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AN INVESTIGATION OF THE CELLULAR UPTAKE AND MECHANISMS OF ACTION OF MGN-3 ON INFLAMMATORY PROCESSES

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A thesis submitted in fulfilment of the requirements of the Manchester Metropolitan University for the degree of Master of Science (by Research)

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Manchester Metropolitan University

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List of abbreviations

A	Absorbance
ANOVA	Analysis of variance
APC	Antigen presenting cell
Au	Gold
AX	Arabinoxylan
Bcl-2	B-cell lymphoma 2
BRM	Biological response modifier
Cdc42	Cell division control protein 42
CD	Cluster of differentiation
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD11b	Cluster of differentiation 11b
CD11c	Cluster of differentiation 11c
CD14	Cluster of differentiation 14
CD16	Cluster of differentiation 16
CD33	Cluster of differentiation 33
CD69	Cluster of differentiation 69
CFU	Colony forming unit
CM	Complete medium
C3a	Complement 3a
C5a	Complement 5a

Da	Dalton
DAMPs	Damage associated molecular patterns
DC	Dendritic cell
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
FAE	Follicle-associated epithelium
FBS	Foetal Bovine Serum
Fc	Fragment crystallisable
FITC	Fluorescein isothiocyanate
FL1-A	Fluorescence parameter 1
FSC	Forward scattered light
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GI	Gastrointestinal
GTPase	Guanosine triphosphatase
HBSS	Hanks balanced salt solution
HPBM	Human peripheral blood monocytes
IFN-γ	Interferon-gamma
IgG	Immunoglobulin type G
IL	Interleukin
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta

IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
iNOS	Inducible nitric oxide synthase
IRF-3	Interferon response factor-3
LD50	Lethal dose 50%
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
M cells	Microfold cells
MD2	Myeloid differentiation protein 2
MFI	Median fluorescent intensity
MGN-3	Maeda, Ghoneum, Ninomiya-3
MHC-II	Major histocompatibility complex class II
MRSA	Methicillin resistant *Staphylococcus aureus*
MyD88	Myeloid differentiation factor 88
M0	Macrophage type 0
M1	Macrophage type 1
M2	Macrophage type 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B

NK	Natural killer
NO	Nitric oxide
P	Probability
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
PP	Peyer's patches
PMA	Phorbol 12-myristate 13-acetate
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
SEM	Scanning electron microscopy
SSC	Side scattered light
Syk	Spleen tyrosine kinase
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TLR-2	Toll-like receptor 2
TLR-4	Toll-like receptor 4
TNFα	Tumour necrosis factor alpha

TRIF	TIR domain-containing adaptor inducing interferon-beta
VD-3	1,25-dihydroxyvitamin D3
VEGF	Vascular endothelial growth factor
WGP	Whole glucan particles
WGPD	Whole glucan particles (WGP) dispersible
WGPS	Whole glucan particles (WGP) soluble

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Abstract

The alarming rate at which pathogens are becoming resistant to conventional therapies is a reason for the urgent need to develop alternative strategies to combat bacterial infections. MGN-3 (biobran) was discovered and named after the three scientists: Maeda, Ghoneum and Ninomiya. It is an enzymatically modified rice bran arabinoxylan, which can enhance the host immune system by mediating macrophage function. The aim of this study was to investigate the effect of MGN-3 on the clearance of methicillin-resistant *Staphylococcus aureus* (MRSA) by human U937 macrophages. The uptake of MGN-3 by U937 macrophages was measured by a phloroglucinol colorimetric assay and visualised by confocal microscopy. An *in vitro* host-pathogen assay (n=24) was performed following exposure of macrophages to MGN-3 (0.5 to 2.0 mg/ml) and/or lipopolysaccharide (LPS) at 1.0 μg/ml or 5.0 μg/ml for 24 hours. The assay was repeated in the presence of receptor inhibitors, to identify potential molecular mechanisms of action. Bacterial internalisation by U937 macrophages was visualised by scanning electron microscopy (SEM).

The study demonstrated approximately 29% of total available MGN-3 was taken up by U937 macrophages. There was a significant ($P<0.05$) dose-dependent increase in clearance of MRSA by U937 macrophages in the presence of MGN-3 compared to the negative control. SEM confirmed internalisation of MRSA by U937 macrophages following MGN-3 treatment. However, MGN-3 inhibited LPS-induced phagocytosis, suggesting both polysaccharides may compete as ligands for the same receptor(s) but that LPS is more potent at inducing phagocytosis. TLR-4 and dectin-1 receptor inhibition reversed the effects of MGN-3 or LPS treatment, suggesting both MGN-3 and LPS mediate phagocytosis at least in part through these receptors.

In conclusion, this study established that MGN-3 significantly promotes phagocytosis of MRSA by U937 macrophages in a dose-dependent manner via TLR-4 and dectin-1. Moreover, MGN-3 mediated LPS-induced phagocytosis, suggesting competition for the same receptor(s) and highlighting the potential of MGN-3 to modulate excessive LPS-induced pro-inflammatory responses. Collectively, these findings may ultimately lead to novel potential therapeutic strategies that can be used alongside antibiotic treatments to combat MRSA infection, thereby reducing the over reliance on antibiotic usage.

1. Introduction

1.1 Dietary fibre

Naturally, dietary fibre is present in various foods such as vegetables, cereals and nuts (Dhingra et al., 2012). It is a complex carbohydrate composed of soluble and insoluble fibre (Zhang et al., 2016b). Dietary fibre is analogous carbohydrates or edible plant materials, which are fermented partially or completely within the large intestine, but resistant to hydrolysis in humans by the small intestines (Li and Komarek, 2017). Short chain fatty acids, which are linked to anti-carcinogenic activity, are produced after fermentation (Lattimer and Haub, 2010).

Components of dietary fibre include polysaccharides, oligosaccharides, lignin, pectin, cellulose, hemicellulose, waxes and gums (Dhingra et al., 2012). Dietary fibre is typically broken down into two main categories, with differing physiological properties based on water solubility (Li and Komarek, 2017). Soluble fibre such as pectin and pentosans can regulate blood glucose, lower cholesterol and postpone gastric emptying, whilst insoluble fibre such as cellulose and lignin can encourage intestinal flora proliferation and increase laxation (Dai and Chau, 2017; Li and Komarek, 2017).

Hemicelluloses are cell wall polysaccharides that contain β-1, 4 glucosidic linkages, bound to glucose unit backbones (Dhingra et al., 2012). Within its structure is a variety of sugars, most commonly, arabinose, xylose and mannose (Dhingra et al., 2012).

Nutrients such as vitamins and minerals are sourced from dietary fibre and whole grains, along with phytochemicals such as β-glucan, inulin and phenolics (Lattimer and Haub, 2010).

Consumption of dietary fibre is associated with improved human health, including reduced risk of cardiovascular disease, type 2 diabetes mellitus and colon cancer (Mendis et al., 2016)

1.2 Gastrointestinal (GI) physiology

The gastrointestinal (GI) tract functions to absorb nutrients from foods, following a series of digestive processes such as mechanical breakdown and salivary and gastric enzyme secretion (Peate, 2018). The small intestine is the main area of absorption, in which digestible macronutrient subunits, micronutrients, vitamins and minerals are absorbed. The large intestine is primarily involved in water and microfloral metabolite uptake, including uptake of ammonia, urea and short chain fatty acids, and remaining nutrients (usually at low levels) (Brownlee, 2011).

