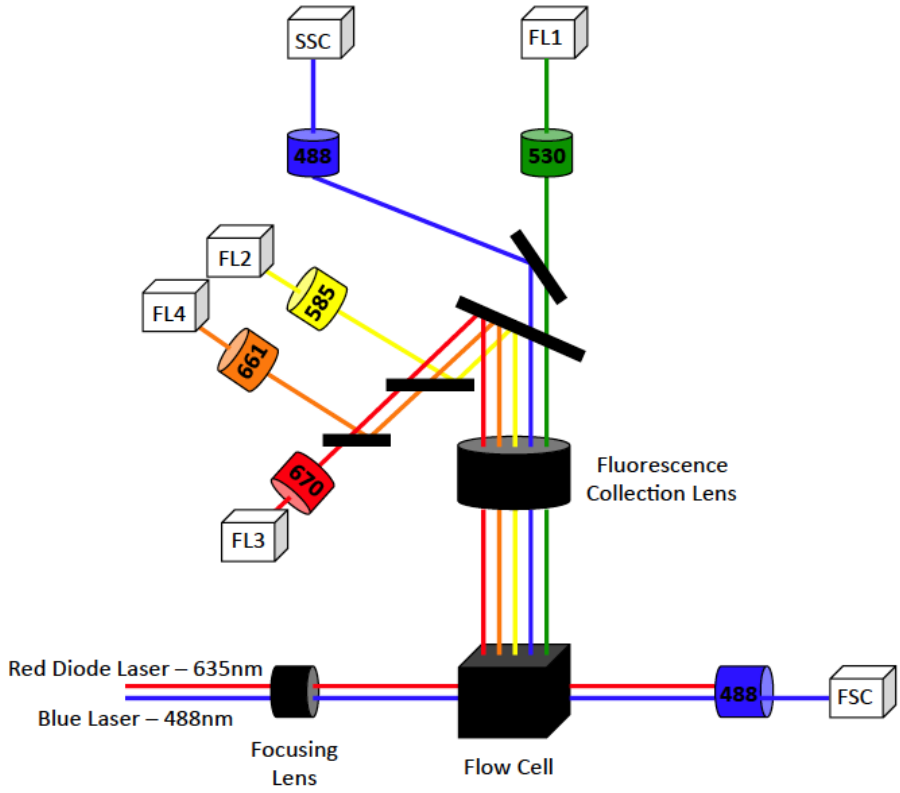


Figure 2.7: Schematic of the fluorescence system inside a Flow Cytometer. Fluorochromes are excited by light emitted from the lasers and release light of a different wavelength; this is passed through a series of filters and refracted to the correct lens for detection.

The amount of fluorescent signal detected is proportional to the number of fluorochromes and therefore the number of antigens on the cell and can hence be used to quantify the expression of antigens. A range of different fluorochromes that emit at different wavelengths can be used on the same sample to identify multiple antigens on the cells as long as care is taken to ensure they do not require the same lens for detection. Lenses that detect similar wavelengths (for example FL1 and FL2) can experience fluorescence spill over and thus compensation is required to prevent artefacts appearing in samples containing more than one fluorochrome. This can be performed before or after running the samples as long as single stain controls are used.

2.2.8 AMNIS ImageStream^X Mark II

The ImageStream is a highly sensitive imaging cytometer that is able to capture multifluorescent images of cells as they pass through the flow cell allowing visual assessment of the internalisation of particles within cells. Samples are prepared using

the same method as previously described for the Flow Cytometer and once again compensation it important to prevent fluorescence spill over.

2.2.9 Lectin Binding Assay

To determine the glycophenotype on the neutrophil cell surface at different stages of transmigration a validated panel of lectins was used that are known to have specificities for a range of terminal residues on N- and O-glycans as described in Table 2.3 and shown diagrammatically in **Figure 2.8**. All lectins were initially titrated to obtain the optimal working concentrations; these are given in **Table 2.3**.

Lectin	Glycan Binding	Working concentration
Ricinus communis Agglutinin$_{120}$ (RCA$_{120}$)	Terminal β-galactose residues (Baenziger and Fiete, 1979)	0.156μg/mL
Peanut Agglutinin (PNA)	Terminal galactose residues bound to N-acetylglucosamine in an α-linkage (LacNAc) (Lotan et al., 1975)	50μg/mL
Lycopersicon esculentum Lectin (LEL)	Poly-LacNAc residues with a minimum of 3 repeats, can be at the end of a glycan chain or capped with a sialic acid molecule (Merkle and Cummings, 1987)	0.625μg/mL
Sambucus nigra Agglutinin (SNA)	Terminal sialic acid in an α-2,6 linkage to an underlying galactose (Rogerieux et al., 1993)	0.156μg/mL
Ulex europaeus Agglutinin I (UEA I)	Terminal α-linked fucose residues bound to an underlying galactose (Hormia et al., 1983)	20μg/mL
Phaseolus vulgaris Leucoagglutinin (PHA-L)	Complex tri- and tetra-antennary N-glycans (Cummings and Kornfeld, 1982)	20μg/mL

Table 2.3: Plant lectins used to determine the glycophenotype. Glycan binding preferences and working concentrations are reported.

Lectin binding enables the investigator to study changes in the glycophenotype; this involves the addition of plant-derived lectins to the cell of interest and quantification of their binding. Plant-derived lectins are used because they are easy to isolate and purify and have well-defined binding specificities therefore various laboratories assessing galectin binding have employed this technique as a surrogate to assess galectin binding preferences (Muglia et al., 2011, Toscano et al., 2007).

Plant lectins that are commonly used for the assessment of the glycophenotype in respect to galectin binding include R*icinus communis* agglutinin$_{120}$ (RCA$_{120}$), Peanut agglutinin (PNA), *Lycopersicon esculentum* lectin (LEL), *Sambucus nigra* agglutinin

(SNA), *Ulex europaeus* agglutinin I (UEA I) and *Phaseolus vulgaris* leucoagglutinin (PHA-L) and their binding preferences are shown in **Figure 2.8**.

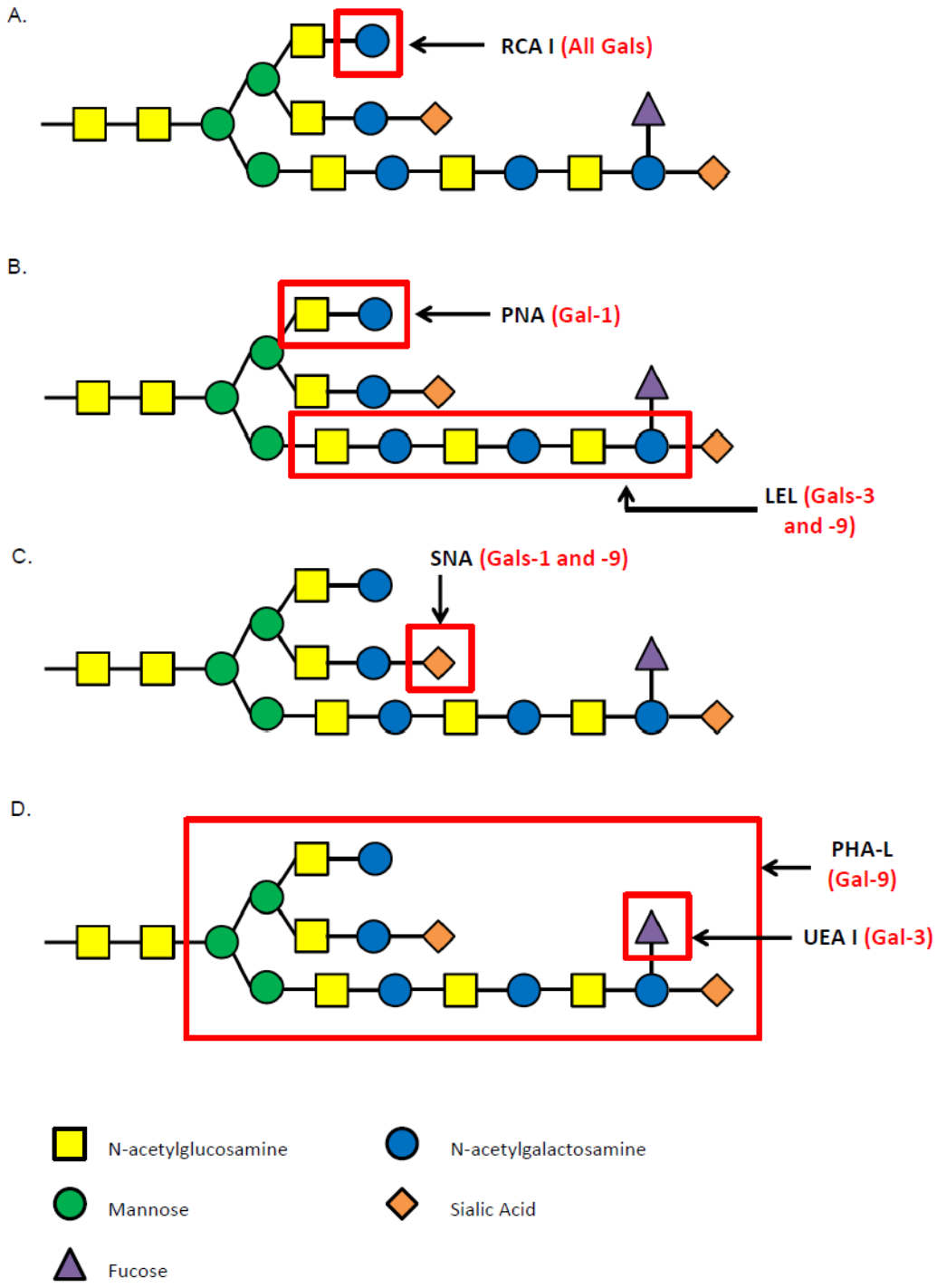


Figure 2.8: Schematic for lectin binding specificities. A. Galactose-specific lectin RCA_{120} binds β-linked galactose, B. LacNAc-specific lectins PNA and LEL bind mono- and poly-LacNAc respectively, C. Sialic acid-specific lectin SNA binds α2,6-linked sialic acid and D. Other common glycan-binding lectins UEA I and PHA-L bind α-linked fucose and complex tri- and tetra-antennary N-glycans respectively. Red text describes galectin binding permissibility indicated by expression of the glycan residues.

RCA_{120} is a lectin that binds to β-linked galactose residues; galectins are defined by their binding to β-galactoside therefore this can be employed to assess the overall galectin binding potential of the cell of interest (Goldstein et al., 1981, Baenziger and Fiete, 1979).

PNA binds specifically to mono-LacNAc residues following the removal of their α2,6-linked sialic acid cap, this is indicative of permissibility to galectin-1 binding (Stowell et al., 2004, Leppänen et al., 2005, Ahmad et al., 2002). LEL binds specifically to poly-LacNAc residues with greater than three repeats of the disaccharide structure in the absence or presence of sialic acid, which is identical to the binding preferences of galectin-3 and similar to that of galectin-9 (Rabinovich and Toscano, 2009, Lotan et al., 1975, Merkle and Cummings, 1987).

Expression of a sialic acid capping structure is important as it can inhibit the binding of galectins-1 and -9 but has no effect on the binding of galectin-3, as this binds internal residues (Leppänen et al., 2005). SNA binds α2,6-linked sialic acid (Rogerieux et al., 1993).

Other common glycan modifications of the cell that affect galectin binding include the expression of α1,3-linked fucose residues; fucose is a component of the sialyl Lewis x motif and therefore has a role in selectin-dependent rolling however they are also large monosaccharides that protrude from the cell surface and are therefore able to block the binding of galectins-1 and -9 via steric hindrance (Stowell et al., 2004, Erbe et al., 1993). Galectin-3 binding is increased in the presence of α-linked fucose **(Stowell, 2008)**. UEA I binding indicates the presence of α1,3-linked fucose residues (Hormia et al., 1983).

The final common modification involved in galectin binding is the expression of complex tri- and tetra-antennary N-glycans as galectin-9 preferentially binds N-acetyllactosamine residues expressed on these structures; a lectin specific for these is PHA-L (Rabinovich and Toscano, 2009, Cummings and Kornfeld, 1982).

Fixed PMN from the 6-well or transwell transmigration assays were re-suspended at a density of 2 x 10^5 cells per well in PBS (+0.1% BSA) and then plated on a 96-well U-bottomed plate. The lectins were added in lectin buffer (10.9mM HEPES sodium salt and 158.4mM sodium chloride in deionised water) at 50μL per well leaving an unlabelled and a secondary only well without lectin. This was left to incubate on ice in the dark for 45 minutes. Following this incubation the cells were washed twice in PBS

(+0.1% BSA) and the secondary antibody (Streptavidin-PE) was added for all necessary wells at a concentration of 120ng/mL in 30μL PBS (+0.1% BSA) and incubated for 30 minutes on ice in the dark. The cells were then washed twice in PBS (+0.1% BSA) and stored at 4°C until ready for analysis by Flow Cytometry. This assay was also performed on buccal neutrophils that had been fixed overnight and on murine peripheral blood and peritoneal exudate leukocytes in conjunction with anti-Ly6G (clone – 1A8) to identify neutrophils following 4 hour peritonitis with 1mg zymosan per mouse as described (**section 2.3.4.1**).

Adherent PMN from the transwell transmigration assay that had been treated with Accutase showed a markedly modulated binding of PNA, UEA I and PHA-L when compared to the non-adherent and transmigrated populations. Freshly isolated neutrophils were therefore treated with Accutase and assessed for their binding of these lectins before and after. As shown in **Figure 2.9** there was a large difference in the binding of lectins to neutrophils following treatment with Accutase suggesting it is modulating glycan expression on the surface of the PMN. This led to the transmigrated PMN in the 6-well assay and the adherent PMN from the transwell assay being excluded from the data as these had been treated with Accutase.

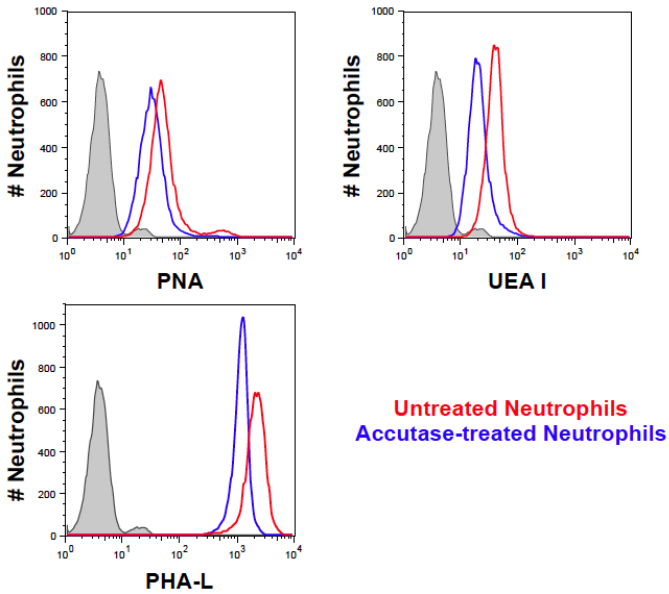


Figure 2.9: Accutase treatment of freshly isolated neutrophils. Freshly isolated neutrophils were treated for 10 minutes with Accutase or PBS at 37˚C and then analysed for the binding of PNA, UEA I and PHA-L.

To eliminate the human serum in the complete HUVEC medium as a modulator of the glycophenotype PMN were isolated and either fixed immediately or treated with

complete HUVEC medium for 90 minutes and then fixed. The following day the glycophenotype of the PMN was assessed as described. No differences could be seen for any of the lectins bound to the cell following treatment with complete HUVEC medium (**Figure 2.10**).

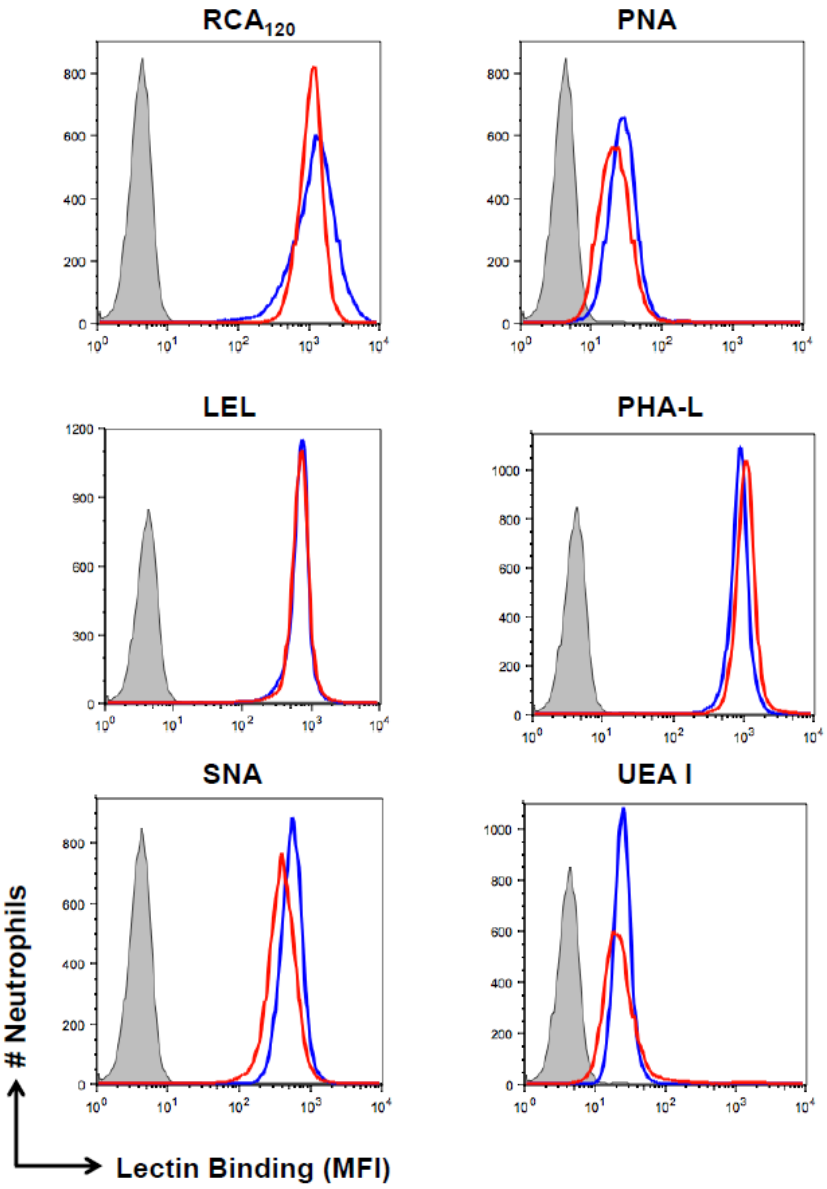


Figure 2.10: Lectin binding to freshly isolated PMN and those that had been treated with complete HUVEC medium for 90 minutes. PMN were isolated from healthy volunteers and either fixed immediately or incubated with complete HUVEC medium for 90 minutes and then fixed. The PMN were then assessed for their binding of a validated panel of lectins. Grey – secondary antibody only, blue – untreated PMN and red – complete HUVEC medium. Data are expressed as histograms of median fluorescence intensity.

In some experiments PMN were isolated from healthy donors and treated with vehicle (PBS), 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C to mimic the period for which the PMN were transmigrated in the transwell transmigration assay. PMN were also isolated from the Tobasco mouthwash protocol outlined in **section 2.2.3**. PMN from the secreted factor diffusion assay were also assayed for lectin binding. All PMN were fixed overnight in 1% PFA before lectin binding was analysed.

2.2.10 Neutrophil Activation Marker Expression Assay

Unfixed PMN collected from the transwell and 6-well assays were re-suspended in PBS (+0.1% BSA) and plated onto a 96-well U-bottomed plate at a density of 2 x 10^5 cells per well. CD11b (clone ICRF44) and CD62L (clone DREG-56) antibodies were added in PBS (+0.1% BSA), as were isotype controls and left to incubate for 30 minutes on ice in the dark before washing the cells twice. The cells were then stored in 1% PFA at 4°C until ready to analyse by Flow Cytometry.

2.2.11 Granule Release Assay

Unfixed PMN collected from the transwell assay were re-suspended in PBS (+0.1% BSA) and then plated at a density of 2 x 10^5 cells per well to allow detection of antigens released from granules – CD35 (clone E11) for secretory vesicles and CD66b (clone 80H3) for secondary granules. The antibodies including isotype controls were added at 50μL per well in PBS (+0.1% BSA) and left to incubate on ice in the dark for 30 minutes. Following incubation the cells were washed twice in PBS (+0.1% BSA) and stored in 1% PFA at 4°C until ready for analysis by Flow Cytometry.

2.2.12 Galectin Expression Assay

2.2.12.1 Isolated PMN – Cell Surface

Unfixed PMN from the transwell assay were re-suspended in PBS (+0.1% BSA) and plated in 96-well U-bottomed plates at a density of 2 x 10^5 per well. The purified galectin-1, -3 and -9 antibodies (polyclonal) were added at 50μL per well in PBS (+0.1% BSA) and left to incubate on ice in the dark for 30 minutes. Following this incubation the cells were washed twice in PBS (+0.1% BSA) and the secondary antibody (chicken anti-goat IgG – alexa-Fluor 488) was added at a concentration of 120ng/mL in 30μL per well (including a secondary antibody only well) and left to incubate on ice in the dark for 30 minutes. Following this incubation the cells were

washed twice in PBS (+0.1% BSA) and stored in 1% PFA at 4°C until ready for analysis by Flow Cytometry.

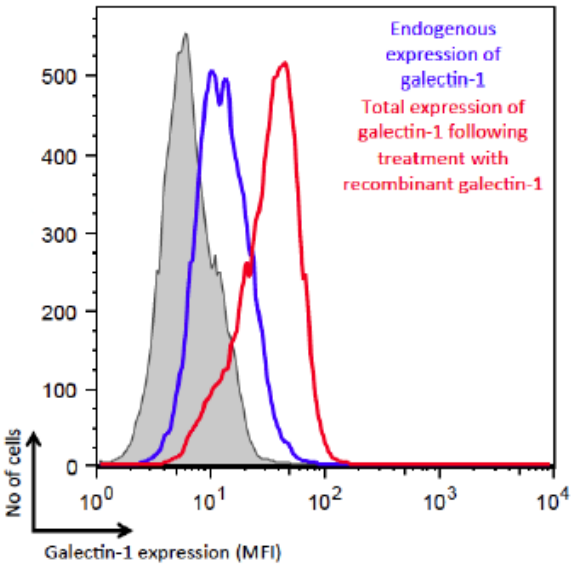
2.2.12.2 Isolated PMN - Intracellular

PMN from the buccal cavity and those isolated from the peripheral circulation were taken from healthy volunteers and paired for each donor, these were washed twice in PBS (+0.1% BSA) and then plated in 96-well U-bottomed plates at a density of 2 x 10^5 per well. Anti-CD66b (clone 80H3) antibodies were added to all wells except the unlabelled and secondary antibody only to differentiate PMN from buccal epithelial cells and these were incubated on ice in the dark for 30 minutes. Cells were washed twice and then incubated in fixation buffer for 20 minutes at room temperature in the dark. Once fixed permeabilisation buffer was added to the cells and they were washed three times. Galectin-1, -3, and -9 antibodies (polyclonal) were diluted in permeabilisation buffer then added to the cells at 50μL per well and then incubated on ice in the dark for 45 minutes. The cells were then washed twice and incubated with the appropriate secondary antibody (chicken anti-goat IgG alexa-Fluor 488) for 30 minutes on ice. Following this the cells were washed and re-suspended in PBS (+0.1% BSA) and stored at 4°C until ready for analysis by Flow Cytometry.

2.2.13 Galectin Binding Assay

PMN from the transwell assay and freshly isolated PMN were washed twice in PBS (+0.1% BSA) and re-suspended in DPBS (with calcium and magnesium) and 0.1% BSA; the PMN were treated with either recombinant human galectin-1, -3 or -9 at 10nM and left to incubate in a water bath at 37°C for 10 minutes. Following this the cells were plated onto a 96-well U-bottomed plate at a density of 2 x 10^5 per well and stained as in the galectin expression assay above.

Galectin binding median fluorescence intensities (MFI) were obtained by subtracting the MFI of the galectin binding assay from the MFI of the galectin expression assay for the same protocol using the same donor. This gave a value for the bound galectin whilst subtracting that endogenously expressed by the cell as shown in **Figure 2.11**.



Bound Galectin = Total Galectin Expression – Endogenous Galectin Expression

Figure 2.11: Calculation used to assess the binding of recombinant galectins. Isolated PMN were allowed to transmigrate across a confluent monolayer of HUVECs on a 3μm transwell insert and the pre- and post-transmigration populations were collected. The population was divided and half were treated with recombinant galectins-1, -3 or -9 for 15 minutes at 37°C. All PMN were then labelled with galectin antibodies and their levels assessed by Flow Cytometry. Once MFI values had been obtained the bound galectin could be ascertained by subtracting the expression of galectin of the PMN from the total expression seen following treatment with the recombinant protein.

2.2.14 Galectin-3 Internalisation Assay

Freshly isolated PMN were obtained from healthy volunteers and re-suspended in DPBS with calcium and magnesium (+0.1% BSA) at 1 x 10^6 per mL, the cells were treated with either recombinant galectin-3 (10nM or 1μM) or vehicle (PBS). One treated and one untreated group was incubated at 37°C and another at 4°C for 2 hours. The cells were then incubated with 30mM lactose for 15 minutes at 37°C to remove cell surface bound galectins. The cells were then plated onto a 96-well U-bottomed plate at a density of 2 x 10^5 cells per well and the intracellular expression of galectin-3 assessed following section 2.2.12.1.

2.3 In Vivo Methods

2.3.1 Mice

Male C57BL/6 mice were obtained from Charles River, Margate, UK. Breeding pairs of galectin-3 null mice (B6.Cg-*Lgals3*^{tm1Po1}/J) were provided by the Consortium for Functional Glycomics (http://functionalglycomics.org) and a colony was established at

Charles River, Margate, UK. These mice were on a C57BL/6 background and age- and sex-matched controls were used for all experimental work. All animals were fed standard laboratory chow and water *ad libitum* and were maintained on a 12 hour light-dark cycle under specific pathogen-free conditions. All experiments were performed with mice 6-7 weeks old, strictly following U.K. Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act 1986).

2.3.2 Genotyping

Tail clips from the galectin-3 null colony were sent from Charles River and the DNA was extracted using the REDExtract-N-Amp tissue PCR kit (Sigma). Tail clippings smaller than 1cm long were incubated with 50μL extraction solution and 12.5μL tissue preparation solution at room temperature for 10 minutes. The solutions were then heated to 95°C for 5 minutes and then 50μL of neutralisation solution was added; the tail clippings were removed and the DNA was amplified using PCR.

The DNA (approximately 0.1μg per reaction) was added to a 0.2mL PCR tube along with 10μL REDExtract-N-Amp PCR Reaction Mix and galectin-3 primers at 1μM as seen in **Table 2.4** and the solution was made up to 20μL with RNAse free water.

Genotype	Primer Sequence
Wild Type	GAGGAGGGTCAAAGGGAAAG
Heterozygous	GACTGGAATTGCCCATGAAC
Homozygous	TCGCCTTCTTGACGAGTTCT

Table 2.4: Primers used for genotyping galectin-3 null mice.

Samples were put in the PCR machine and the following cycle was used; 94°C for 2 minutes and then followed a cycle of (94°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds) x 35; this was followed by incubation at 72°C for 3 minutes. Once complete the amplified DNA was run on a gel immediately.

2.3.3 Gel Electrophoresis

A 1.5% agarose gel was made in TAE buffer with 2μL of gel red added to allow visualisation of the DNA. Once set the gel was placed in an Electrophoresis machine filled with TAE buffer and 10μL of the amplified DNA was loaded into the wells, along with a 1 kilobase DNA ladder. The gel was run at 80 constant volts until the dye front

reached the end of the gel. This was then read on the Protein Simple FluorChem E machine using ultraviolet illumination for 100ms.

2.3.4 Inflammatory Mouse Models

2.3.4.1 Resolving Zymosan-Induced Peritonitis Mouse Model

Zymosan is a cell wall sugar derived from yeast (*Saccharomyces cereviseae*) composed of a β1,3-glucan core linked to chitin and β1,6-glucans that has been used for many years to study inflammation and phagocytosis. It exerts its effects on the cells by activating the TLR2 pathway, which is expressed on monocytes, macrophages, neutrophils, and endothelial cells (Underhill, 2003). Zymosan binding results in an increased production of pro-inflammatory cytokines and this induces an inflammatory response. It has been previously shown in our lab and by others that 1mg zymosan per mouse induces an inflammatory response characterised by high recruitment of neutrophils and monocytes that resolves within 72-96 hours and therefore this protocol was utilised for the purposes of this study (Ajuebor et al., 1999, Navarro-Xavier et al., 2010).

Male C57BL/6 mice were injected intraperitoneally on the left hand side with 1mg zymosan in 1mL sterile, filtered PBS as previously described by Doherty et al in 1985, 0-hour mice received no treatment (Doherty et al., 1985). At 4, 24, 48, 72 and 96 hours post injection the mice were anaesthetised, along with the 0-hour controls, with isoflurane and a cardiac puncture was performed with a 25-gauge needle and a 1mL syringe containing 0.1mL sodium citrate and the blood was stored in a falcon tube on ice. The mice were then sacrificed by cervical dislocation.

Peritoneal lavages were performed with a 23-gauge needle and syringe containing 4mL PBS (+3mM EDTA); a small incision was made in the skin taking care not to penetrate the lining of the peritoneal cavity, the solution was injected and washed around the cavity to collect exudated cells, the exudate was then collected and stored in a falcon tube on ice. Following the lavage the right leg was removed and the femurs isolated and cleaned with sterile gauze; the ends of the femur were then removed and the bone flushed with 1mL PBS (+3mM EDTA) using a 23-gauge needle and a 2mL syringe. The bone marrow cells were collected into a falcon tube and stored on ice.

A lobe of lung was collected from 0 hour control mice for RNA extraction by cutting into the ribs to open the chest cavity, the heart and lungs were then lifted gently using

forceps to prevent damage occurring and a lobe of one of the lungs was removed, this was then placed into 1mL Qiagen RNAlater and frozen at -80°C until ready for use.

In some experiments zymosan-induced peritonitis was performed on male and female galectin-3 null mice at age 6 weeks with age and sex matched wild type controls. Peritoneal lavage fluid was collected from these mice at 48, 72 and 96 hours post zymosan administration in order to assess differences at the resolution phase of the inflammatory response. Differential leukocyte counts and apoptosis assays were performed on these samples as described later.

2.3.4.2 Murine Dorsal Air Pouch Model

Male C57BL/6 mice were injected subcutaneously with 2.5mL of sterile filtered air into the dorsal skin using a 23-gauge needle and a 45μm filter; 3 days later mice were given a second injection of 2.5mL air into the pre-existing pouch. At day 6 mice were injected with 1mg zymosan in 500μL sterile filtered PBS into the pouch or with PBS alone and left for 4 or 24 hours. Mice were sacrificed using increasing concentrations of carbon dioxide. The air pouch was lavaged using 2mL PBS (+3mM EDTA) using a 23-gauge needle and a 2mL syringe without opening the skin and the cells were collected and stored on ice. Death was then confirmed using cervical dislocation.

2.3.4.3 Adoptive Transfer

Male C57BL/6 mice (10 weeks old) were anaesthetised with isoflurane and a cardiac puncture was performed with a 25-gauge needle and a 1mL syringe containing 0.1mL sodium citrate and the blood was stored in a falcon tube on ice. Neutrophils were isolated from the blood using negative selection as described in section 2.3.8; isolated neutrophils were re-suspended at 100 x 10^6 per mL in sterile PBS and labelled with CellTrace™ Carboxyfluorescein succinimidyl ester (CFSE).

Male galectin-3 null mice were placed in a warming box set to 30°C for approximately 10 minutes and then immediately transferred into a restrainer and 100μL of neutrophil suspension was injected intravenously per mouse. 15 minutes after this the mice were injected intraperitoneally with zymosan as previously described; 4 hours later the mice were sacrificed and the peritoneal cavities lavaged to collect recruited leukocytes. CFSE-positive leukocytes were assessed for their intracellular galectin-3 expression.

2.3.5 Flow Cytometry

2.3.5.1 Peripheral Blood

An aliquot (10μL) of the peripheral blood was removed for counting; this was diluted in Turks solution and counted using a haemocytometer to obtain total cell counts per mouse. BD FACS lysing solution was used to lyse erythrocytes in the remaining blood and the leukocytes were pelleted by centrifugation at 300*g* for 10 minutes. Following re-suspension in PBS (+0.1% BSA) cells were plated onto a 96-well U-bottomed plate at a density of 2 x 10^5 cells per well for staining.

2.3.5.2 Exudate

Aliquots (10μL) of the peritoneal and air pouch exudates were removed for counting (in Turks). The remainder of the exudate was centrifuged at 300*g* for 10 minutes to pellet the cells, the supernatant was collected and frozen for ELISA analysis and the cells were then re-suspended in PBS (+0.1% BSA) and plated onto a 96-well U-bottomed plate at a density of 2 x 10^5 cells per well for staining.

2.3.5.3 Bone Marrow

Flushed bone marrow was put through a 70μm cell strainer to remove any bone fragments or non-cellular debris and the collected solution was centrifuged at 300*g* for 10 minutes to pellet the cells. The cells were re-suspended in PBS (+0.1% BSA) and an aliquot (10μL) was removed for counting (in Turks). The remaining cells were plated onto a 96-well U-bottomed plate at a density of 2 x 10^5 cells per well for staining.

2.3.5.4 Intracellular Cell Staining

The cells were washed twice in PBS (+0.1% BSA) and incubated with an antibody to CD16/CD32 (clone 83) at 0.25μg/mL to prevent non-specific binding for 10 minutes on ice in the dark. The peripheral blood and peritoneal exudate were then incubated with Ly6G (clone 1A8) to label neutrophils, F4/80 (clone BM8) to label monocytes/macrophages and Gr-1 (clone RB6-8C5) to differentiate between classical and non-classical monocytes/macrophages. The bone marrow cells were incubated with Ly6G and CXCR4 (clone 2B11) to label bone marrow neutrophils and Ly6C (clone HK1.4) to label bone marrow monocytes. The cells were incubated on ice in the dark for 30 minutes.

The cells were then washed twice in PBS (+0.1% BSA) and fixed in 100µL BD fixation buffer per well for 10 minutes at room temperature. Once fixed the cells were re-suspended and washed three times in BD permeabilisation buffer to allow intracellular staining. The cells were then incubated with antibodies for galectin-1 (polyclonal), galectin-3-PE (clone M3/38) and galectin-9 (polyclonal) on ice in the dark for 45 minutes, the cells were washed twice and a secondary antibody for galectins-1 and -9 (chicken anti-goat IgG alexa-Fluor 488) was added for 30 minutes on ice in the dark. Following this cells were washed twice and re-suspended in PBS at 4°C until ready for analysis by Flow Cytometry.

Leukocytes could be differentiated into neutrophils and classical/non-classical monocytes/macrophages using specific antibodies by Flow Cytometric assessment**.** Once the cells had been defined as neutrophils or monocytes/macrophages using the specific antibodies the expression of the galectins could be assessed using double staining as in **Figure 2.12**.

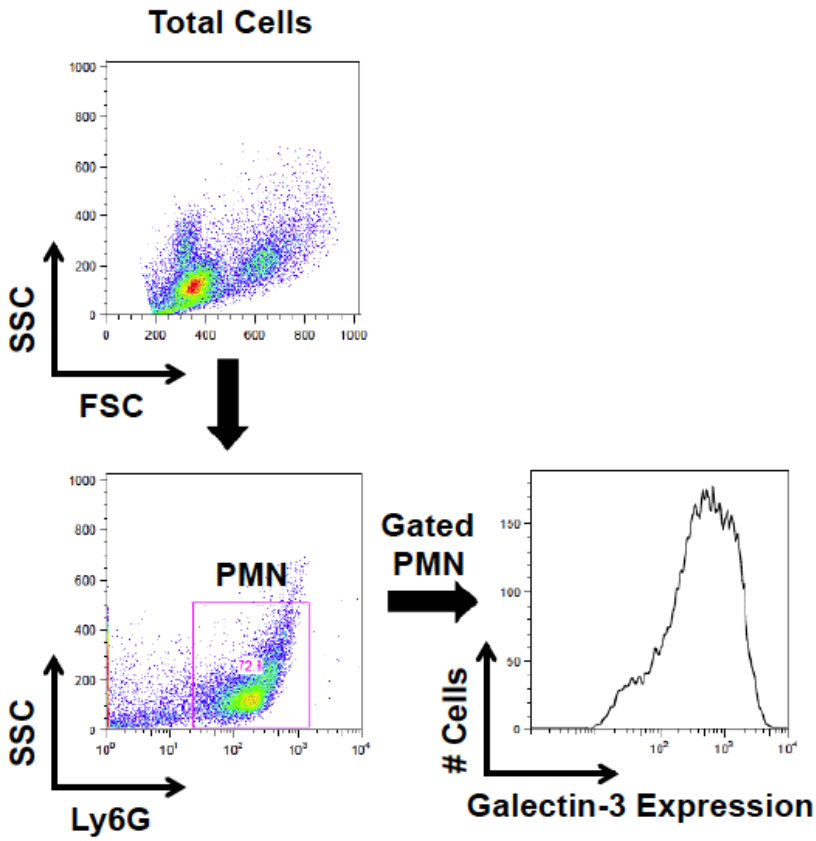


Figure 2.12: Galectin-3 expression in Ly6G positive murine neutrophils. Mice were injected with 1mg zymosan i.p. and leukocytes were collected from the peritoneal cavity 4-hours post injection. Leukocytes were double stained for Ly6G (clone 1A8) and galectin-3 (clone M3/38) and assessed by Flow Cytometry. Initially the cells were gated for Ly6G^{high} expression and this population was then analysed for galectin-3 expression.

Following the analysis of galectin expression on monocytes it was seen that there were large variations in expression levels between cells. It was therefore important to differentiate between classical and non-classical monocytes/macrophages as these different subsets may express different levels of the galectins. An antibody targeted to Gr-1 (clone RB6-8C5), which binds both Ly6G and Ly6C residues, was used as a double stain along with F4/80 (clone BM8) as Ly6C is highly expressed on classical monocytes/macrophages and is expressed in lower levels on non-classical monocytes/macrophages as shown in **Figure 2.13**.

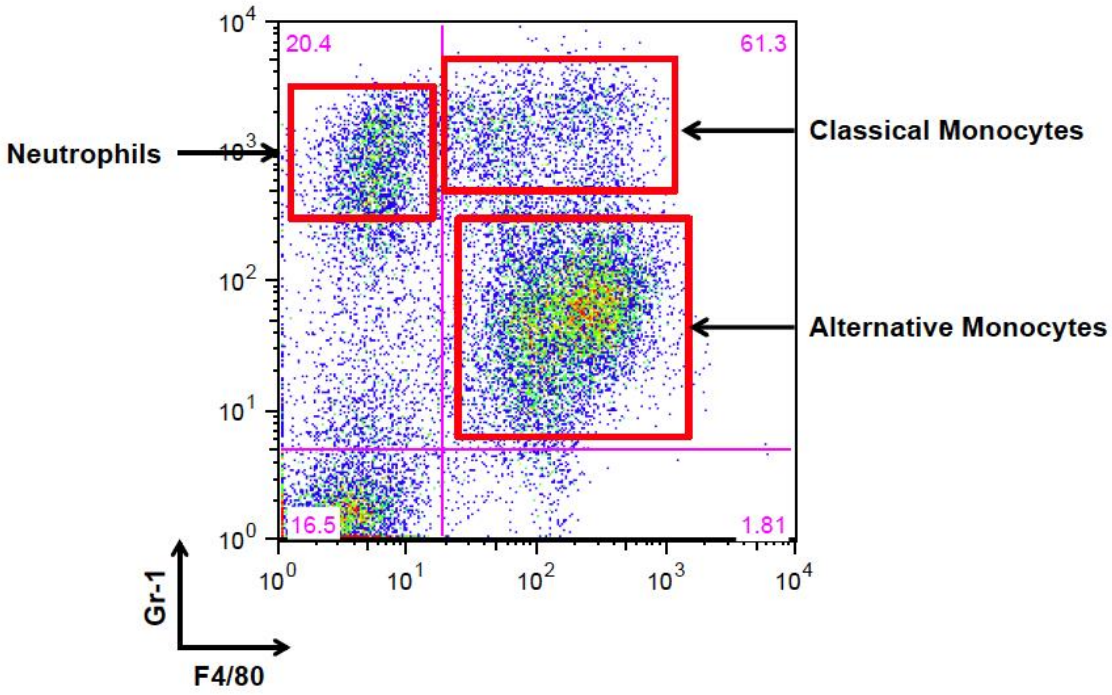


Figure 2.13: Identification of classical and non-classical populations of monocytes and macrophages. Murine leukocytes collected from peritoneal lavage 72-hours post 1mg zymosan-induced peritonitis were assessed for their expression of F4/80 and Gr-1. Classical monocytes/macrophages are F4/80^{high} and Gr-1^{high} while non-classical monocytes/macrophages are F4/80^{high} and Gr-1^{low}. Neutrophils can also be identified using these antibodies as they are F4/80^{negative} and Gr-1^{high}.