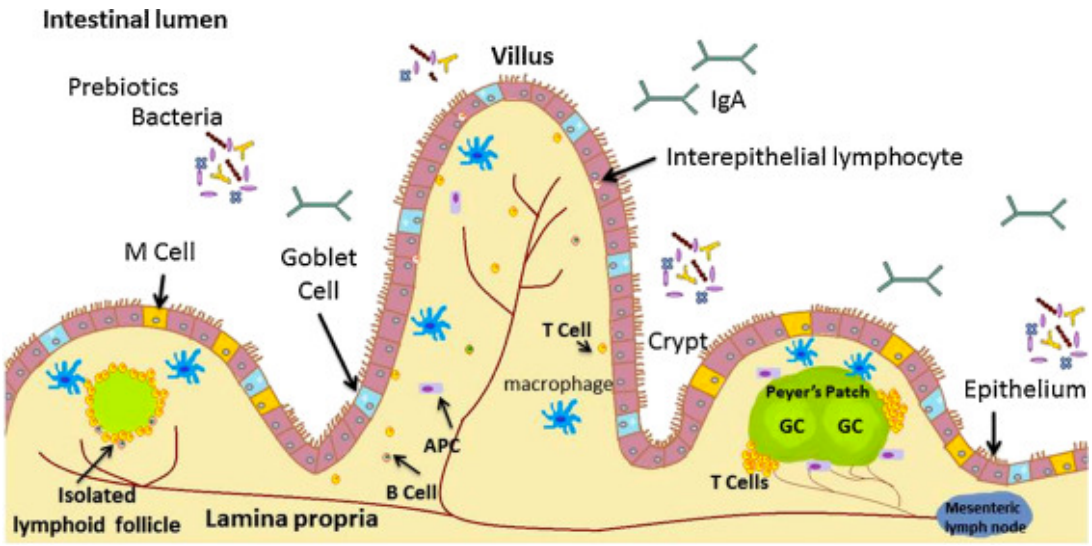

Figure 1: The gut-associated lymphoid tissue (GALT). Epithelial boarder between immune cells, Peyer's Patches and mesenteric lymph nodes etc. and the intestinal lumen (Mendis et al., 2016).

1.2.1 GI immune function

The GI mucosa acts as a barrier, keeping bacteria, toxins and digestive enzymes from interaction with the rest of the body (Kim et al., 2010). Tolerance to benign microorganisms by the gut-associated lymphoid tissue (GALT) is vital for homeostasis (Brownlee, 2011). The GALT (Figure 1) encompasses the Peyer's Patches (PP), lamina propria and mesenteric lymph nodes that contain dendritic cells and lymphocytes (Kiyohara et al., 2010). The follicle-associated epithelium (FAE) surrounds aggregated lymphoid tissues to give rise to the PP (in the small intestine) (Jung et al., 2010). Chronic inflammatory disorders can be a result of loss of tolerance, leading to conditions such

as Crohn's and coeliac disease (Bain and Mowat, 2014). Immunosenescence is also associated with decreased or loss of tolerance (Kobayashi et al., 2013).

M (microfold) cells are specialised enterocytes, contained within the FAE, along with an abundance of infiltrated macrophages, dendritic cells (DCs) and B- and T- cells. Transcytosis of bacteria and antigens by M cells, to underlying immune cells and PP sampling can either initiate or inhibit an immune response. However, PP are also a possible point of systemic entry for pathogens. Several bacterial species, such as *Escherichia coli* and *Salmonella typhimurium* are able to gain systemic access via adherence to M cells (Jung et al., 2010).

The protective potential of the colonic mucosal barrier appears to be increased by various types of dietary fibre, including wheat bran and alginate. Dietary fibre has been suggested to reduce the number of potentially harmful metabolites (Brownlee, 2011). It has been proposed that M cells are able to transport dietary fibre to the underlying immune cells, through the PP to activate local cytokine production (Volman et al., 2008; Mendis et al., 2016).

1.3 Systemic immune function

The initial protection provided by the innate immune system is via epithelial barriers, the lining of the gastrointestinal and urinary tract and the skin (Volman et al., 2008). Leukocytes involved in the acute stages of innate immunity include macrophages, neutrophils and natural killer (NK) cells (Volman et al., 2008).

Complement is involved in pathogen defence, inflammation and maintenance of homeostasis as part of the innate immune system (Merle et al., 2015). Complement activation occurs immediately after pathogen encounter and is initiated through either classical, alternative or mannose-activated pathways (Merle et al., 2015; Silawal et al., 2018). In addition to its involvement in innate immunity, T cell, B cell and antigen presenting cell (APC) function is regulated by complement (Wills-Karp, 2007).

Adaptive immunity can be activated by interaction with the innate immune system (Mendis et al., 2016). Adaptive immunity is highly specific, with slower initiation compared to the non-specific innate immunity (Volman et al., 2008). Once activated, B-

lymphocytes produce antigen specific antibodies, which upon repetitive interaction with the same antigen, responses are enhanced (Volman et al., 2008). Cytotoxic and helper T-lymphocytes are involved in cell lysis and cytokine production respectively (Volman et al., 2008).

1.3.1 Inflammation

Inflammation is a tightly regulated protective immune response to potentially harmful stimuli (Guo et al., 2015). Acute inflammation is an immediate local response to infection or tissue damage, usually lasting hours or days (Peate, 2011). Acute inflammation presents as pain, swelling, redness, heat and impaired function (Peate, 2011). Chronic inflammation, on the other hand, usually causes a dampened, constant response, ultimately leading to tissue damage (Franceschi and Campisi, 2014). Persistent pathogen infection can be a result of insufficient inflammation (Guo et al., 2015).

Inducers of inflammation (Figure 2) include pathogen associated molecular patterns (PAMPs) which are microbial surface molecules that are targeted by the innate immune system and damage-associated molecular patterns (DAMPs) which are released from damaged host cells (Mendis et al., 2016; Varela et al., 2018). Mannose and toll-like receptors (TLRs), for example, are pattern recognition receptors (PRRs) able to distinguish PAMPs (Mendis et al., 2016). Activation of TLRs subsequently stimulates protein kinase recruitment and activation, followed by the activation of transcription factors such as interferon response factor-3 (IRF-3) and nuclear factor κB (NF-κB), leading to enzyme, protein and cytokine transcription (Mendis et al., 2016). The inflammatory cytokines that are released include interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα) (Varela et al., 2018). During this process, an inflammatory response is initiated, followed by resolution and repair (Italiani and Boraschi, 2014).

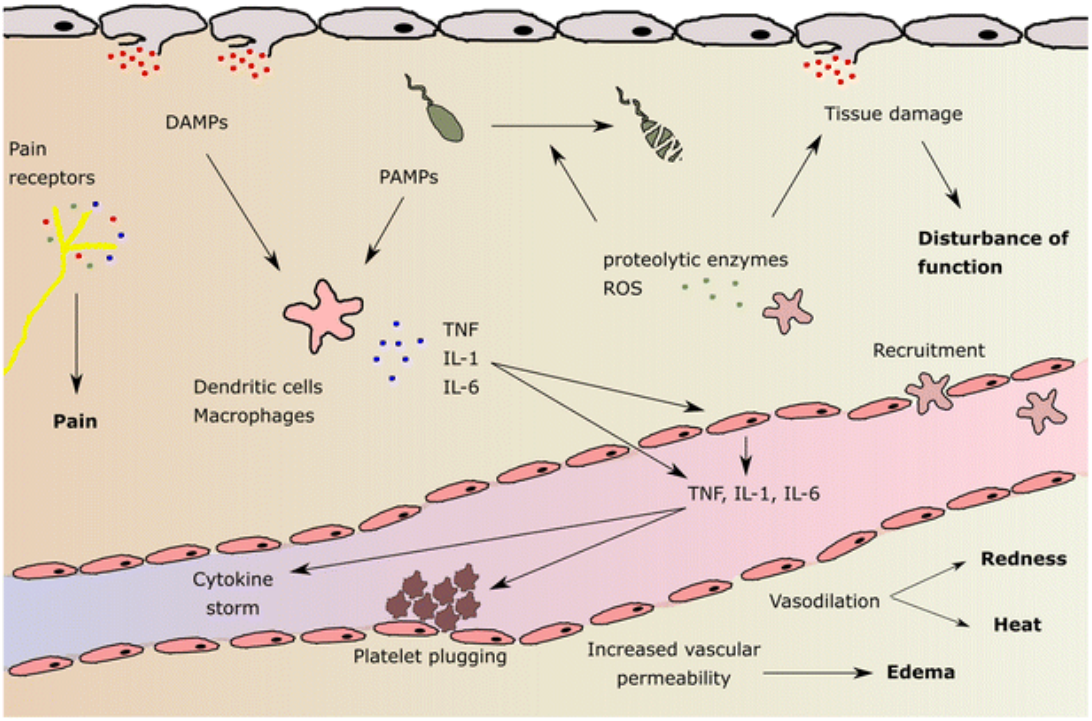

Figure 2: The inflammatory process (Varela et al., 2018). The diagram shows the process of inflammation starting from pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) receptor stimulation and how the five signs of inflammation occur.

Neutrophils are the first cells present during an inflammatory response, they recruit monocytes to the area via chemoattractants (Varela et al., 2018). Both neutrophils and macrophages synthesise reactive oxygen species (ROS) after stimulation (Varela et al., 2018).

A crucial component of the innate system and inflammation is the activation of an inflammasome, which are multimeric complexes (Sharma and Kanneganti, 2016). Formation of the inflammasome is facilitated by intracellular ROS (Varela et al., 2018). Inflammasomes essentially causes caspase-1 activation to induce pyroptosis (inflammatory cell death) via production of interleukin-18 (IL-18) and interleukin-1 beta (IL-1β) (Sharma and Kanneganti, 2016).

Anaphylatoxins such as complement 3a (C3a) and complement 5a (C5a) in the complement cascade, can stimulate inflammation by initiating an oxidative burst in neutrophils and macrophages (Merle et al., 2015). C3a also shows anti-inflammatory properties in some cells (Merle et al., 2015).

1.3.2 Monocytes and Macrophages

Circulating blood monocytes are precursors of macrophages and represent ~10% of leukocytes in the human body, whilst macrophages constitute 10-15% of total cell counts in normal conditions, with numbers peaking during an inflammatory response (Italiani and Boraschi, 2014). Monocytes are heterogenous cells, with the ability to differentiate into macrophages and dendritic cells as they exit the vascular endothelium via diapedesis, in the presence of infection (Yang et al., 2014; Boyette et al., 2017). They provide innate host defences in the form of pathogen recognition, phagocytosis, production of nitric oxide (NO), ROS, inflammatory cytokines and myeloperoxidase (Italiani and Boraschi, 2014; Fang et al., 2017). Macrophages are one of the major cell types involved during pathogen encounter and inflammation (Ginhoux and Jung, 2014). Wound healing, debris clearance, immune surveillance, pathogen defence and inflammatory response induction and resolution are the collective functions of tissue macrophages (Italiani and Boraschi, 2014).

Uncommitted (M0) macrophages can be polarised to pro- and anti-inflammatory phenotypes, known as M1 and M2 macrophages respectively (Italiani and Boraschi, 2014; Tarique et al., 2015). M1 macrophages present during acute periods of infection/injury and produce cytokines such as TNFα, inducible nitric oxide synthase (iNOS) and IL-6 to induce inflammation (Olingy et al., 2017). M2 macrophages are seen at latter stages and encourage arteriogenesis via production of interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF) (Olingy et al., 2017). NO produced by M1 macrophages acts as an effector for microbicidal activity, whilst ornithine, produced by M2 macrophages stimulates fibrosis, collagen and polyamine synthesis for cell proliferation and repair (Italiani and Boraschi, 2014). The M1 and M2 monocytic subtypes can be identified by the following protein expression profiles in human blood; $CD14^{hi}CD16^{-}$ and $CD14^{+}CD16^{+}$, respectively (Olingy et al., 2017). Metabolism affects the phenotype of macrophages, M1 macrophages are glycolytic but M2 macrophages oxidise fatty acids (Varela et al., 2018).

Most antigen encounters occur in the human intestine. In the intestinal mucosa, macrophages are amongst the most abundant leukocytes and are crucial for homeostasis (Bain and Mowat, 2014). Intestinal macrophages express cluster of

differentiation 11c (CD11c) along with major histocompatibility complex class II (MHC-II) (Bain and Mowat, 2014). Unlike macrophages present elsewhere, contact to stimuli such as ingested bacteria, does not cause pro-inflammatory reactions by mucosal macrophages. They also lack the ability of nitric oxide generation and respiratory burst (Bain and Mowat, 2014).

1.3.3 Phagocytosis

Phagocytosis (Figure 3) is the recognition and cellular uptake of particles larger than 0.5 μm by phagocytes, into a phagosome using a process called endocytosis (Gordon, 2016; Fang et al., 2017). Pathogen recognition causes extension of pseudopodia which surrounds the particle (Richards and Endres, 2014). Following internalisation, the phagosome maturates, by fusing with lysosomes to produce a phagolysosome: a microbicidal vacuole (Rosales and Uribe-Querol, 2017). Particles are destroyed via secretion of lysozyme and proteases from the phagolysosome and generation of toxic reactive oxygen compounds by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (Merle et al., 2015).

This process is important for pathogen ingestion but also for homeostasis and clearance of apoptotic cells (Rosales and Uribe-Querol, 2017). Monocytes, macrophages, dendritic cells and neutrophils are very efficient at phagocytosis and are described as professional phagocytes (Arandjelovic and Ravichandran, 2015). Non-professional phagocytes include epithelial and endothelial cells that are unable to engulf microorganisms but can eliminate apoptotic cells (Rosales and Uribe-Querol, 2017).