2.3.5.5 Cell Surface Staining

Peritoneal exudate cells were washed twice in PBS (+0.1% BSA) and then re-suspended in a CD16/CD32 antibody (clone 83) at 0.25μg/mL to prevent non-specific binding; this was incubated for 10 minutes on ice in the dark. Following this the cells were incubated with a combination of Ly6G (clone 1A8) to label neutrophils and Galectin-3-PE (clone M3/38) for 30 minutes on ice in the dark. The cells were then

washed twice in PBS (+0.1% BSA) and stored in 1% PFA until ready for analysis by Flow Cytometry.

2.3.6 Apoptosis Assay

Peritoneal exudate leukocytes from galectin-3 null mice and wild type controls were plated onto a 96-well U-bottomed plate at a density of 1 x 10^6 cells per well and washed twice in PBS (+0.1% BSA) and then re-suspended in a CD16/CD32 antibody (clone 83) at 0.25μg/mL for 10 minutes on ice in the dark. Following this the neutrophils were labelled with Ly6G (clone 1A8) for 30 minutes on ice in the dark. The cells were washed twice in ice cold PBS (+0.1% BSA) and re-suspended in 100μL 1x Annexin V binding buffer and transferred to FACS tubes; leukocytes were incubated with 5μL Annexin V and 5μL PI (along with single stain controls) at room temperature in the dark for 15 minutes. Following this 400μL of 1x Annexin V binding buffer was added to each tube and analysed immediately by Flow Cytometry.

2.3.7 Enzyme-Linked Immunosorbent Assay (ELISA)

A high-binding 96-well plate was coated with 100μL per well capture antibody (Rat, anti-mouse galectin-3, 2μg/mL in PBS), covered with parafilm and left at 4°C overnight. The following morning the coating buffer was aspirated off and the plate washed three times by filling each well with PBS (+0.05% Tween-20) for 30 seconds and discarding; the final wash was aspirated off to ensure wells were empty. The wells were then blocked with 200μL PBS (+1% BSA) and incubated at room temperature for 1 hour. The PBS was aspirated and the plate washed three times as previously described. The recombinant mouse galectin-3 standard was diluted to 1000pg/mL in PBS (+1% BSA) and six serial dilutions were performed to create a standard curve to 15.625pg/mL. 100μL of each standard dilution was added in duplicate leaving a pair of wells without galectin-3 to act as a blank for the analysis.

The supernatants from the peritoneal exudates were then added in duplicate to the rest of the wells after a 1:5 dilution in PBS (+1% BSA) and the plate was incubated at room temperature for 2 hours. The samples were aspirated and the plate washed three times as previously described. The detection antibody (Goat, anti-mouse galectin-3, 200ng/mL in PBS +1% BSA) was added to each well (100μL) and incubated at room temperature for 2 hours. The detection antibody was aspirated and the plate washed three times as previously described. 100μL Streptavidin-HRP was then added to the

wells and this was incubated at room temperature for 20 minutes. The Streptavidin-HRP was aspirated and the plate washed three times as previously described.

100μL Tetramethylbenzidine (TMB) solution was added to each well and incubated for approximately 20 minutes, 50μL 1M phosphoric acid was then added to each well to stop the reaction and the plate was immediately read using a Labsystems MultiScan Bichromatic Plate Reader using the wavelength subtraction mode which allows the plate to be read at 450nm but with readings at 570nm subtracted to reduce background absorbance values.

The galectin-3 concentrations of the cell-free supernatants from the peritoneal exudate were interpolated from the standard curve shown in **Figure 2.14**.

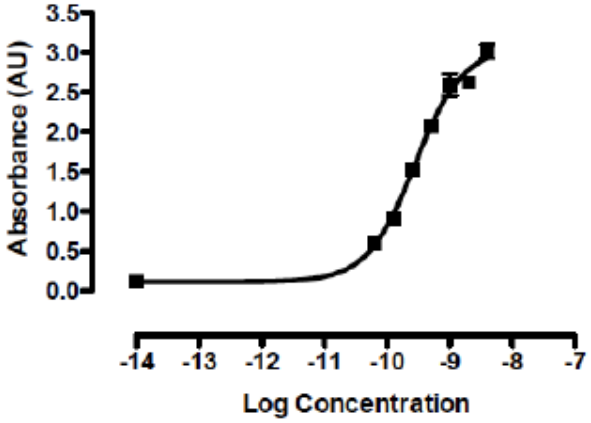


Figure 2.14: Standard curve produced for galectin-3 ELISA.

2.3.8 Neutrophil Isolation by Negative Selection

Peritoneal exudate cells and bone marrow cells were collected from mice following a 4 hour zymosan-induced peritonitis protocol as described in section 2.3.5.2 an aliquot (10μL) of each population was taken for counting in Turks solution to obtain the total cell number and another aliquot (200μL) was taken to stain for Ly6G to assess starting neutrophil purity.

The cells were centrifuged at 300*g* for 10 minutes to obtain a pellet and this was re-suspended in PBS (+2% BSA) at a concentration of 1 x 10^8 cells per mL, normal rat serum was then added to each sample at 50μL/mL of cell suspension. The volumes of

each sample were made up to 500μL in PBS (+2% BSA). 25μL of the mouse neutrophil enrichment cocktail was added to each sample and this was incubated on ice for 15 minutes, the tube was then filled with PBS (+2% BSA) and this was centrifuged at 300*g* for 10 minutes. The pellet was re-suspended in 500μL PBS (+2% BSA) and 25μL biotin selection cocktail was added to the sample, this was incubated on ice for 15 minutes.

The magnetic particles were vortexed for 30 seconds to ensure there were no aggregates and 75μL of this was added to each sample, and incubated on ice for 10 minutes. The entire sample was then transferred to a 5mL polystyrene round bottomed tube and made up to 5mL with PBS (+2% BSA), this was all placed in an Invitrogen DynaMag™-15 magnet for 5 minutes to allow all cells that are not neutrophils to be bound to the magnet leaving the neutrophils in suspension. The neutrophils were collected into a falcon and the negative fraction was re-suspended in PBS (+2% BSA). This process was repeated twice more to collect as many neutrophils from the supernatant as possible.

Following this the neutrophil fraction was also placed in the magnet in an attempt to remove any contaminating cells that would affect the purity. From this fraction an aliquot (10μL) of each population was taken for counting in Turks solution to obtain the yield and another aliquot (200μL) was taken to stain for Ly6G to assess final neutrophil purity.

Both the neutrophil fraction and the negative fraction were re-suspended in 350μL Qiagen RLT Buffer containing 10μL β-mercaptoethanol/mL to lyse the cells ready for RNA extraction. This was then frozen at -80°C until ready to extract the RNA.

2.3.9 Neutrophil Isolation by Positive Selection

Negative selection resulted in a low yield and purity for both the bone marrow and the peritoneal exudate cells and therefore a positive selection method was attempted in order to increase both these parameters.

Bone marrow and peritoneal exudate were collected as previously described, an aliquot (10μL) was taken for counting in Turks solution on a haemocytometer and the cells were centrifuged at 300*g* for 10 minutes to pellet the cells. The pellet was re-suspended in PBS (+0.1% BSA) to a final concentration of 1×10^7 cells per mL and to this 10μg/mL purified rat anti-mouse Ly6G antibody was added and incubated on ice in the dark for 20 minutes.

During this incubation period 100μL sheep anti-rat Dynabeads® per mL of sample were washed in 1mL PBS (+0.1% BSA) by placing in the magnet, leaving for 2 minutes and pouring off the supernatant, once washed the beads were re-suspended in PBS (+0.1% BSA) at their original volume.

Once the incubation was completed 2mL PBS (+0.1% BSA) was added to each sample and they were centrifuged at 300*g* for 10 minutes. The pellet was then re-suspended to 1 x 10^7 cells per mL in PBS (+0.1% BSA) and 100μL Dynabeads added per mL of sample. The sample was rotated gently for 20 minutes and then the total volume was increased by 1mL to limit the trapping of unbound cells, this was placed in the magnet for 2 minutes and the supernatant kept to analyse as the negative fraction. The remaining neutrophils were washed three times by re-suspending in 1mL PBS (+0.1% BSA) and placing in the magnet for 1 minute then pouring off the supernatant.

An aliquot of the sample (100μL) was then put on a slide and allowed to air dry and this was stained with Haematoxylin and Eosin to confirm no eosinophil contamination, the rest of the sample was transferred to an eppendorf, centrifuged at 8000*g* for 1 minute and the pellet was re-suspended in 750μL Trizol reagent and frozen at -80°C until ready to isolate RNA.

2.3.10 RNA Extraction using the RNeasy Mini-Kit

The lung tissue positive control, isolated neutrophils and negative fractions were defrosted on ice and the lung was weighed to ensure the tissue was less than 30mg. This was then placed in a homogenisation tube with 600μL Qiagen RLT buffer containing 10μL β-mercaptoethanol/mL and homogenised using a Precellys 24 homogeniser at 6800rpm for 1 minute.

The neutrophil and negative fractions were syringed with a 1mL 27-gauge tuberculin syringe at least 5 times per sample to ensure all cells were lysed and then 350μL 70% molecular biology grade ethanol was added to each sample (including the lung tissue) and this was mixed until clear. The entire volume was then transferred to a Qiagen column and centrifuged at 8000*g* for 15 seconds and the flow through discarded, 700μL of RW1 buffer was added to the column and this was centrifuged at 8000*g* for 15 seconds and again the flow through was discarded.

500μL RPE buffer containing 1 volume of ethanol was added to the column and again centrifuged at 8000*g* for 15 seconds and the flow through was discarded. This stage

was repeated with another 500μL RPE buffer and centrifuged at 8000*g* for 2 minutes and this time the collection tube was discarded and replaced with a new one. This was spun at 8000*g* for 1 minute and the column was then placed into a 1.5mL RNAse-free eppendorf, 30μL RNAse-free water was added directly to the membrane and allowed to sit for 1 minute before centrifuging at 8000*g* for 1 minute. The column was then discarded and 1μL of RNA was assessed on an ND-1000 Nanodrop Spectrometer; a beam of near-monochromatic light is passed through the droplet of RNA and the light transmitted through the sample is measured. The ratio of sample absorbance at 260 and 280nm is given and this is used to assess the purity of the RNA with 2 being considered pure, deviations from this number can suggest contamination with protein or phenol. A second ratio of sample absorbance at 260 and 230nm is given and this is a secondary measure of purity. The Nanodrop also provides a concentration for the RNA present in the sample in ng/μL. The RNA was then frozen at -80°C until ready for cDNA synthesis.

2.3.10.1 QiaShredder Column

Assessment of the 260/280 and 260/230 values obtained from the Nanodrop readings described in section 2.3.10 showed high levels of contamination and low levels of RNA from the neutrophils, this may be due to the difficulty in fully lysing neutrophils that has been reported in the literature. The protocol was therefore repeated using a QiaShredder column, which is designed to help lyse cells more efficiently than syringing.

700μL of the neutrophil fraction in Qiagen RLT Buffer containing 10μL β-mercaptoethanol/mL was added to the top of the QiaShredder column and this was centrifuged at 8000*g* for 2 minutes, the column was discarded and the eluted material loaded onto the top of a Qiagen column and the RNeasy Mini-Kit protocol followed as above.

2.3.11 RNA Extraction using Trizol

Nanodrop readings following use of the QiaShredder column were still low for RNA levels and still exhibited high levels of salt contamination therefore a Trizol procedure was attempted in order to obtain higher levels of RNA from the neutrophils.

Once the neutrophils had been isolated the cell pellet was re-suspended in 750μL Trizol reagent and this was frozen at -80°C until ready to extract the RNA.

The samples were defrosted on ice and then 200μL of chloroform was added and the sample shaken vigorously by hand for 15 seconds. This was incubated at room temperature for 10 minutes and then centrifuged at 8000*g* for 15 minutes at 4°C. The aqueous phase was transferred to a clean eppendorf and 500μL 2-propanolol was added and mixed, this was incubated at room temperature for 10 minutes and then centrifuged at 8000*g* for 10 minutes at 4°C. The supernatant was discarded and the cells washed in 1mL 75% ethanol, this was centrifuged at 8000*g* for 5 minutes at 4°C, the majority of the ethanol was aspirated and the rest left to air-dry. The pellet was re-suspended in 40μL RNAse-free water and the RNA concentration determined on the Nanodrop. The RNA was then frozen at -80°C.

This provided the best yield of RNA from the neutrophils (although the salt contamination levels were still high) and therefore was chosen as the method from which cDNA synthesis was performed.

2.3.12 Complementary DNA (cDNA) synthesis

The RNA was defrosted on ice and then 11μL was mixed with 1μL oligo$(DT)_{15}$ and 1μL dNTP mix, and incubated in a MWG Biotech Primus-96 plus PCR machine at 65°C for 5 minutes. Following a 5 minute incubation on ice each sample then received 4μL first strand buffer, 1μL DTT, 1μL RNAse out and 1μL Superscript (controls were included which received everything but Superscript). This was all incubated in the PCR machine at 50°C for 1 hour and then 70°C for 15 minutes. Once the cDNA had been produced this was frozen at -20°C until ready for PCR.

2.3.13 Polymerase Chain Reaction (PCR)

The cDNA was defrosted on ice and then 1μL of each sample was mixed with 10μL ReddyMix PCR MasterMix and 1μL of each gene specific primer required (Qiagen Quantitec Primer Assay®), the primers used were for *Lgals3*, *Lgals9*, *Ly6g*, *Cd68* and *Rpl32*. This was incubated in the PCR machine at 94°C for 5 minutes and then followed a cycle of (94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds) x 35; this was followed by incubation at 72°C for 10 minutes. Once complete the amplified DNA was stored at -20°C or run on a 2% agarose gel immediately as described in **section 2.3.3**.

2.4 Statistical Analysis

Statistical significance was assessed using SPSS computer software. Data is expressed as mean ± standard error of the mean (SEM) of n experiments. All data were tested for normal distribution and power calculations were performed using G*Power software (Faul et al., 2009). A Grubbs test was used to identify statistical outliers and these were removed before tests were performed. Statistical differences were analysed by two-tailed T-test for 2 groups, one-way analysis of variance (ANOVA) followed by a Bonferroni or Dunnetts post hoc test (depending on if comparing all values or each value to a control respectively) or two-way ANOVA followed by Bonferroni post hoc test. In assays where repeated measures were performed a repeated measures test was utilised. In all cases a P value ≤ 0.05 was considered significant to reject the null hypothesis and differences were considered significant.

CHAPTER 3:

RESULTS (1): MODULATION OF THE NEUTROPHIL GLYCOPHENOTYPE AND GALECTIN BINDING PROFILE

3 Modulation of the Neutrophil Glycophenotype and Galectin Binding Profile

3.1 Introduction

The glycosylation of adaptive immune cells is known to be of particular importance to their recruitment, activation and clearance from an inflammatory site (Wright and Cooper, 2014); the glycophenotype is modulated dependent on the extracellular environment, which allows binding of lectins to take place and affect cell functions.

Galectins-1, -3 and -9 are known to be immunomodulatory as they bind to, and elicit actions from immune cells. Galectins-1 and -9 are predominantly anti-inflammatory as they decrease leukocyte recruitment and skew towards a Th2 phenotype during disease (Rabinovich et al., 2000, La et al., 2003, He et al., 2009), while galectin-3 is considered pro-inflammatory as it increases leukocyte recruitment and skews towards a Th1/Th17 phenotype (Colnot et al., 1998b, Hsu et al., 2000).

The actions of galectins are determined not only by the cellular expression of the proteins but also by the target cell permissibility to galectin binding, the glycophenotype of the cell. Work has been performed on the glycophenotype of adaptive immune cells and how this pertains to galectin binding but the innate immune system has not been characterised in this way.

This study therefore set out to delineate the effects of inflammation on the neutrophil glycophenotype, as well as elucidate the temporal and spatial modulation of galectins during resolving inflammation.

The aims of this section were therefore to characterise the modulation of the glycophenotype of human and murine neutrophils following an inflammatory stimulus and/or cell trafficking using a lectin binding assay with a validated panel of lectins.

Following this the binding of galectins-1, -3 and -9 will be assessed following the same inflammatory stimuli and/or cell trafficking to determine whether this correlates with the glycophenotypic changes seen.

Initially freshly isolated human PMN were assessed at varying stages of trafficking across an endothelial monolayer that had been pre-treated for 4 hours with 10ng/mL hrTNF-α to induce activation and up-regulate E-selectin on the HUVEC surface. To distinguish changes caused by PMN activation and those due to transendothelial migration the glycophenotype of human PMN was characterised following activation with soluble mediators such as hrTNF-α (10ng/mL) and fMLP (1μM); stimulants that release secretory vesicles and secondary granules respectively. Human PMN were also treated with conditioned medium from activated endothelial cells to determine the role of endothelial cell-derived soluble factors on PMN glycophenotype. A novel human model of PMN extravasation was also utilised in which neurogenic inflammation was used to recruit PMN to the buccal cavity within 1 hour.

Assessment of the glycophenotype is able to provide an approximation of the permissibility for galectin binding to neutrophils. In order to determine the accuracy of lectin binding, the binding of recombinant galectins to PMN under the same conditions was assayed.

To characterise the modulation in a pathophysiological setting an *in vivo* murine zymosan-induced peritonitis model was used, which results in robust neutrophil recruitment to the peritoneal cavity within 4 hours of administration. The glycophenotype of murine neutrophils collected from the peripheral circulation was compared to those collected from the peritoneal cavity following 4 hour zymosan-induced peritonitis.

3.1.1 Glycophenotype of Freshly Isolated Human Neutrophils

Human PMN were isolated from whole blood of healthy volunteers as described in section 2.2.2, fixed in 1% PFA overnight and a validated panel of lectins was used to label specific terminal glycosylation motifs as shown in Table 2.3, lectin binding was assessed by Flow Cytometry as shown in **Figure 3.1**.

Basally, human PMN express a glycophenotype characterised by low binding levels of PNA (55.87 ± 14.53) and UEA I (13.14 ± 3.123) indicative of low expression of terminal mono-LacNAc and α1,3-linked fucose residues. Whereas high binding levels of RCA_{120} (711.4 ± 145.4), LEL (726.5 ± 216.2), PHA-L (930.4 ± 150.3) and SNA (310.6 ± 116.6) were observed, which are indicative of high expression of β-linked galactose, poly-LacNAc chains with α2,6-linked sialic acid residues expressed on complex tri- and tetra-antennary N-glycans.

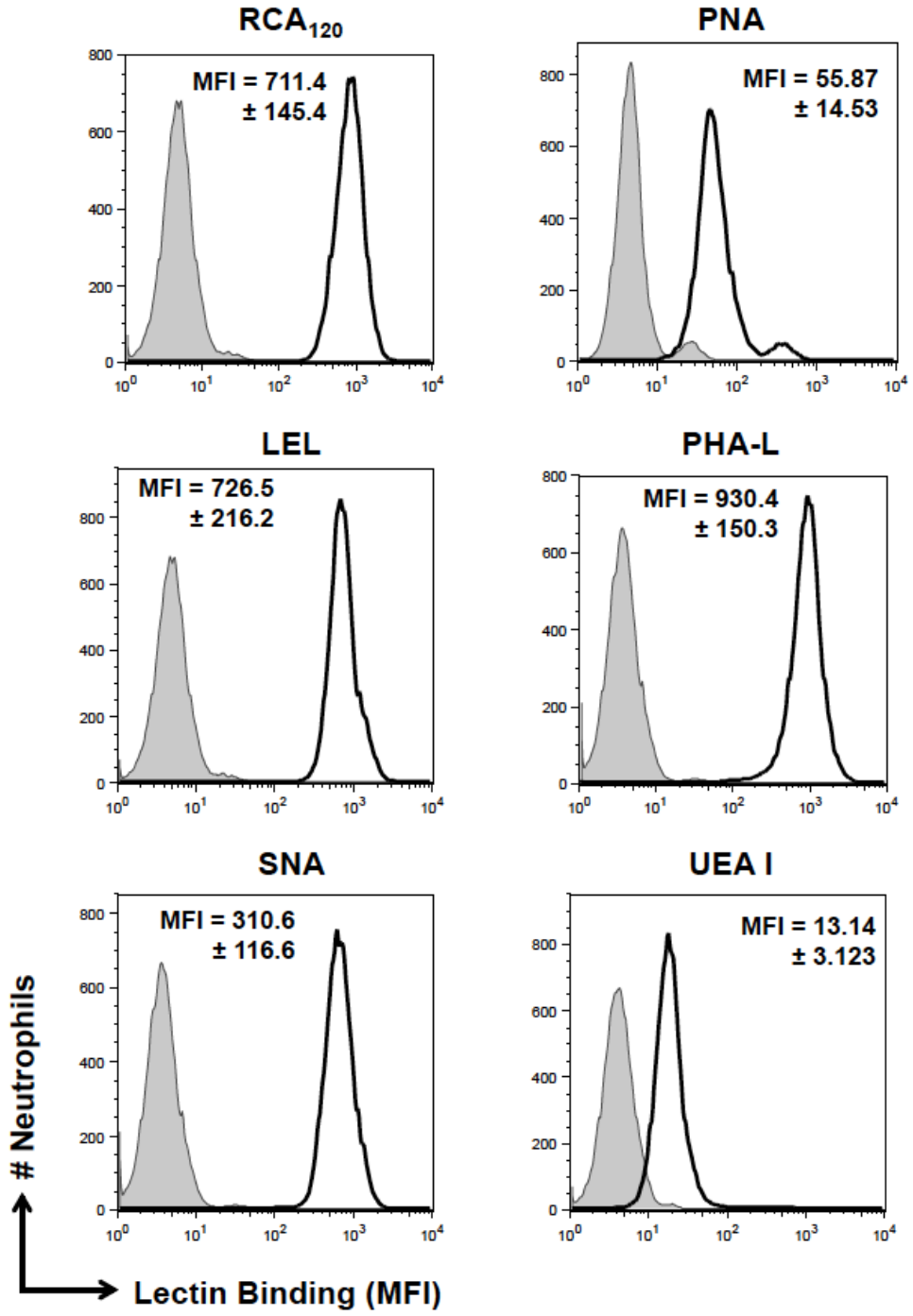


Figure 3.1: Representative histograms showing basal lectin binding on freshly isolated human PMN. Blood was taken from healthy volunteers and PMN were isolated by dextran sedimentation and fixed overnight. PMN were then incubated with plant lectins for specific glycans and binding was analysed by Flow Cytometry. Shaded peak = secondary antibody only. N = 5 per group, MFI = mean ± SEM.

3.1.2 Modulation of the Neutrophil Glycophenotype during Adhesion to Endothelial Cells

An assay was performed to assess the glycophenotype of PMN during the process of adhesion to an endothelial monolayer in a 6-well plate. Isolated PMN from healthy

volunteers were added to a well in which a confluent monolayer of HUVECs had been pre-treated for 4 hours with 10ng/mL hrTNF-α. The cells were incubated together for 30 minutes before non-adherent and adherent PMN were collected from the plate and analysed for their expression of neutrophil activation markers (CD11b and CD62L), freshly isolated PMN were also used as a control as shown in **Figure 3.2**.

The expression of CD62L was high on freshly isolated PMN (536.5 ± 77.94) and this decreased by more than 97% in non-adherent and adherent cells (13.06 ± 2.541, P = 0.0026 and 8.457 ± 1.729, P = 0.0025 respectively); while the expression of CD11b was high in freshly isolated PMN (984.2 ± 45.37) and remained unchanged during co-culture with, and adhesion to HUVECs (1034 ± 52.12 and 901.3 ± 36.07). Taken together this suggests that the non-adherent and adherent PMN were primed yet not fully activated.

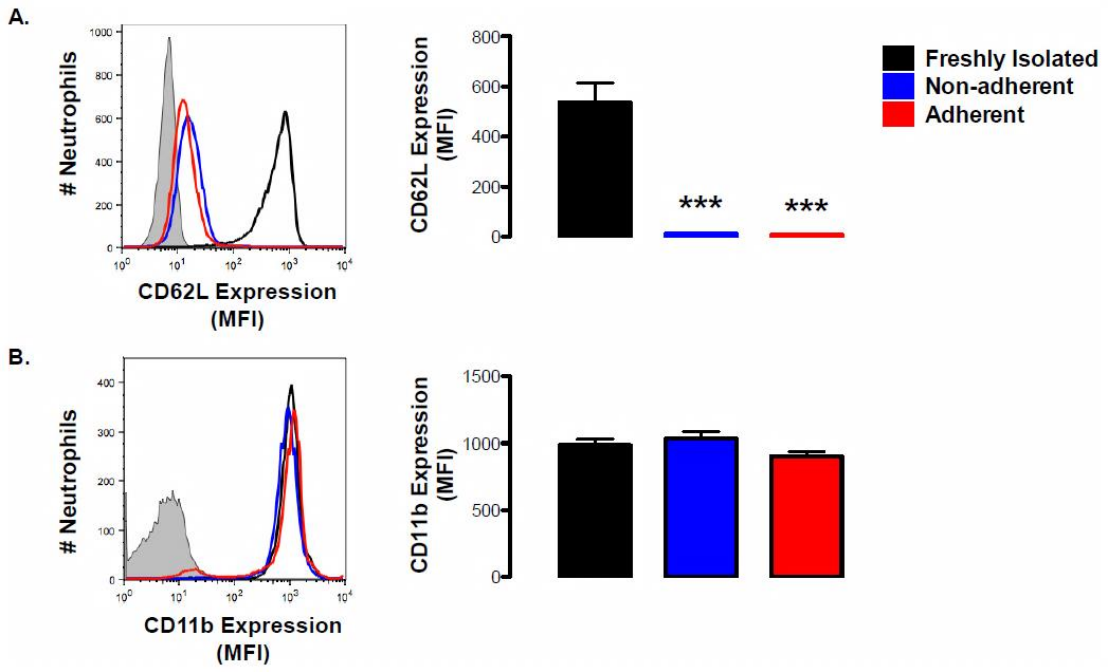


Figure 3.2: Neutrophil activation marker expression in human PMN collected from activated endothelial cells. Blood was taken from healthy volunteers, PMN were isolated and added to a 6-well plate in which a confluent monolayer of HUVECs had been pre-treated for 4 hours with 10ng/mL hrTNF-α. The cells were incubated together for 30 minutes, adherent and non-adherent PMN were collected and the expression of CD62L and CD11b assessed by Flow Cytometry in comparison with freshly isolated PMN. A. Representative histogram and graph of CD62L expression and B. Representative histogram and graph of CD11b expression. Shaded peak = isotype control. Data are expressed as mean ± SEM, N = 3 per group, *** P < 0.001 vs freshly isolated PMN as analysed by one-way ANOVA with Bonferroni post hoc test.

The glycophenotype of these PMN was then assessed by lectin binding. A 69% reduction in RCA_{120} binding could be seen between non-adherent and adherent PMN indicating a significant loss of β-linked galactose residues as PMN adhere to an endothelial monolayer (1832 ± 477.98 vs 573.7 ± 530.1, P = 0.003) **Figure 3.3.**

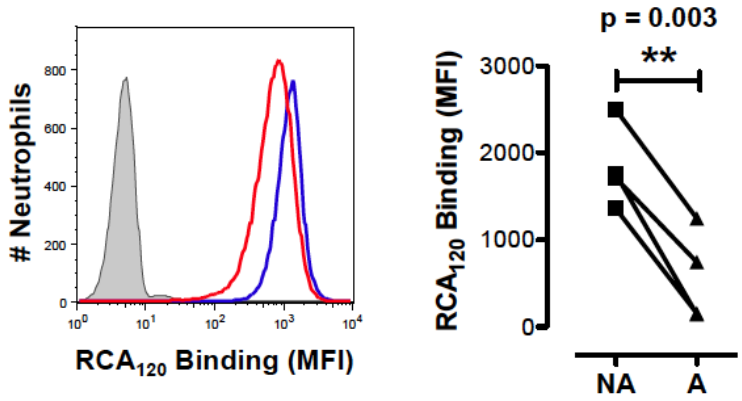


Figure 3.3: Binding of a β-linked galactose-specific lectin to non-adherent and adherent human PMN following incubation with HUVECs. Freshly isolated PMN were incubated for 30 minutes with HUVECs that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent (NA) and adherent (A) PMN were collected from the well and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. Figure shows representative histogram and graph of RCA_{120} binding. Grey = secondary antibody binding, blue = non-adherent PMN and red = adherent PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 4 per group. ** P < 0.01 as analysed by two-tailed paired T test.

An overall trend was seen with lectin binding that was indicative of galectin binding permissibility with a reduction in lectin binding following PMN adhesion to the endothelial cell monolayer. A 50% reduction in PNA binding (14.18 ± 7.59 vs 7.79 ± 5.60, P = 0.026); a 30% decrease in LEL binding (463.9 ± 263.2 vs 327.8 ± 185.2, P = 0.03) and a 28% reduction in PHA-L binding (462.0 ± 169.3 vs 334.7 ± 108.5, P = 0.025; **Figure 3.4**) was observed. This indicated of a loss of mono- and poly-LacNAc residues as well as complex tri- and tetra-antennary N-glycans as neutrophils adhere to the endothelial monolayer.

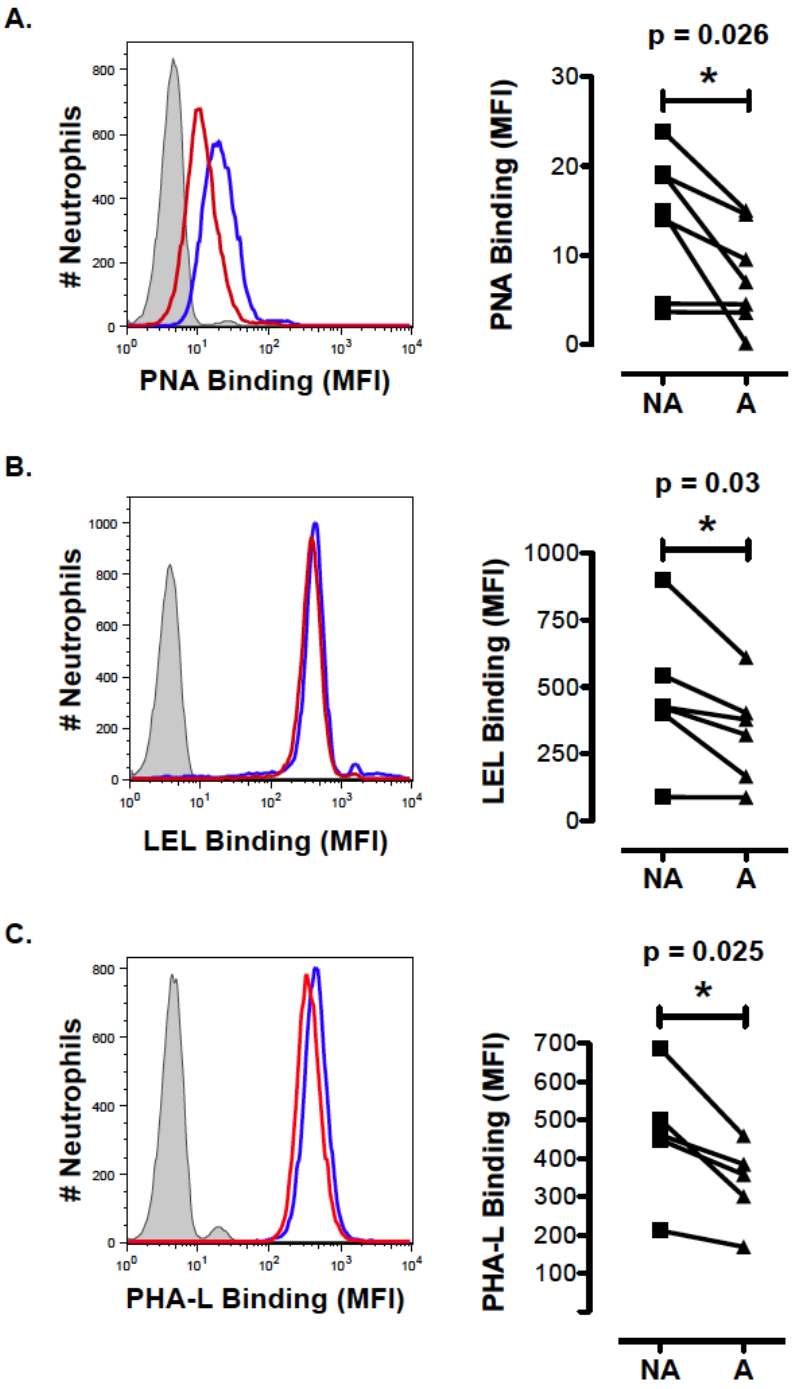


Figure 3.4: Binding of lectins indicative of galectin binding permissibility to non-adherent and adherent human PMN following incubation with HUVECs. Freshly isolated PMN were incubated for 30 minutes with HUVECs that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent (NA) and adherent (A) PMN were collected from the well and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. A. Representative histogram and graph of PNA binding, B. Representative histogram and graph of LEL binding and C. Representative histogram and graph of PHA-L binding. Grey = secondary antibody binding, blue = non-adherent PMN and red = adherent PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 5-7 donors per group. * $P < 0.05$ as analysed by two-tailed paired T test.

There was an overall decrease in α2,6-linked sialic acid expression following PMN adhesion as indicated by a 42% reduction in SNA binding when compared to non-adherent PMN (149.3 ± 82.72 vs 87.03 ± 43.28, P = 0.014) **Figure 3.5**.

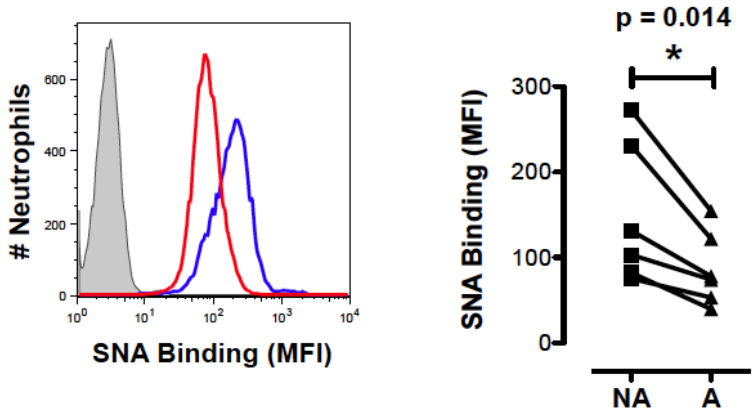


Figure 3.5: Binding of an α2,6-linked sialic acid-specific lectin to non-adherent and adherent human PMN following incubation with HUVECs. Freshly isolated PMN were incubated for 30 minutes with HUVECs that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent (NA) and adherent (A) PMN were collected from the well and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. Figure shows representative histogram and graph of SNA binding. Grey = secondary antibody binding, blue = non-adherent PMN and red = adherent PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 5 per group. * P < 0.05 as analysed by two-tailed paired T test.

The expression of α-linked fucose residues was significantly reduced following PMN binding to endothelial cells as indicated by a 53% decrease in UEA I binding (3.44 ± 1.69 vs 1.63 ± 1.16, P = 0.035) **Figure 3.6.**

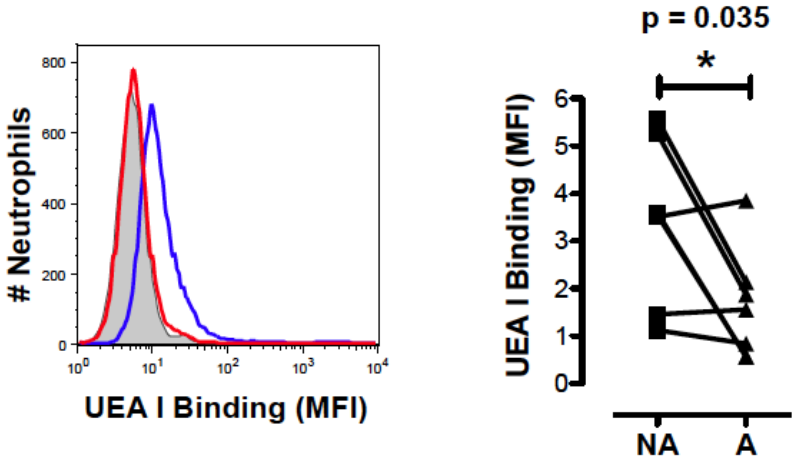


Figure 3.6: Binding of α-linked fucose-specific lectin to non-adherent and adherent human PMN following incubation with HUVECs. Freshly isolated PMN were incubated for 30 minutes with HUVECs that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent (NA) and adherent (A) PMN were collected from the well and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. Figure shows representative histogram and graph of UEA I binding. Grey = secondary antibody binding, blue = non-adherent PMN and red = adherent PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 6 per group. * P < 0.05 as analysed by two-tailed paired T test.

3.1.3 Galectin Binding to Isolated Neutrophils during Adhesion to Endothelial Cells

The binding of recombinant galectins-1, -3 and -9 (10nM) to PMN that were non-adherent and adherent collected from the 6-well adhesion assay was assessed. PMN were collected as previously described and incubated with recombinant galectins at 37°C for 15 minutes to allow binding; following this the PMN were washed and assessed for the expression of galectin on the cell surface compared to those that were not treated with recombinant protein. The total binding was calculated by subtracting the endogenous galectin expression from that seen in treated cells from the same donor.

Galectin-1 binding to freshly isolated PMN was low (5.194 ± 1.497) however this increased by approximately 4-fold in PMN that were in contact with, but not adherent to, the endothelium (18.43 ± 2.791) and this decreased back to basal levels upon adhesion to the endothelial monolayer (7.537 ± 2.515) as shown in **Figure 3.7**.

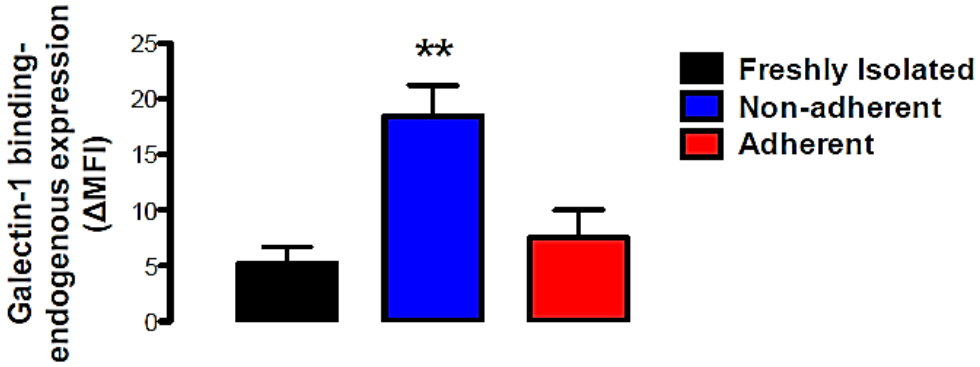


Figure 3.7: Recombinant galectin-1 binding to PMN during adhesion to an endothelial monolayer. Freshly isolated PMN were left to adhere for 30 minutes to a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent and adherent PMN were treated with recombinant galectin-1 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-1 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-1 was subtracted from the total level to obtain the bound galectin-1 per group. Figure shows graph of recombinant galectin-1 binding. Data are expressed as mean ± SEM, n = 3 per group, ** $P < 0.01$ vs freshly isolated as analysed by one-way ANOVA with Bonferroni post hoc test.

The binding of galectin-3 to freshly isolated PMN was higher than that of galectin-1 (110 ± 16.75) and although significance was not reached this appears to decrease as cells come into contact with, and adhere to the endothelium (49.63 ± 14.37 and 49.00 ± 20.09 respectively) as shown in **Figure 3.8**.