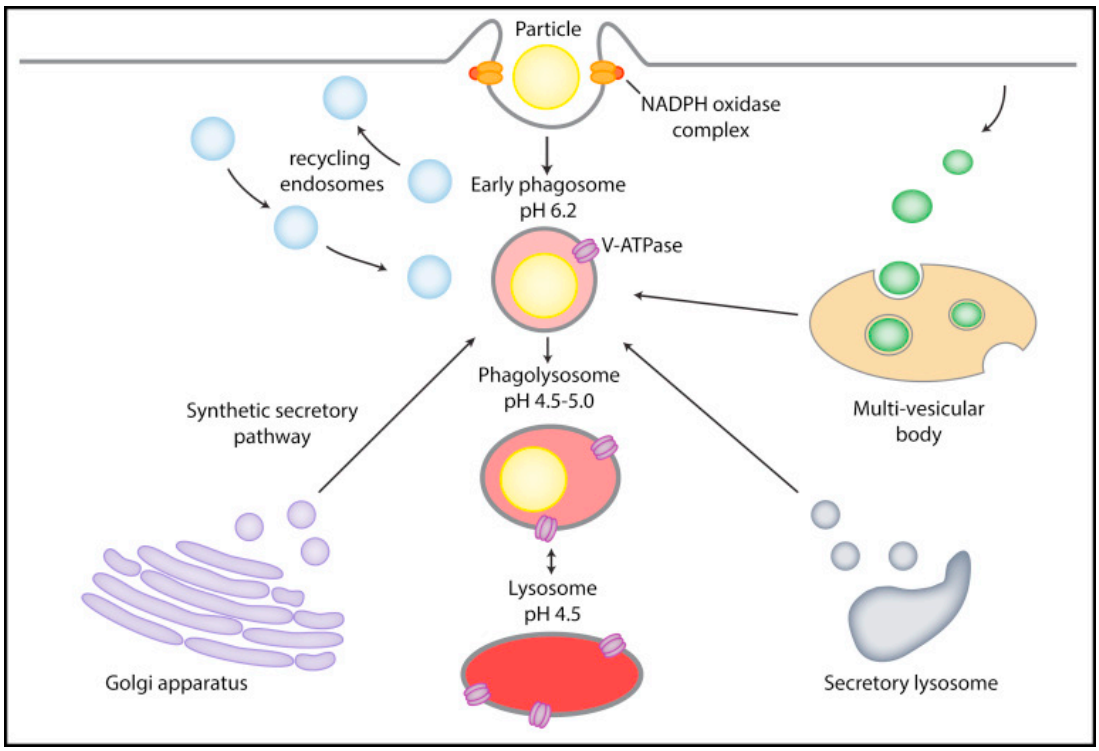

Figure 3: The Phagocytic Pathway (Gordon, 2016). A diagram showing the process of phagocytosis; internalisation into the early phagosome, which matures into a phagolysosome with help of the Golgi apparatus and fusion with lysosomes. pH of the phagolysosome lowers to acidic conditions for microbicidal activity.

Receptors involved in phagocytosis can be opsonic or non-opsonic. Opsonic receptors recognise opsonins such as complement, antibodies and mannose-binding lectin from the host, which bind and target microorganisms (Rosales and Uribe-Querol, 2017). These opsonic receptors include, the fragment crystallisable (Fc) and complement receptors (Rosales and Uribe-Querol, 2017). Non-opsonic receptors include CD33, Dectin-1 and scavenger receptors; these recognise microorganisms directly (Rosales and Uribe-Querol, 2017).

TLRs are sensors, they do not enable phagocytic entry, but instead they stimulate uptake and signalling by cooperating with other non-opsonic receptors (Gordon, 2016).

Opsonization by C3 fragments initiates different cellular processes compared to immunoglobulin type G (IgG) opsonization (Merle et al., 2015). Activation by complement receptor 3 (CR3) causes a slower reaction involving guanosine triphosphatase (GTPase) Rho dependant actin polymerization, whilst Fcγ receptor activation results in quicker engulfment via GTPase Rac and cell division control protein 42 (Cdc42) mediated membrane extensions (Merle et al., 2015).

1.4 Arabinoxylan (AX)

Arabinoxylan (AX) is a hetro-polysaccharide, composed of β-(1-4)-linked D-xylopyranosyl residues connected to α-L-arabinofuranose side chains (Hald et al., 2016; Fadel et al., 2017). The arabinose functional groups are often linked to the C(O)-2 and/or C(O)-3 positions of the xylose units, depending on the source of AX (Sahasrabudhe et al., 2016). The C(O)-5 position of arabinose may also consist of ester linked phenolic acids (Mendis et al., 2017).

Dietary AX is primarily found in cereal grains including corn, wheat and rice bran, and is a major component in whole grain dietary fibres (Lattimer and Haub, 2010; Sahasrabudhe et al., 2016). The abundance of AX in these food groups, which constitutes a large proportion of the human diet, suggests that AX is introduced in large quantities to the GI tract (Mendis et al., 2016). AX is quickly fermented by colon microflora, much like soluble fibres, in the GI tract (Lattimer and Haub, 2010).

AX exerts immunomodulatory properties such as phagocytosis augmentation and anti-tumour activities in mice (Zhou et al., 2010; Cao et al., 2011).

Xylose is metabolised in the pentose phosphate pathway, via two main routes of entry; the xylose isomerase pathway and the redox pathway, common amongst bacteria and eukaryotes respectively (Mendis et al., 2016).

The similarities between lipopolysaccharide (LPS) and AX from rice bran and corn husk, in terms of molecular structure suggests that TLR-4 might be a possible receptor for AX (Zhang et al., 2015).

1.5 MGN-3/Biobran

MGN-3/Biobran, hereby referred to as MGN-3, is an enzymatically modified arabinoxylan extracted from rice bran (Ghoneum and Jewett, 2000; Ooi et al., 2017). Rice bran is acquired from milling rice, and is a source of dietary fibre, vitamins, proteins and lipids, with links to numerous health benefits (Ghoneum and Agrawal, 2011). Shitake mushroom (*Lentinus edodes mycelia*) derived carbohydrate hydrolysing enzymes are used for MGN-3 modification (Perez-Martinez et al., 2015). AX is the major

chemical structure of MGN-3, consisting of a xylose and an arabinose polymer in the main and side chain respectively (Noaman et al., 2008).

The safety of MGN-3 has been confirmed and is stated as a non-toxic agent. It presented negative for mutagenicity in the Ames test, genotoxic, antigenicity and subchronic toxicity testing in guinea pig and rat models have also established the non-toxic state of MGN-3 (Ghoneum and Gollapudi, 2011). It has been shown that >36g/kg is classed as the lethal dose 50% (LD50) for MGN-3 (Ghoneum and Gollapudi, 2011).

1.5.1 MGN-3 and immunomodulation

MGN-3 is a potent biological response modifier (BRM), which can enhance host immune system function by augmentation of macrophage, NK, T and B cell function and interferon-gamma (IFN-γ) and TNF-α production (Gollapudi and Ghoneum, 2008).

Anticancer properties of MGN-3 include: *in vivo* cancer cell apoptosis stimulation, tumour growth inhibition in mice and human breast cancer sensitisation to chemotherapy agents (Badr El-Din et al., 2008; Noaman et al., 2008; Ghoneum et al., 2014). MGN-3 with conventional cancer treatment, has been shown to have synergistic anti-tumour properties against certain cancers, for example hepatocellular carcinoma and breast cancer (Perez-Martinez et al., 2015). In addition to interventional cancer therapies, MGN-3 has been described to improve overall survival of hepatocellular carcinoma adult patients in a clinical trial (Bang et al., 2010). A study by Ghoneum and Gollapudi showed enhancement of yeast-induced apoptosis of breast cancer (MCF-7) cells, whilst an earlier study indicated MGN-3 induced sensitisation to apoptosis by decreasing B-cell lymphoma 2 (Bcl-2) expression in human leukemic cells (Ghoneum and Gollapudi, 2003; Ghoneum and Gollapudi, 2005).

Induction of NK cell cytotoxicity against adult tumour cells both *in vitro* and *in vivo* can also be achieved (Ghoneum and Gollapudi, 2011; Perez-Martinez et al., 2015). Paediatric tumour cell lines have also been tested; an improvement in NK cytotoxicity *in vivo* and *in vitro* was also observed upon MGN-3 stimulation (Perez-Martinez et al., 2015). MGN-3 can enhance maturation and activate human dendritic cells *in vitro* for cytokine production (Cholujova et al., 2009; Ghoneum and Agrawal, 2011). Dendritic cells,

activated by MGN-3 can prime CD8+ cells to express higher levels of granzyme B, encourage CD4+ T cell proliferation and cytokine production (IL-10, 17 and IFN-γ) (Ghoneum and Agrawal, 2014).

High doses of MGN-3 resulted in M0 to M1 macrophage modification, which released pro-inflammatory cytokines, TNFα, IL-6 and IL-8 (Perez-Martinez et al., 2015).

MGN-3 mediated increase of TNFα production in peripheral blood lymphocytes is dose dependant, the increase being higher at the concentration of 1 mg/ml compared to 0.1 mg/ml (Ghoneum and Jewett, 2000). In peripheral blood lymphocytes, MGN-3 caused increased CD69 expression and also IFN-γ; however this was at lower levels in comparison to TNFα (Ghoneum and Jewett, 2000).

Discovery of radio-resistance properties have suggested potential uses for MGN-3 (Ghoneum et al., 2013). An *in vivo* mice study found that MGN-3 treatment provided protection to the parameters of the complete blood count, complete bone marrow cellularity and weight of the spleen, against irradiation-induced damage (Ghoneum et al., 2013). MGN-3 can also enhance antioxidant scavenging enzyme activity along with free radical reduction in tumour bearing mice with antioxidant disturbances (Noaman et al., 2008; Ghoneum et al., 2013).

Another *in vivo* study involving mice with Ehrlich carcinoma showed tumour inhibitory activity by MGN-3 (Ghoneum and Agrawal, 2011). These anti-cancer properties were associated to the T and NK cell activation and T regulatory lymphocyte generation abilities in patients with metastatic or locally restricted tumours (Lissoni et al., 2008).

The safety of MGN-3 was confirmed upon human investigation in clinical trials on cancer patients and analysis of blood chemistry (Ghoneum and Matsuura, 2004; Ghoneum et al., 2013).

A recent study demonstrated that one month of daily MGN-3 supplementation (500 mg/day) increased the activity of NK cells in the geriatric population, in a randomised, double-blind, placebo controlled human trial (Elsaid et al., 2018). The mechanism of action is currently unknown; however, it is suggested that MGN-3 may activate protein kinase C via stimulation of early signalling events or cause an increase of NK granzyme B and granular perforin levels (Elsaid et al., 2018).

1.5.2 Beta-glucan (β-glucan)

β-glucan is a natural polysaccharide and functional food ingredient, which is present in MGN-3 (Ghoneum et al., 2013; Stier et al., 2014). It is a carbohydrate linked with glucose molecules which is present in the cell walls of fungi and yeast, as a main structural component (Volman et al., 2008).

The macromolecular structure of β-glucan differs depending on the source (Volman et al., 2008). In oat and barley, β-glucans are unbranched with 1,3 and 1,4 β-linked glycopyranosyl residues, whereas the unbranched β-glucan from bacterial cell walls consists of only 1,3 β-linked glycopyranosyl residues (Volman et al., 2008).

β-glucan has been shown to express antioxidant properties, anticancer activity and modulate immune responses (Ghoneum et al., 2013; Byun et al., 2016). Human peripheral blood mononuclear cell proliferation can be induced by β-glucan, along with monocyte derived dendritic cell maturation (Chan et al., 2009). Short chain fatty acid production from β-glucan is suggested to be a plausible mechanism for its metabolic effects (Lattimer and Haub, 2010).

1.6 Cell surface receptors

Dietary fibre is recognised by PRRs such as TLRs and C type lectin receptors such as Dectin-1 (Sahasrabudhe et al., 2016). Receptors allow the cells to interact with the external environment for nutrient transport and signal transduction (Shankaran et al., 2007). Physical properties of ligands affect receptor behaviour such as binding specificity (Shankaran et al., 2007).

1.6.1 Toll-like receptors (TLRs)

TLRs are PRRs which are required for immuno-regulation and mediation of inflammatory cytokine production, found on the surface of monocytes, macrophages and dendritic cells (Fang et al., 2017). TLRs found in humans comprise TLR1 – 10, some of which are located on the cell surface whilst others are intracellular (Kawasaki and Kawai, 2014).

Bacterial and viral agents are recognised by TLRs, these include nucleic acids, proteins and lipids (Sahasrabudhe et al., 2016). Many bacterial lipopolysaccharides are recognised by TLRs, leading to activation of signalling pathways involved in endocytosis (Mendis et al., 2016; Fang et al., 2017). TLR-2 can be activated by inulin-type fructan to prevent pathogen entry via intestinal barrier enhancement (Sahasrabudhe et al., 2016). The structure of TLR-4 is comprised of a toll/interleukin-1 receptor (TIR) domain linked to an extracellular domain composed of leucine-rich repeats (Kuzmich et al., 2017). TLR signalling can coordinate both the innate and adaptive immunity through the stimulation of cytokine and chemokine expression (Plato et al., 2013). Two signalling pathways can be activated by TLRs after engagement, including the MyD88-dependant pathway and the TIR domain-containing adaptor inducing interferon-beta (TRIF)-dependant pathway (Kawasaki and Kawai, 2014). Both pathways lead to the activation of mitogen-activated protein kinases (MAPKs) and NF-κB (Kawasaki and Kawai, 2014).

1.6.2 Dectin-1 receptor

β-glucan is proven to bind to the Dectin-1, a type II transmembrane protein receptor that binds and recognises the β-1-3 and 1-6 linkages (Chan et al., 2009; Sahasrabudhe et al., 2016). These specific PAMPs activate NF-κB and are expressed by *Aspergillus fumigatus* and *Candida albicans* for example (Geijtenbeek and Gringhuis, 2009). Dectin-1A and Dectin-1B are correspondingly, the full length and stalk-less variants of the Dectin-1 receptor. Not only is Dectin-1 activated by β-glucan but by AX also (Sahasrabudhe et al., 2016).

Dectin-1, when activated can mediate phagocytic pathogen detection, phagocytosis and cytokine production (Chan et al., 2009; Gordon, 2016). This receptor is expressed on innate immune cells such as macrophages, DCs and neutrophils (Chan et al., 2009).

1.7 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a powerful inducer of inflammation, found in the outer membrane of gram-negative bacteria such as *Escherichia coli* (*E. coli*) (Zhang et al., 2013; de Lima et al., 2014). There are three components that make up LPS; the O-antigen

polysaccharide, lipid A and core oligosaccharide (Shimada et al., 2012). Immune cells, especially macrophages are stimulated by the lipid A fraction of LPS (Shimada et al., 2012).

LPS is recognised by TLR-4, which leads to pro-inflammatory cytokine production (de Lima et al., 2014). LPS interaction requires myeloid differentiation protein 2 (MD2) to associate with TLR-4 to form a heterodimer called the TLR4-MD2 complex (Choi et al., 2009). TLR4-MD2-LPS complex formation stimulates myeloid differentiation factor 88 (MyD88) to initiate pro-inflammation signalling (Fang et al., 2017). These signalling cascades subsequently activate protein kinase B (PKB) and phosphatidylinositol-3-kinase (PI3K) (Fang et al., 2017).

1.8 Methicillin resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus (*S. aureus*) is a non-motile, gram positive coccus with a diameter of around 0.6 μm and is usually found on human skin (Green et al., 2012; Vitko and Richardson, 2013). It is both a commensal and an opportunistic pathogenic bacterium (Tong et al., 2015).

Methicillin resistant *Staphylococcus aureus* (MRSA), are strains of *Staphylococcus* that has established a resistance against β-lactam antibiotics, including cephalosporins and penicillins due to the production of β-lactamase (Lovering et al., 2012; Moellering, 2012; Pathare et al., 2015). MRSA was identified initially in England in 1961, a few years after methicillin development, and is a common cause of infection in healthcare and the community (Green et al., 2012; Habib et al., 2015). Almost 50% of all *S. aureus* infections is caused by MRSA (Adani et al., 2018). It can cause toxic shock syndrome, bacteraemia, septicaemia and surgical site infections (Vitko and Richardson, 2013).

The prognosis of infection with MRSA is poor, especially in MRSA related bacteraemia with a mortality rate of 90 days (Bal et al., 2017). *S. aureus* is one of the leading causes of bacteraemia, showing elevated levels of mortality and morbidity and costs in association with MRSA (Adani et al., 2018).