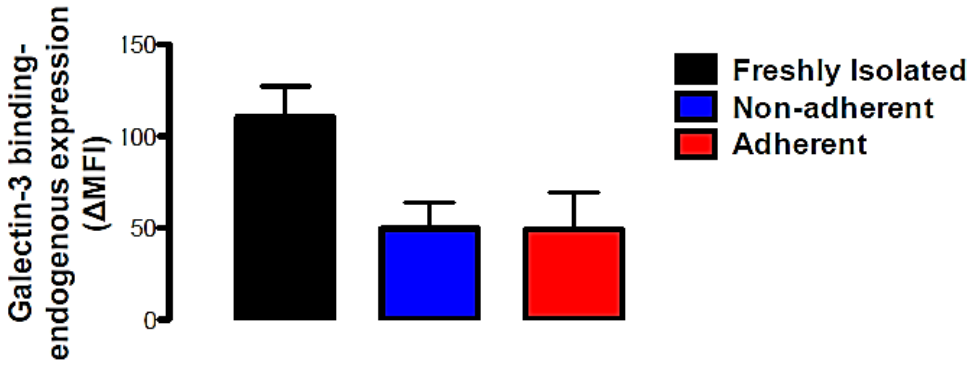


Figure 3.8: Recombinant galectin-3 binding to PMN during adhesion to an endothelial monolayer. Freshly isolated PMN were left to adhere for 30 minutes to a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent and adherent PMN were treated with recombinant galectin-3 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-3 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-3 was subtracted from the total level to obtain the bound galectin-3 per group. Figure shows graph of recombinant galectin-3 binding. Data are expressed as mean ± SEM, n = 3 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

Galectin-9 binding was high in freshly isolated PMN (294.5 ± 64.5) and this was not significantly modulated upon contact with, or adhesion to a HUVEC monolayer (357. 8 ± 57.68 and 271.1 ± 59.97 respectively) as shown in **Figure 3.9**.

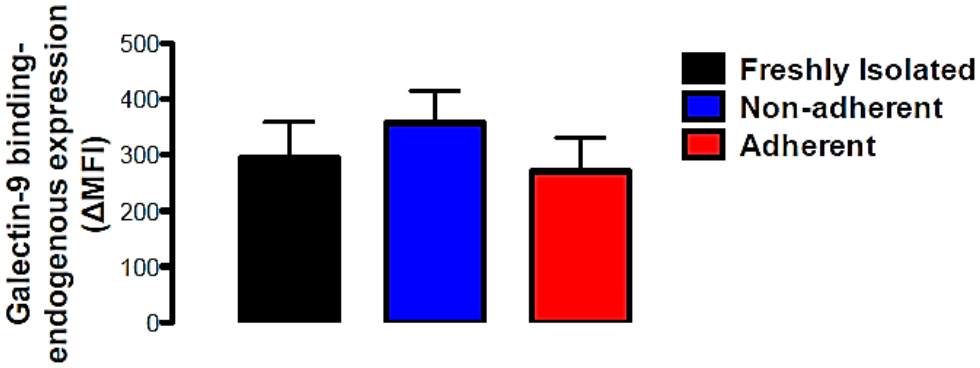


Figure 3.9: Stable, recombinant galectin-9 binding to PMN during adhesion to an endothelial monolayer. Freshly isolated PMN were left to adhere for 30 minutes to a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-adherent and adherent PMN were treated with stable, recombinant galectin-9 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-9 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-3 was subtracted from the total level to obtain the bound galectin-9 per group. Figure shows graph of stable, recombinant galectin-9 binding. Data are expressed as mean ± SEM, n = 3 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

3.1.4 Modulation of the Neutrophil Glycophenotype during Transendothelial Migration

Freshly isolated PMN were assessed for the modulation of their glycophenotype during transendothelial migration using a transwell system. HUVECs were seeded onto a transwell insert with 3μm pores and left to become fully confluent (typically overnight), the cells were then stimulated for 4 hours with 10ng/mL hrTNF-α to up-regulate cell adhesion molecules required for PMN adhesion and transmigration. Isolated PMN were added to the top of the transwell and allowed to transmigrate for 90 minutes. PMN were then collected from the top of the transwell (non-transmigrated) and from under the transwell (transmigrated). The collected populations of PMN were then assessed for their expression of activation markers (**Figure 3.10**).

Similar to that seen in the 6-well assay freshly isolated PMN expressed high levels of CD62L (601.8 ± 84.91) and this was decreased by approximately 98% in non-transmigrated and transmigrated PMN (12.0 ± 1.167, P = 0.0025 and 6.967 ± 0.718, P = 0.0025 respectively). CD11b was also highly expressed by freshly isolated PMN (250.2 ± 25.29) but this was not modulated upon transmigration through an endothelial monolayer (343.8 ± 19.96 and 282.3 ± 3.65).

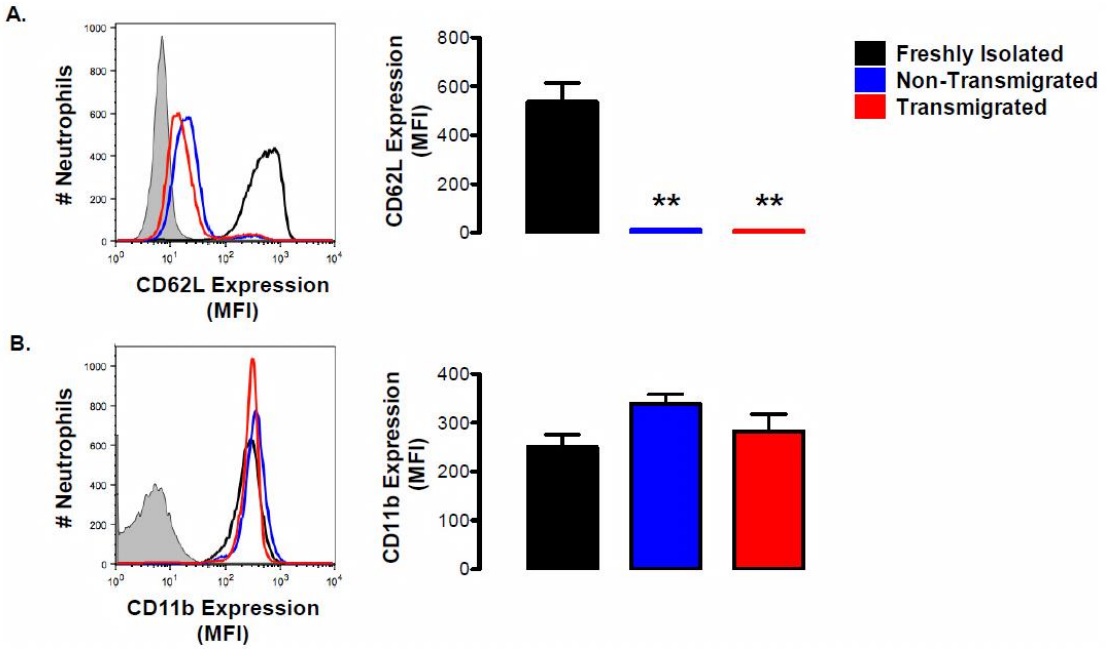


Figure 3.10: Neutrophil activation marker expression in human PMN collected from the transwell assay. Blood was taken from healthy volunteers, PMN were isolated and added to a transwell on which a confluent monolayer of HUVECs had been pre-treated for 4 hours with 10ng/mL hrTNF-α. The cells were allowed to transmigrate for 90 minutes and the expression of CD62L and CD11b was assessed by Flow Cytometry and compared to PMN that had not transmigrated (top well of transwell) as well as control PMN (that had not come into contact with HUVECs). A. Representative histogram and graph of CD62L expression and B. Representative histogram and graph of CD11b expression. Data are expressed as mean ± SEM, N = 3 per group, ** P < 0.01 vs freshly isolated PMN as analysed by one-way ANOVA with Bonferroni post hoc test.

Non-transmigrated and transmigrated PMN were then assessed by lectin binding. The expression of β-linked galactose residues was significantly reduced as PMN transmigrated through an endothelial monolayer as shown by a 52% decrease in RCA_{120} binding (2109.67 ± 937.16 vs 1020.22 ± 450.53, P = 0.009) **Figure 3.11**.

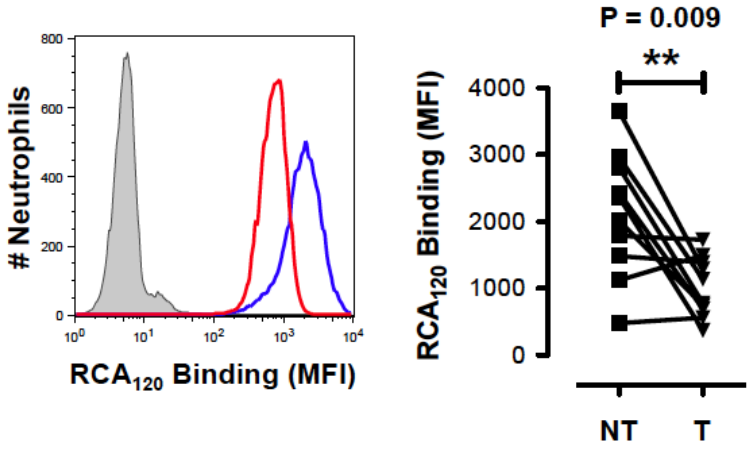


Figure 3.11: Binding of a β-linked galactose-specific lectin to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated (NT) and transmigrated (T) PMN were collected and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. Figure shows representative histogram and graph of RCA_{120} binding. Grey = secondary antibody binding, blue = non-transmigrated PMN and red = transmigrated PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 10 per group. ** P < 0.01 as analysed by two-tailed paired T test.

As with the adherent cells a trend towards decreased lectin binding was seen between non-transmigrated and transmigrated PMN. A 46% reduction in PNA binding was seen which was indicative of a decrease in the expression of mono-LacNAc residues as PMN traverse the endothelial monolayer (25.91 ± 13.64 vs 14.06 ± 7.78, P = 0.015) **Figure 3.12a**. No significant modulation could be seen when comparing LEL binding between non-transmigrated and transmigrated PMN suggesting no statistically significant difference in the expression of poly-LacNAc residues, however a trend towards decreased expression on transmigrated PMN was observed with 2 out of 6 donors (269.2 ± 123.0 vs 137.6 ± 53.68, P = 0.127) **Figure 3.12b**. A decrease in the expression of complex tri- and tetra-antennary N-glycans was seen as indicated by a 49% decrease in the binding of PHA-L when comparing non-transmigrated and transmigrated PMN (838 ± 163.8 vs 420.7 ± 44.84, P = 0.039) **Figure 3.12c**.

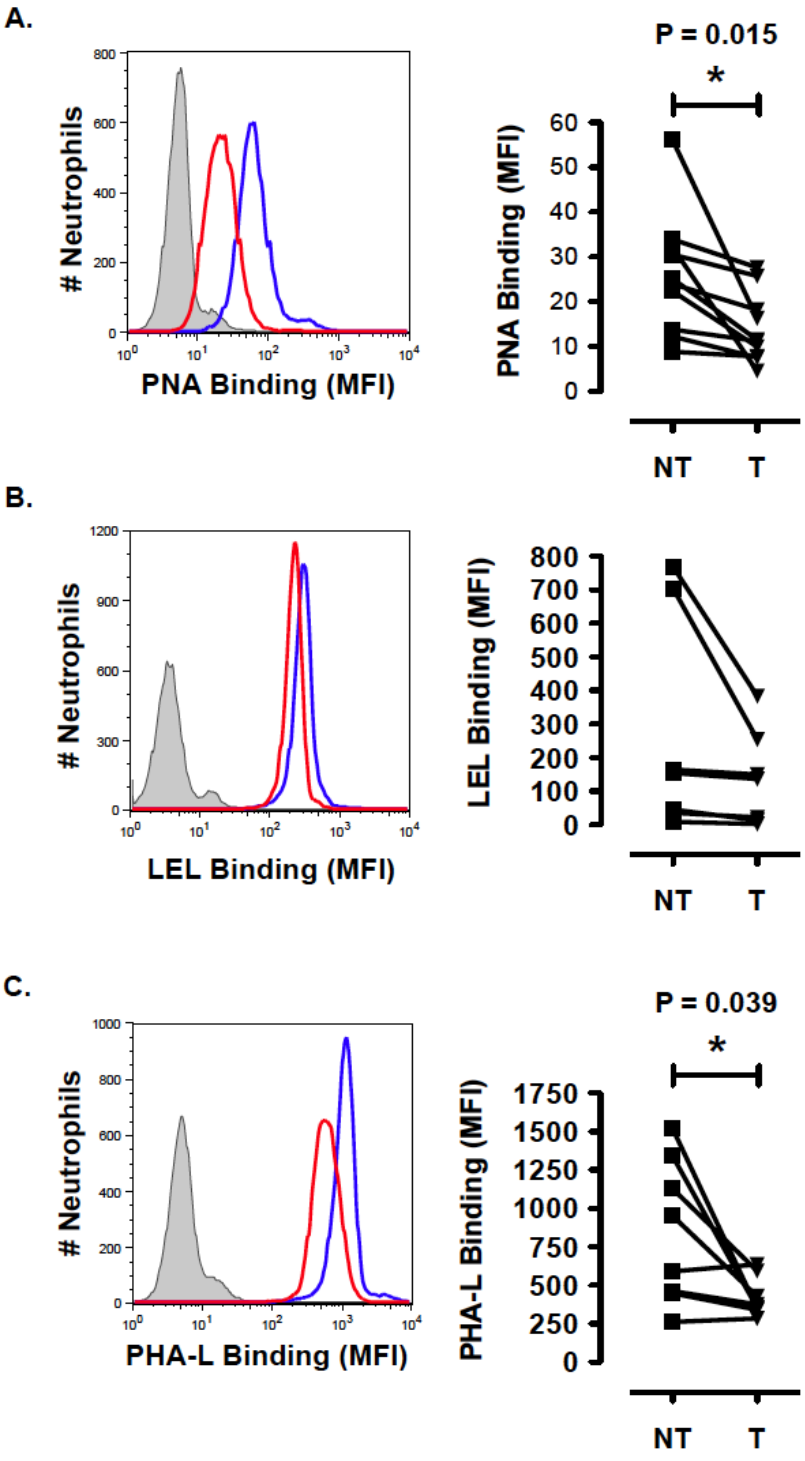


Figure 3.12: Binding of lectins indicative of galectin binding permissibility to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated (NT) and transmigrated (T) PMN were collected and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. A. Representative histogram and graph of PNA binding, B. Representative histogram and graph of LEL binding and C. Representative histogram and graph of PHA-L binding. Grey = secondary antibody binding, blue = non-transmigrated PMN and red = transmigrated PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 10 per group for PNA, 9 per group for LEL and 10 per group for PHA-L. * $P < 0.05$ as analysed by two-tailed paired T test.

SNA binding decreased by 59% when comparing non-transmigrated and transmigrated PMN indicative of a decrease in the expression of α2,6-linked sialic acid residues upon transendothelial migration (469.30 ± 348.58 vs 194.80 ± 55.57, P = 0.048) **Figure 3.13**.

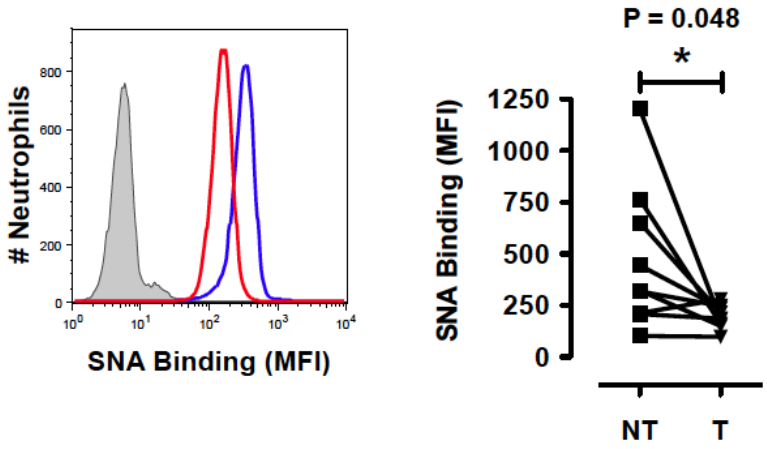


Figure 3.13: Binding of an α2,6-linked sialic acid-specific lectin to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated (NT) and transmigrated (T) PMN were collected and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. Figure shows representative histogram and graph of SNA binding. Grey = secondary antibody binding, blue = non-transmigrated PMN and red = transmigrated PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 9 per group. * P < 0.05 as analysed by two-tailed paired T test.

The expression of fucose was significantly reduced in PMN that have transmigrated through an endothelial monolayer compared to those that didn't as indicated by a 71% decrease in UEA I binding (21.99 ± 14.08 vs 6.27 ± 4.26, P = 0.006) **Figure 3.14**.

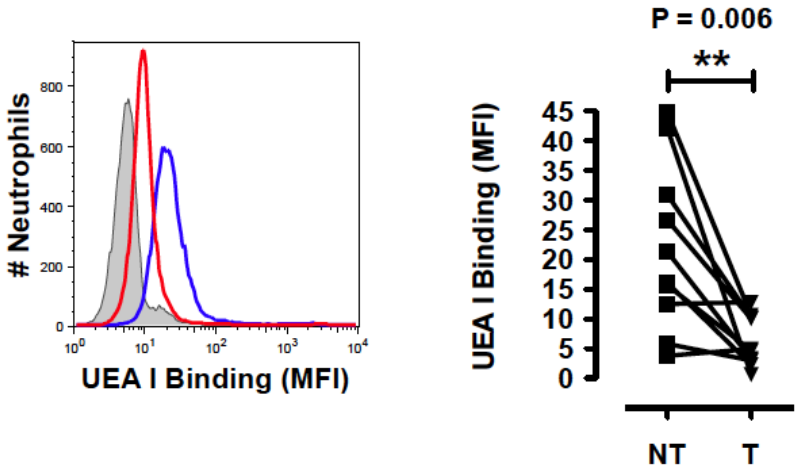


Figure 3.14: Binding of an α-linked fucose-specific lectin to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated (NT) and transmigrated (T) PMN were collected and fixed overnight in 1% PFA before lectin binding took place. Flow Cytometry was used to assess the expression of bound lectin. Figure shows representative histogram and graph of UEA I binding. Grey = secondary antibody binding, blue = non-transmigrated PMN and red = transmigrated PMN. Data are expressed as median fluorescence intensity per donor for paired samples, n = 10 per group. ** P < 0.01 as analysed by two-tailed paired T test.

3.1.5 Galectin Binding to Isolated Neutrophils Pre- and Post-Transendothelial Migration

To assess the binding of galectins-1, -3 and -9 to human neutrophils following transendothelial migration the model of transwell transmigration was utilised once more as described above (**section 2.2.5**).

Galectin-1 binding was low in freshly isolated PMN (5.658 ± 2.399); this increased by approximately 4-fold in non-transmigrated PMN and this increased again by 1.5-fold as PMN transmigrated (21.52 ± 6.70 and 32.30 ± 8.17 respectively) suggesting an increase in binding sites for galectin-1 becoming available as PMN transmigrate though an endothelial monolayer as shown in **Figure 3.15**.

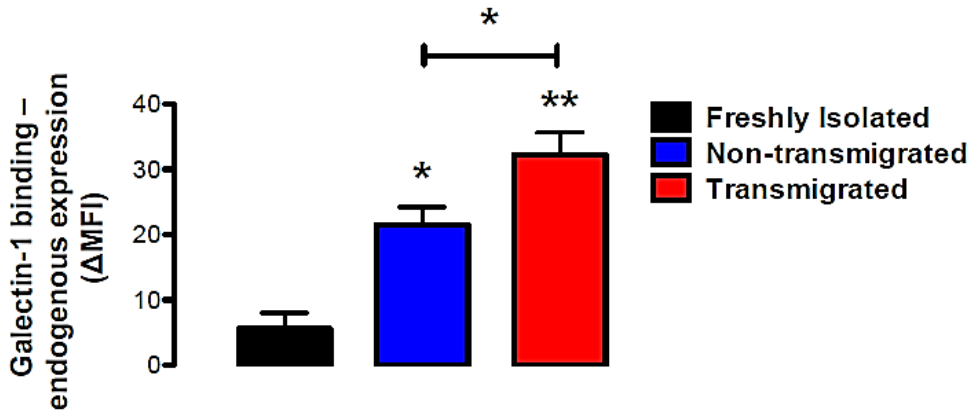


Figure 3.15: Binding of recombinant galectin-1 to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated and transmigrated PMN were treated with recombinant galectin-1 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-1 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-1 was subtracted from the total level to obtain the total bound galectin-1 per group. Figure shows graph of recombinant galectin-1 binding. Data are expressed as mean ± SEM, n = 6 per group, * $P < 0.05$ and ** $P < 0.01$ vs freshly isolated as analysed by one-way ANOVA with Bonferroni post hoc test.

Galectin-3 bound to freshly isolated PMN at high levels compared to galectin-1 (108.7 ± 29.8) and this was not modulated significantly by contact with, or transmigration through endothelial cells (143.02 ± 105.94 and 112.05 ± 42.99 respectively) suggesting the number of counter-receptors for galectin-3 were unchanged upon PMN transendothelial migration as shown in **Figure 3.16**.

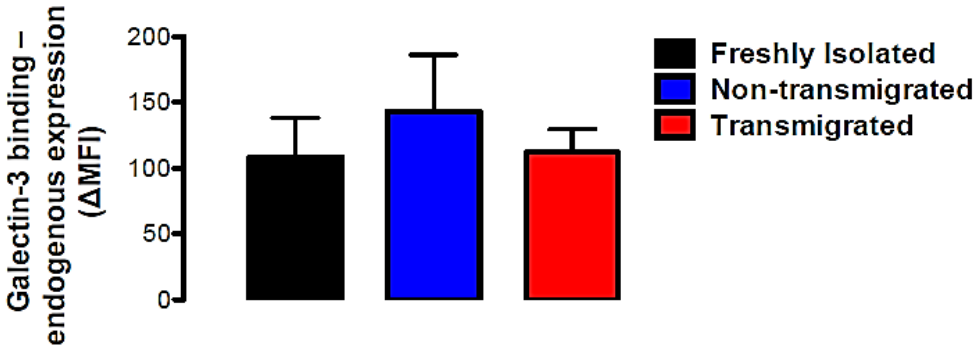


Figure 3.16: Binding of recombinant galectin-3 to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated and transmigrated PMN were collected, treated with recombinant galectin-3 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-3 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-3 was subtracted from the total level to obtain the total bound galectin-3 per group. Figure shows graph of recombinant galectin-3 binding. Data are expressed as mean ± SEM, n = 6 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

Very high galectin-9 binding could be seen in freshly isolated PMN (851.4 ± 342.9) however this was not modulated as PMN come into contact with, and traffic through an endothelial monolayer (816.58 ± 438.01 and 521.8 ± 151.09, P = 0.075) suggesting that, similar to galectin-3, the number of counter-receptors for galectin-9 were unchanged as shown in **Figure 3.17**.

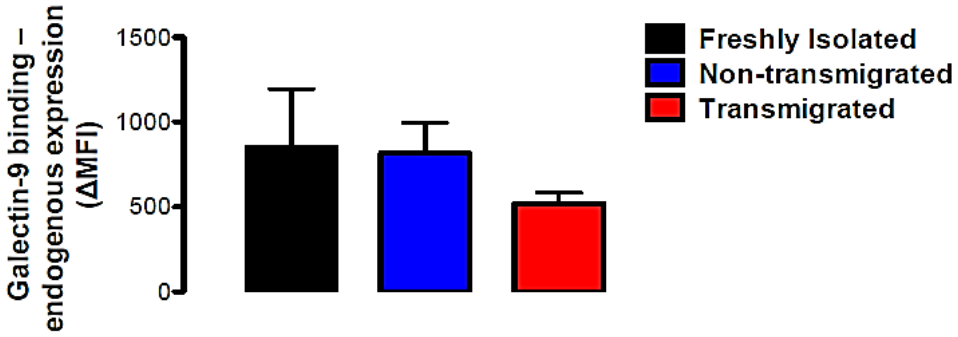


Figure 3.17: Binding of stable, recombinant galectin-9 to non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated and transmigrated PMN were collected, treated with stable, recombinant galectin-9 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-9 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-9 was subtracted from the total level to obtain the total bound galectin-9 per group. Figure shows graph for stable, recombinant galectin-9 binding. Data are expressed as mean ± SEM, n = 6 per group and analysed by one-way ANOVA with Bonferroni post hoc test.

3.1.6 Identification of Granule Release during Neutrophil Transendothelial Migration

As the glycophenotype was modulated by transendothelial migration (**section 3.1.4**) it was important to ascertain whether the changes were arising via PMN granule release or via interaction with the endothelial cells. Thus PMN were treated with stimulants that induce granule release similar to that seen in the transwell transmigration assay and the glycophenotype compared.

Non-transmigrated and transmigrated PMN were collected following the transwell transmigration assay and without fixation were analysed for their expression of granule release markers, CD35 (a marker for secretory vesicles) and CD66b (a marker for secondary granules). hrTNF-α (10ng/mL) and fMLP (1μM) were used as positive controls to stimulate freshly isolated PMN as these induce the release of secretory vesicles and secondary granules respectively (Norling et al., 2012).

Freshly isolated PMN express low levels of CD35 (68.93 ± 12.86) and there was a significant increase in expression in non-transmigrated (198.7 ± 35.06, P = 0.0156) and transmigrated PMN (164.0 ± 24.89, P = 0.0189) as shown in **Figure 3.18**.

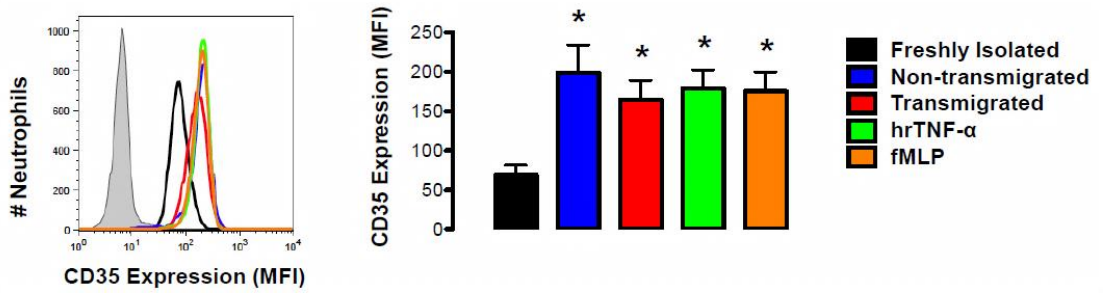


Figure 3.18: Secretory vesicles are released following activation of human PMN. PMN were isolated from healthy volunteers and either allowed to transmigrate for 90 minutes through a confluent monolayer of HUVEC cells that had been pre-treated with hrTNF-α (10ng/mL) for 4 hours or directly stimulated with 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C. PMN were collected from each treatment and assessed for their expression of CD35 using Flow Cytometry compared to freshly isolated PMN. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 3-5 per group. * P < 0.05 vs control as analysed using One-Way ANOVA with Dunnetts post-hoc test.

CD66b was expressed by freshly isolated PMN at low levels (32.09 ± 4.599), this was not significantly modulated in PMN that have not transmigrated through the monolayer (56.57 ± 12.55), however a significant increase in the expression of CD66b was seen in PMN that had transmigrated through the endothelial monolayer by approximately 3 fold (92.77 ± 9.742, P = 0.026). The positive control fMLP also induced CD66b up-regulation as expected (**Figure 3.19**). These results indicate that transendothelial migration promotes the release of secondary granules from PMN.

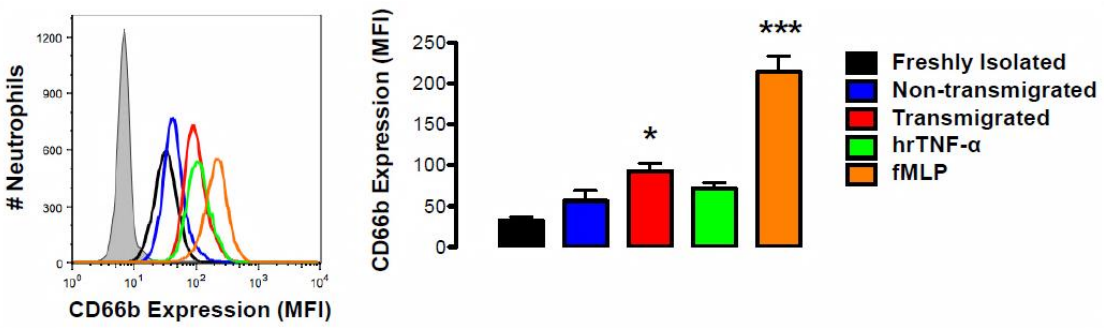


Figure 3.19: Neutrophil secondary granules are released following transmigration. PMN were isolated from healthy volunteers and either allowed to transmigrate for 90 minutes through a confluent monolayer of HUVEC cells that had been pre-treated with hrTNF-α (10ng/mL) for 4 hours or directly stimulated with 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C. PMN were collected from each treatment and assessed for their expression of CD66b using Flow Cytometry compared to freshly isolated PMN. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 3-5 per group. * $P < 0.05$ and *** $P < 0.001$ vs control as analysed by One-Way ANOVA with Dunnetts post-hoc test.

Non-transmigrated PMN release their secretory vesicles while those that have transmigrated through begin to release their secondary granules. Thus freshly isolated PMN were treated with 10ng/mL hrTNF-α (which induces the release of secretory vesicles only) or 1μM fMLP (which induces the release of secretory vesicles, tertiary granules and secondary granules) for 90 minutes at 37°C. The PMN were fixed overnight in 1% PFA and then incubated with the lectins shown in **Table 2.3** to assess whether granule release induced the changes seen in the PMN glycophenotype upon transendothelial migration.

RCA_{120} binding was high in freshly isolated PMN (711.4 ± 145.4) and this did not change in control PMN, which were incubated at 37°C for 90 minutes (695.8 ± 140.6). Mobilisation of PMN granules using 10ng/mL hrTNF-α and 1μM fMLP was unable to induce changes in the expression of β-linked galactose residues on the PMN following 90-minute incubation as demonstrated by no modulation of binding of RCA_{120} (731.7 ± 165.4 and 713.5 ± 139.8 respectively) (**Figure 3.20**).

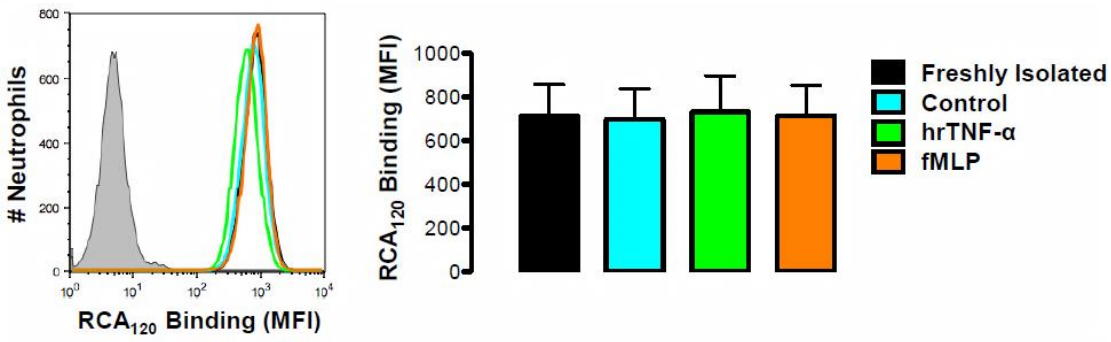


Figure 3.20: Binding of RCA_{120} to human PMN following 90-minute stimulation with hrTNF-α or fMLP. PMN were isolated from healthy volunteers and either fixed immediately (freshly isolated) or incubated with vehicle (PBS), 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C. Following this the cells were washed twice in PBS and fixed overnight in 1% PFA. The fixed PMN were incubated with lectins and the binding assessed using Flow Cytometry. Figure shows representative histogram and graph for RCA_{120} binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, N = 7 per group and are analysed by repeated measures one-way ANOVA with Bonferroni post hoc test.

PNA binding was low on freshly isolated PMN (55.87 ± 14.53) and this was unaltered in control, hrTNF-α or fMLP treated cells (52.72 ± 17.49, 70.64 ± 20.44 and 61.94 ± 18.92 respectively). LEL binding was high on freshly isolated PMN (726.5 ± 216.2) and, similar to PNA was not modulated in control, hrTNF-α or fMLP treated cells (686.3 ± 205.4, 609.1 ± 169 and 594.1 ± 172.2 respectively). PHA-L binding was also high on freshly isolated PMN (930.4 ± 150.3) and this was also unaltered in control, hrTNF-α or fMLP treatment (868.2 ± 152.5, 1100 ± 195 and 1030 ± 173.6 respectively). Taken together this suggests granule release does not cause the changes in mono- or poly-LacNAc residues or complex tri- and tetra-antennary N-glycans seen during transmigration (**Figure 3.21**).

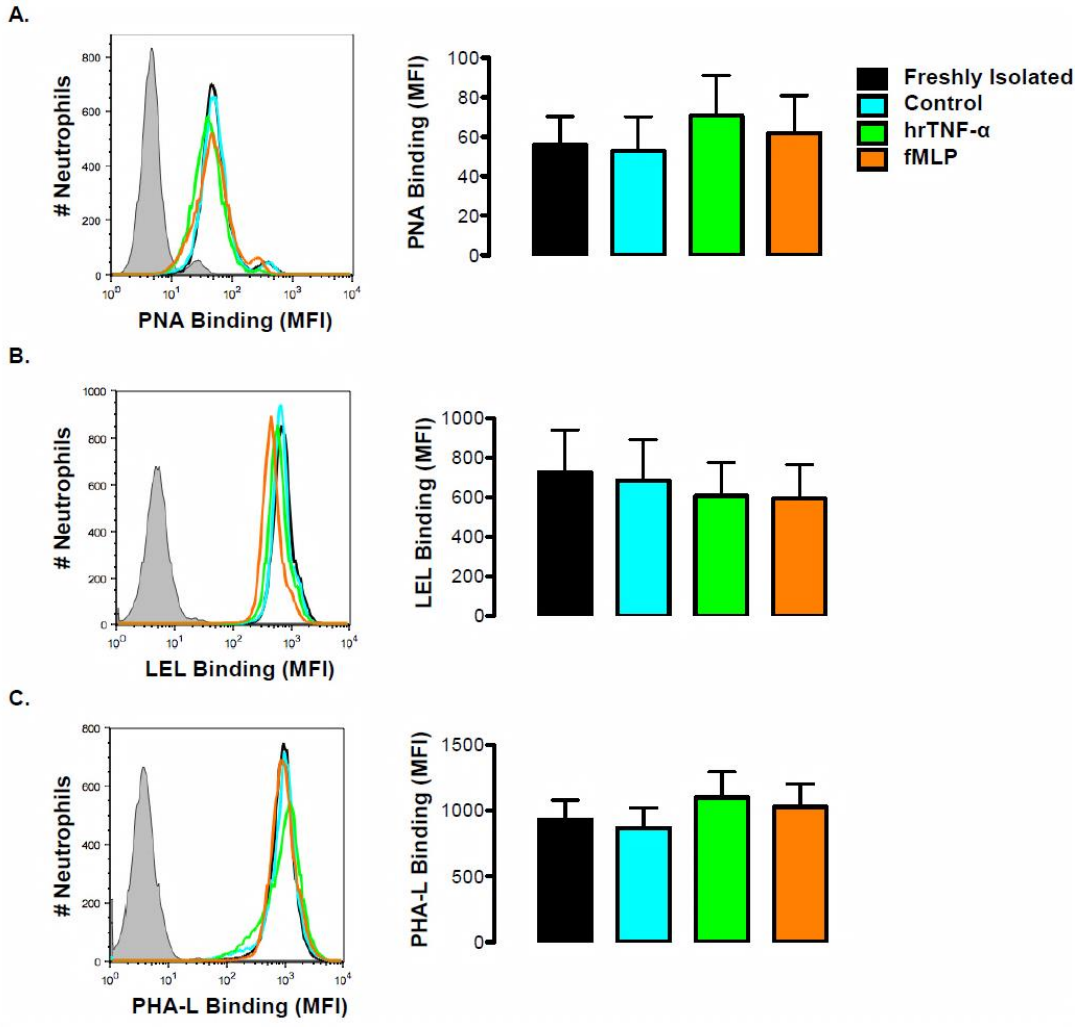


Figure 3.21: Binding of lectins indicative of galectin binding permissibility to human PMN following 90-minute stimulation with hrTNF-α or fMLP. PMN were isolated from healthy volunteers and either fixed immediately (freshly isolated) or incubated with vehicle (PBS), 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C. Following this the cells were washed twice in PBS and fixed overnight in 1% PFA. The fixed PMN were incubated with lectins and the binding assessed using Flow Cytometry. A. Representative histogram and graph for PNA binding, B. Representative histogram and graph for LEL binding and C. Representative histogram and graph for PHA-L binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 7 per group and are analysed by repeated measures one-way ANOVA with Bonferroni post hoc test.

Sialic acid was expressed at high levels by freshly isolated PMN (310.6 ± 176.6) and although large deviations were detected within data sets it appears that the expression of α2,6-linked sialic acid was not modulated by incubation at 37˚C or stimulation of human PMN with 10ng/mL hrTNF-α or 1μM fMLP as shown by no change in the binding of SNA (511.3 ± 325.6, 605.6 ± 393.2 and 497 ± 288.2 respectively) (**Figure 3.22**).

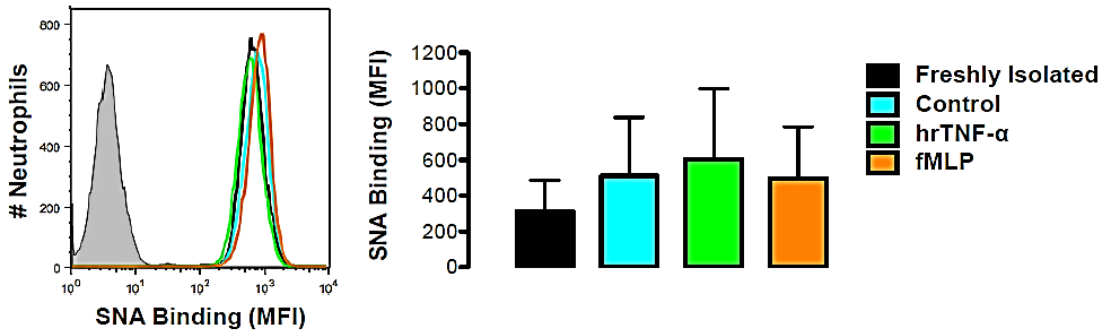


Figure 3.22: Binding of SNA to human PMN following 90-minute stimulation with hrTNF-α or fMLP. PMN were isolated from healthy volunteers and either fixed immediately (freshly isolated) or incubated with vehicle (PBS), 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C. Following this the cells were washed twice in PBS and fixed overnight in 1% PFA. The fixed PMN were incubated with lectins and the binding assessed using Flow Cytometry. Figure shows representative histogram and graph for SNA binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 7 per group and are analysed by repeated measures one-way ANOVA with Bonferroni post hoc test.

UEA I binding was low on freshly isolated PMN (13.14 ± 3.123) and there was no modulation of the binding of UEA I and thus expression of fucose residues on human PMN following stimulation with vehicle, 10ng/mL hrTNF-α or 1μM fMLP (11.08 ± 2.701, 15.82 ± 3.365 and 13.1 ± 3 respectively) (**Figure 3.23**).

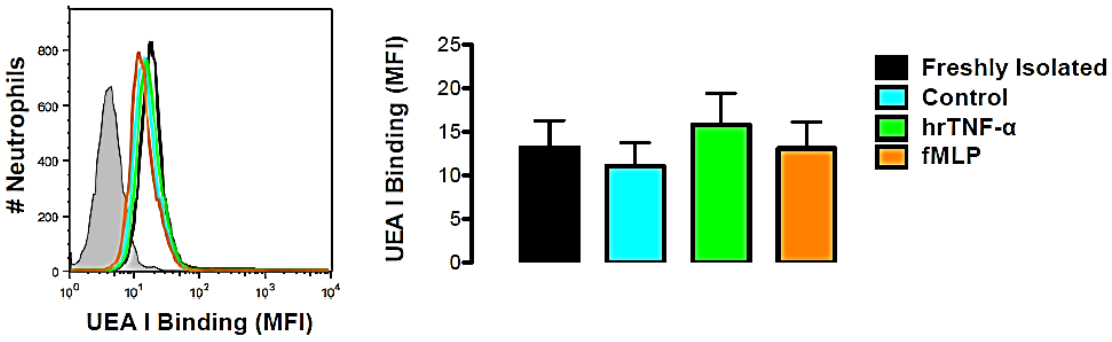


Figure 3.23: Binding of UEA I to human PMN following 90-minute stimulation with hrTNF-α or fMLP. PMN were isolated from healthy volunteers and either fixed immediately (freshly isolated) or incubated with vehicle (PBS), 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C. Following this the cells were washed twice in PBS and fixed overnight in 1% PFA. The fixed PMN were incubated with UEA I and the binding assessed using Flow Cytometry. Figure shows representative histogram and graph for UEA I binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 7 per group and are analysed by repeated measures one-way ANOVA with Bonferroni post hoc test.

3.1.7 Galectin Binding to Isolated Neutrophils following Granule Release

The binding of recombinant galectins-1, -3 and -9 was then assessed following 90-minute stimulation with 10ng/mL hrTNF-α or 1μM fMLP to determine the effect of secretory vesicle and secondary granule release respectively on the binding of the galectins and compared to freshly isolated PMN.

The binding of galectin-1 to freshly isolated PMN was low (1.828 ± 0.705) and this was unchanged in PMN that were treated with 10ng/mL hrTNF-α (2.738 ± 1.085) or 1μM fMLP (1.2 ± 0.7517, P = 0.031) for 90 minutes suggesting no change in galectin-1 counter-receptors occurred following release of secretory vesicles or secondary granules (**Figure 3.24**).

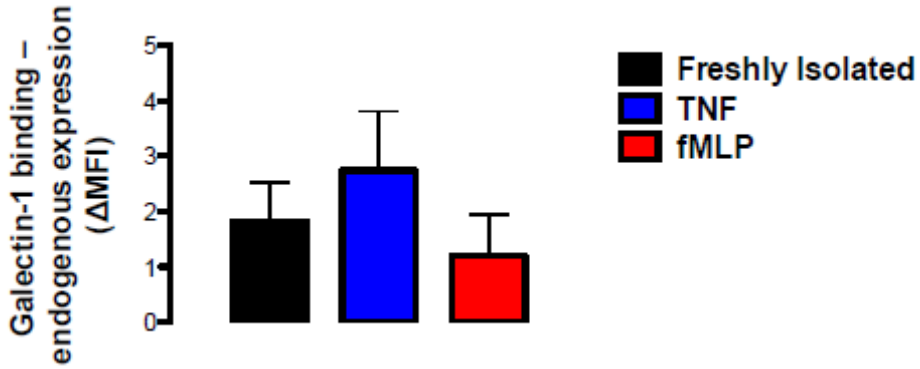


Figure 3.24: Binding of recombinant galectin-1 to isolated PMN following 90-minute stimulation with hrTNF-α or fMLP. Freshly isolated PMN were incubated with 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C; PMN were collected, treated with recombinant galectin-1 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-1 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-1 was subtracted from the total level to obtain the total bound galectin-1. Figure shows graph of recombinant galectin-1 binding. Data are expressed as mean ± SEM, n = 4 per group, * P < 0.05 as analysed by one-way ANOVA with Bonferroni post hoc test.

Binding of galectin-3 to freshly isolated PMN was higher than that seen for galectin-1 (47.38 ± 10.2); this was not modulated following 90-minute treatment with either 10ng/mL hrTNF-α or 1μM fMLP (37.06 ± 21.43 and 53.16 ± 21.49 respectively) suggesting no change in the binding sites for galectin-3 following granule release (**Figure 3.25**).

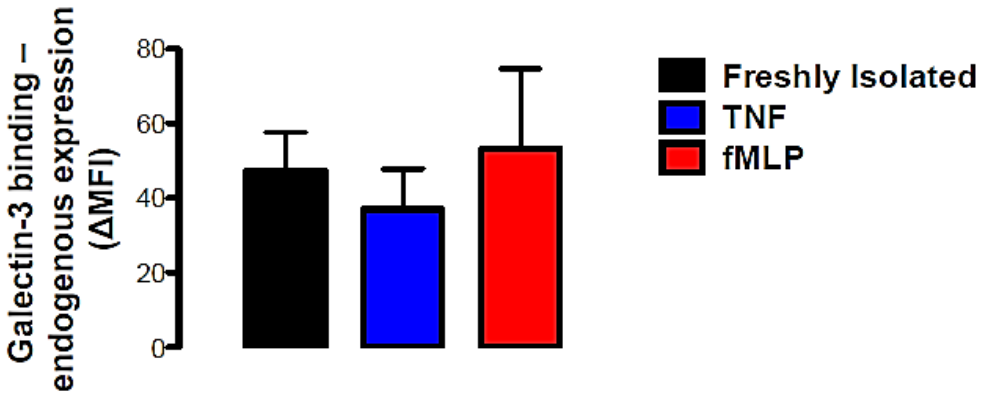


Figure 3.25: Binding of recombinant galectin-3 to isolated PMN following 90-minute stimulation with hrTNF-α or fMLP. Freshly isolated PMN were incubated with 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C; PMN were collected, treated with recombinant galectin-3 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-3 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-3 was subtracted from the total level to obtain the total bound galectin-3. Figure shows graph of recombinant galectin-3 binding. Data are expressed as mean ± SEM, n = 4 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

Galectin-9 binds highly to freshly isolated PMN (388.1 ± 62.67) and this level does not change following 90-minute stimulation with 10ng/mL hrTNF-α (397.7 ± 12.22) however the binding significantly increases upon treatment with 1μM fMLP (576.4 ± 112.1, P = 0.015). This suggests under basal conditions receptor(s) for galectin-9 are present on the PMN and these either increase or additional receptors are translocated to the cell surface upon release of secondary granules (**Figure 3.26**).

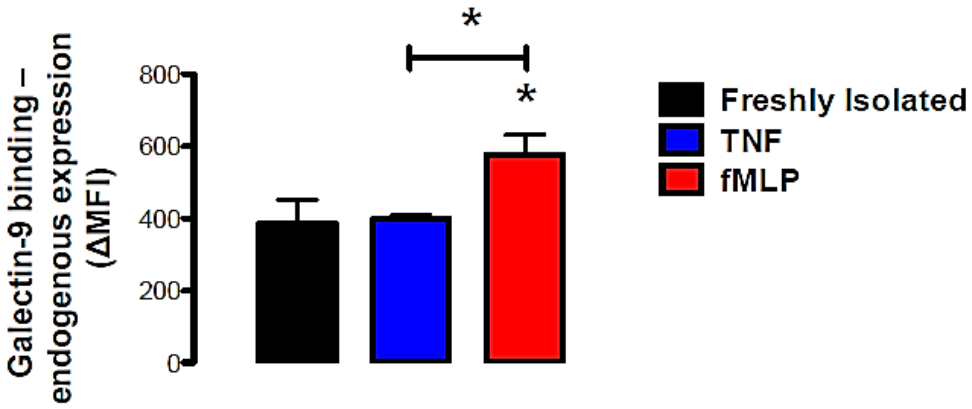


Figure 3.26: Binding of stable, recombinant galectin-9 to isolated PMN following 90-minute stimulation with hrTNF-α or fMLP. Freshly isolated PMN were incubated with 10ng/mL hrTNF-α or 1μM fMLP for 90 minutes at 37°C; PMN were collected, treated with stable, recombinant galectin-9 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-9 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-9 was subtracted from the total level to obtain the total bound galectin-9. Figure shows stable, recombinant galectin-9 binding. Data are expressed as mean ± SEM, n = 4 per group, * P < 0.05 vs freshly isolated unless otherwise indicated as analysed by one-way ANOVA with Bonferroni post hoc test.

3.1.8 Effect of Endothelial Cell-Derived Factors on the Glycophenotype of Neutrophils

As the changes in human PMN glycophenotype was not a consequence of granule release (**section 3.1.6**) the role of the endothelium and endothelial-derived factors were therefore assessed.

Freshly isolated PMN were placed in the bottom well of 6-well plate and a confluent monolayer of HUVECs on a transwell insert that had been pre-treated with 10ng/mL hrTNF-α for 4 hours was added above the PMN. The cells were incubated together at 37°C for 90 minutes to mimic the conditions seen in the transwell transmigration assay, following this the PMN were collected, fixed overnight and assessed for their glycophenotype as previously performed.

As described before, the binding of RCA_{120} was high in freshly isolated PMN and this did not change following 90 minute incubation at 37°C. However, a significant increase occurred as a consequence of endothelial-derived factors (922.1 ± 178.1 vs 2068 ± 511.5, P = 0.019), which matches the increase seen in cells that come into contact with the HUVEC but neither adhere nor transmigrate confirming that these changes were due to a secreted factor from the activated endothelium (**Figure 3.27**).

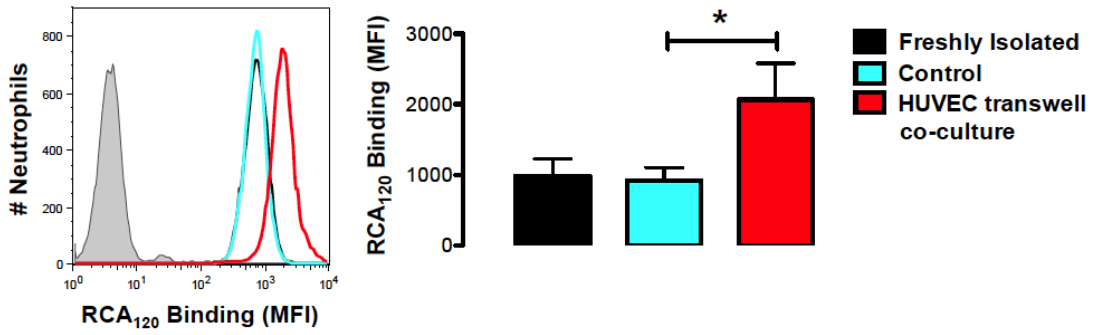


Figure 3.27: RCA_{120} binding to PMN that were separated from direct cell-cell contract with HUVECs by a transwell. PMN were isolated from healthy volunteers and placed in the lower well of a 6-well plate with a pre-activated confluent monolayer of HUVEC cells on a transwell on top. This was incubated at 37°C for 90 minutes and the binding of RCA_{120} was assessed in comparison to freshly isolated PMN and those that had been incubated in the absence of HUVECs. Figure shows representative histogram and graph of RCA_{120} binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 5 per group, * P < 0.05 as analysed by one-way ANOVA with Bonferroni post hoc test.

The expression of mono-LacNAc residues was low in freshly isolated cells compared to the expression of poly-LacNAc residues and complex tri- and tetra-antennary N-glycans as determined by binding of PNA, LEL and PHA-L respectively (52.22 ± 9.871, 874.4 ± 197.9 and 958.4 ± 12.7). No significant modulation of expression of these residues was observed following stimulation with endothelial cell-derived secreted factors; this was particularly evident for PNA binding (54.28 ± 10.43). However, a trend towards increased expression was seen with LEL (1288 ± 263.5) and PHA-L (1388 ± 21.2) but this did not reach statistical significance as shown in **Figure 3.28**. This opposes the findings from non-adherent and non-transmigrated PMN as these demonstrate decreased binding of PNA, LEL and PHA-L compared to freshly isolated cells and thus this cannot be attributed to a secreted endothelial factor.

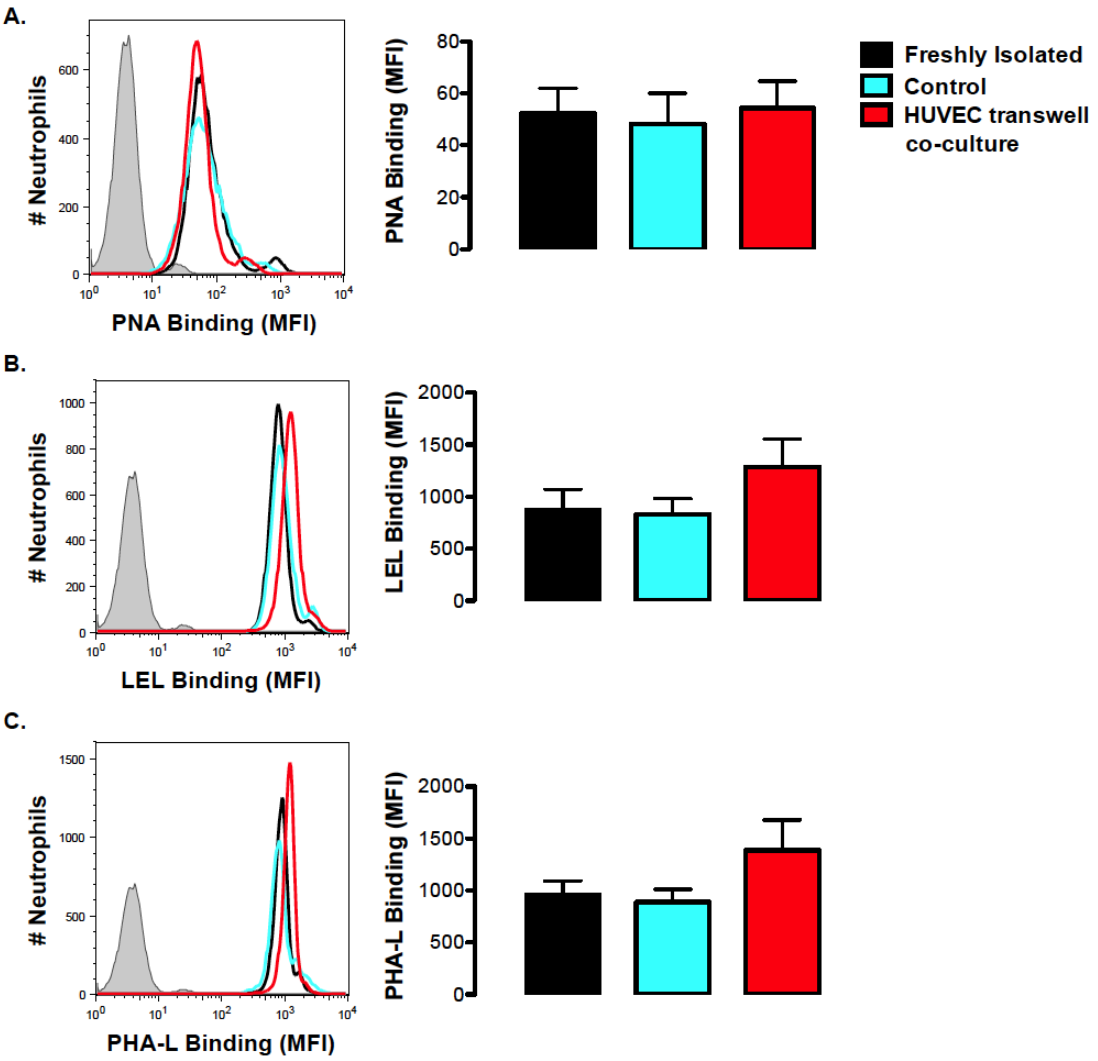


Figure 3.28: Galectin-permissive lectin binding to PMN that were separated from direct cell-cell contact with HUVECs by a transwell. PMN were isolated from healthy volunteers and placed in the lower well of a 6-well plate with a pre-activated confluent monolayer of HUVEC cells on a transwell on top. This was incubated at 37°C for 90 minutes and the binding of lectins was assessed in comparison to freshly isolated PMN and those that had been incubated in the absence of HUVECs. A. Representative histogram and graph showing binding of PNA, B. Representative histogram and graph showing binding of LEL and C. Representative histogram and graph showing binding of PHA-L. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 5 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

The expression of α2,6-linked sialic acid was high on freshly isolated PMN as demonstrated by SNA binding (393.2 ± 136.1) and, similar to that seen with LEL and PHA-L, the binding of SNA appeared to follow a trend towards an increase upon incubation with factors secreted from activated HUVECs (859.7 ± 181) as shown in **Figure 3.29**. This correlated with the finding from PMN in the transwell assay that have not transmigrated as these also demonstrated an increase in the expression of α2,6-linked sialic acid; PMN that were non-adherent to the endothelium exhibit decreased SNA binding however these were in contact with the HUVECs for a shorter period of time.

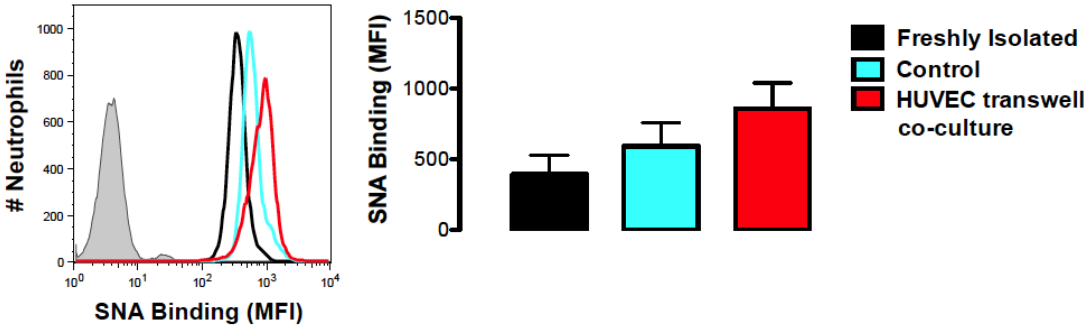


Figure 3.29: SNA binding to PMN that were separated from direct cell-cell contact with HUVECs by a transwell. PMN were isolated from healthy volunteers and placed in the lower well of a 6-well plate with a pre-activated confluent monolayer of HUVEC cells on a transwell on top. This was incubated at 37°C for 90 minutes and the binding of SNA was assessed in comparison to freshly isolated PMN and those that had been incubated in the absence of HUVECs. Figure shows representative histogram and graph of SNA binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 5 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

The binding of UEA I was low in freshly isolated PMN suggesting low expression of α-linked fucose residues however upon contact with mediators that were released from activated HUVECs this increased approximately 3-fold (27.61 ± 9.02 vs 101.6 ± 19.97, P = 0.017) as seen in **Figure 3.30**. As with SNA binding this closely mimicked the response seen in PMN that did not transmigrate through the HUVECs in the transwell assay but not those that were non-adherent in the 6-well assay suggesting endothelial cell-derived factors were causing these effects but may take a longer time frame than 30 minutes to do so.

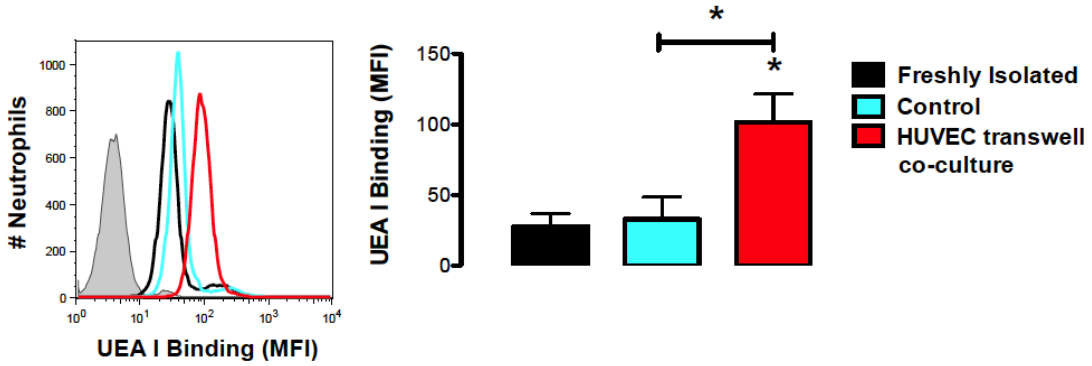


Figure 3.30: UEA I binding to PMN that were separated from direct cell-cell contact with HUVECs by a transwell. PMN were isolated from healthy volunteers and placed in the lower well of a 6-well plate with a pre-activated confluent monolayer of HUVEC cells on a transwell on top. This was incubated at 37°C for 90 minutes and the binding of UEA I was assessed in comparison to freshly isolated PMN and those that had been incubated in the absence of HUVECs. Figure shows representative histogram and graph of UEA I binding. Shaded peak = isotype control. Data are expressed as mean ± SEM, n = 5 per group, * P < 0.05 vs freshly isolated PMN as analysed by one-way ANOVA with Bonferroni post hoc test.

3.1.9 Modulation of the Glycophenotype of Neutrophils during Exudation to the Buccal Cavity

Although the transwell transmigration assay is able to provide information on the effect of PMN transendothelial migration it is unable to accurately portray the recruitment of PMN that occurs pathologically. During inflammation PMN respond to chemokines and cytokines by transmigrating through an endothelial monolayer to leave the circulation and then traffic to an inflammatory site through the extracellular matrix coming into contact with many other cell types; once at the site of inflammation the primary granules are released and the PMN are phenotypically very different to those in the circulation (Chilvers et al., 2000). In order to provide a more pathophysiological model of human PMN recruitment healthy volunteers were utilised to collect PMN that had recruited to the Buccal cavity in response to mild inflammation.

Healthy volunteers performed the Tobasco mouth rinse as described in **section 2.2.3** and the exudated PMN were collected (approximately 5-10 x 10^6 per donor), fixed overnight in 1% PFA and assessed for their lectin binding compared to paired peripheral blood samples.

No difference was seen in the binding of RCA_{120} when comparing peripheral blood neutrophils to those recruited in response to neurogenic inflammation suggesting no modulation of the expression of β-linked galactose residues (760.2 ± 138.7 vs 782.8 ± 145.1) **Figure 3.31**.

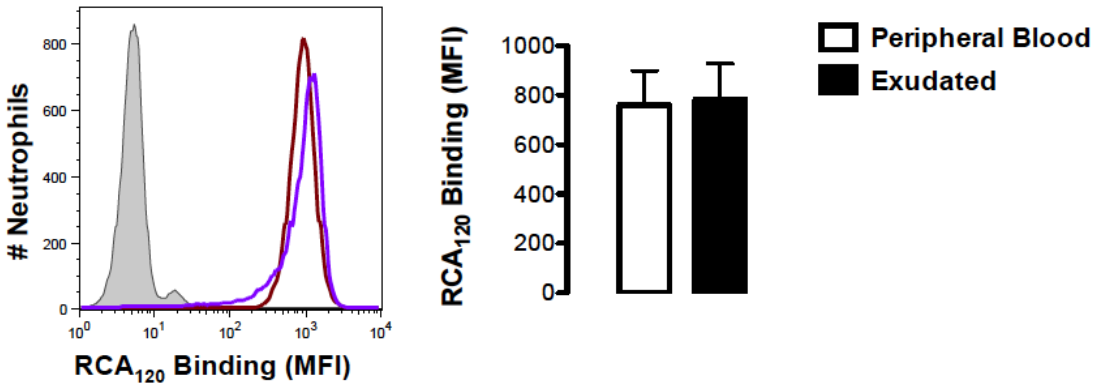


Figure 3.31: Lectin binding profile for β-linked galactose residues on human peripheral blood and exudated PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour exudated PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were washed twice and fixed overnight in 1% PFA. The fixed PMN were incubated with lectins and the binding was assessed using Flow Cytometry. Figure shows representative histogram and graph for RCA_{120} binding. Grey = secondary antibody only, burgundy = peripheral blood PMN and purple = exudated PMN. Data are expressed as mean ± SEM, n = 7 per group, and are analysed by two-tailed T test.

The expression of mono-LacNAc residues on recruited PMN was significantly higher than peripheral blood PMN as shown by a 9-fold increase in binding of PNA (27.83 ± 3.498 vs 264.3 ± 89.78, P = 0.0411) **Figure 3.32a**. The binding of LEL and PHA-L was not significantly modulated between the two groups suggesting no significant differences could be seen in the expression of poly-LacNAc residues (1095 ± 199.1 vs 858.2 ± 239.0) or of complex tri- and tetra-antennary N-glycans (850 ± 175.9 vs 936.4 ± 82.02) **Figure 3.32**.

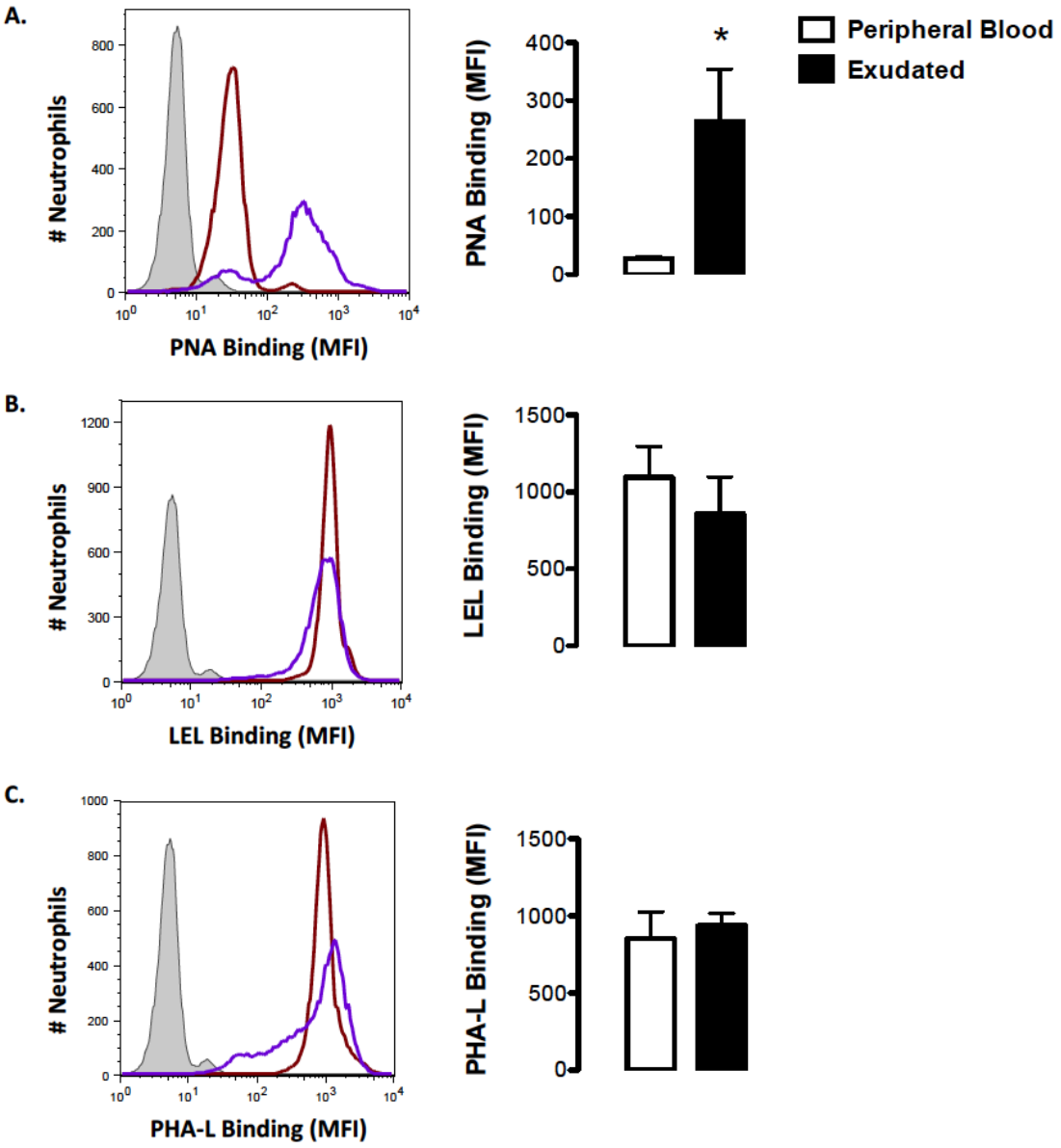


Figure 3.32: Lectin binding profile for galectin binding-permissive residues on human peripheral blood and exudated PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour exudated PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were washed twice and fixed overnight in 1% PFA. The fixed PMN were incubated with lectins and the binding was assessed using Flow Cytometry. A. Representative histogram and graph for PNA binding, B. Representative histogram and graph for LEL binding and C. Representative histogram and graph for PHA-L binding. Grey = secondary antibody only, burgundy = peripheral blood PMN and purple = exudated PMN. Data are expressed as mean ± SEM, n = 7 per group, * $P < 0.05$ vs peripheral blood as analysed by two-tailed T test.

The expression of α2,6-linked sialic acid was significantly increased in PMN that have trafficked to the buccal cavity compared to those in the peripheral blood with a 2 fold increase in SNA binding (333.2 ± 16.76 vs 747.1 ± 56.6, P = 0.0146) **Figure 3.33**.

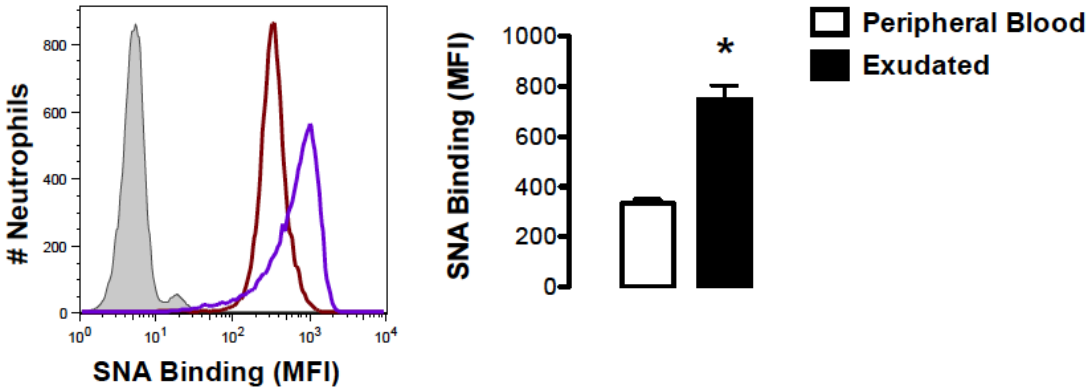


Figure 3.33: Lectin binding profile for α2,6-linked sialic acid-specific residues on human peripheral blood and exudated PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour exudated PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were washed twice and fixed overnight in 1% PFA. The fixed PMN were incubated with lectins and the binding was assessed using Flow Cytometry. Figure shows representative histogram and graph for SNA binding. Grey = secondary antibody only, burgundy = peripheral blood PMN and purple = exudated PMN. Data are expressed as mean ± SEM, n = 7 per group, * P < 0.05 vs peripheral blood as analysed by two-tailed T test.

Fucose residues were not significantly modulated when comparing recruited PMN to those in the peripheral circulation as demonstrated by the lack of difference in UEA I binding (26.62 ± 6.606 vs 16.24 ± 5.860) **Figure 3.34**.

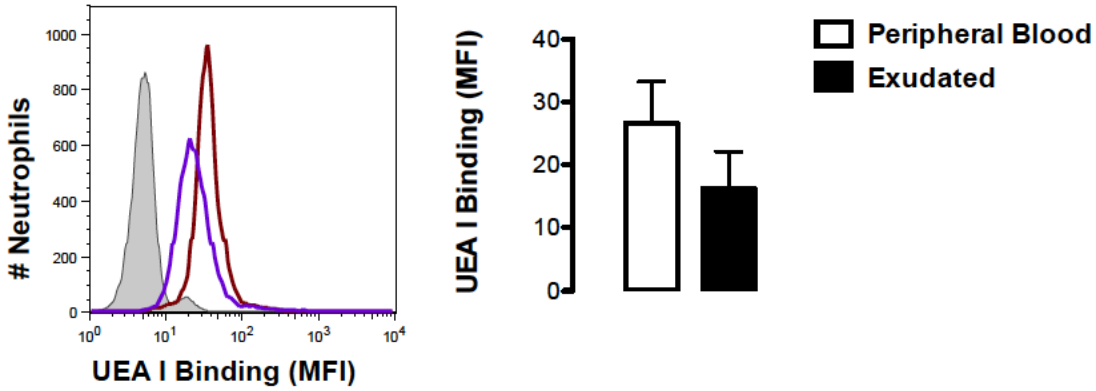


Figure 3.34: Lectin binding profile for α-linked fucose residues on human peripheral blood and recruited PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour exudated PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were washed twice and fixed overnight in 1% PFA. The fixed PMN were incubated with UEA I and the binding was assessed using Flow Cytometry. Figure shows representative histogram and graph for UEA I binding. Grey = secondary antibody only, burgundy = peripheral blood PMN and purple = exudated PMN. Data are expressed as mean ± SEM, n = 7 per group, Data are analysed by two-tailed T test.

3.1.10 Galectin Binding to Neutrophils during Exudation to the Buccal Cavity

Galectin binding was compared between PMN isolated from the peripheral circulation of healthy volunteers and those that had trafficked to the buccal cavity in response to pro-inflammatory stimulation (Tobasco mouth rinse). PMN were collected from paired donors and incubated with recombinant galectins-1, -3 or -9 for 15 minutes at 37˚C; following this the cells were washed and analysed for the level of bound galectin by subtracting the endogenous expression (PMN untreated with recombinant galectins) from the total expression.

The binding of galectin-1 to peripheral blood PMN was low as previously determined however PMN that extravasated to the buccal cavity had significantly diminished galectin-1 binding (3.860 ± 1.191 vs 0.1167 ± 0.04, P = 0.0348) as shown in **Figure 3.35**.

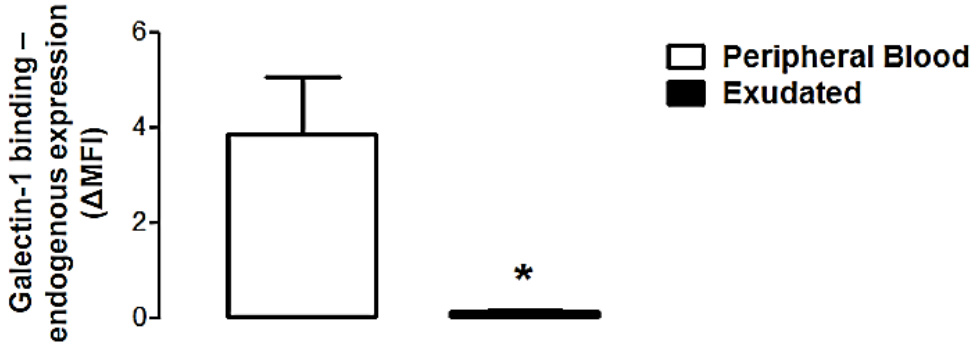


Figure 3.35: Binding of recombinant galectin-1 to peripheral blood and exudated human PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour recruited PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were treated with recombinant galectin-1 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-1 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-1 was subtracted from the total level to obtain the total bound galectin-1. Figure shows graph of recombinant galectin-1 binding. Data are expressed as mean ± SEM, n = 4 per group, * $P < 0.05$ as analysed by one-way ANOVA with two-tailed T-test.

Galectin-3 bound to peripheral blood PMN at a higher level than galectin-1 and this significantly increased by almost 65% upon exudation of the PMN to an inflammatory site (128.6 ± 5.953 vs 209.6 ± 25.4, P = 0.0361) as shown in **Figure 3.36**.

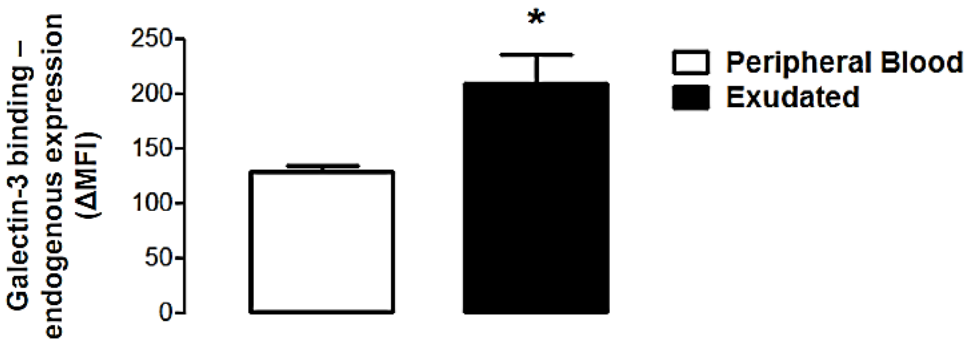


Figure 3.36: Binding of recombinant galectin-3 to peripheral blood and exudated human PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour recruited PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were treated with recombinant galectin-3 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-3 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-3 was subtracted from the total level to obtain the total bound galectin-1. Figure shows graph of recombinant galectin-3 binding. Data are expressed as mean ± SEM, n = 4 per group, * P < 0.05 as analysed by one-way ANOVA with two-tailed T-test.

Galectin-9 binding was high on peripheral blood PMN however, similar to galectin-3, this significantly increased by approximately 95% upon exudation to the buccal cavity in response to Tobasco stimulation (223.6 ± 19.46 vs 432.4 ± 39.47, P = 0.009) as shown in **Figure 3.37**.

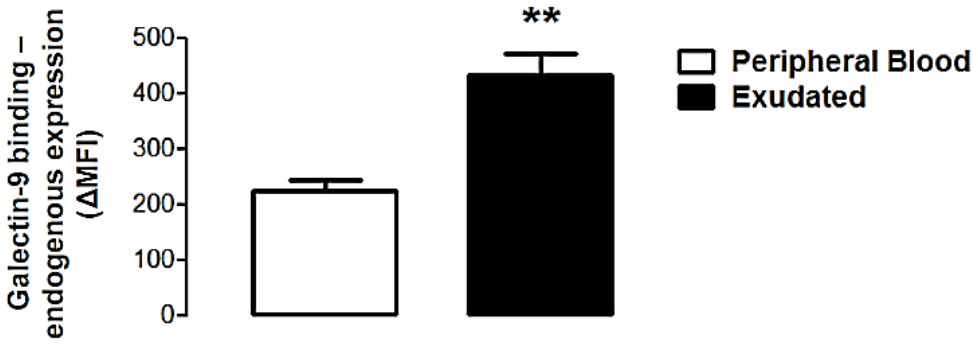


Figure 3.37: Binding of stable, recombinant galectin-9 to peripheral blood and exudated human PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour recruited PMN were collected by saline wash, peripheral blood PMN were also isolated by dextran sedimentation. Both groups of PMN were treated with stable, recombinant galectin-9 (10nM) or vehicle for 15 minutes at 37°C and assessed for their level of galectin-9 on the cell surface using Flow Cytometry compared to freshly isolated PMN. Endogenous expression of galectin-9 was subtracted from the total level to obtain the total bound galectin-9. Figure shows graph of stable, recombinant galectin-9 binding. Data are expressed as mean ± SEM, n = 4 per group, ** P < 0.01 as analysed by one-way ANOVA with two-tailed T-test.

In summary, galectin-1 binding was not modulated upon human PMN adhesion to an endothelial monolayer but increases following transendothelial migration; this contradicts the fMLP stimulation assay as those PMN had decreased galectin-1 binding, as do the cells that have trafficked to an inflammatory site.

Galectin-3 binding to human PMN was not significantly modulated by adhesion to or transmigration through an endothelial monolayer or by stimulation of the cells with hrTNF-α or fMLP; however the binding was significantly increased to PMN that were recruited to the buccal cavity in response to pro-inflammatory stimulation (Tobasco mouth wash).

The binding of stable, recombinant galectin-9 to human PMN was high on freshly isolated cells and this was not significantly modulated by adhesion or transmigration through a HUVEC monolayer. Upon activation of the PMN with fMLP or trafficking to an inflammatory site binding increases, potentially suggesting additional counter-receptor(s) for galectin-9 are released under these conditions.

3.1.11 Modulation of the Glycophenotype of Murine Neutrophils from the Peripheral Blood to the Peritoneal Cavity

A murine model of acute inflammation was utilised to study the differences in lectin binding on murine neutrophils; comparing peripheral blood neutrophils to recruited cells within the peritoneal cavity. Zymosan (1mg/mouse) was administered intraperitoneally into male 6-week old C57BL/6 mice and after 4 hours the mice were anaesthetised and cardiac punctures and peritoneal lavages were performed to obtain peripheral blood and exudated neutrophils respectively. Neutrophils were identified with a specific Ly6G antibody and a validated panel of lectins were used to assess the neutrophil glycophenotype.