1.8.1 Types of MRSA

Healthcare-associated/nosocomial MRSA are usually confined to healthcare facilities and results in bloodstream, respiratory and urinary tract infections (Boswihi and Udo, 2018). The risk factors of healthcare-associated MRSA include prolonged hospitalisation, especially in intensive care, old age, antibiotic use and use of intramuscular devices (Boswihi and Udo, 2018). The rate of MRSA-related nosocomial pneumonia has seen an increase over the past fifty years, especially in the ageing population (Kawanami et al., 2016). Significant mortality rates and substantial healthcare costs are associated with this type of infection (Shorr et al., 2010). Treatment for MRSA infection is often challenging in the older population as antimicrobial therapy is typically less effective, and due to immunosenescence present with high mortality (Pomorska-Wesołowska et al., 2017).

Community-associated MRSA is seen in individuals without a history of hospitalisation. These were most commonly presented as soft tissue and skin infections but post-influenza pneumonia, necrotising fasciitis and bacteraemia are amongst some of the more serious infections that have been reported (Boswihi and Udo, 2018).

1.8.2 Treatments and limitations

MRSA is not only resistant to methicillin but also to a large proportion of non-β-lactam antibiotics (Boswihi and Udo, 2018). Vancomycin was the most effective treatment for MRSA infections in the 1980s, until an isolate of *S. aureus* was discovered in 1997 with reduced susceptibility to this antibiotic, followed by the discovery of a resistant strain (Chang et al., 2003).

Vancomycin is the standard first-line treatment for MRSA infections and can still be used to treat pneumonia, bacteraemia and acute skin infections. Linezolid and daptomycin are used to treat pneumonia and bacteraemia respectively along with skin infections (Bal et al., 2017; Adani et al., 2018). However, antibiotics are related to common adverse effects including abdominal pain, nausea, itching and headache (Green et al., 2012).

Resistance to current treatments may materialise over time, further limiting the number of treatments available, which highlights the importance of the discovery of new strategies to overcome this problem.

1.9 Effect of MGN-3 on the phagocytosis of MRSA

Antibiotic resistance is a global danger to human health, associated with high rates of mortality and morbidity (Frieri et al., 2016). The death rate from nosocomial infections caused by resistant bacteria in Europe alone is estimated at 25,000 people per year (MacGowan and Macnaughton, 2017). The alarming rate at which pathogens are becoming resistant to conventional therapies (Frieri et al., 2016) is a reason for the urgent need to develop new or alternative strategies to combat bacterial infections.

Enhancing the host's natural innate immunity to problematic pathogens, especially when used in conjunction with traditional antibiotic therapies, may increase the success rate of clearing persistent infections. Dietary fibres such as AX and β-glucan have been shown to stimulate innate immunity and promote phagocytosis (Cao et al., 2011; Byun et al., 2016). Thus, one potential strategy would be to encourage the dietary intake of foods containing AX or supplements such as MGN-3. This could be beneficial, especially for immunocompromised individuals as constant, low level immune system stimulation could assist in clearing potential pathogens before infection presents. Given the increase in the older population (>65 years) (MacGowan and Macnaughton, 2017) immune system enhancement could help in the reduction of hospitalisation; where patients are more susceptible to infection, and therefore a reduction in antibiotic use and healthcare costs.

The mechanisms by which dietary fibres promote the function of phagocytes are largely unknown. Uncovering the mechanisms of action could lead to the production and/or enhancement of targeted drugs and therapies. Therefore, this study focused on the effect of MGN-3 on macrophage phagocytosis and potential receptors that may mediate bacterial clearance. Moreover, there has been little research to determine the influence of dietary fibres on the clearance of antibiotic-resistant strains of bacteria such as MRSA. MGN-3 is known to stimulate systemic immune activities (Gollapudi and Ghoneum,

2008), suggesting it may be of therapeutic benefit not only to clear pathogens that gain host entry via the gut but also bacteria that infect non-intestinal tissues such as wound pathogens like MRSA. Indeed, the transport of dietary fibres across the gut lining into non-intestinal tissues of the immune system has been recently reviewed (Mendis et al., 2016). Rice et al. (2005) isolated fluorescently labelled β-glucan from a subpopulation of the intestinal epithelial cells and Peyer's patches after oral administration, thus suggesting dietary fibres can be taken up from the intestinal lumen by M-cells and transported to underlying lymphoid tissue. Mucosal DC uptake of dietary fibres occurs through sampling and interacting with gut contents locally via cellular projections that cross the epithelium (Valzasina et al., 2001; Sandvik et al., 2007). In addition, murine studies have shown that orally administered dietary fibres are taken by GI macrophages and transported to the spleen, lymph and bone marrow (Hong et al., 2004). Thus, the principal mechanisms through which dietary fibre is taken up from the diet appear to be via by M-cell, DC and/or macrophage transportation across the mucosal epithelium. Once transported to the spleen, lymph tissues and bone marrow dietary fibres such a MGN-3 could modulate phagocyte responses in non-intestinal, peripheral tissues by interacting with inflammatory cells recruited from circulation, such as monocytes that differentiate into tissue macrophages at sites of infection (Ginhoux and Jung, 2014).

1.10 Hypotheses

Alternative hypothesis: MGN-3 significantly promotes the clearance of MRSA by U937 macrophages.

Null hypothesis: MGN-3 has no significant effect on the clearance of MRSA by U937 macrophages.

1.11 Aim

To investigate the effect of MGN-3 on the clearance of MRSA by human U937 macrophages.

1.12 Objectives

The following specific objectives were formulated to achieve the aim:

1. To generate and confirm the differentiation of U937 monocytes to CD11c+ macrophages.
2. To investigate the internalisation of MGN-3 by human U937 macrophages.
3. To investigate the effect of MGN-3 on the phagocytosis of MRSA by human U937 macrophages.
4. To visualise MGN-3 induced internalisation of MRSA by human U937 macrophages.
5. To investigate the mechanism of action by which MGN-3 promotes phagocytosis in U937 macrophages.

2. Methodology

2.1 Materials

U937 human monocyte cell line, isolated from a histiocytic lymphoma of a 37-year-old male *(Health Protection Agency Culture Collections, Salisbury)*

MGN-3/Biobran *(Revital, Middlesex)*

RPMI-1640 media with L-glutamine and 25 mM HEPES *(Thermo Fisher Scientific, Loughborough)*

Foetal Bovine Serum (FBS) *(Sigma-Aldrich, Dorset)*

Penicillin-Streptomycin *(Thermo Fisher Scientific, Loughborough)*

Phorbol 12-myristate 13-acetate (PMA) *(Applichem, Darmstadt)*

Lipopolysaccharide (LPS) *(Sigma-Aldrich, Dorset)*

Methicillin Resistant *Staphylococcus aureus* (MRSA) – strain 11, a patient specimen collected at Withington Hospital, Manchester.