RCA_{120} binding was low in murine peripheral blood neutrophils compared to human cells and binding decreased by approximately 60% when comparing those in the peripheral circulation to those that exudated suggesting a loss of β-linked galactose upon trafficking (20.84 ± 3.10 vs 8.18 ± 3.20, P = 0.009) **Figure 3.38**.

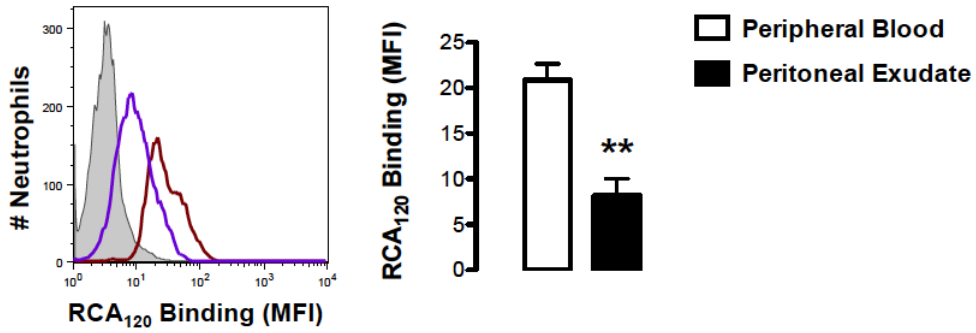


Figure 3.38: Lectin binding profile for β-linked galactose residues on murine peripheral blood and peritoneal exudate recruited neutrophils. Peritonitis was induced following administration of 1mg zymosan ip. and peripheral blood (burgundy) and peritoneal exudate (purple) was collected 4 hours later. Neutrophils were labelled with Ly6G and double stained for RCA_{120} and the binding of lectins to neutrophils was analysed by Flow Cytometry. Figure shows representative histogram and graph for RCA_{120} binding. Data are expressed as mean ± SEM, N = 3 per group, ** P < 0.01 vs Blood as analysed by two-tailed T test.

Human peripheral blood PMN bind low levels of PNA and high levels of LEL and PHA-L; this is altered in murine peripheral blood neutrophils that bind high levels of PNA and PHA-L and low levels of LEL. An overall trend towards decreased lectin binding was seen in exudated neutrophils compared to those in the peripheral blood with a 81% decrease in the binding of PNA (201.53 ± 4.73 vs 38.01 ± 18.51, P = 0.007), a 44% decrease in LEL binding (19.01 ± 2.07 vs 10.71 ± 1.89, P = 0.001) and an 86% decrease in PHA-L binding (3900.21 ± 193.94 vs 551.78 ± 125.01, P = 0.0001) **Figure 3.39**. This was indicative of a significant loss of

mono- and poly-LacNAc residues and complex tri- and tetra antennary N-glycans as neutrophils trafficked to an inflammatory site.

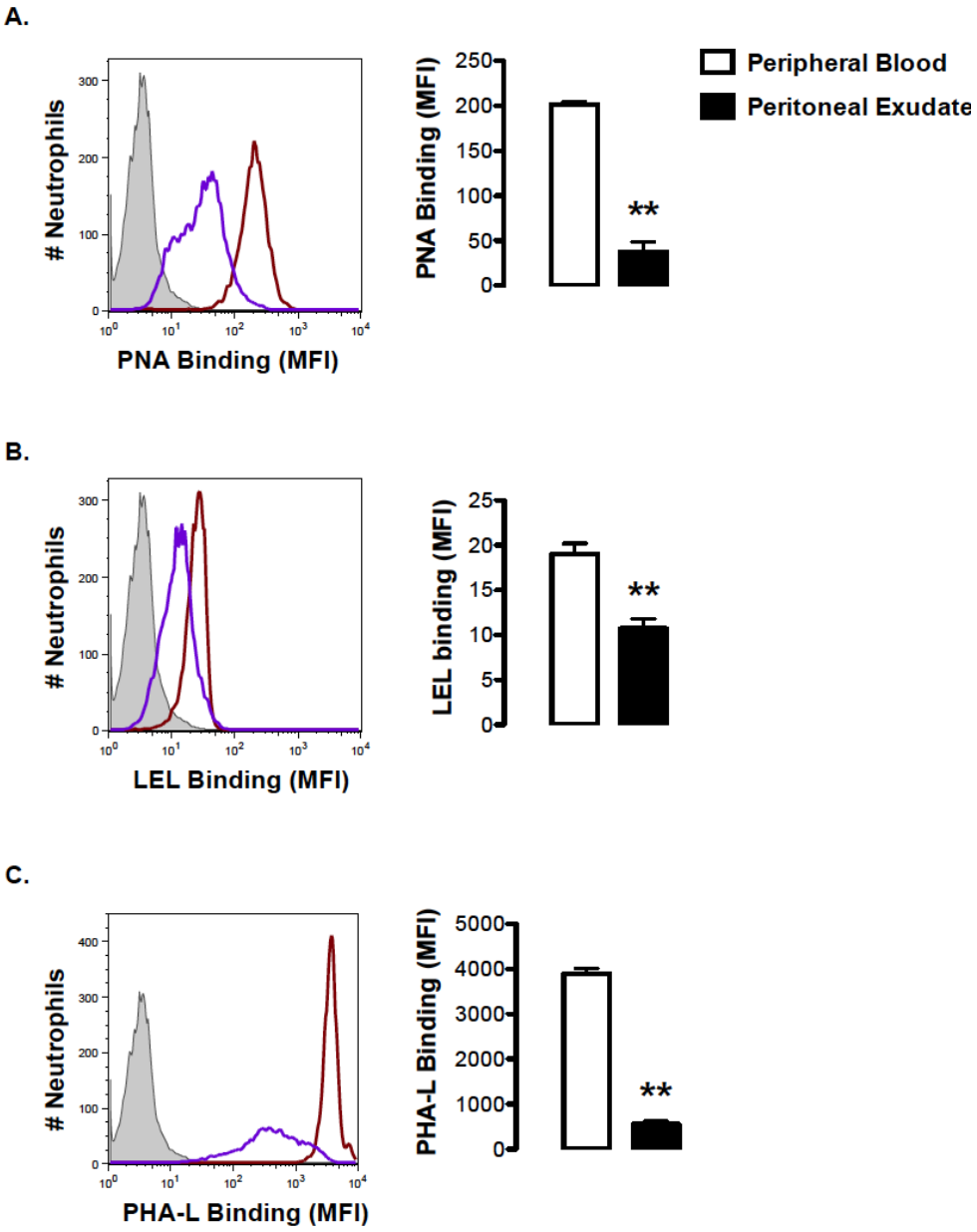


Figure 3.39: Lectin binding for residues permissive for galectin binding on murine peripheral blood and peritoneal exudate recruited neutrophils. Peritonitis was induced following administration of 1mg zymosan ip. and peripheral blood (burgundy) and peritoneal exudate (purple) was collected 4 hours later. Neutrophils were labelled with Ly6G and double stained for lectins and the binding of lectins to neutrophils was analysed by Flow Cytometry. A. Representative histogram and graph for PNA binding, B. Representative histogram and graph for LEL binding and C. Representative histogram and graph for PHA-L binding. Data are expressed as mean ± SEM, N = 3 per group, ** $P < 0.01$ vs blood as analysed by two-tailed T test.

Murine peripheral blood neutrophils express lower levels of α-linked sialic acid than human PMN as demonstrated by lower SNA binding. A significant loss of α2,6-linked sialic acid was observed on exudated neutrophils compared to those in the peripheral circulation as demonstrated by a 93% loss of SNA binding (49.51 ± 5.32 vs 3.55 ± 1.85, P = 0.006) **Figure 3.40**.

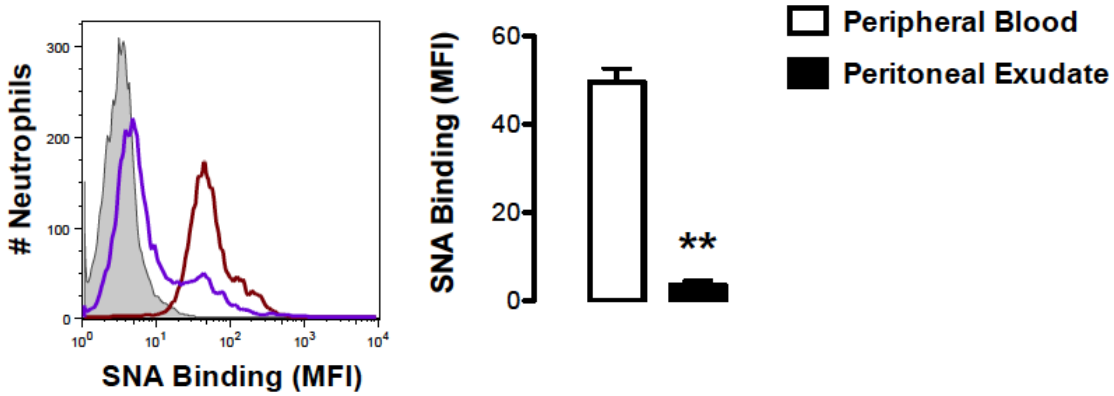


Figure 3.40: Lectin binding for α2,6-linked sialic acid residues on murine peripheral blood and peritoneal exudate recruited neutrophils. Peritonitis was induced following administration of 1mg zymosan i.p. and peripheral blood (burgundy) and peritoneal exudate (purple) was collected 4-hours later. Neutrophils were labelled with Ly6G and double-stained for SNA binding and the binding of SNA to neutrophils was analysed by Flow Cytometry. Figure shows representative histogram and graph for SNA binding. Data are expressed as median fluorescence intensity per sample for paired donors, N = 3 per group, ** P < 0.01 vs blood as analysed by two-tailed paired T test.

Collectively, the data gathered from human and murine studies should be compared to assess whether any species differences are observed in neutrophil glycophenotype. Murine neutrophils in the peripheral circulation expressed higher levels of α-linked fucose compared to human cells. The expression of fucose residues was significantly diminished upon neutrophil trafficking to a site of inflammation as demonstrated by a 75% loss of UEA I binding to neutrophils in the peritoneal cavity compared to those in the peripheral circulation (94.68 ± 5.81 vs 22.98 ± 3.98, P = 0.005) **Figure 3.41**.

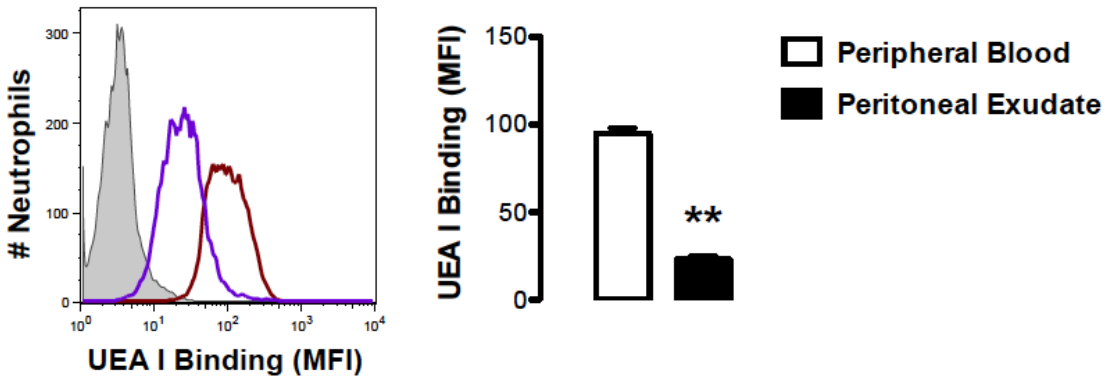


Figure 3.41: Lectin binding for α-linked fucose residues on murine peripheral blood and peritoneal exudate recruited neutrophils. Peritonitis was induced following administration of 1mg zymosan ip. and peripheral blood (burgundy) and peritoneal exudate (purple) was collected 4-hours later, Neutrophils were labelled with Ly6G and double-stained for UEA I binding and the binding of UEA I to neutrophils was analysed by Flow Cytometry. Figure shows representative histogram and graph for UEA I binding. Data are expressed as median fluorescence intensity per sample for paired donors, N = 3 per group, ** P < 0.01 vs blood as analysed by two-tailed paired T test.

3.2 Discussion

The glycophenotype of human PMN was modulated by co-culture with, adhesion to and transmigration through an endothelial monolayer; this was not a direct consequence of granule release, or of endothelial cell-derived mediators as demonstrated by an inability of granule release or conditioned medium to mimic the phenotype changes. Thus the modulation seen was most likely a combination of the two factors and also a direct consequence of transendothelial migration.

Murine and human models of pathophysiological inflammation were used to collect neutrophils that had migrated to an inflammatory site and although it was hypothesised that the lectin binding profile of exudated murine neutrophils would be similar to that of human exudated neutrophils this was not the case and large variation was seen with many of the lectins.

This study suggests that analysis of the glycophenotype of neutrophils is not a suitable marker for galectin binding permissibility, this may be due to the loss of heavily glycosylated proteins from the cell surface upon activation skewing the results rather than the action of specific glycosylation enzymes as is seen with adaptive immune cells.

It was initially postulated that the differential responses seen to galectin binding were dependent either on the modulation of the glycophenotype of the neutrophil affecting galectin binding permissibility or the differential expression of galectins during an inflammatory response thus the next chapter addresses the second part of this hypothesis by assessing the galectin expression profile in both isolated human neutrophils that have been transmigrated through an endothelial monolayer and murine leukocytes *in vivo* using a resolving model of acute inflammation.

CHAPTER 4:

RESULTS (2): MODULATION OF THE GALECTIN EXPRESSION PROFILE OF LEUKOCYTES

4 Modulation of the Galectin Expression Profile of Leukocytes

4.1 Introduction

Galectins-1, -3 and -9 are all known to be expressed by leukocytes of the innate immune system (Gil et al., 2006a, Truong et al., 1993, Sanchez-Cuellar et al., 2012, Sato and Hughes, 1994), however these lectins have opposing actions. Galectins-1 and -9 act on cells of the innate immune system to promote a reduction in recruitment (Cooper et al., 2008, Rabinovich et al., 2000, La et al., 2003, He et al., 2009) while galectin-3 increases the recruitment of leukocytes to an inflammatory site (Colnot et al., 1998b, Hsu et al., 2000). It can therefore be hypothesised that the expression of each galectin is tightly controlled to ensure leukocytes are not activated during homeostasis.

Published studies have also demonstrated that the localisation of the galectin affects the function with intracellular galectin-3 binding to Bcl-2 proteins in Jurkat T cells and preventing apoptosis (Akahani et al., 1997) while the same lectin bound to the surface of CD4+ T cells promotes their apoptosis (Fukumori et al., 2003). Therefore it can be hypothesised that the cellular expression is also tightly controlled to ensure the correct cellular processes are occurring.

The aims of this study were therefore to investigate how the expression of galectins alters in human neutrophils following an inflammatory stimulus and/or cell trafficking.

Secondly this study aimed to investigate how the expression of galectins-1, -3 and -9 are modulated over the course of a resolving inflammatory response using a murine model of zymosan-induced peritonitis, a model which has been well-characterised to resolve over a 96-hour time period.

The expression of galectins-1, -3 and -9 were assessed in PMN that had transmigrated through an endothelial monolayer, both at the cell surface and intracellularly. The modulation of expression of galectins-1, -3 and -9 was also characterised in a human model of neutrophil recruitment using a Tobasco mouth wash and compared to paired peripheral blood PMN. Finally galectin expression was assessed in an *in vivo* murine model of inflammation to characterise how the expression of the galectins is altered on myeloid cells during a resolving inflammatory process.

4.1.1 Cell Surface Galectin Expression of Isolated Neutrophils Pre- and Post-Transendothelial Migration

Isolated PMN were assessed for their cell surface expression of galectins-1, -3 and -9 following transendothelial migration using a model of transwell transmigration as described previously in **section 2.2.5**.

Non-transmigrated human PMN express low levels of galectin-1 and this was further decreased upon transmigration through an endothelial monolayer as demonstrated by a 55% decrease in the binding of anti-galectin-1 antibodies (7.77 ± 3.68 vs 3.52 ± 1.45, P = 0.012) as seen in **Figure 4.1**.

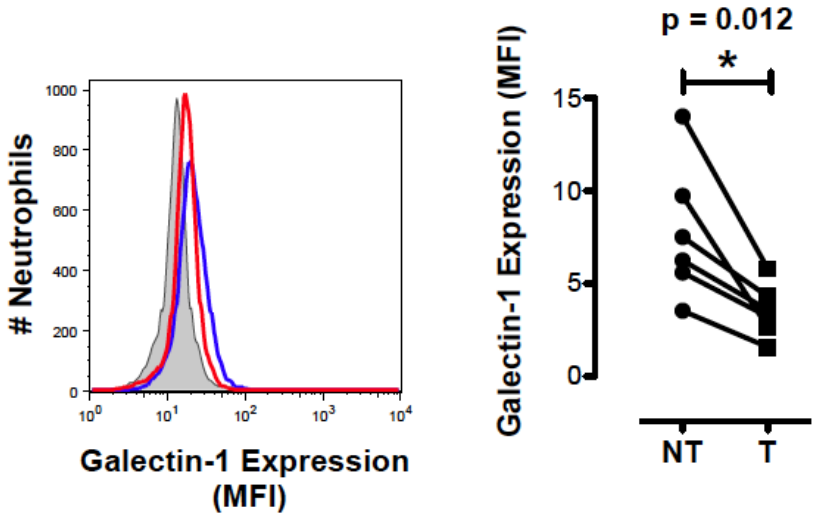


Figure 4.1: Cell surface expression of galectin-1 on non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α for 4 hours. Non-transmigrated (NT) and transmigrated (T) PMN were collected from the upper and lower chamber respectively and washed before staining with an anti-galectin-1 antibody took place and Flow Cytometry was used to assess the expression. Figure shows representative histogram and graph for galectin-1 expression. Data are expressed as median fluorescence intensity per sample for paired donors, n = 6 per group, * P < 0.05 as analysed by two-tailed paired T-test.

The basal expression of galectin-3 on non-transmigrated human PMN was higher than galectin-1 however this expression was not significantly altered by transmigration through an endothelial monolayer (15.82 ± 6.03 vs 14.75 ± 2.31) **Figure 4.2**.

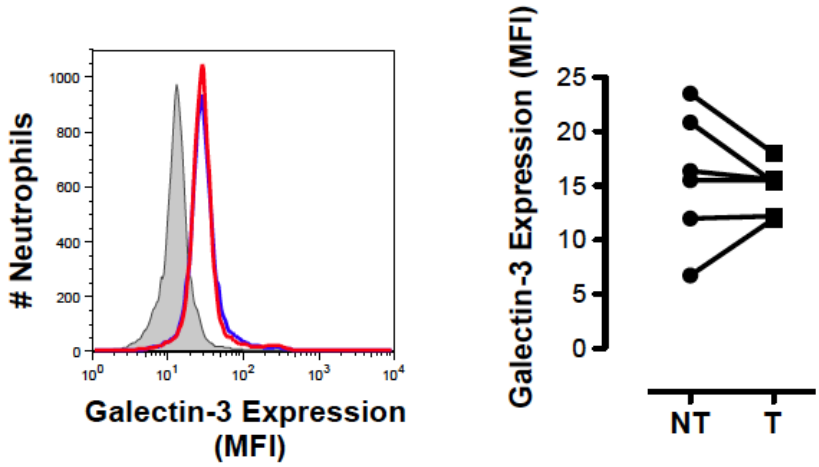


Figure 4.2: Cell surface expression of galectin-3 on non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α for 4 hours. Non-transmigrated (NT) and transmigrated (T) PMN were collected from the upper and lower chamber respectively and washed before staining with an anti-galectin-3 antibody took place and Flow Cytometry was used to assess the expression. Figure shows representative histogram and graph for galectin-3 expression. Data are expressed as median fluorescence intensity per sample for paired donors, n = 6 per group and are analysed by two-tailed paired T-test.

Similar to galectin-1, the cell surface expression of galectin-9 was very low on non-transmigrated PMN and this also decreased by 59% following transendothelial migration (3.3 ± 2.23 vs 1.34 ± 1.08, P = 0.014) as demonstrated by decreased binding of an anti-galectin-9 antibody (**Figure 4.3)**.

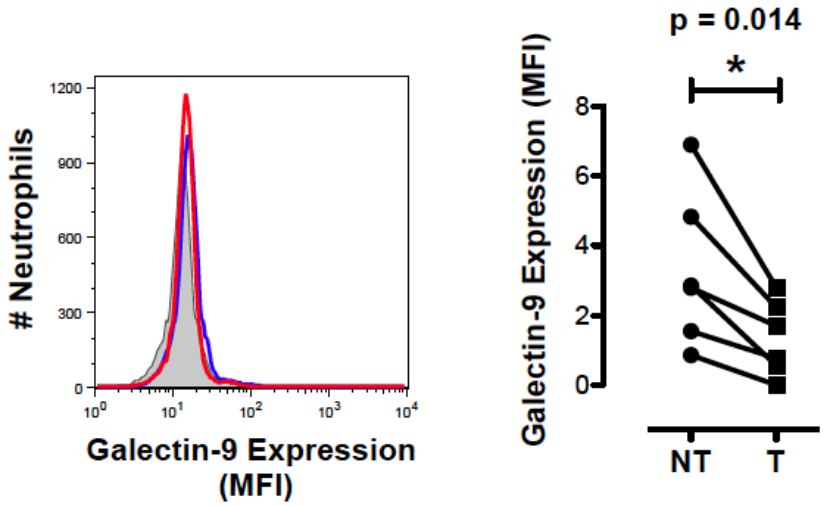


Figure 4.3: Cell surface expression of galectin-9 on non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated (NT) and transmigrated (T) PMN were collected from the upper and lower chamber respectively and washed before staining with an anti-galectin-9 antibody took place and Flow Cytometry was used to assess the expression. Figure shows representative histogram and graph for galectin-9 expression. Data are expressed as median fluorescence intensity per sample for paired donors, n = 6 per group, * P < 0.05 as analysed by two-tailed paired T-test.

4.1.2 Intracellular Galectin Expression of Isolated Neutrophils Pre- and Post-Transendothelial Migration

PMN from the transwell transmigration assay were also assessed for their intracellular expression of galectins-1, -3 and -9 using Flow Cytometry by permeabilising cells before galectin antibodies were added.

Low levels of galectin-1 were expressed by freshly isolated PMN (2.373 ± 0.765) and this was not modulated in non-transmigrated (1.083 ± 1.044) or transmigrated PMN (1.030 ± 0.156), although a trend towards decreased expression could be seen this did not reach statistical significance (**Figure 4.4**).

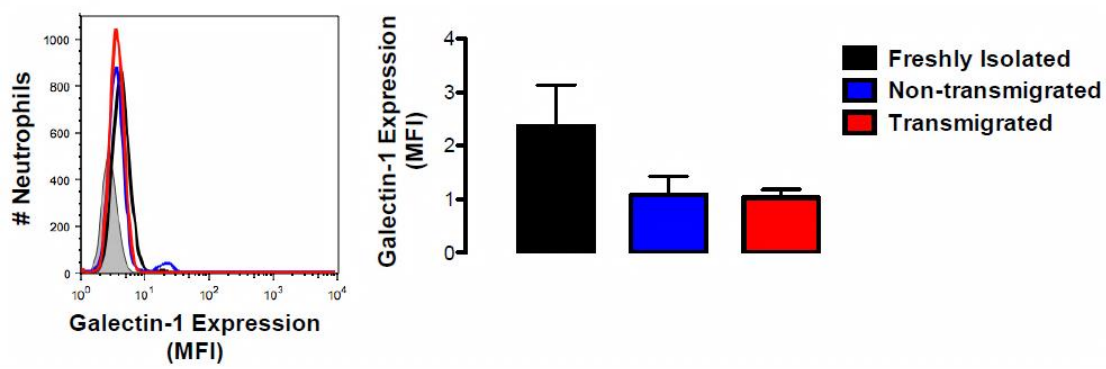


Figure 4.4: Intracellular expression of galectin-1 on non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated and transmigrated PMN were collected from the upper and lower chamber respectively along with freshly isolated PMN. PMN were fixed and permeabilised before staining with an anti-galectin-1 antibody took place and Flow Cytometry was used to assess the expression. Figure shows representative histogram and graph for galectin-1 expression Data are expressed as mean ± SEM, n = 3 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

Galectin-3 was expressed at higher levels than galectin-1 by freshly isolated PMN (13.01 ± 3.96), however this was not significantly modulated in non-transmigrated (8.777 ± 1.044) and transmigrated PMN (7.333 ± 1.713) **Figure 4.5**. Intracellular levels of galectin-3 were comparable to those seen at the cell surface suggesting all galectin-3 expressed by human PMN is localised at the cell surface.

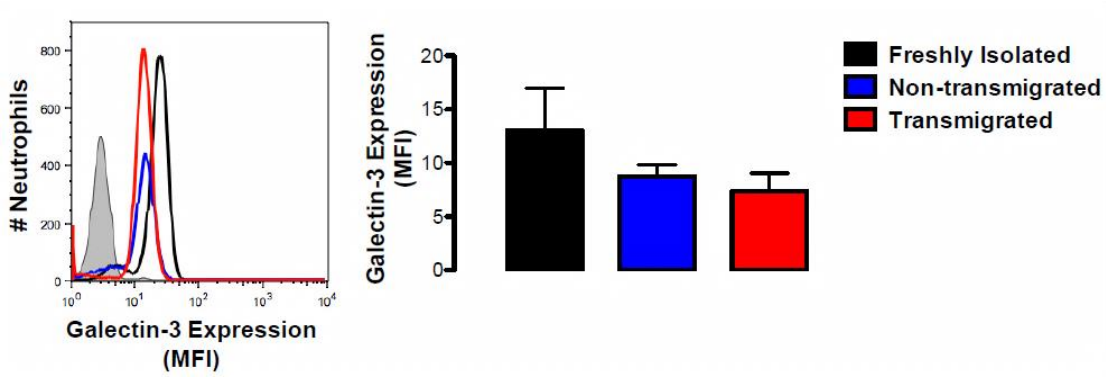


Figure 4.5: Intracellular expression of galectin-3 on non-transmigrated and transmigrated human PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated and transmigrated PMN were collected from the upper and lower chamber respectively along with freshly isolated PMN. PMN were fixed and permeabilised before staining with an anti-galectin-3 antibody took place and Flow Cytometry was used to assess the expression. Figure shows representative histogram and graph for galectin-3 expression. Data are expressed as mean ± SEM, n = 3 per group and are analysed by one-way ANOVA with Bonferroni post hoc test.

Galectin-9 expression in freshly isolated PMN is negligible (1.413 ± 0.206) and although significant decreases are noted in non-transmigrated PMN (0.580 ± 0.150, P = 0.012) and transmigrated PMN (0.700 ± 0.134, P = 0.027), due to the extremely low levels of expression (MFI values of less than one) these minimal differences can be discounted as shown in **Figure 4.6**.

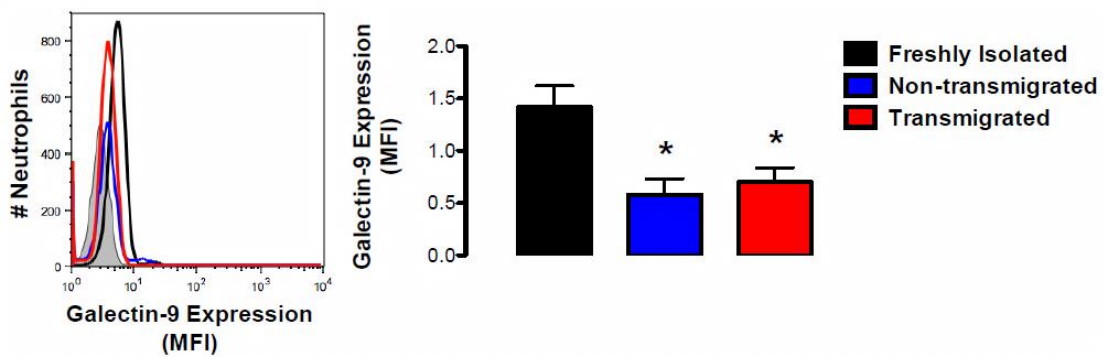


Figure 4.6: Intracellular expression of galectin-9 on non-transmigrated and transmigrated PMN. Freshly isolated PMN were left to transmigrate for 90 minutes through a HUVEC monolayer that had been pre-treated with 10ng/mL hrTNF-α. Non-transmigrated and transmigrated PMN were collected from the upper and lower chamber respectively along with freshly isolated PMN. PMN were fixed and permeabilised before staining with an anti-galectin-9 antibody took place and Flow Cytometry was used to assess the expression. Figure shows representative histogram and graph for galectin-9 expression. Data are expressed as mean ± SEM, n = 3 per group, * P < 0.05 vs freshly isolated PMN as analysed by one-way ANOVA with Bonferroni post hoc test.

4.1.3 Modulation of the Intracellular Galectin Expression of Neutrophils during Exudation to the Buccal Cavity

Healthy volunteers performed the Tobasco wash as described in **section 2.2.3** and the exudated PMN collected were paired with isolated peripheral blood PMN from the same donor. The PMN were fixed overnight in 1% PFA and then permeabilised before anti-galectin-1, -3 and -9 antibodies were used to assess intracellular galectin expression of exudated neutrophils compared to those in the peripheral circulation.

As previously demonstrated galectin-1 was expressed at low levels by PMN in the peripheral circulation and this was not significantly modulated by transmigration to the buccal cavity (2.172 ± 0.3474 vs 1.846 ± 0.4532) **Figure 4.7**.

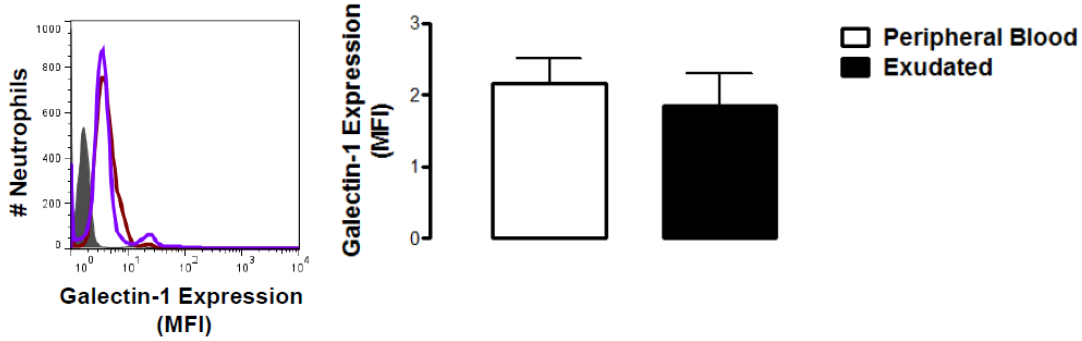


Figure 4.7: Expression of galectin-1 in human peripheral blood and exudated PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour recruited PMN were collected by saline wash (purple), peripheral blood PMN were also isolated by dextran sedimentation (burgundy). Both groups of PMN were washed twice and fixed overnight in 1% PFA, the following day the PMN were permeabilised and labelled with galectin-1 antibodies and the expression was assessed using Flow Cytometry. Figure shows representative histogram and graph for galectin-1 expression. Data are expressed as mean ± SEM, n = 5 and are analysed by two-tailed T test.

Galectin-3 was expressed at higher levels by PMN in the peripheral circulation than galectin-1 however this was also not significantly modulated by exudation to an inflammatory site (13.07 ± 1.852 vs 15.78 ± 2.074) **Figure 4.8**.

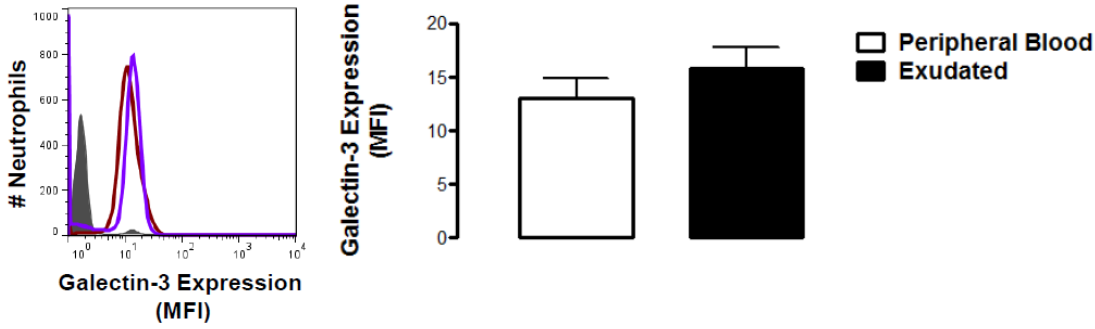


Figure 4.8: Expression of galectin-3 in human peripheral blood and exudated PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour recruited PMN were collected by saline wash (purple), peripheral blood PMN were also isolated by dextran sedimentation (burgundy). Both groups of PMN were washed twice and fixed overnight in 1% PFA, the following day the PMN were permeabilised and labelled with galectin-3 antibodies and the expression was assessed using Flow Cytometry. Figure shows representative histogram and graph for galectin-3 expression. Data are expressed as mean ± SEM, n = 5 and are analysed by two-tailed T test.

As previously demonstrated galectin-9 was expressed in human peripheral blood PMN at low levels, this was reduced further by transmigration to the buccal cavity (1.378 ± 0.2122 vs 0.760 ± 0.1425, P = 0.0420), again due to the extremely low expression levels this must be taken into account (**Figure 4.9**).

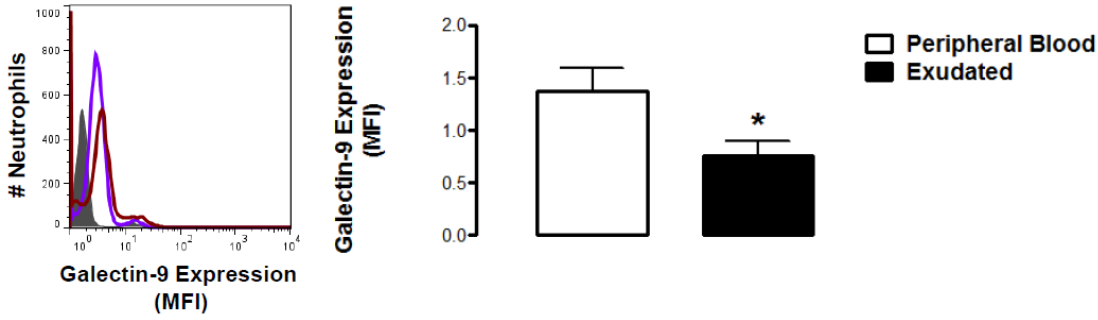


Figure 4.9: Expression of galectin-9 in human peripheral blood and exudated PMN. Healthy volunteers rinsed their mouths with a 10% Tobasco solution and following 1 hour recruited PMN were collected by saline wash (purple), peripheral blood PMN were also isolated by dextran sedimentation (burgundy). Both groups of PMN were washed twice and fixed overnight in 1% PFA, the following day the PMN were permeabilised and labelled with galectin-9 antibodies and the expression was assessed using Flow Cytometry. Figure shows representative histogram and graph for galectin-9 expression. Data are expressed as mean ± SEM, n = 5, * P < 0.05 vs blood as analysed by two-tailed T test.

4.1.4 Modulation of Galectin Expression in Myeloid Cells during Acute, Resolving Inflammation

Zymosan-induced peritonitis (1mg/mouse) is a model used to study the inflammatory process from induction to resolution, as it is a well-defined, reproducible model of inflammation. The inflammation is characterised by a neutrophil and monocyte-driven response that resolves completely over a 96-hour period.

Male C57BL/6 mice were injected with 1mg zymosan ip and during a 96h time-course samples were collected (at 0, 4, 24, 48, 72 and 96 hours post injection); cardiac punctures were performed to obtain peripheral leukocytes and peritoneal lavages were collected for exudated leukocytes. Femurs were also collected, flushed with PBS and this was filtered through a 70μm cell strainer to collect bone marrow leukocytes. Total leukocyte counts were performed in Turk's solution for all time points and antibodies specific for neutrophils (Ly6G, clone 1A8) and monocyte/macrophages (F4/80, clone BM8) were used to differentiate between the two populations using Flow Cytometry (bone marrow neutrophils were identified using Ly6G and CXCR4 while bone marrow monocytes were labelled with Ly6C).

There was a rapid increase in the number of total leukocytes in the peritoneal cavity within 4-24 hours of zymosan administration and this remained elevated over the course of the response with the increased leukocytes still observed at 96 hours compared to 0 hours (**Figure 4.10a**). The neutrophil number in the peritoneal cavity increased sharply over 4-24 hours and then returned back to basal levels within the 96 hour time course. In contrast the monocyte/macrophage numbers declined within the first 4 hours of zymosan treatment and then re-populated the peritoneal cavity over the time course ending with a significantly higher number of monocyte/macrophages than were present at 0 hour (**Figure 4.10b**). This correlates with previous studies of zymosan-induced peritonitis (Ajuebor et al., 1999).

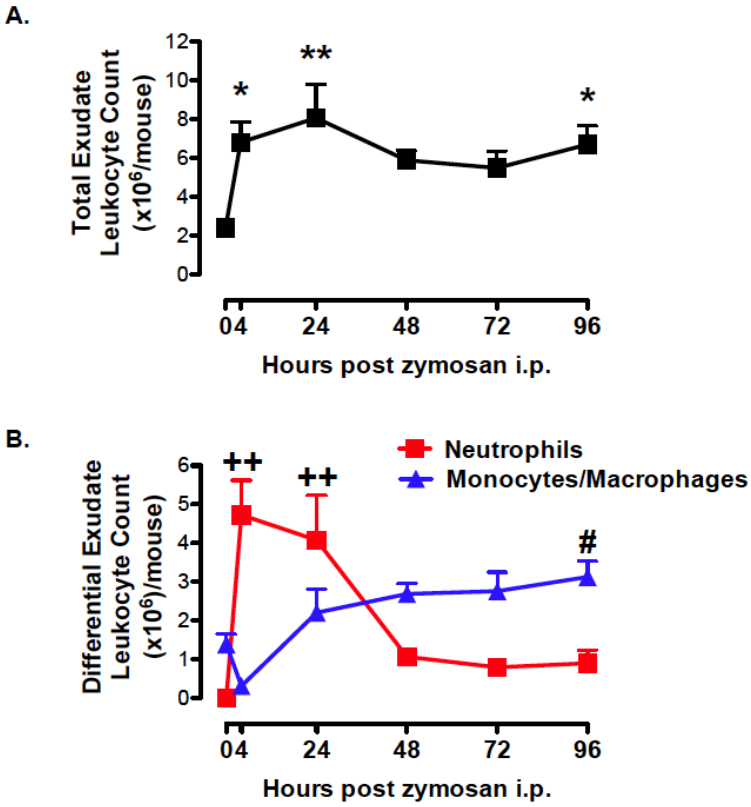


Figure 4.10: Total and differential leukocyte counts from peritoneal exudate over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were given 1mg zymosan ip and over a 96-hour time course leukocytes were collected from the peritoneal exudate using peritoneal lavage. A. Total leukocytes were stained with Turk's solution and counted using a light microscope and B. Differential leukocyte counts were performed using anti-Ly6G (specific for neutrophils) and anti-F4/80 (specific for monocyte/macrophages). Data are expressed as mean ± SEM, N = 12 per group. * $P < 0.05$ and ** $P < 0.01$ vs 0hr time point for total leukocytes, ++ $P < 0.01$ vs neutrophils at 0hr time point and # $P < 0.05$ vs monocytes at 0hr time point as analysed using One-Way ANOVA with Dunnetts post hoc test.

The number of total leukocytes within peripheral blood decreased rapidly following induction of peritonitis, yet returned to basal levels over the course of the response (**Figure 4.11a**). Monocytes account for the decrease in total cells seen at 4 hours, which return to basal levels over the time course. Whilst there were no significant modulations in the numbers of neutrophils in the peripheral blood, a trend can be seen that mirrors the changes in the peritoneal cavity with a decrease as cells migrate from the peripheral blood to the peritoneal cavity and then an increase as the peripheral blood re-populates (**Figure 4.11b**).

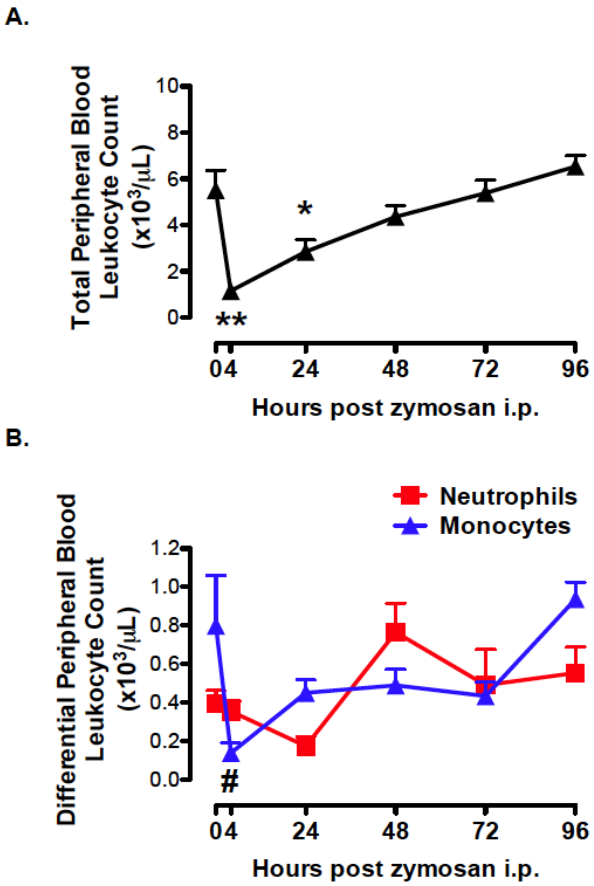


Figure 4.11: Total and differential leukocyte counts from peripheral blood over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were given 1mg zymosan ip and over a 96-hour time course peripheral blood was collected via cardiac puncture, the red cells were lysed and the leukocytes assessed. A. Total leukocytes were stained with Turk's solution and counted using a light microscope and B. Differential leukocyte counts were performed using anti-Ly6G (specific for neutrophils) and anti-F4/80 (specific for monocytes). Data are expressed as mean ± SEM, N = 8 per group. * $P < 0.05$ and ** $P < 0.01$ vs 0-hour time point for total leukocytes. # $P < 0.05$ vs 0-hour monocytes as analysed by One-Way ANOVA with Dunnetts post-hoc test.

Although no significant modulation of the leukocyte number was observed within the bone marrow, a similar yet delayed trend followed the peripheral blood neutrophils with a decrease in the number of cells in the bone marrow approximately 24-hours later than that seen in the peripheral blood. This is likely to account for leukocyte mobilisation to repopulate the peripheral circulation (**Figure 4.12**).

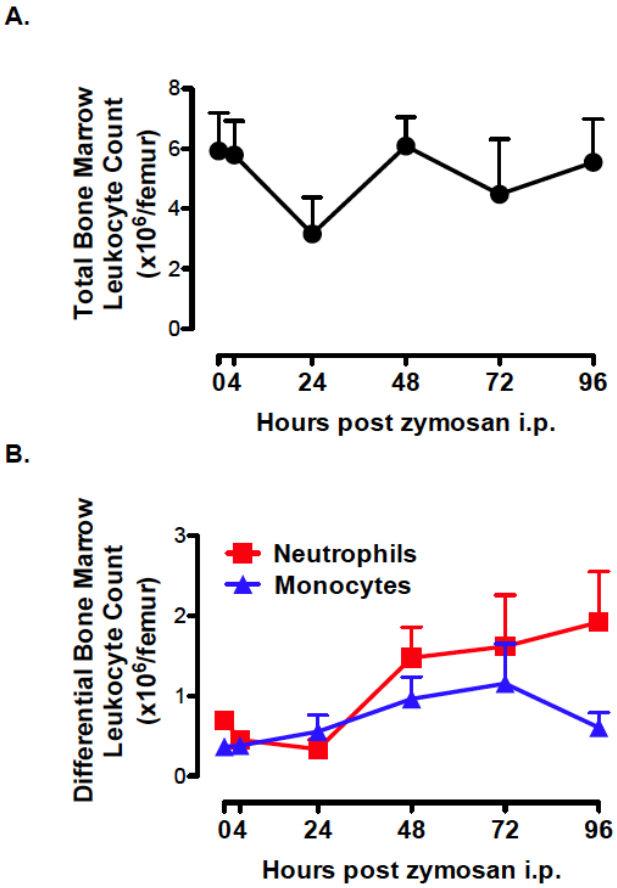


Figure 4.12: Total and differential leukocyte counts from bone marrow over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were given 1mg zymosan ip and over a 96-hour time course bone marrow was collected via removal of the femur and flushing through with PBS, the leukocyte were collected by passing the bone marrow through a 70μm cell strainer. A. Total leukocytes were stained with Turk's solution and counted using a light microscope and B. Differential leukocyte counts were performed using anti-Ly6G and anti-CXCR4 (specific for bone marrow neutrophils) and anti-Ly6C (specific for bone marrow monocytes). Data are expressed as mean ± SEM, N = 4 per group. Analysed by One-Way ANOVA with Dunnetts post-hoc test.

It is known that galectins have differing actions on leukocyte recruitment, for instance galectin-1 inhibits neutrophil trafficking while galectin-3 activates neutrophils and increases neutrophil-endothelial cell interactions (Cooper et al., 2008, Karlsson et al., 1998, Sato et al., 2002b). Given that galectins function both endogenously and exogenously their expression during a resolving inflammatory response must be both spatially and temporally modulated.

Supernatants from peritoneal exudates were taken at each time point and assessed for galectin-1 and -3 concentrations by ELISA analysis. Galectin-1 expression was high in untreated mice (1.701 ± 0.324 ng/mL) and this rapidly decreased by approximately 50% following the onset of inflammation (0.809 ± 0.103 ng/mL) by 24 hours post zymosan. A second peak was seen at 48 hours where levels were similar to that seen in control animals (1.804 ± 0.266 ng/mL) and this once again decreased and remained low at 96h (**Figure 4.13**).

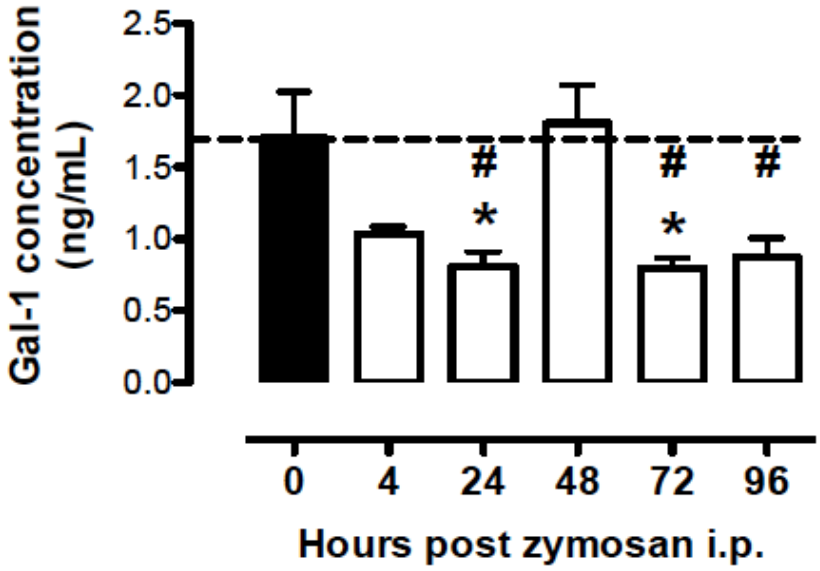


Figure 4.13: Galectin-1 concentration in peritoneal exudate fluid over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were given zymosan ip (1mg/mouse) and peritoneal lavages were performed at 0, 4, 24, 48, 72 and 96 hours post injection. Peritoneal exudate was centrifuged at 300g for 10 minutes and the cell-free supernatant was collected and assayed for the concentration of galectin-1 by ELISA. Data are expressed as mean ± SEM, n = 4-7 per group, * $P < 0.05$ vs 0 hour and # $P < 0.05$ vs 48 hour as analysed by one-way ANOVA with Bonferroni post hoc test.

Galectin-3 was also detected in the peritoneal wash of mice that did not receive any zymosan (2.155 ± 0.450 ng/mL) and levels increased over the time course with a peak approximately 4-fold higher than time zero at 72 hours. At 96 hours the galectin-3 concentration was not significantly different from control levels suggesting a return to homeostasis had begun (**Figure 4.14**).

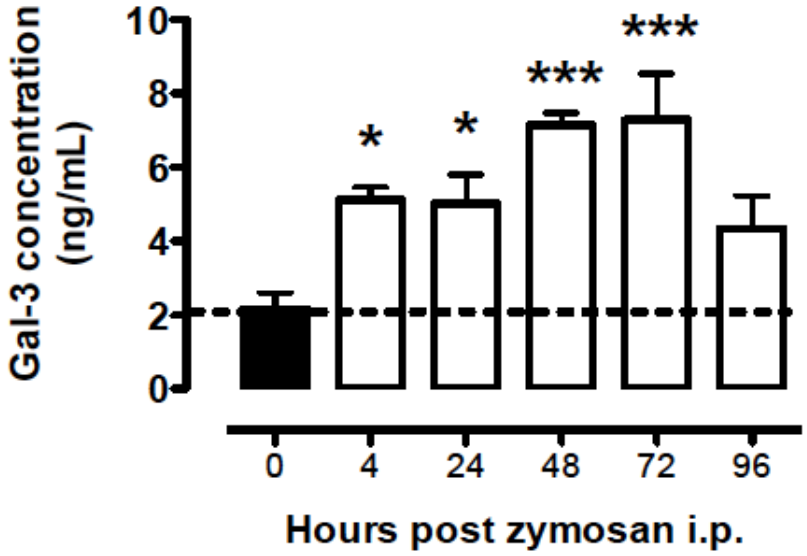


Figure 4.14: Galectin-3 concentration in peritoneal exudate fluid following zymosan-induced peritonitis. Mice were given zymosan ip (1mg/mouse) and peritoneal lavages were performed at 0, 4, 24, 48, 72 and 96 hours post injection. Peritoneal exudate was centrifuged at 300g for 10 minutes and the cell-free supernatant was collected and assayed for the concentration of galectin-3 by ELISA. Data are expressed as mean ± SEM, n = 4-7 per group, * $P < 0.05$ and *** $P < 0.001$ vs 0 hour as analysed by one-way ANOVA with Dunnetts post hoc test.

4.1.4.1 Modulation of Galectin Expression in Murine Neutrophils

To assess the modulation of galectin expression in neutrophils they were specifically labelled with Ly6G (clone 1A8), fixed and then permeabilised and galectin expression was assessed using specific antibodies. Murine neutrophils showed no expression of galectins-1 or -9 in peritoneal exudate, peripheral blood or bone marrow when assessed over a 96-hour time course following zymosan-induced peritonitis (**Figure 4.15**).

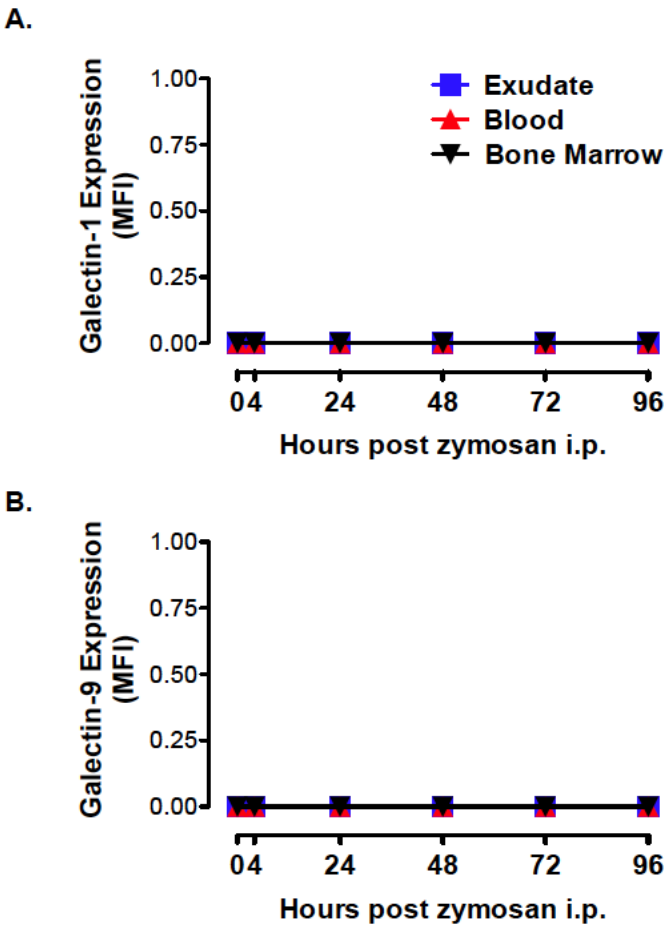


Figure 4.15: Galectin-1 and galectin-9 expression in PMN from peritoneal exudate, peripheral blood and bone marrow over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were given 1mg zymosan ip and over a 96-hour time course leukocytes were collected from the peritoneal cavity, peripheral blood and bone marrow. The cells were double stained for Ly6G to identify neutrophils and galectin-1 or -9 and the expression was assessed using Flow Cytometry. A. Galectin-1 expression and B. Galectin-9 expression. Data are expressed as Mean ± SEM, n = 12 per group for peritoneal exudate, 8 per group for peripheral blood and 4 per group for bone marrow. Analysed by Two Way ANOVA with Bonferroni post hoc test.

In contrast to galectins-1 and -9, galectin-3 expression within neutrophils was found to be modulated in cells recruited to the peritoneal cavity. Low levels of galectin-3 were detected in neutrophils from the peripheral blood and bone marrow, however this was significantly increased in exudated neutrophils over 72 hours; levels then declined at 96 hours at which point homeostasis was restored as seen in **Figure 4.16**.

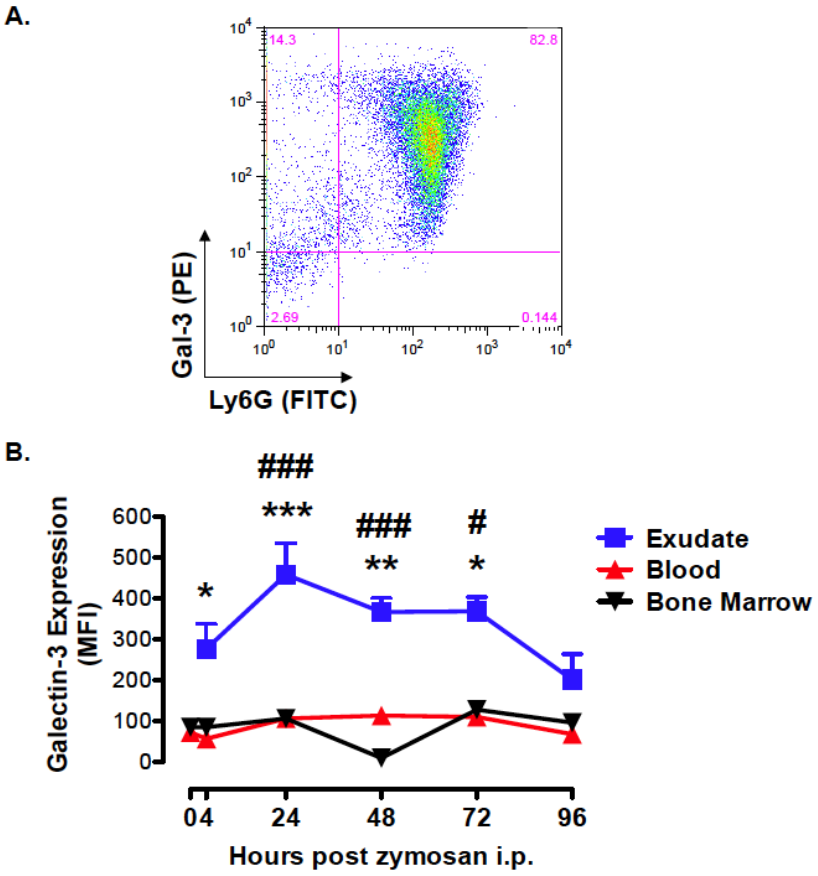


Figure 4.16: Galectin-3 expression in PMN from peritoneal exudate, peripheral blood and bone marrow over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip and over a 96-hour time course leukocytes were collected from the peritoneal cavity, peripheral blood and bone marrow. A. Representative histogram showing neutrophils double-stained for Ly6g and galectin-3 expression at 4 hours post zymosan. B. Graph showing galectin-3 expression in neutrophils from the peritoneal exudate, peripheral blood and bone marrow over 96 hours. Data are expressed as mean ± SEM, n = 12 per group for peritoneal exudate, 8 per group for peripheral blood and 4 per group for bone marrow. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs peripheral blood at same time point, # $P < 0.05$ and ### $P < 0.001$ vs bone marrow at same time point as analysed by Two Way ANOVA with Bonferroni post hoc test.

4.1.4.2 Modulation of Galectin Expression in Murine Monocytes/Macrophages

Initially cells from the peritoneal exudate and peripheral blood were labelled with an anti-F4/80 antibody to differentiate monocyte/macrophages from other leukocytes. The

cells were double stained with galectin-1, -3 or -9 antibodies and the expression assessed by Flow Cytometry.

Low levels of galectin-1 were detected in peripheral blood and bone marrow monocytes, however this was significantly increased in monocyte/macrophages recruited to the peritoneal cavity from 24 to 72 hours of zymosan treatment that peaked at 72 hours (**Figure 4.17**).

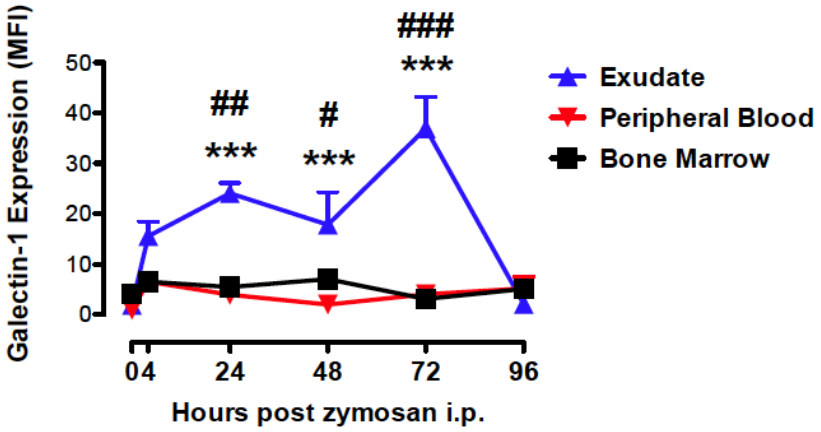


Figure 4.17: Galectin-1 expression in murine monocytes/macrophages over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip. and peritoneal exudates, peripheral blood and bone marrow leukocytes were collected from 0-96 hours. Permeabilised monocytes/macrophages were labelled with anti-F4/80 or anti-Ly6C for bone marrow monocytes (clone – HK1.4) and galectin-1 antibodies. Figure shows graph for galectin-1 expression. Data are expressed as mean ± SEM, n = 8 per group for peritoneal exudate and peripheral blood and 4 per group for bone marrow, *** $P < 0.001$ vs peripheral blood, # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ vs bone marrow at same time point as analysed by Two-Way ANOVA with Bonferroni post hoc test.

Similar to neutrophils, the levels of galectin-3 in peripheral blood and bone marrow monocytes was relatively low compared to monocyte/macrophages that exudated to the peritoneal cavity. The levels of galectin-3 were raised at 24-72 hours, similar to galectin-1, again with a peak in the expression at 72 hours as seen in **Figure 4.18**.

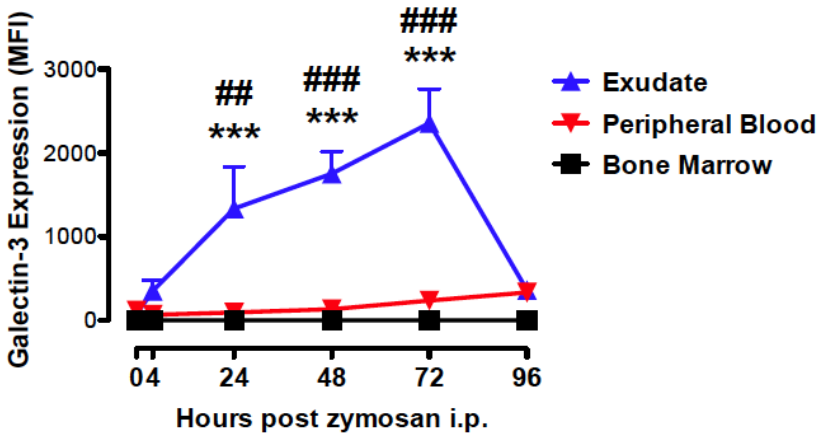


Figure 4.18: Galectin-3 expression in murine monocytes/macrophages over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip. and peritoneal exudates, peripheral blood and bone marrow leukocytes were collected from 0-96 hours. Permeabilised monocytes/macrophages were labelled with anti-F4/80 or anti-Ly6C and galectin-3 antibodies. Figure shows graph for galectin-3 expression. Data are expressed as mean ± SEM, n = 8 per group for peritoneal exudate and peripheral blood and 4 per group for bone marrow, *** $P < 0.001$ vs peripheral blood, ## $P < 0.01$ and ### $P < 0.01$ vs bone marrow at same time point as analysed by Two-Way ANOVA with Bonferroni post hoc test.

The levels of galectin-9 in bone marrow monocytes was low and this was not significantly modulated over the course of zymosan peritonitis; unlike all other galectins the expression of galectin-9 increased in peripheral blood monocytes at 4 hours before returning to basal levels, although this did not reach significance. Galectin-9 expression in peritoneal exudate monocyte/macrophages gradually increased from 4 hours until 72 hours at which point it was significantly different from the levels in the peripheral blood and bone marrow monocytes; levels then declined to basal at 96 hours (**Figure 4.19**).

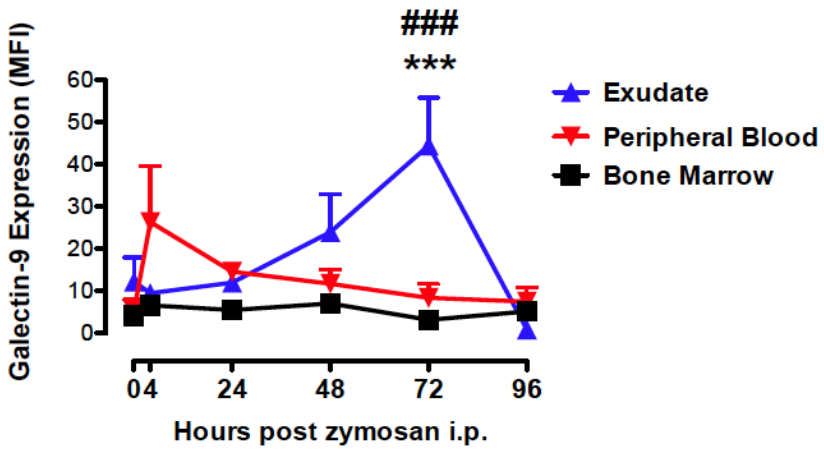


Figure 4.19: Galectin-9 expression in murine monocytes/macrophages over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip. and peritoneal exudates, peripheral blood and bone marrow leukocytes were collected from 0-96 hours. Permeabilised monocytes/macrophages were labelled with anti-F4/80 or anti-Ly6C for bone marrow and galectin-9 antibodies. Figure shows graph for galectin-9 expression. Data are expressed as mean ± SEM, n = 8 per group for peritoneal exudate and peripheral blood and 4 per group for bone marrow, *** $P < 0.001$ vs peripheral blood and ### $P < 0.001$ vs bone marrow at same time point as analysed by Two-Way ANOVA with Bonferroni post hoc test.

Within the monocyte/macrophage populations of peripheral blood and peritoneal exudate there was variation in the galectin expression suggesting that more than one population of cells may be present and thus the monocyte/macrophages were phenotypically sorted into classical and non-classical subtypes based on Ly6C expression. Classical monocytes express high levels of Ly6C while non-classical monocytes express low levels of Ly6C (Gordon and Taylor, 2005).

Cells from the peritoneal exudate and peripheral blood were collected and labelled with F4/80 and Gr-1 (an antibody specific to both Ly6G and Ly6C antigens). Leukocytes that were F4/80 positive and Gr-1 high were considered classical monocyte/macrophages and those that were F4/80 positive and Gr-1 low were considered non-classical monocyte/macrophages.

In the peritoneal exudate non-classical monocyte/macrophages were detected at time 0 in significantly higher numbers than classical monocyte/macrophages. These resident cells rapidly depleted within 4-24 hours, followed by an increase that peaked at 72 hours at which point the number of monocyte/macrophages returned to approximately basal levels by 96 hours. Classical monocyte/macrophages were not present at 0 hours however they increased until a peak was reached at 24 hours; the cells then declined again by 96 hours (**Figure 4.20a**).

Both subtypes of monocytes were present at 0 hours in peripheral blood but a significantly larger number of non-classical monocytes were seen. The number of classical monocytes in the peripheral circulation remained almost constant while the number of non-classical monocytes decreased until 24 hours and then increased to numbers above that seen at 0 hours (**Figure 4.20b**).

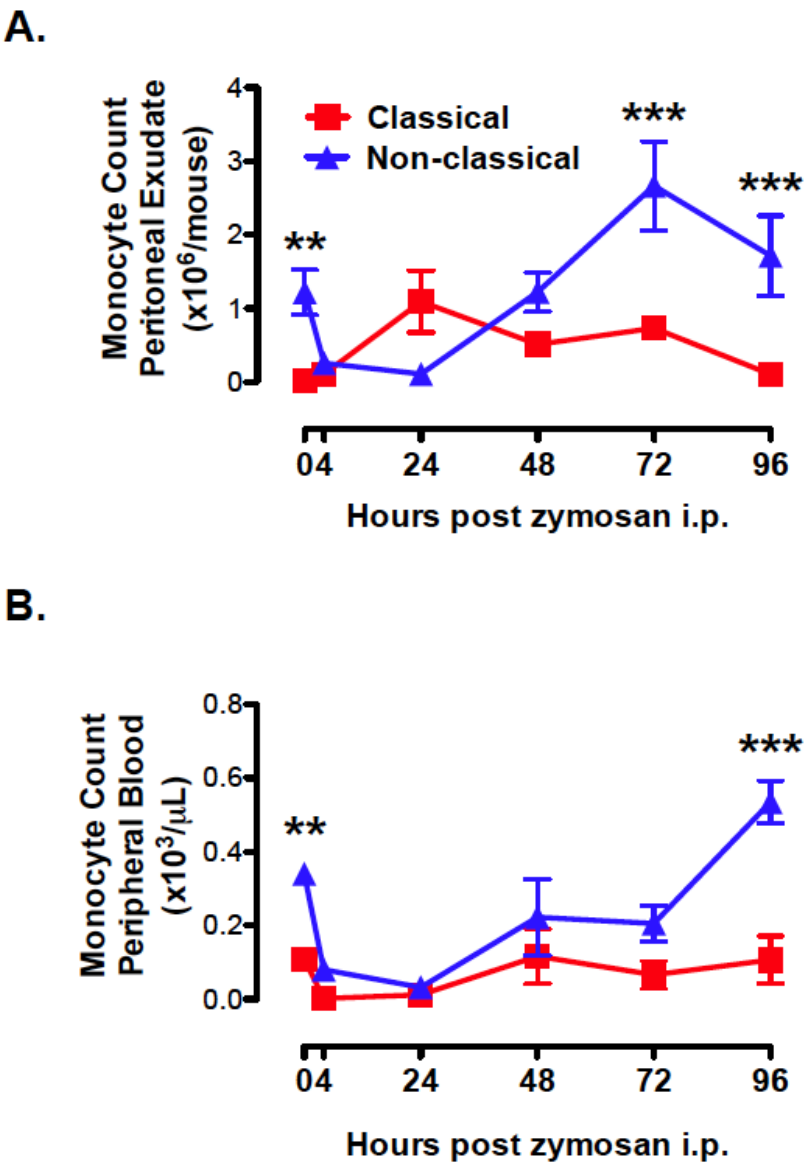


Figure 4.20: Differential counts for classical and non-classical monocytes from the peritoneal exudate and peripheral blood taken over a 96-hour time course following 1mg zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip. and peritoneal exudates and peripheral blood were collected from 0-96 hours. Leukocytes were double stained with anti-F4/80 and anti-Gr-1 and the total numbers of classical and non-classical monocytes calculated. A. Peritoneal exudate monocyte/macrophages counts and B. Peripheral blood monocyte counts. Data are expressed as mean ± SEM, n = 4 per group, ** $P < 0.01$ and *** $P < 0.001$ vs classical monocyte/macrophages at same time point as analysed by two-way ANOVA with Bonferroni post hoc test.

At 48 and 72 hours both subtypes of monocyte/macrophages were present and thus peritoneal exudate and peripheral blood cells were collected at these time points and analysed for their intracellular expression of galectins-1, -3 and -9 using specific antibodies.

Low levels of galectin-1 were detected in both non-classical and classical monocytes in peripheral blood; this was not significantly modulated between 48 and 72 hours although a trend towards increased levels of galectin-1 was observed in both subtypes (**Figure 4.21a**).

Very low levels of galectin-1 were detected in all monocyte/macrophages in the peritoneal exudate at 48 hours however levels significantly increased over the time course (classical 0.513 ± 0.594 vs 17.25 ± 4.239, P = 0.019; non-classical 1.223 ± 1.133 vs 30.35 ± 8.707, P = 0.000001). The non-classical monocytes/macrophages in the peritoneal exudate also have significantly higher levels of galectin-1 than classical monocytes/macrophages at 72 hours post zymosan injection (30.35 ± 4.35 vs 17.25 ± 2.12, P = 0.004) **Figure 4.21b**.

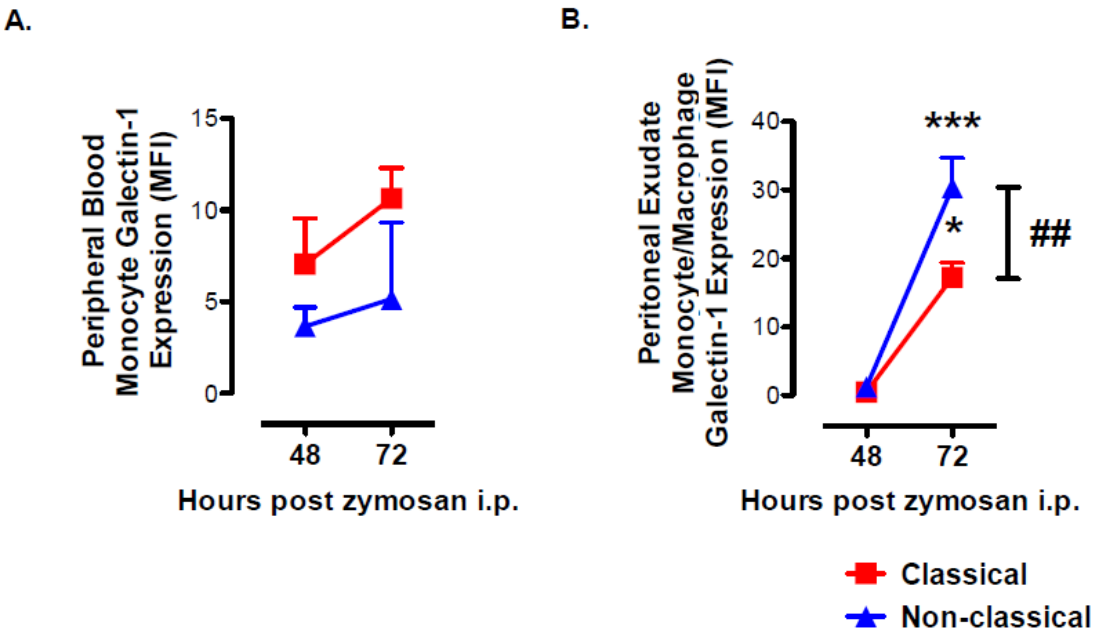


Figure 4.21: Galectin-1 expression in classical and non-classical monocytes/macrophages collected from the peritoneal cavity and peripheral blood at 48 and 72 hours post zymosan ip. Mice were injected with 1mg zymosan ip. and peritoneal exudates and peripheral blood were collected at 48 and 72 hours. Leukocytes were triple stained with anti-F4/80, anti-Gr-1 and anti-galectin-1. The expression of galectin-1 was assessed using Flow Cytometry. A. Peripheral blood monocytes and B. Peritoneal exudate monocyte/macrophages. Data are expressed as mean ± SEM, n = 4 per group, * P < 0.05 and *** P < 0.001 vs 48 hour, ## P < 0.01 as analysed by two-way ANOVA with Bonferroni post hoc test.

Peripheral blood monocytes expressed relatively low levels of galectin-3 compared to exudated cells at 48 and 72 hours (Figure 4.18). When analysing galectin-3 expression based on monocyte/macrophage phenotype, non-classical cells were found to express significantly higher levels of galectin-3 at both 48 and 72 hour time points (48-hour

25.05 ± 11.14 vs 81.18 ± 34.75, P = 0.007; 72-hour 26.03 ± 12.59 vs 82.00 ± 29.88, P = 0.007) with no modulation in levels over time (**Figure 4.22a**).

In the peritoneal exudate levels of galectin-3 in monocyte/macrophages were much higher than those in the peripheral blood however at 48 hours the levels were comparable between non-classical and classical monocyte/macrophages. Non-classical monocyte/macrophages do not display significant modulation of galectin-3 at 72 hours but non-classical monocyte/macrophages have an increase of approximately 2.25-fold (562.7 ± 143.6 vs 1716 ± 246.1, P = 0.00001) and thus expressed significantly higher levels of galectin-3 compared to non-classical monocyte/macrophages at 72 hours post zymosan administration (762.3 ± 132.0 vs 1716 ± 246.1, P = 0.00001) **Figure 4.22b**.

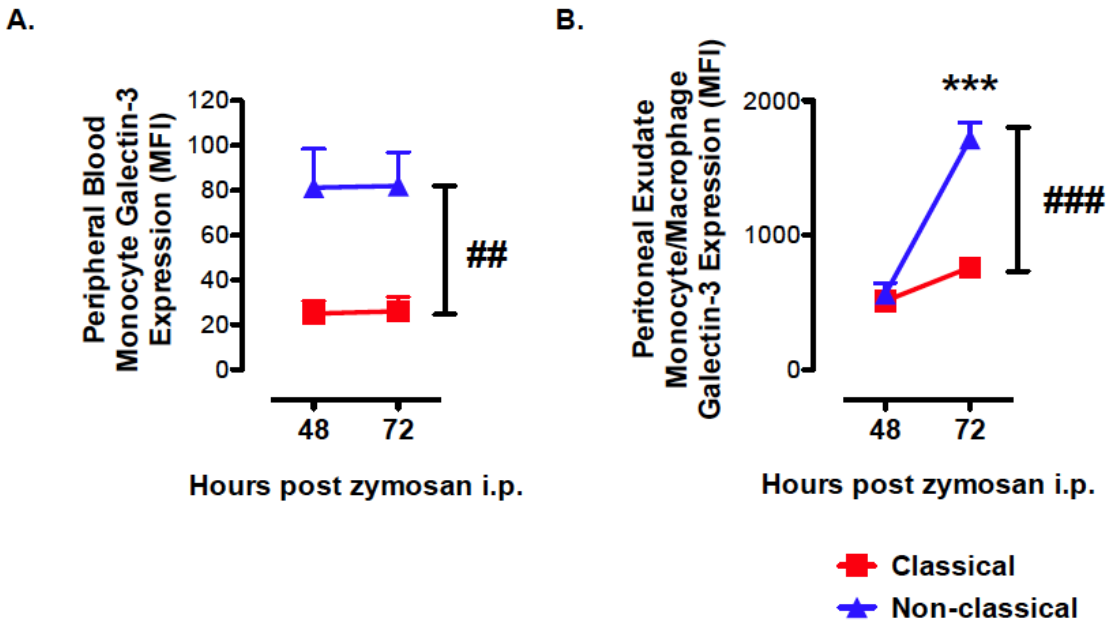


Figure 4.22: Galectin-3 expression in classical and non-classical monocytes/macrophages collected from the peritoneal cavity and peripheral blood at 48 and 72 hours post zymosan ip. Mice were injected with 1mg zymosan ip. and peritoneal exudates and peripheral blood were collected at 48 and 72 hours. Leukocytes were triple stained with anti-F4/80, anti-Gr-1 and anti-galectin-3. The expression of galectin-3 was assessed using Flow Cytometry. A. Peripheral blood monocytes and B. Peritoneal exudate monocyte/macrophages. Data are expressed as mean ± SEM, n = 4 per group. *** P < 0.001 vs 48 hour, ### P < 0.001 as analysed by two-way ANOVA with Bonferroni post hoc test.

In the peripheral blood both non-classical and classical monocytes expressed low levels of galectin-9 at similar levels that were not modulated over the time course (**Figure 4.23a**).

Neither phenotype of monocytes/macrophages expressed galectin-9 at 48 hours, however levels significantly increased by 72 hours for both peripheral blood and peritoneal cavity cells. The levels of galectin-9 increased more in non-classical monocyte/macrophages compared to classical monocyte/macrophages and therefore a significant difference in the galectin-9 levels was detected between the two subtypes at 72 hours (6.993 ± 0.8472 vs 21.9 ± 5.758, P = 0.00001) **Figure 4.23b**.

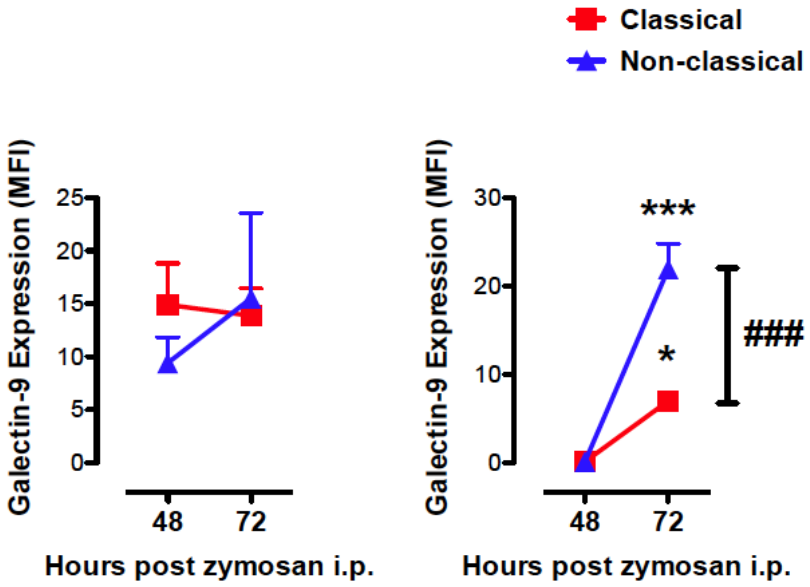


Figure 4.23: Galectin-9 expression in classical and non-classical monocytes/macrophages collected from the peritoneal cavity and peripheral blood at 48 and 72 hours post zymosan ip. Mice were injected with 1mg zymosan ip. and peritoneal exudates and peripheral blood were collected at 48 and 72 hours. Leukocytes were triple stained with anti-F4/80, anti-Gr-1 and anti-galectin-9. The expression of galectin-9 was assessed using Flow Cytometry. A. Peripheral blood monocytes and B. Peritoneal exudate monocyte/macrophages. Data are expressed as mean ± SEM, n = 4 per group. * P < 0.05 and *** P < 0.001 vs 48 hour, ### P < 0.001 as analysed by two-way ANOVA with Bonferroni post hoc test.

4.2 Discussion

Levels of galectins-1, -3 and -9 were significantly higher in peritoneal cavity recruited leukocytes compared to peripheral blood or bone marrow. This spatial modulation suggests a local role for the galectins on leukocytes or that leukocyte-derived galectins are required at the inflammatory site and leukocytes deliver these. The expression was also temporally modulated with a high level of galectin-3 seen in recruited neutrophils at the peak of inflammation (4-24 hours) although this did not decrease back to basal levels during the resolution period as may be expected.

Monocyte/macrophages also demonstrated a temporal modulation of galectins with all three increased at 72 hours post zymosan in non-classical monocyte/macrophages. As classical monocyte/macrophages are known to secrete high levels of the galectins (Novak et al., 2012) this provides an explanation for the difference in levels between subsets.