Nutrient Broth *(Oxoid, Basingstoke)*

Nutrient Agar *(Oxoid, Basingstoke)*

Saline *(Sigma-Aldrich, Dorset)*

Trypsin EDTA 0.25% *(Thermo Fisher Scientific, Loughborough)*

0.4% Trypan blue *(Sigma-Aldrich, Dorset)*

Phosphate buffered saline (PBS) *(Fisher Scientific, Loughborough)*

Phloroglucinol *(Sigma-Aldrich, Dorset)*

Hydrochloric acid (concentrated) *(Thermo Fisher Scientific, Loughborough)*

D-(+)-Xylose *(Sigma-Aldrich, Dorset)*

Acetic acid (glacial) *(Sigma-Aldrich, Dorset)*

D-(+)-Glucose *(Sigma-Aldrich, Dorset)*

Ethanol (99%) *(Sigma-Aldrich, Dorset)*

Whole glucan particles (WGP) soluble *(Invivogen, France)*

Whole glucan particles (WGP) dispersible *(Invivogen, France)*

TLR-4 inhibitor (TLR4-IN-C34) *(Sigma-Aldrich, Dorset)*

Heteroxylan primary antibody (*Abcam, Cambridge)*

Fluorescein isothiocyanate (FITC) Goat Anti-Rat IgM mu chain *(Abcam, Cambridge)*

Normal goat serum *(Vector Laboratories, Peterborough)*

Hanks balanced salt solution (HBSS) *(Sigma-Aldrich, Dorset)*

Fluorescein isothiocyanate (FITC) conjugated anti-human CD11c antibody *(Clone Bu15; BioLegend, UK)*

Glutaraldehyde *(Sigma-Aldrich, Dorset)*

Paraformaldehyde *(Sigma-Aldrich, Dorset)*

Silicon wafers *(Sigma-Aldrich, Dorset)*

TritonX-100 *(Sigma-Aldrich, Dorset)*

Nutrient agar *(Thermo Fisher Scientific, Loughborough)*

Nutrient broth *(Thermo Fisher Scientific, Loughborough)*

2.2 Ethics

The ethical application (SE171862; Appendix 1) for this project was approved by the Manchester Metropolitan University ethics committee on 23/04/18.

2.3 Media and solutions

Complete medium (CM) – RPMI-1640 1X medium (with L-Glutamine and 25 mM HEPES), supplemented with 10% FBS and 2% penicillin-streptomycin.

Antibiotic-free medium – RPMI-1640 1X medium (with L-Glutamine and 25 mM HEPES), supplemented with 10% FBS.

Colourless RPMI medium – RPMI-1640 1X medium (without phenol red), supplemented with 10% FBS.

MGN-3 solution – MGN-3 was dissolved in antibiotic-free medium, using heat and sonication to ensure the sample was fully dissolved. The sample was filtered using a 0.2 μm sterile filter before dilution to the following concentrations – 0.5, 1 and 2 mg/ml. The solutions were stored at 4°C but heated up to 37°C prior to cell culture use.

LPS – LPS (from E.coli) was diluted in antibiotic-free medium to make up concentrations of 1 and 5 μg/ml. The samples filtered using a 0.2 μm sterile filter and stored at 4°C but heated up to 37°C prior to cell culture use.

TLR-4 inhibitor – A stock solution was prepared in HBSS. The stock solution was diluted in antibiotic-free medium to form a concentration of 250 ng/ml. The samples were put through a 0.2 μm sterile filter and stored at 4°C but heated up to 37°C prior to cell culture use.

WGP soluble (Dectin-1 receptor inhibitor) – A stock solution was prepared in PBS at a concentration of 1 mg/ml before further dilution in PBS at 100 μg/ml. Antibiotic-free medium was used to further dilute the solution for cell culture use at 2 and 10 μg/ml. The samples were sterile filtered (0.2 μm) and stored at 4°C but heated up to 37°C prior to cell culture use.

WGP dispersible (Dectin-1 receptor activator) – A stock solution at 1 mg/ml was prepared in antibiotic-free medium. A further dilution in antibiotic-free medium was carried out to give a concentration of 200 μg/ml. The samples were sterile filtered (0.2 μm) and stored at 4°C but heated up to 37°C prior to cell culture use.

2.4 Cell culture

U937 monocytes were cultured in CM and incubated at 37°C with 5% CO_2. The cells were re-suspended in fresh CM every two days and set at 0.5 x 10^6 viable cells/ml for routine growth. For experimental purposes, the cells were cultured at 1 x 10^6 viable cells/ml. The cells were centrifuged at 1000 RPM for 7 minutes to collect cell pellet before re-suspension.

2.4.1 U937 monocyte differentiation into macrophage-like cells

U937 monocytes, set at a concentration of 1 x 10^6 cells/ml, were differentiated into adherent macrophage-like cells with the addition of Phorbol 12-myristate 13-acetate (PMA) at 50 ng/ml, followed by a 72-hour incubation at 37°C and 5% CO_2 (Sproston et al., 2018).

PMA is a phorbol ester used to promote monocyte/macrophage differentiation, via protein kinase C activation to alter gene expression as described previously (Song et al., 2015).

Cell viability was maintained above 80% during culture. Cell growth and viability were evaluated using a Biorad TC10 automated cell counter and trypan blue (0.4% in PBS) cell staining. A volume of cell suspension was mixed with an equal amount of trypan blue dye. The negative charge of trypan blue results in non-interaction with intact cell membranes, therefore, the cells that stain are considered non-viable (Tran et al., 2011).

2.5 Flow cytometry – CD11c analysis

The cells were cultured and differentiated as described in section 2.4.

Once differentiated, the cells were detached from plate wells using 40 µl trypsin EDTA for 5 minutes followed by 40 µl antibiotic-free medium for neutralisation. The cells were then centrifuged for 5 minutes at 500g before resuspending the pellet in 500 µl CM. The cells were then centrifuged at 500g for 5 minutes before washing twice with PBS. Subsequently, the cells were fixed in 4% paraformaldehyde at room temperature for 10

– 15 minutes. Following incubation, cells were centrifuged immediately for 5 minutes at 500g and washed twice in PBS, discarding supernatant. FITC conjugated anti-human CD11c antibody was prepared at a 1:50 concentration, using wash buffer (10% FBS in PBS) as a diluent. The antibody was added to the cell pellet and incubated for 30 minutes at room temperature. The cells were washed twice in PBS before resuspension in 500 μl PBS for analysis.

The expression of CD11c (cell surface marker) was assessed on 10,000 live, individual cells with a BD Accuri C6F1 Cytometer using BD Accuri C6 software. The data was analysed after gating events in the forward scattered light (FSC)/side scattered light (SSC) and fluorescence parameter 1 (FL1-A). Data was displayed as the median fluorescent intensity (MFI) relative to unstained monocytes (negative control).

2.6 MGN-3 internalisation

U937 monocytes were cultured in CM and set at a concentration of 1 x 10^6 cells/ml as stated in section 2.4. The cells were differentiated in the presence of PMA (100 ng/ml) in T75 cell culture flasks for 24 hours at 37°C with 5% CO_2. The culture medium was changed after 24 hours and incubated in the same conditions for a further 48 hours.

The adherent macrophage-like cells were treated with MGN-3 at 2 mg/ml, LPS at 5 μg/ml and CM as a control for 24 hours at 37°C with 5% CO_2.

The cells were washed four times following treatment incubation using colourless RPMI medium. All washes were stored for analysis. Trypsin EDTA was used to lift adherent cells before analysing cell counts and viability as described in section 2.4.1.

The cells were then centrifuged at 1000 RPM for 7 minutes. The supernatant was discarded, and the cells were resuspended in PBS, followed by centrifugation at 1000 RPM for 7 minutes. The supernatant was discarded, and the cells were resuspended in 0.5 ml sterile water, ready for analysis.

MGN-3 solution in CM at concentrations of 0.2, 2 and 4 mg/ml and CM alone were prepared for analysis, in order to determine the amount of xylose actually added to the cells and any CM interference.

2.6.1 Pentosan determination

Extracting solution was prepared by mixing 110 ml acetic acid (glacial), 2 ml hydrochloric acid (concentrated), 5 ml phloroglucinol (20% w/v in ethanol) and 1 ml glucose solution (1.75% in distilled water).

1 ml of sample containing cells was mixed with 5 ml extracting solution in glass test tubes before placing in a vigorously boiling water bath for 25 minutes to observe for a change in colour (dependant on pentosan concentration). Afterwards, the samples were rapidly cooled on ice for analysis.

The samples were analysed on a spectrophotometer. The absorbance (A) was read at 552 nm (A_{552nm}) and corrected by subtracting the absorbance at 510nm (A_{510nm}) for each sample.