This study demonstrates that the expression of galectins-1, -3 and -9 are modulated over the course of an inflammatory response, potentially to ensure that the leukocytes are only ever in contact with the galectin which will promote the correct response for the stage of inflammation. This study also demonstrated an up-regulation of galectin-3 later in the inflammatory process, a time in which it would be expected that the pro-inflammatory lectin would have been cleared to prevent unwanted actions. Thus it can be hypothesised that galectin-3 plays a role not only in the induction of an inflammatory response but also in the clearance; this was tested in the next section.

CHAPTER 5:

RESULTS (3): ROLES OF GALECTIN-3 IN THE RESOLUTION OF INFLAMMATION

5 Roles of Galectin-3 in the Resolution of Inflammation

5.1 Introduction

Many studies using galectin-3 null mice have indicated a pro-inflammatory role of this protein. This genotype exhibits defective leukocyte recruitment in models of thioglycollate-induced peritonitis, pneumococcal pneumonia, respiratory tularaemia and parasitic protozoan cutaneous inflammation (Colnot et al., 1998b, Hsu et al., 2000, Farnworth et al., 2008, Mishra et al., 2013, Bhaumik et al., 2013).

Contrary to the pro-inflammatory role of galectin-3, the deficient mice also exhibit defective alternative activation of macrophages, which display reduced phagocytic capabilities compared to wild type cells (MacKinnon et al., 2008, Sano et al., 2003). Recombinant galectin-3 (20μM) induces the exposure of phosphatidylserine on the surface of neutrophils, which does not coincide with increased apoptosis but promotes the efferocytosis of neutrophils by monocytes (Stowell et al., 2008a). These findings suggest a role for galectin-3 in resolution of the innate inflammatory response and this was further investigated firstly to confirm this hypothesis and secondly to elucidate a mechanism for this.

The aim of this section was to determine the role of galectin-3 on murine leukocytes during a resolving inflammatory response.

Initially galectin-3 null mice were used to assess the resolution phase of zymosan-induced peritonitis and determine if a deficiency was present. Following this, wild type mice were used to determine the localisation of galectin-3 in murine neutrophils during an inflammatory response and attempts were then made to establish whether murine neutrophils could in fact produce galectin-3 as this is controversial in the literature (Farnworth et al., 2008, Sato et al., 2002b).

5.1.1 Effects of Galectin-3-Deficiency during Resolution

The model of zymosan-induced peritonitis used (1mg/mouse) is well characterised by Navarro-Xavier and colleagues who determined peak leukocyte recruitment to be at 24 hours and the resolution interval (time taken for total leukocytes to reduce by 50%) to

be 24 hours and therefore the resolution phase in this model begins at 48 hours post zymosan (Navarro-Xavier et al., 2010).

Initially galectin-3 null mice and age- and sex-matched C57BL/6 controls were given zymosan i.p. (1mg/mouse) and following 48, 72 and 96 hours peritoneal lavages were performed. Total leukocyte counts were then performed. At 48 hours post zymosan, total leukocyte counts were comparable for wild type and galectin-3 null mice however at 72 hours there was a trend towards more leukocytes in the peritoneal cavities of galectin-3 null mice although this did not reach statistical significance. At 96 hours no difference was seen in the total leukocyte count comparing the two strains of mice (**Figure 5.1**).

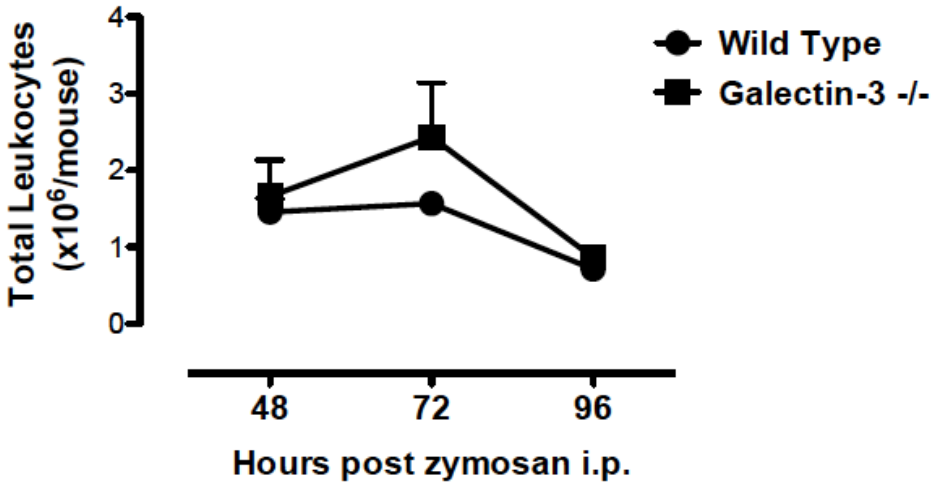


Figure 5.1: Total leukocyte count for galectin-3 null mice with age- and sex-matched wild type controls during the resolution phase of zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip and following 48, 72 and 96 hours peritoneal lavages were performed to collect exudates. An aliquot of leukocytes was taken prior to staining and counted in Turk's solution using a light microscope. Data are expressed as mean ± SEM, n = 3-4 per group. Data are analysed using two-way ANOVA with Bonferroni post hoc test.

The leukocytes were double-stained for F4-80 (clone BM8) and Gr-1 (clone RB6-8C5) to allow the percentages of neutrophils (Gr-1 high, F4/80 negative), classical monocyte/macrophages (Gr-1 high, F4/80 positive) and non-classical monocyte/macrophages (Gr-1 low, F4/80 positive) to be distinguished.

In wild type mice neutrophils accounted for approximately 10% of total leukocytes during the resolution phase of zymosan-induced peritonitis (48-96 hours). In contrast the proportion of neutrophils in the exudates of galectin-3 null mice significantly increased from 48 to 96 hours with approximately 40% of leukocytes recovered being neutrophils (**Figure 5.2**).

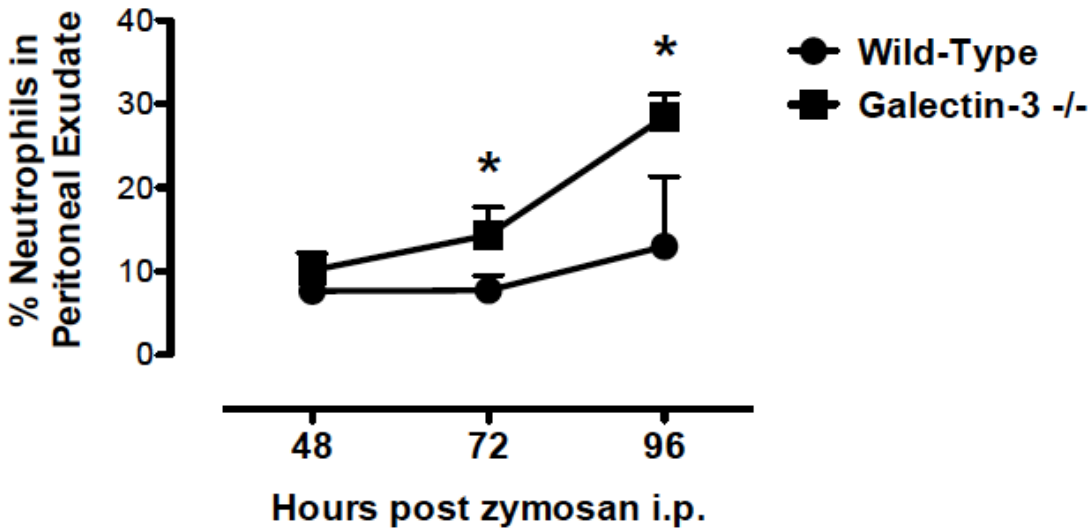


Figure 5.2: Neutrophil percentage collected from peritoneal exudate during the resolution phase of zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip and following 48, 72 and 96 hours peritoneal lavages were performed to collect exudates. Leukocytes were labelled with antibodies for F4/80 and Gr-1 and those cells that were Gr-1 high and F4/80 negative were identified as neutrophils. Data are expressed as mean ± SEM, n = 3-4 per group, * $P < 0.05$ vs wild type at same time point as analysed by two-way ANOVA with Bonferroni post hoc test.

At 48 hours 20% of the cells recovered from the peritoneum were classical monocytes/macrophages in both genotypes and this was not significantly altered at 72 hours although a trend towards a reduced number was seen in the wild type mice with approximately 15% of total leukocytes being classical monocyte/macrophages. By 96 hours no classical monocyte/macrophages could be detected in either strain of mice (**Figure 5.3**).

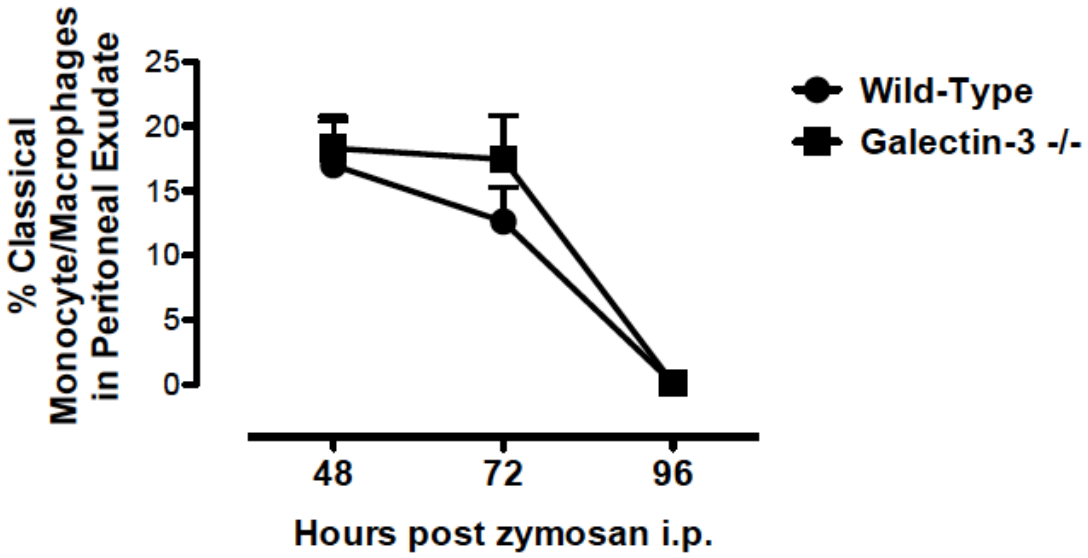


Figure 5.3: Classical monocyte/macrophage percentage collected from peritoneal exudate during the resolution phase of zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip and following 48, 72 and 96 hours peritoneal lavages were performed to collect exudates. Leukocytes were labelled with antibodies for F4/80 and Gr-1 and those cells that were Gr-1 high and F4/80 positive were identified as classical monocyte/macrophages. Data are expressed as mean ± SEM, n = 3-4 per group and analysed by two-way ANOVA with Bonferroni post hoc test.

At 48 hours approximately 40% of cells recovered were non-classical monocytes/macrophages in both genotypes and this 40% remained stable in galectin-3 null mice. In contrast the number of non-classical monocytes/macrophages significantly increased in wild type mice at 96 hours (**Figure 5.4**).

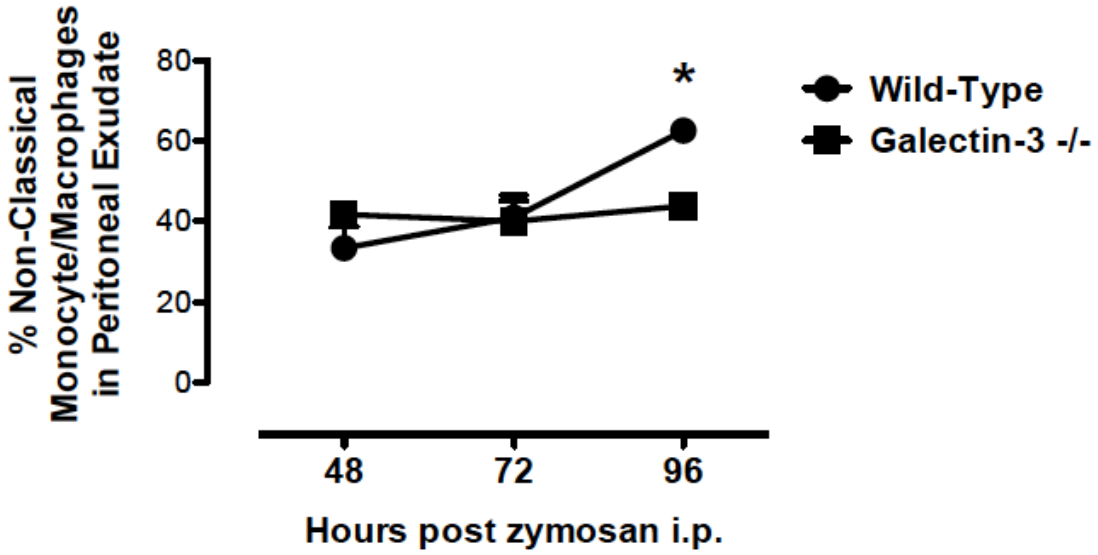


Figure 5.4: Non-classical monocyte/macrophage percentage collected from peritoneal exudate during the resolution phase of zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip and following 48, 72 and 96 hours peritoneal lavages were performed to collect exudates. Leukocytes were labelled with antibodies for F4/80 and Gr-1 and those cells that were Gr-1 low and F4/80 positive were identified as non-classical monocyte/macrophages. Data are expressed as mean ± SEM, n = 3-4 per group, * $P < 0.05$ vs galectin-3 null mice at same time point as analysed by two-way ANOVA with Bonferroni post hoc test.

Galectin-3 null mice have more neutrophils and less non-classical monocyte/macrophages at the later stages of zymosan-induced peritonitis suggesting that resolution is impeded in these mice. A previous report demonstrated that alternative activation of monocyte/macrophages is defective in galectin-3 null mice (MacKinnon et al., 2008) which may explain the reduced numbers of non-classical monocytes/macrophages found in this model. The mechanism behind the increased PMN counts observed during late stages of peritonitis in galectin-3 null mice is not known and therefore is further investigated here.

Galectin-3 is linked to neutrophil clearance through its ability to induce phosphatidylserine exposure, in the absence of apoptosis, on the neutrophil surface(Stowell et al., 2008b). A defect in this mechanism might therefore result in retention, or lack of clearance of PMN at the inflammatory site.

Leukocytes from the peritoneal exudate were labelled with Ly6G (clone 1A8) to identify neutrophils and then with Annexin V (AnxV) and propidium iodide (PI) to identify cells that were Annexin V positive and PI negative as these are not yet apoptotic, yet have exposed phosphatidylserine.

At 48 and 72 hours after zymosan injection approximately 20% of murine neutrophils in the peritoneal cavity were early apoptotic (Annexin V positive, PI negative) and this was comparable between wild type and galectin-3 null mice. Of the remaining neutrophils approximately 70% were positive for both Annexin V and PI and were thus apoptotic, while 10% were viable cells (Annexin V negative, PI negative). At 96 hours approximately 5% of neutrophils were early apoptotic and again this was comparable between the two strains of mice while the remaining 95% of neutrophils were viable cells (**Figure 5.5**).

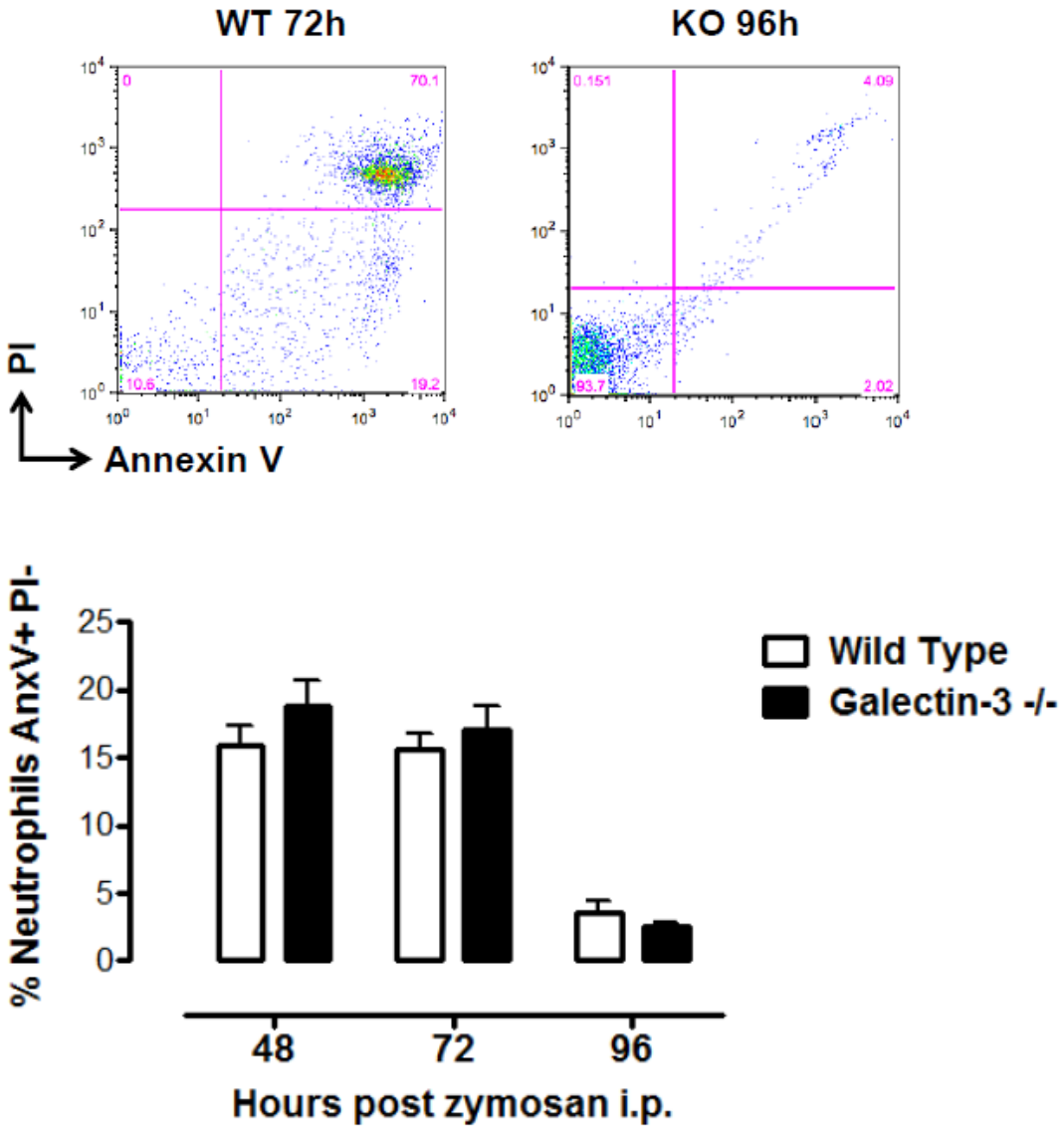


Figure 5.5: Exposure of phosphatidylserine of murine neutrophils in galectin-3 null and wild type mice following zymosan-induced peritonitis. Mice were injected with 1mg zymosan ip and following 48, 72 and 96 hours peritoneal lavages were performed to collect leukocytes. Neutrophils were specifically labelled with Ly6G antibodies and then with Annexin V and propidium iodide and the percentage of neutrophils that were Annexin V positive and PI negative was calculated. Representative dot plots showing Annexin V and PI double staining for wild type mice at 72 hours and KO mice at 96 hours. Data are expressed as mean ± SEM, n = 3-4 per group and analysed by two-way ANOVA with Bonferroni post hoc test.

A decrease in the number of neutrophils with exposed phosphatidylserine between 72 and 96 hours without an increase in apoptotic cells may indicate that an increase in efferocytosis occurs between these time points. To test this hypothesis leukocytes were collected from the peritoneal cavity at 96 hours post zymosan administration and labelled with an F4/80 antibody to specifically identify monocyte/macrophages; following this the cells were fixed and permeabilised using permeabilisation buffer

before labelling with a Ly6G antibody. These leukocytes were analysed using an AMNIS ImageStream X MK II, cells positive for F4/80 were assessed for their intracellular expression of Ly6G, which was indicative of efferocytosed neutrophils.

Only a small number of monocyte/macrophages had phagocytosed neutrophils visible inside the cell, approximately 2% in wild type and null mice. Detection of very low numbers could possibly be due to antigens on phagocytosed neutrophils being destroyed before staining occurred and thus substantial differences between genotypes could not be determined (**Figure 5.6**).

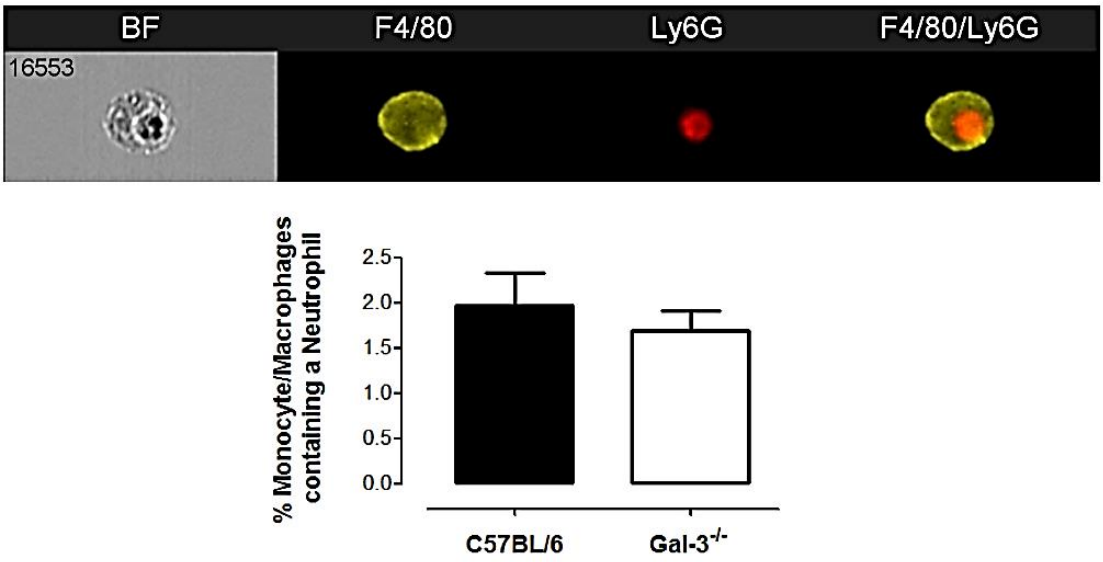


Figure 5.6: Efferocytosis of neutrophils by monocyte/macrophages at 96 hours post zymosan peritonitis. Mice were injected with 1mg zymosan ip and following 96 hours peritoneal lavages were performed to collect leukocytes. Monocyte/macrophages were specifically labelled with F4/80 and then fixed and permeabilised before labelling with Ly6G for intracellular neutrophils. The internalisation of neutrophils by monocyte/macrophages was assessed using an AMNIS ImageStream X MK II. Data are expressed as mean ± SEM, n = 4 per group and analysed by two tailed T test.

5.1.2 Localisation of Galectin-3 in Murine Neutrophils Following Recruitment to the Peritoneal Cavity

Galectin-3 is expressed throughout all cellular compartments and its localisation determines its function. When bound to the cell surface of neutrophils it is able to induce the exposure of phosphatidylserine without inducing apoptosis and also increases the production of reactive oxygen species and degranulation (Stowell et al., 2008b, Yamaoka et al., 1995). When expressed intracellularly in Jurkat T cells galectin-3 is able to bind Bcl-2 proteins and inhibit the apoptosis of the cells (Akahani et al., 1997). The actions of intracellular galectin-3 in neutrophils has not been elucidated

primarily because it is believed to be expressed at low levels in these cells, however as neutrophils also express Bcl-2 proteins this is a potential mechanism of action for galectin-3 within murine neutrophils (Moulding et al., 2001).

Murine leukocytes were collected from the peritoneal cavity of 6-week old C57BL/6 mice at 4, 24 and 48 hours post zymosan administration (peak neutrophil time points) and Ly6G was used as a specific marker for identifying neutrophils. Leukocytes were double-stained for the expression of galectin-3 and Ly6G on the cell surface or were permeabilised and the intracellular expression of galectin-3 assessed. The cytosolic expression was calculated by subtracting the cell surface expression from the total expression.

The localisation of galectin-3 was modulated in neutrophils over the course of the inflammatory response. At 4 hours galectin-3 was predominantly expressed in the cell cytosol (488.5 ± 10.243 vs 17.125 ± 0.491, P = 0.00001). At 24 hours post zymosan, galectin-3 was expressed on both the neutrophil surface and in the cytosol at equal levels (459.209 ± 76.538 vs 402.667 ± 34.801). At 48 hours post zymosan galectin-3 was once again expressed predominantly in the cytosol of the neutrophil (367.571 ± 33.788 vs 11.237 ± 3.741, P = 0.00001) **Figure 5.7**.

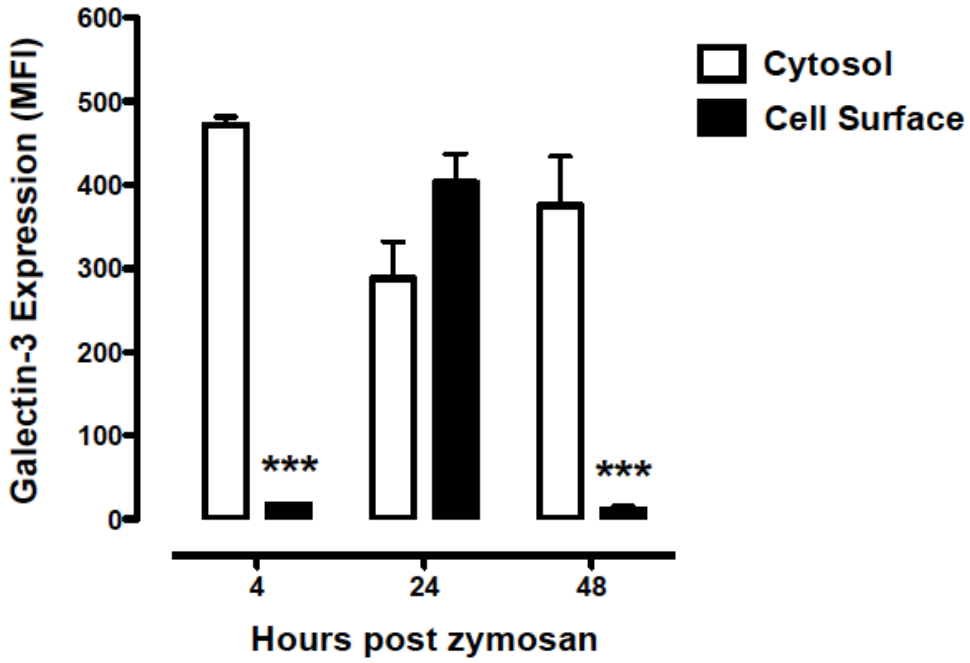


Figure 5.7: Cytosolic and cell surface expression of galectin-3 in murine neutrophils taken from the peritoneal exudate at 4, 24 and 48 hours post zymosan ip. Mice were injected with 1mg zymosan ip. and peritoneal exudates were collected at 4, 24 and 48. Leukocytes were double stained with anti-Ly6G (clone – 1A8) and anti-galectin-3 (clone – M3/38) on the cell surface and following permeabilisation to give total expression. The expression of galectin-3 was assessed using Flow Cytometry and the cytosolic galectin-3 expression calculated by subtracting the cell surface expression from the total expression. Data are expressed as mean ± SEM, n = 4 per group. *** P < 0.001 vs cytosol at same time point as analysed by two-way ANOVA with Bonferroni post hoc test.

5.1.3 Do Murine Neutrophils Produce Galectin-3?

Murine neutrophils expressed high levels of galectin-3 following extravasation to the peritoneal cavity (Figure 4.16), which was located inside the cell at 4 hours post zymosan suggesting the cells are capable of producing the lectin upon activation/transmigration.

To determine whether neutrophils express mRNA for galectin-3 a pure population of neutrophils was required. Macrophages and monocytes produce high levels of galectin-3 and thus express *lgals3* and would contaminate the sample even at low numbers. In order to obtain a pure population of neutrophils leukocytes were isolated from the peritoneal cavity and bone marrow of male 6-week old C57BL/6 mice as previously described (**section 2.3.4.1**) following 4-hour zymosan-induced peritonitis; the leukocyte suspensions were then subjected to negative selection for neutrophils. An aliquot of isolated neutrophils (200μL) was taken for purity assessment using Ly6G and Flow Cytometry; this demonstrated that approximately 80% purity could be reached for peritoneal leukocytes and 23% for bone marrow leukocytes as shown in Figure 5.8; therefore this isolation method was not suitable for collection of a pure population of murine neutrophils (**Figure 5.8**).

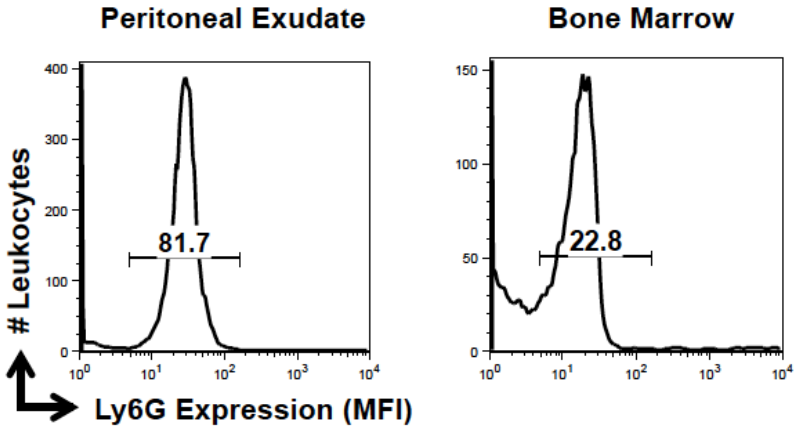


Figure 5.8: Neutrophil population purity following negative selection. Leukocytes were collected from peritoneal exudate and bone marrow following 4-hour zymosan-induced peritonitis. Leukocytes were negatively selected for neutrophils and an aliquot of the remaining neutrophil-rich sample was assessed for purity using a Ly6G antibody by Flow Cytometry. Bar shows percentage of sample positive for Ly6G.

Positive selection of neutrophils was then attempted using Dynabeads®, again on leukocytes collected from the peritoneal cavity and bone marrow following 4-hour zymosan-induced peritonitis on male 6-week old C57BL/6 mice. As the beads remain attached to the neutrophils it was not possible to assess the purity by Flow Cytometry as with the negative selection and thus PCR was used to assess purity as well as determine mRNA levels of *lgals3*. RNA was extracted using the RNeasy kit and the levels and purity were quantified using a Nanodrop as described in **section 2.3.10**. From this cDNA was synthesised and PCR performed using primers for *Ly6g, cd68* and *lgals9* for neutrophils, monocytes and eosinophil contamination respectively, *rpl32*

was used as a housekeeping gene and *lgals3* levels were determined. This data showed that all samples were contaminated with monocytes and eosinophils (**Figure 5.9**) and thus a pure population of neutrophils could not be isolated using positive selection.

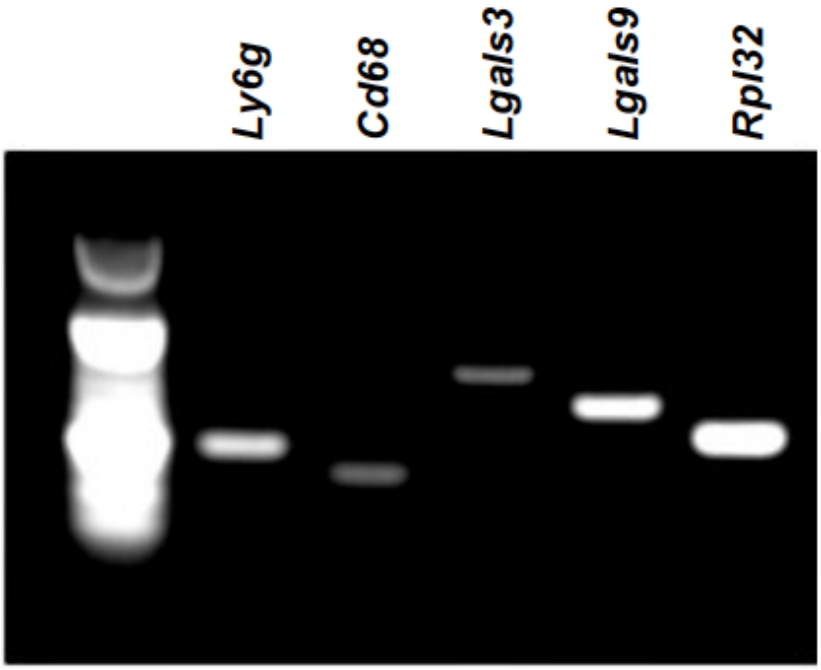


Figure 5.9: Neutrophil population purity following positive selection. Leukocytes were collected from peritoneal exudate following 4-hour zymosan-induced peritonitis. Leukocytes were positively selected for neutrophils and the neutrophil-rich sample was assessed for purity using PCR. Primers for *ly6g, cd68* and *lgals9* were used to determine levels of neutrophil, monocyte and eosinophils in the sample respectively. *Lgals3* was used to assess galectin-3 levels and *rpl32* was used as a housekeeping gene.

5.1.4 Do Murine Neutrophils Bind Galectin-3 Following its Release From Other Sources?

As it was not possible to isolate a pure population of murine neutrophils without monocyte contamination it could not be confidently determined that murine neutrophils produce galectin-3 however the lectin was detectable inside exudated neutrophils at 4 hours. If the galectin-3 was derived from other cell types and being bound by murine neutrophils upon their transmigration to the peritoneal cavity the lectin must be internalised by the cells and this was assessed using human neutrophils due to the difficulties in obtaining large numbers of peripheral blood neutrophils from mice.

Human PMN were incubated with either 10nM recombinant galectin-3, a concentration which is detected under pathophysiological conditions (Iurisci et al., 2000), or 1μM as a

positive control for 2 hours at either 37°C allowing cellular processes to occur or 4°C inhibiting them; the PMN were then washed with lactose to remove all surface bound lectin. The intracellular expression of galectin-3 was analysed using Flow Cytometry compared to vehicle-treated cells.

At concentrations close to that seen pathophysiologically (10nM) human neutrophils were unable to internalise galectin-3, as demonstrated by no change in the intracellular expression following incubation at 37°C compared to 4°C, however when an excess of galectin-3 was added the neutrophils internalised the lectin as shown in **Figure 5.10**.

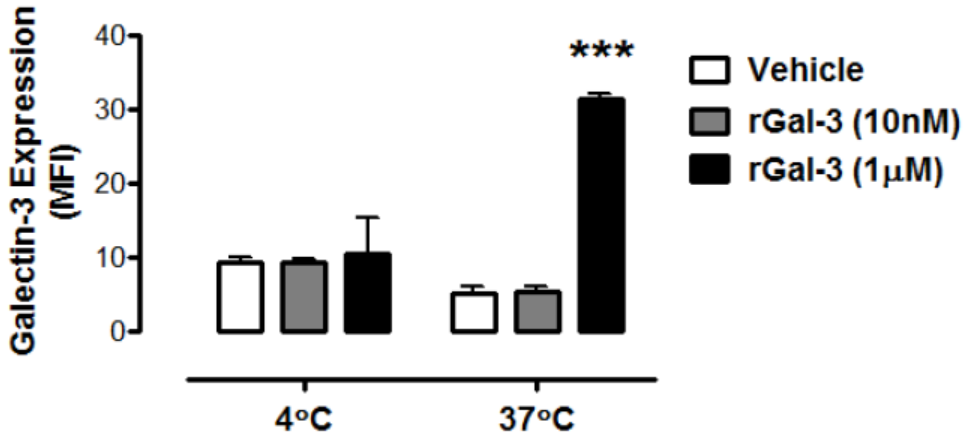


Figure 5.10: Internalisation of galectin-3 by activated human neutrophils. Human neutrophils were isolated from healthy volunteers using dextran sedimentation and then incubated with recombinant galectin-3 (10nM or 1μM) for 2 hours at either 4°C or 37°C, following this cells were washed with lactose to remove any surface bound galectin and then fixed overnight at 4°C. Fixed cells were permeabilised and labelled with antibodies for galectin-3 and the expression assessed by Flow Cytometry. Data are expressed as mean ± SEM, n = 3 per group, *** $P < 0.001$ vs all other treatments as analysed by two-way ANOVA with Bonferroni post hoc test.

An adoptive transfer model was used to determine whether murine neutrophils were able to bind or produce galectin-3. Bone marrow neutrophils were isolated from C57BL/6 mice using negative selection and labelled with CFSE, these cells were then injected into galectin-3 knock out mice via tail vein injection and 15 minutes later peritonitis was induced with 1mg zymosan i.p. Four hours later the peritoneal cavities were lavaged and the leukocytes obtained fixed in 1% PFA overnight; the following day the leukocytes were permeabilised and labelled with galectin-3 antibodies. Cells which were positive for CFSE were assessed for their expression of galectin-3.

Murine bone marrow neutrophils express low levels of galectin-3 compared to those that extravasate to the peritoneal cavity in response to zymosan-induced peritonitis.

When comparing galectin-3 expression levels from bone marrow neutrophils and CFSE-positive neutrophils collected from the peritoneal cavity of galectin-3 null mice following adoptive transfer a decrease was observed (**Figure 5.11**). This decrease can be explained by secretion of the lectin into the surrounding tissues during trafficking to the peritoneal cavity.

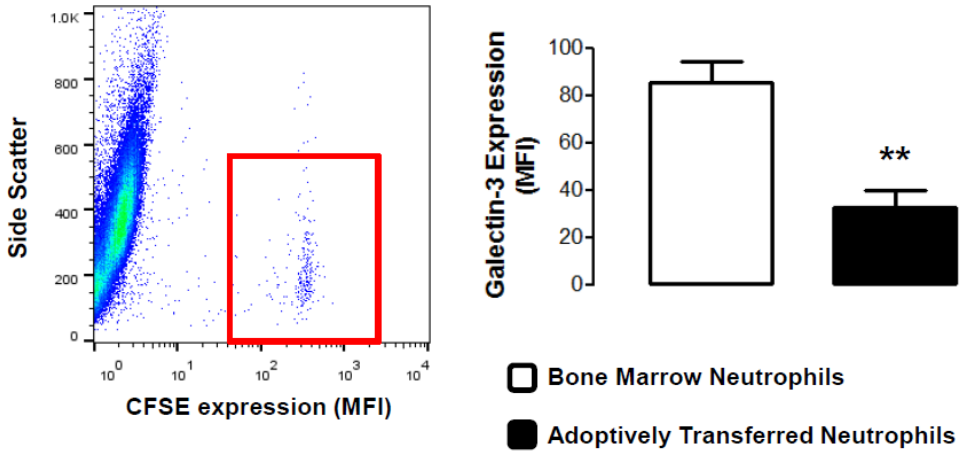


Figure 5.11: Galectin-3 expression in murine neutrophils following adoptive transfer of C57BL/6 neutrophils to galectin-3 null mice. Bone marrow neutrophils were isolated from male 10-week old C57BL/6 mice using negative selection and labeled with CFSE; these cells were transferred into galectin-3 null mice using i.v. tail vein injection and 15 minutes later peritonitis was induced using 1mg zymosan. Four hours later peritoneal lavages were performed and the collected leukocytes were fixed, permeabilised and assessed for their intracellular expression of galectin-3. Figure shows CFSE-positive cells as analysed by Flow Cytometry and graph comparing bone marrow neutrophils to adoptively-transferred neutrophils galectin-3 expression. Data are expressed as mean ± SEM, n = 4-6 per group, ** $P < 0.01$ vs bone marrow neutrophils as determined by two-tailed T-test.

This data strongly suggests that murine neutrophils do not produce galectin-3 but rather bind it following its release from other cellular sources. In order to determine the source of the galectin-3 an air pouch model of inflammation was used. The murine dorsal air pouch model is a manufactured cavity induced by injection of sterile air subcutaneously into the dorsal flank of the mouse; this cavity is absent of resident macrophages and thus a useful model removing a major source of galectin-3 that could bind to exudated neutrophils. Briefly 3mL sterile filtered air was injected subcutaneously into the back at day 0 and again at day 3, at day 6 zymosan (1mg/mouse) was injected into the pouch and following 4 and 24 hours the mice were sacrificed and the pouches lavaged. Collected leukocytes were labeled with Ly6G (clone 1A8) to identify neutrophils and then fixed and permeabilised to assess the intracellular expression of galectin-3 in these neutrophils.

Initially a differential leukocyte count was performed using Ly6G and F4/80 antibodies, this demonstrated that, similar to the peritonitis assay, neutrophils were the predominant leukocyte type at 4 and 24 hours post zymosan administration (**Figure 5.12**).

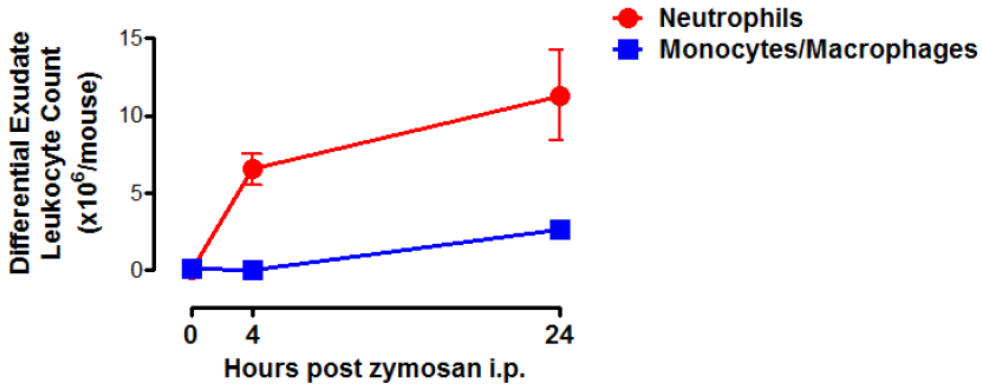


Figure 5.12: Differential leukocyte count for air pouch exudate at 4 and 24 hours post zymosan administration. An air pouch was established by injection of 3mL sterile, filtered air at days 0 and 3, at day 6 zymosan (1mg/mouse) was injected into the pouch and after 4 and 24 hours the mice were sacrificed and the air pouches lavaged. The collected leukocytes were labelled with Ly6G and F4/80 to determine numbers of neutrophils and monocyte/macrophages respectively. Data are expressed as mean ± SEM, n =4 per group.

The intracellular expression of galectin-3 was assessed by labelling fixed and permeabilised cells with galectin-3 antibodies. Galectin-3 levels in murine neutrophils collected from the air pouch were similar to the peritonitis model of inflammation with high levels of galectin-3 expressed at 4 hours post zymosan administration, which increased at 24 hours as shown in **Figure 5.13**.

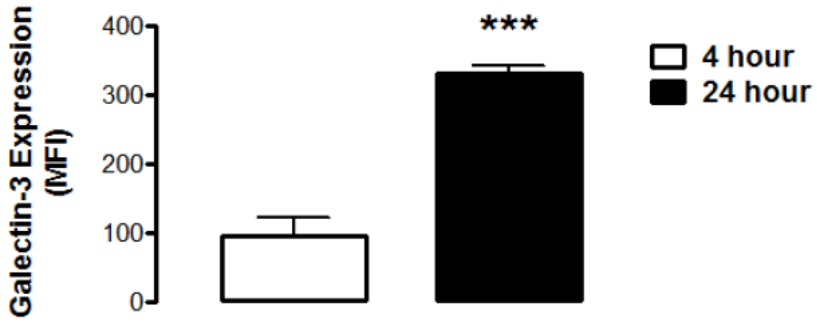


Figure 5.13: Galectin-3 expression in neutrophils recruited to the murine dorsal air pouch in response to 1mg zymosan over 4 and 24 hours. An air pouch was established by injection of 3mL sterile, filtered air at days 0 and 3, at day 6 zymosan (1mg/mouse) was injected into the pouch and after 4 and 24 hours the mice were sacrificed and the air pouches lavaged. The collected leukocytes were labelled with Ly6G and then fixed and permeabilised prior to labelling with galectin-3 antibodies. The intracellular expression of galectin-3 was determined by Flow Cytometry. Data are expressed as mean ± SEM, n =4 per group, *** $P < 0.001$ vs 4 hour as analysed by two-tailed T-test.

5.3 Discussion

Using a resolving model of inflammation, a difference in leukocyte recruitment was evident between control C57BL/6 and galectin-3 null mice with the latter demonstrating higher neutrophil and lower non-classical monocyte/macrophage percentages in the peritoneal cavity during the resolution phase. This difference in neutrophil number could not be explained by a deficiency in phosphatidylserine exposure. Imagestream analysis was utilised to identify whether efferocytosis levels were different between genotypes, however only low percentages of monocyte/macrophages contained neutrophils (approximately 2% in both genotypes) potentially due to antigen loss as a result of efferocytosis.

It is clear that neutrophils express galectin-3 intracellularly at 4 hours post zymosan injection and therefore neutrophils must either produce the lectin or internalise it following binding to the neutrophil surface. Unfortunately a pure population of neutrophils could not be obtained using either negative or positive selection in order to assess mRNA expression and therefore it was important to see if galectin-3 could be bound by cells following release from other cellular sources.

An adoptive transfer model demonstrated that in the absence of galectin-3 from other cellular sources (using null recipient mice), wild-type bone marrow neutrophils exhibited lower galectin-3 levels following extravasation into the peritoneal cavity. This is in contrast to the higher expression levels observed in peritonitis experiments using wild-type mice adding support to the hypothesis that galectin-3 is released from other sources that subsequently neutrophils can bind. Resident macrophages were unlikely to be the source of this galectin-3 as increased expression is still seen in neutrophils recruited to a manufactured air pouch that lacks resident macrophages.

CHAPTER 6:

DISCUSSION

6 Discussion

Galectins-1, -3 and -9 play important roles in inflammation via interactions with leukocytes. Various animal models of inflammation have demonstrated roles for galectins-1 and -9 in the attenuation of inflammation due to reduced leukocyte recruitment (Cooper et al., 2012, La et al., 2003, Sakai et al., 2011, He et al., 2009); while others show that a genetic knockdown of galectin-3 leads to decreased leukocyte recruitment, demonstrating a role for this lectin in perpetuating inflammatory responses (Colnot et al., 1998b, Hsu et al., 2000).

As myeloid cells are able to bind galectins-1, -3 and -9, yet these galectins may have opposing effects, it can be hypothesised that mechanisms are in place to allow specific galectins to be dominant dependent on the environment at a given point in time. Such mechanisms may include modulation of the cellular glycophenotype in response to the inflammatory milieu or specific cell-cell interactions leading to modifications of the binding permissibility for particular galectins, as they exhibit different affinities for different carbohydrate residues. This can be seen with naïve thymocytes which are unable to bind galectin-1 until treated with neuraminidase due to the presence of an α2,6-linked sialic acid capping structure on a galectin-1 receptor (Earl et al., 2010). Another possibility may be a modulation of the expression of galectins by the cells pertinent to the inflammatory response over the course of inflammation. This is seen with endothelial cells, which express increased levels of galectins-1 and -9 in response to activation while levels of galectin-3 are not affected (Thijssen et al., 2008).

The major aims of this thesis were to assess the PMN glycophenotype in response to transendothelial migration and activation along with the binding profile of galectins-1, -3 and -9. The galectin expression profile was also assessed in an *in vivo* resolving model of inflammation to determine the spatio-temporal expression of each of the galectins within myeloid cells. Finally an investigation into the cellular localisation and potential role of galectin-3 in the resolution process was performed.

It was demonstrated that the PMN glycophenotype is modulated by transendothelial migration; this modification occurs as a direct consequence of trafficking through the endothelium as it could not be mimicked by neutrophil activation or treatment of the neutrophils with conditioned medium from activated HUVECs. Secondly it was demonstrated that although lectin binding can estimate galectin binding permissibility for neutrophils it wasn't accurate enough to confidently determine binding, although this

has been previously demonstrated for T cells and endothelial cells (Toscano et al., 2007, Mercer et al., 2009, Croci et al., 2014). It can be hypothesised that neutrophil glycophenotypic changes are a result of a modulation of the glycoproteins expressed at the cell surface upon activation and not due to the actions of glycosylation enzymes within the cell. This discrepancy with T cells may be due to the shorter lifespan of the neutrophil or the more limited functionality of the neutrophils compared to T cells, which can differentiate into Th1 or Th2 cells depending on the extracellular milieu and thus may require more glycophenotypic modulation. It was found that the expressions of galectins-1, -3 and -9 are modulated over the course of a resolving inflammatory response allowing different galectins to be dominant at different times in the response. Finally a role was determined for galectin-3 in the resolution of inflammation as galectin-3 null mice demonstrated a failure to clear neutrophils and an excess of non-classical monocyte/macrophages at late time points (96 hours) suggesting a dysregulated resolution process compared to age- and sex-matched C57BL/6 controls.

6.1 Glycophenotype and Galectin Binding Profile of Neutrophils

Plant-derived lectins are a useful model for determining the cellular glycophenotype as they have well-defined specificities for glycan residues; these can also be used to assess lectin binding permissibility. Galectins-1, -3 and -9 exhibit different binding preferences for glycan residues and therefore a combination of lectins can be used to estimate galectin binding permissibility. Galectins are defined by their ability to bind β-linked galactose and thus RCA_{120} can be utilised as a marker for the overall permissibility for galectin binding (Goldstein et al., 1981, Baenziger and Fiete, 1979). Galectin-1 binds mono-LacNAc residues following removal of the α2,6-linked sialic acid capping structure and thus an increase in PNA binding and a concurrent decrease in SNA binding could be indicative of increased permissibility for galectin-1 binding (Stowell et al., 2004, Leppänen et al., 2005, Ahmad et al., 2002). Galectin-3 binds poly-LacNAc residues and the binding is enhanced by α-linked fucose residues and thus increased binding of LEL and UEA I could be indicative of increased galectin-3 binding permissibility (Rabinovich and Toscano, 2009, Merkle and Cummings, 1987). Galectin-9 binds poly-LacNAc residues expressed on complex tri- and tetra-antennary N-glycans following removal of the α2,6-linked sialic acid capping structure and thus increased binding of LEL and PHA-L with decreased binding of SNA can be used to

indicate an increase in the permissibility for galectin-9 binding (Rabinovich and Toscano, 2009, Sato et al., 2002a).

Studies have been previously published that utilise lectin binding assays to determine galectin binding permissibility in T cells and endothelial cells. Rabinovich et al demonstrated that Th1 cells have a lower level of sialylation compared to Th2 cells, which results in increased binding of galectin-1 to the Th1 cells and increased cell death (Toscano et al., 2007). Similar results were seen when an assessment of the glycophenotype of human duodenal samples was performed; Th1 and Th17 cells within the duodenum bound PNA with an increased affinity compared to Th2 cells while these Th2 cells had higher SNA reactivity suggesting increased sialylation of Th2 cells and reduced galectin-1 binding (Mercer et al., 2009). Another study demonstrated that glycophenotypic changes in the endothelium can impact galectin binding to these cells; treatment of the endothelium with immunosuppressive cytokines (TGF-β and IL-10) led to an increase in the expression of tri- and tetra-antennary N-glycans and poly-LacNAc residues with a concomitant decrease in α2,6-linked sialic acid, which led to an increase in the binding permissibility for galectin-1 (Croci et al., 2014). Taken together these data indicate that galectin binding permissibility can be extrapolated from changes in the cellular glycophenotype for T cells and endothelial cells and thus theoretically the binding of galectins to other cell types may also be deduced from lectin binding assays.

As the initial aims of this project were to characterise the glycophenotype and galectin binding phenotype of human neutrophils and to determine whether modulation occurs upon trafficking a panel of lectins was carefully selected that bind the most common terminal residues on glycan chains, particularly those which bind residues that are bound by galectins-1, -3 and -9 (Baenziger and Fiete, 1979, Lotan et al., 1975, Merkle and Cummings, 1987, Rogerieux et al., 1993, Hormia et al., 1983, Cummings and Kornfeld, 1982). Following this freshly isolated PMN were incubated with recombinant galectins to determine whether the binding matches the pattern indicated by the lectin binding assay.

It was initially demonstrated in this study that human PMN express a glycophenotype characterised by low levels of mono-LacNAc and α1,3-linked fucose residues and high expression of β-linked galactose residues, poly-LacNAc chains and α2,6-linked sialic acid with complex tri- and tetra-antennary N-glycans (**Figure 3.1**). This correlates with the findings of Babu et al in 2009, who showed via mass spectrometry that human

neutrophils have predominantly core 2 O-glycans and complex bi-, tri- and tetra-antennary N-glycans, both with sialylated and fucosylated branches (Babu et al., 2009, Haslam et al., 2008). The high level of sialylation present on freshly isolated human neutrophils is indicative of a low level of permissibility for galectins-1 and -9 binding (Stowell et al., 2004, Leppänen et al., 2005, Ahmad et al., 2002, Rabinovich and Toscano, 2009, Sato et al., 2002a). The low level binding of galectin-1 was confirmed by recombinant galectin binding assays however the binding of stable, recombinant galectin-9 to freshly isolated PMN was higher than indicated in the lectin binding assay. This would suggest a receptor for galectin-9 is constitutively expressed on the neutrophil cell surface and this corroborates findings from Vega-Carrascal et al who demonstrated the expression of Tim-3 on naïve human neutrophils (Vega-Carrascal et al., 2014).

Two populations of PMN were collected from the 6-well adhesion assay; those that had not adhered to the endothelial surface following 30 minute incubation and those that had. Non-adherent PMN had extremely high RCA_{120} reactivity and thus β-galactoside expression suggesting a high permissibility for galectin binding but no other significant modulations from the freshly isolated PMN glycophenotype were seen. This modulation is induced by an endothelial cell-derived factor as it is also seen in PMN that are in co-culture, but isolated from direct cell-cell contact with HUVECs. As the activated endothelium is known to be an important source of galectins it is possible that PMN that are trafficking PMN bind these more readily than those in the circulation (Thijssen et al., 2008).

The population of PMN that adhered to the endothelial monolayer following 30 minute incubation demonstrated significantly less binding of all lectins when compared to those that didn't adhere. The activation of neutrophils is a two-step process in which resting neutrophils are primed by a stimulant, including bacterial products, cytokines and chemokines; these primed neutrophils are then trafficked to an inflammatory site where activation with a secondary stimulant results in full activation (Hallett and Lloyds, 1995).

The phenotype of primed neutrophils differs significantly from naïve neutrophils in the peripheral circulation due to many factors; granules are mobilised to the surface upon priming leading to an increase in specific cell surface molecules including CD11b and CD16 (Faurschou and Borregaard, 2003) and the expression of metalloproteinases at the cell surface of the neutrophil also increases (i.e. ADAM17) resulting in cleavage of

proteins from the cell surface such as L-selectin (Walcheck et al., 2006). As L-selectin is a heavily glycosylated protein it can be hypothesised that the loss of this molecule and others may result in the overall decrease in glycosylation seen by the neutrophils.

Binding of galectins-1, -3 and -9 were not modulated by adhesion of the neutrophil to an endothelial surface; this was expected from the lectin binding results as although SNA reactivity decreased so did PNA and LEL reactivity suggesting that the reduction in α2,6-linked sialic acid residues was not exposing underlying mono- or poly-LacNAc residues. This somewhat supports the hypothesis that the decreased lectin reactivity in adherent compared to non-adherent PMN is due to a removal of cell surface glycoproteins and is not a result of glycosylation enzyme activity.

It is also pertinent to consider the role of plasma proteins in the modulation of the neutrophil glycophenotype; studies have shown that plasma proteins such as albumin and α1-antitrypsin bind to neutrophils in the circulation, these proteins are heavily glycosylated and are also shed from the cell upon activation and transmigration (McCarthy et al., 2014). Therefore it can be postulated that the release of these glycosylated proteins from the neutrophil cell surface is another cause of the decrease in glycosylation seen in the transmigration assay.

In the transwell transmigration assay two populations of neutrophils were collected once again; those that had neither adhered nor transmigrated and those that had trafficked through the endothelial monolayer. Although it could be theorised that the PMN that didn't traffic would have a similar glycophenotype to non-adherent cells in the 6-well assay this was not the case. This was most likely due to the increased length of incubation (90 minutes as opposed to 30 minutes) and thus potentially the prolonged activity of endothelial cell-derived factors on the PMN. These non-transmigrated PMN did not mimic the increase in β-galactoside residues seen in the 6-well assay but an increase in SNA reactivity was seen perhaps indicating a capping of newly exposed β-galactose residues following 90 minute incubation. An increase in fucose expression was also demonstrated in these non-transmigrated neutrophils, which was not seen in the 6-well assay. Neutrophils require an up-regulation of sialyl Lewis x motifs in order to tether and roll on the endothelium as demonstrated by the loss of these functions in mice lacking ST3Gal-IV and FucT-VII enzymes (Ellies et al., 2002, Malý et al., 1996); thus it can be hypothesised that following 90 minute incubation with activated HUVECs glycoproteins have been mobilised to the surface which potentiate trafficking.

A more effective way to determine the significance of these changes would be to use a single transwell transmigration assay to collect non-adherent, adherent and transmigrated PMN and assess thee glycophenotypic changes between the subsets.

When assessing PMN that had transmigrated through the endothelial monolayer an identical pattern was seen to those that had adhered to the HUVECs with all lectin reactivity significantly decreased. A similar pattern of decreased glycosylation was seen in murine PMN when comparing those in the peripheral circulation to those recruited to the peritoneal cavity in response to zymosan.

PMN that transmigrated through the endothelium demonstrated increased binding of recombinant galectin-1 compared to those that didn't adhere; this is consistent with previous reports that demonstrated that primed neutrophils have an increased propensity to bind galectin-1 (Almkvist et al., 2002). Transmigrated PMN had a primed phenotype characterised by release of secretory vesicles in this study. Transmigrated PMN did not demonstrate any increased binding of recombinant galectin-3 compared to those that did not transmigrate; galectin-3 is known to bind preferentially to activated neutrophils (Karlsson et al., 1998) and thus may explain the lack of reactivity seen in this assay as the neutrophils did not become fully activated. The binding of stable, recombinant galectin-9 was not changed when comparing PMN that did not transmigrate through an endothelial monolayer to those that did.

An attempt to recapitulate this in a human model of PMN recruitment to an inflammatory site using capsaicin-induced neurogenic inflammation gave significantly different results from both the *in vitro* trafficking assays and the *in vivo* murine assays. Fundamental differences can be noted between these assays including that the capsaicin-induced inflammation is neurogenic and thus driven by CGRP and substance P while both the *in vitro* and *in vivo* assays are driven by cytokine-induced inflammation (Helme and McKernan, 1985). A difference in the inflammatory site is also important as both the *in vitro* and *in vivo* studies were performed under sterile conditions while the capsaicin-induced inflammation recruited PMN to the buccal cavity, an area highly colonised by bacteria. Although no studies have yet been performed to assess the variation in neutrophil glycophenotype between sterile and infectious inflammation it was postulated that a difference may occur due to the observation in zebrafish embryos that neutrophils recruited in response to sterile inflammation have a higher propensity for reverse transmigration than those induced in response to infectious agents (Mathias et al., 2006, Kolaczkowska and Kubes, 2013).

PMN that had transmigrated to an inflammatory site, the buccal cavity in response to capsaicin demonstrated an almost complete loss of galectin-1 binding permissibility. As galectin-1 has anti-inflammatory effects on neutrophils (Stowell et al., 2009b, Dias-Baruffi et al., 2010, Cooper et al., 2008, La et al., 2003) it can be postulated that fully activated neutrophils lose their reactivity for galectin-1 to prevent these anti-inflammatory effects inhibiting the actions of the neutrophils. This may also explain the failure of resolution in chronic inflammation as anti-inflammatory pro-resolving proteins cannot bind to neutrophils as readily. These recruited PMN demonstrated a significant increase in the binding of recombinant galectin-3 compared to freshly isolated PMN, as galectin-3 is known to bind more readily to activated neutrophils (Karlsson et al., 1998) and thus it would be expected that neutrophils at the inflammatory site are more permissible for galectin-3 binding. The binding of stable, recombinant galectin-9 to neutrophils significantly increased at the inflammatory site compared to those in the circulation; high binding of galectin-9 is seen on freshly isolated neutrophils however this did not increase during adhesion or trafficking and was only evident at the inflammatory site suggesting the presence of additional receptor(s) for galectin-9 that are mobilised to the cell surface of the neutrophil upon activation. This may indicate that the trafficking process initiates changes in the glycophenotype that are modulated further depending on the local inflammatory milieu.

As a significantly different glycophenotype was seen in trafficked vs freshly isolated PMN it was important to delineate the cause of this modulation. PMN in the circulation have a naïve phenotype and have yet to release their granules (Borregaard et al., 1993) however upon activation these follow a specific pattern of granule release resulting in a sequential modulation of cell surface proteins to perform specific functions. As demonstrated by **Figure 3.18** and **Figure 3.19** trafficking through an endothelial monolayer promotes the release of secretory vesicles and secondary granules from the cells while just being in the presence of activated endothelial cells for 90 minutes is enough to promote the release of the secretory vesicles from the neutrophil. Thus the glycophenotypic changes seen during transmigration may be a result of granule release from the cell; unfortunately no work has yet been performed on the expression of glycosylation enzymes in neutrophil granules however it is known that many of the proteins translocated to the cell surface in response to granule release are glycosylated and thus may be the source of the modulation (Borregaard et al., 1993). To test this hypothesis freshly isolated PMN were treated with hrTNF-α or fMLP to induce the release of secretory vesicles and secondary granules respectively; no

changes were seen in the binding of any of the lectins suggesting granule release is insufficient to cause the modulations seen.

PMN treated with hrTNF-α and fMLP also did not demonstrate modulated binding of recombinant galectins-1 and -3 compared to freshly isolated cells suggesting that, as with the glycophenotypic changes, this is mediated by transmigration and not merely by granule release from the neutrophil. Galectin-9 binding was significantly increased upon treatment with fMLP, but not hrTNF-α; this adds evidence to the hypothesis that additional galectin-9 receptor(s) are translocated to the cell surface upon activation and further demonstrates that the secondary granules are most likely the location of this receptor(s).

It was then hypothesised that the modulations seen in response to trafficking were due to the actions of a HUVEC-derived factor on the neutrophil and thus freshly isolated PMN were incubated in co-culture with HUVECs but separated from direct cell-cell contact by a transwell insert. These conditions failed to modulate the PMN glycophenotype to the same extent as trafficking however as a control of unstimulated HUVECs co-cultured with freshly isolated PMN was not used it cannot be confidently stated that direct cell-cell contact mediates these changes.

The binding of galectins-1, -3 and -9 are all modulated on human neutrophils under the specific conditions analysed however the lectin binding assay was unable to predict these changes accurately; this is most likely due to the modulation being a result glycoprotein expression changes and not glycosylation enzyme activity. The leukocyte adhesion cascade for neutrophil recruitment is well documented and it is known that a great number of receptors are upregulated and downregulated from the neutrophil surface upon adhesion and transmigration (Wright and Cooper, 2014); as many of these are glycosylated and yet not galectin counter-receptors the limitations of using whole cell surface lectin binding assays to determine galectin binding permissibility to neutrophils become clear. As demonstrated for galectins-1, -3 and -9 the indicated permissibility of lectin binding to neutrophils is only an estimate and the results are different when galectin binding assays are used.

6.2 Galectin Expression of Leukocytes

The expression of galectins-1 and -3 in both human and murine myeloid cells during inflammation is well characterised by various groups (Novak et al., 2012, Ho and Springer, 1982, Sato and Hughes, 1994, MacKinnon et al., 2008, Gil et al., 2006b,

Truong et al., 1993, Farnworth et al., 2008). However the modulation of these proteins has not been fully investigated; thus this project aims to determine how their expression is modulated following transendothelial migration, inflammatory stimulation or recruitment to an inflammatory site. The expression of galectin-9 in human neutrophils has not yet been investigated and therefore this study provides novel information on the expression of this protein during inflammation.

Initially isolated human PMN were assessed for their expression of galectins at the cell surface and intracellularly and it was determined that galectins-1 and -9 are lowly expressed under basal conditions at both locations; this further decreases upon transendothelial migration, which correlates with the findings of Gil et al who determined that adhesion of human PMN to an endothelial monolayer decreases the expression of galectin-1 (Gil et al., 2006b)(Gil et al., 2006b). It has been postulated that galectin-9 can compensate for a loss of galectin-1 during inflammation following a study that showed increased galectin-9 mRNA in galectin-1 null mice challenged with carrageenan paw oedema (Iqbal et al., 2011); thus it can be hypothesised that these proteins share many properties and would be expressed at similar times during inflammation.

Galectin-3 is expressed at higher levels than galectins-1 and -9 in neutrophils and the expression is predominantly cell surface as determined by comparable levels of protein in both cell surface and intracellular flow cytometry assays. Transendothelial migration of human PMN did not alter the expression of this lectin.

In a more pathophysiological model of inflammation PMN were recruited to the buccal cavity in response to neurogenic inflammation and the expression of galectins-1, -3 and -9 compared to that seen in peripheral blood. Contrary to that seen in the transendothelial migration assay the levels of galectin-1 were not significantly modulated in PMN that had transmigrated to a site of inflammation and this was also true for galectin-3. Similar to what is seen during transendothelial migration galectin-9 levels significantly decrease upon transmigration to the buccal cavity in human neutrophils.

This study on the expression of galectins-1, -3 and -9 on human PMN confirms what is known in the literature; that the lectin expressions are modulated by inflammatory stimuli, however it is still unclear at which stage of inflammation these are important (Liu et al., 1995, Gil et al., 2006b). It can be hypothesised that different galectins are expressed predominantly at different stages of the inflammatory process and this

allows specificity of their actions. This was therefore investigated further using a model of zymosan-induced peritonitis in mice, a model that is well defined and resolves over a 96-hour time course (Navarro-Xavier et al., 2010, Ajuebor et al., 1999).

Following confirmation that the model is resolving, ELISAs were performed for galectins-1 and -3 to determine the peak expression time points for these lectins in the peritoneal cavity. Levels of both galectins were similar in untreated mice however upon inflammatory stimulation galectin-1 rapidly decreases while galectin-3 increases dramatically suggesting at this time point galectin-3 is dominant in the response. Galectin-3 is proposed to be important in leukocyte recruitment while galectin-1 inhibits this process therefore it would be expected that galectin-3 be dominant at 4-24 hours as this is the peak leukocyte recruitment period (Nieminen et al., 2008, Norling et al., 2008). A second peak in the galectin-1 level can be seen at 48 hours, a time point in which non-classical monocytes are being recruited and the resolution process has begun. This peak in galectin-1 expression at the start of the resolution process was also seen in a model of carrageenan-induced paw oedema (Iqbal et al., 2011) and may be indicative of a decrease in leukocyte recruitment as resolution begins. As galectin-3 is considered pro-inflammatory it would be expected to decrease at this stage however this did not occur and levels of the lectin remained high until 96 hours when resolution had been achieved, possibly suggesting a role for galectin-3 in resolution.

In this model of inflammation murine neutrophils do not express any galectin-1 or -9 at any time point in peritoneal exudate, peripheral blood or bone marrow; galectin-3, on the other hand is expressed by all murine neutrophils at relatively low levels except for those that have trafficked to the peritoneal cavity where levels are greatly increased. This contradicts published work by Sato and colleagues and Farnworth et al who suggested that murine neutrophils do not express any galectin-3 (Sato et al., 2002b, Farnworth et al., 2008); this may be due to collection methods as this assay kept neutrophils on ice until fixed, preventing secretion of the lectin from the cell while Sato and Farnworth incubated neutrophils at 37°C which would allow secretion. This work provides the first confirmation that murine neutrophils do express galectin-3 and that this increases as cells are recruited to a site of inflammation.

Murine monocytes in the bone marrow and peripheral blood express all three galectins at low levels and these are all significantly increased in cells that have trafficked to the inflammatory site. Due to large variation in the levels of each galectin within a time point it was postulated that monocyte/macrophage subtypes may be present that

express galectins differentially depending on their function. This would correlate with the current literature, as it appears that classical monocyte/macrophages secrete galectins-1 and -3 while non-classical monocyte/macrophages do not and thus express higher levels of galectins (Novak et al., 2012, MacKinnon et al., 2008, Sato and Hughes, 1994, Liu et al., 1995). While the expression levels of galectin-9 have not previously been assessed this correlates with the suggested compensation function for galectin-1 as these have similar effects (Iqbal et al., 2011).

It has previously been demonstrated that galectins-1 and -9 are anti-inflammatory proteins that act to decrease leukocyte recruitment and promote conversion of murine monocytes to a pro-resolving phenotype (Barrionuevo et al., 2007, Rostoker et al., 2013, Kashio et al., 2003) and thus increased expression of these proteins by non-classical monocytes/macrophages would be expected as these cells are highly implicated in the resolution process.

This work thus provides the first confirmation that galectin-3 is expressed, or taken up by murine neutrophils during inflammation and this is both temporally and spatially modulated with recruited neutrophils expressing significantly higher levels of the protein. Galectins-1, -3 and -9 are expressed by monocyte/macrophages, also at low levels and this is also significantly increased upon transmigration to an inflammatory site. It has also confirmed that non-classical monocyte/macrophages express higher levels of the galectins compared to classical monocytes possibly due to secretion of the galectins by classical monocyte/macrophages.

6.3 A role for Galectin-3 in Resolution?

The pro-inflammatory role of galectin-3 has been demonstrated in many models of inflammatory diseases; mice lacking this galectin are less susceptible to the development of T cell-driven pathologies including experimental autoimmune encephalomyelitis, hepatic fibrosis and antigen-induced arthritis (Jiang et al., 2009, Henderson et al., 2006, Forsman et al., 2011). This pro-inflammatory role has been further characterised in the early stages of an acute inflammatory response where galectin-3 null mice exhibit reduced leukocyte recruitment in response to thioglycollate-induced peritonitis and parasitic protozoan cutaneous infection (Colnot et al., 1998b, Hsu et al., 2000, Bhaumik et al., 2013).

Contrary to these findings other studies have demonstrated that galectin-3 null macrophages have defective phagocytic capabilities and alternative macrophage

activation; the lectin is also able to induce the exposure of phosphatidylserine and subsequent efferocytosis of human neutrophils without a concurrent increase in apoptosis and when taken together this suggests that galectin-3 may have a pro-resolution effect on leukocytes towards the end of an inflammatory response (Karlsson et al., 2009, Caberoy et al., 2012, Stowell et al., 2008b).

Galectin-3 is found at high levels in neutrophils that have been recruited to the peritoneal cavity following zymosan-induced peritonitis and while this level peaks during the initial stages of the response it remains elevated compared to peripheral blood cells until resolution has been reached at 96 hours (**Figure 4.16**). In monocyte/macrophages recruited to the peritoneal cavity the levels are greater than that seen in the peripheral blood and this peaks at 72 hours (**Figure 4.18**). The expression of galectin-3 at the later stages of the inflammatory response suggests a role for the lectin in the resolution of this response.

Initially it was essential to confirm that the lectin plays a role in the resolution of inflammation; this was assessed by once again utilising the zymosan-induced peritonitis mouse model but with galectin-3 null mice, as well as age- and sex-matched wild type controls, to determine whether the resolution is impaired in these mice. Although no differences could be seen in the number of classical monocyte/macrophages when comparing the wild type and the galectin-3 null mice (**Figure 5.3**), it was shown that the knock out mice had reduced numbers of non-classical monocyte/macrophages at 96 hours (**Figure 5.4**). This data is in line with previous reports showing defective alternative activation of macrophages in galectin-3 null mice (MacKinnon et al., 2008). Galectin-3 null mice also have increased levels of neutrophils at the later stages of inflammation compared to the wild type mice (**Figure 5.2**). This is different to what was seen in a model of thioglycollate-induced peritonitis in which galectin-3 null mice have fewer granulocytes at day 4 post thioglycollate treatment (Colnot et al., 1998b); marked differences are seen in the peritoneal inflammatory responses between thioglycollate and zymosan-induced peritonitis with a significantly prolonged response in thioglycollate-induced peritonitis thus it can be postulated that at day 4 the resolution process has yet to begin and the responses are not comparable at this time point (Davies et al., 2013). This data taken together confirms a difference in the resolution process when comparing galectin-3 null and wild type mice and thus assays were performed to assess the explanation for this difference.

Galectin-3 is known to function as an opsonin on cells, to prepare them for efferocytosis by phagocytes and to promote the exposure of phosphatidylserine (a well characterised “eat-me” signal) on activated neutrophils and thus it was important to determine whether this was defective in neutrophils from galectin-3 null mice (Sano et al., 2003, Karlsson et al., 2009, Stowell et al., 2008b). No difference was seen between the strains of mice when assessing the binding of Annexin V, which identifies cells with exposed phosphatidylserine or the percentage of F4/80 macrophages containing Ly6G-labelled neutrophils suggesting that this mechanism is not defective in these mice.

In order to better characterise the role of galectin-3 on murine neutrophils it was important to determine the cellular localisation of this protein. Galectin-3 is known to promote the efferocytosis of neutrophils when bound to cell surface receptors by inducing the exposure of phosphatidylserine and by acting directly as an opsonin (Sano et al., 2003, Stowell et al., 2008b, Karlsson et al., 2009); galectin-3 can also inhibit apoptosis when bound intracellularly through interactions with Bcl-2 proteins (Akahani et al., 1997). It was therefore important to characterise the location of galectin-3 in murine neutrophils at the peak of neutrophil recruitment. At 4 hours galectin-3 was predominantly intracellular, by 24 hours this had translocated to the cell surface, which may potentially be a mechanism of clearing satiated neutrophils by facilitating efferocytosis. This is supported by the fact that at 48 hours galectin-3 was localised inside the cell, potentially suggesting that any cells that had cell surface lectin were cleared by phagocytes.

Galectin-3 was expressed intracellularly at 4 hours in the peritoneal cavity; this could either be due to production of the lectin by activated neutrophils, or due to binding of the lectin to neutrophil cell surface receptors following its release from other cellular sources and internalisation. Initially attempts were made to isolate a pure population of murine neutrophils from the peritoneal exudate and bone marrow in order to assess differences in the mRNA expression for *Lgals3,* however it was not possible to obtain a pure population of neutrophils. Therefore it was essential to determine whether galectin-3 can be bound and internalised by activated neutrophils. Human neutrophils were isolated from healthy donors as it is easier to obtain a pure population of human neutrophils than murine neutrophils. Following activation of these cells with fMLP the neutrophils were incubated with a physiologically relevant concentration of galectin-3 (10nM) and with a non-physiological concentration as a positive control, whilst it

appears that galectin-3 can be internalised at 1μM any internalisation at physiological concentrations was below the level of detection by Flow Cytometry.

Although it couldn't be clearly demonstrated that galectin-3 binds to neutrophils and is internalised *in vitro* it is still a possibility that could occur *in vivo* and thus it was important to determine whether galectin-3 is secreted from other cell types. Murine bone marrow neutrophils express low levels of galectin-3 compared to those at the inflammatory site and thus these were isolated from wild type mice, fluorescently labelled and administered intravenously to galectin-3 null mice. Peritonitis was induced in galectin-3 null mice and galectin-3 expression was determined in these fluorescent, wild-type neutrophils. If galectin-3 was produced by neutrophils in response to inflammation these cells would demonstrate increased galectin-3 levels, however if the lectin binds following its release from other cell types there would be no change as these mice do not produce galectin-3. The galectin-3 expression of wild type neutrophils did not increase in the galectin-3 null mice; this would be expected only if the neutrophils generated the lectin, therefore the results support the theory that galectin-3 binds following release from other cellular sources.

The role of resident macrophages in the secretion of galectin-3 was easy to assess using a model of murine dorsal air pouch inflammation; a cavity is manufactured in which no resident macrophages are located and zymosan is injected into this cavity. Upon trafficking of neutrophils to this site of inflammation neutrophil galectin-3 levels were assessed and found to be very similar to that seen in the peritoneal cavity.

Although this assay provided evidence that excluded resident macrophages as the source of galectin-3 bound to murine neutrophils it could not discount other cellular sources as in both models the neutrophils must pass through endothelial cells and stromal cells to reach the site of inflammation, where lots of other inflammatory cells are present that are capable of secreting galectin-3 in response to inflammation, for example monocytes and endothelial cells.

CHAPTER 7:

CONCLUSIONS AND FUTURE DIRECTIONS

7 Conclusions and Future Directions

In conclusion this study has demonstrated that human and murine neutrophils exhibit a modulation of their glycophenotype upon trafficking through an endothelial monolayer, however unlike T cells and endothelial cells this does not correspond to changes in the galectin binding profile. This is most likely due to the neutrophil cellular glycoprotein expression being markedly altered upon trafficking rather than actions of glycosylation enzymes as demonstrated in the other cell types. A previous study assessed the glycophenotype of naïve neutrophils by mass spectrometry (Babu et al., 2009) and thus could be a useful tool for determining the changes seen during transmigration as this method determines overall glycosylation changes rather than modulation of the terminal residues only, this may help to determine whether the changes seen are due to removal of ligands from the surface of the neutrophil or enzymatic activity.

Galectin binding permissibility is also modulated upon trafficking, with naïve neutrophils being more permissive to the binding of anti-inflammatory galectin-1 while activated neutrophils at the inflammatory site are more permissive to binding galectin-3 and also galectin-9, a novel finding of this study that has yet to be further investigated. The role of galectin-9 on human neutrophils has yet to be characterised, this study demonstrated that galectin-9 binds to naïve human neutrophils at high levels and this increases dramatically upon activation of the cells thus further investigation should take place using these two populations of cells including:

1. Annexin V and PI staining should be performed on naïve and activated human neutrophils following treatment with stable, recombinant galectin-9 to determine whether this lectin induces apoptosis or the exposure of phosphatidylserine in either population of neutrophil
2. Flow chamber assays should be used to determine the role of galectin-9 on the interaction between HUVECs and neutrophils; galectin-9 has been postulated to act in compensation for galectin-1 (Iqbal et al., 2011) and as it is known that galectin-1 inhibits neutrophil transmigration (Cooper et al., 2008) it can be hypothesised that galectin-9 would perform similar roles
3. The constitutive receptor for galectin-9 on human neutrophils has been identified as Tim-3 (Vega-Carrascal et al., 2014), this study demonstrated a second receptor for galectin-9 is located in the secondary granules of human

neutrophils and thus identification of this receptor would be of great importance for the study of galectin-9 on human neutrophils

The expression of galectins-1, -3 and -9 are spatially and temporally modulated upon trafficking of monocytes from bone marrow to peripheral blood to an inflammatory site, in this study the peritoneal cavity. These are all expressed at low levels by bone marrow and peripheral blood cells but significantly increase upon trafficking to an inflammatory site. Higher levels of these proteins were found in non-classical vs classical monocytes/macrophages as previously determined in the literature. A recent paper demonstrated that the resolution phase of an acute inflammatory response is followed by an influx of monocyte-derived macrophages and resident macrophages that act to bridge the innate and adaptive immune responses; determination of the galectin expression profile of these cells would add a further layer of information to this study and help to fully characterise the modulation of galectin expression over a resolving inflammatory response (Newson et al., 2014).

Galectin-3 is found in murine neutrophils, another novel finding of this study, and the levels significantly increased as the neutrophils trafficked to an inflammatory site; previous studies have designated galectin-3 as a pro-inflammatory lectin however due to increased expression during the resolution phase of zymosan-induced peritonitis it can be postulated that this protein plays a role in resolution. This was further investigated and it was found that the resolution process was dysregulated in galectin-3 null mice however the exact mechanism of this was not delineated in this study; it was demonstrated that neutrophil apoptosis and the exposure of phosphatidylserine were unchanged in these mice suggesting no defect in efferocytosis was occurring. Secondly it was shown that wild type bone marrow neutrophils adoptively transferred into galectin-3 null mice did not demonstrate increased galectin-3 following transmigration to the peritoneal cavity; this suggests that the galectin-3 is being bound to the neutrophil however does not confirm that murine neutrophils are incapable of producing the lectin. Further investigation into the mechanism underlying potential pro-resolution roles of galectin-3 on murine neutrophils should be performed including:

1. *In situ* hybridisation studies or single cell PCR to confidently determine whether galectin-3 is produced by murine neutrophils or binds to the cell following transmigration to the inflammatory site
2. The use of other resolving inflammatory models in galectin-3 null mice to determine whether the resolution is defective in other models including the

K/BxN serum transfer model of arthritis, a model of arthritis in which neutrophils are mediators

3. The determination of galectin-3 expression in neutrophils over the course of a chronic inflammatory response such as zymosan-induced peritonitis (10mg zymosan/mouse) to determine whether a difference in the expression of galectin-3 is seen when resolution does not occur

8 References

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