2.6.2 Standard calibration curve

A standard calibration curve was created using samples prepared following section 2.6.1. D-(+)-Xylose was dissolved in distilled water at a concentration of 2 mg/ml. Standards were prepared at the following concentrations: 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 1.5 mg/ml.

The calibration curve was plotted based on the corrected absorbance ($A_{552nm} - A_{510nm}$) from the spectrophotometer readings of the standards as described in section 1.6.1. The amount of xylose in each sample was then determined by interpolation from the standard calibration curve.

2.7 Confocal microscopy

U937 monocytes were seeded onto sterile silicon wafers (1 cm^2) in a 24-well cell culture plate for differentiation into macrophage-like cells (see section 2.4.1). Treatments include an untreated negative control, MGN-3 at 2 mg/ml and LPS at 5 μg/ml. The cells were incubated with the treatments for 24-hours in 37°C with 5% CO_2.

The silicon wafers were washed in PBS after removal of the supernatant from the wells. The wafers were then fixed with 4% paraformaldehyde for 10 – 15 minutes. The samples were washed using PBS three times to remove remaining paraformaldehyde, then permeabilised with 1% TritonX-100 in PBS for 5 – 8 minutes, followed by repeat washes with PBS. The samples were then blocked using blocking buffer (5% goat serum in 0.1% TritonX-100 in PBS) for one hour. After blocking, the samples were immediately treated with heteroxylan primary antibody at a 1:100 concentration and incubated at 4°C for 24 hours. After several washes with PBS, FITC goat anti-Rat IgM mu chain (secondary antibody) at a 1:50 concentration was incubated with the samples for one hour at room temperature in the dark. Samples were then mounted for visualisation. These antibody concentrations were confirmed as optimal for the detection of MGN-3 fluorescence when the concentrations were experimentally varied.

U937 macrophages were treated with 2.0 mg/ml MGN-3 and 5.0 μg/ml LPS. Untreated macrophage controls were also prepared along with negative controls for the primary and secondary antibody to confirm effectiveness of the assay. The primary antibody negative control was absent of macrophages, but the silicon wafer was treated with MGN-3 2.0 mg/ml. The cells in the secondary antibody negative control was also treated with 2.0 mg/ml MGN-3, however, the primary antibody was exchanged for the blocking buffer in the staining process.

The samples were analysed and captured on a Carl Zeiss, AX10 Imager Z1 fluorescence microscope using AxioVision 4.8 software. Images were captured at 20x and 63x magnifications.

2.8 Host-pathogen interaction assay

2.8.1 MGN-3/LPS treatment

U937 macrophages were treated with solubilized MGN-3 solution at concentrations of 0.5, 1 and 2 mg/ml and LPS at concentrations of 1 and 5 μg/ml. The negative and positive controls were performed, consisting of macrophages and bacteria (no treatment) and bacteria alone in the absence of macrophages respectively. Treatments and the controls

were incubated for 24-hours at 37°C and 5% CO_2. A duplicate plate was prepared to assess cell counts and viability.

Antibiotic-free medium was used from this stage onwards to ensure unaffected bacterial growth.

2.8.2 Cell counts and viability

Macrophage counts obtained from the replica plate. Adhered cells were detached from the well plates using 40 µl trypsin EDTA for 5 minutes followed by 40 µl antibiotic-free medium for neutralisation. Cell counts and viability were measured using 0.4% trypan blue and a Biorad TC10 automated cell counter, as described in section 2.4.1.

2.8.3 Bacterial preparation

MRSA, strain 11, was cultured onto nutrient agar plates from a frozen stock, under aseptic conditions using the streak plating method. This was incubated for 24 hours at 37°C to allow for bacterial growth. One to two separate MRSA colonies from the streak plate were cultured in nutrient broth for 24-hours at 37°C. Serial dilutions of the broth using saline was carried out under aseptic conditions to achieve an optimal concentration of 2 x 10^4 CFU/ml.

2.8.4 Host Pathogen Interaction Assay

MRSA at 1 x 10^4 CFU/ml was added to each well containing U937 macrophages, followed by a 3-hour incubation at 37°C and 5% CO_2 to enable phagocytosis to take place.

The supernatant (100 µl) from the well plate were collected into separate bijoux, in addition to 450 µl of trypsin and 450 µl of antibiotic-free medium. 100 µl of this solution was cultured onto nutrient agar plates, followed by a period of incubation at 37°C for 24 hours. Bacterial colony recovery was collected after this period for quantification.

2.9 Host-pathogen interaction with receptor competition

U937 macrophages were treated, following the protocol in section 2.8. The treatments used were as follows; LPS at 10 μg/ml together with MGN-3 at concentrations of 1, 2 and 4 mg/ml. The treated cells were subsequently incubated for 24 hours at 37°C and 5% CO_2.

2.10 Host-pathogen interaction with Toll-like receptor 4 (TLR-4) inhibitor

Using the protocol in section 2.8, TLR-4 inhibitor (125 ng/ml) was added to the cells in conjunction with the following treatments: MGN-3 (0.5, 1 and 2 mg/ml), LPS (1 and 5 μg/ml) and antibiotic-free medium (untreated control). The same treatments were replicated in the absence of TLR-4 inhibitor.

2.11 Host-pathogen interaction with Dectin-1 inhibitor

Protocol as followed in section 2.8. The controls included a negative control treated with antibiotic-free medium, MGN-3 at concentrations of 0.5, 1 and 2 mg/ml, LPS at 1 and 5 μg/ml and WGP dispersible (WGPD) at 500 μg/ml.

The inhibitor treated cells were incubated at 37°C for 1 hour with WGP soluble (WGPS) at 5 μg/ml, prior to activation with the above treatments for a further 24 hours.

2.12 Scanning electron microscopy (SEM)

Silicon wafers (1 cm^2) were sterilised with 70% ethanol for 30 minutes before washing twice in PBS. Ethanol was washed off thoroughly to avoid adverse effects on the cells. The wafers were air dried in sterile conditions before being placed into a 24 well plate with sterile forceps.

U937 monocytes were differentiated and set at 1 x 10^6 CFU/ml on the silicon wafers (see section 2.4.1). MRSA strain 11 at various concentrations, 1 x 10^4 CFU/ml, 1 x 10^5 CFU/ml

and 1 x 10^6 CFU/ml were seeded onto the wafers and incubated for 3 hours at 37°C with 5% CO_2 to allow for host-pathogen interaction.

Subsequently, the samples were placed in 2.5 – 4% glutaraldehyde for 24 hours at 4°C to fix the samples. Glutaraldehyde was washed off the samples using firstly PBS, then increasing concentrations of ethanol at 20%, 40%, 60%, 80% and 100% for 15 – 30 minutes each. Samples were then left to dry in a vacuum-assisted desiccator for 24 hours.

Samples were coated in gold (Au) metal using a Polaron SEM Coating System after mounting samples onto aluminium pin stubs. The cells were analysed using a Carl Zeiss, Supra 40VP machine with SmartSEM software. Images were obtained using the secondary electron detector, using a working distance of approximately 6 mm and an acceleration voltage of 2kV. 10,000x and 25,000x magnifications were used for image capture.

2.13 Statistical analysis

IBM SPSS Statistics (version 24) was used to perform Statistical data analysis. Significance differences between groups of data was determined using one-way analysis of variance (ANOVA), followed by a Tukey HSD post hoc pairwise comparison tests. Results were considered to be significant if the probability (P) = < 0.05.

3. Results

3.1 Flow cytometry - CD11c analysis

The CD11c cell surface marker (Figure 4, 5) was used to confirm differentiation of PMA-treated U937 monocytes into macrophage-like cells. Unstained monocytes were used as a negative control for comparison.

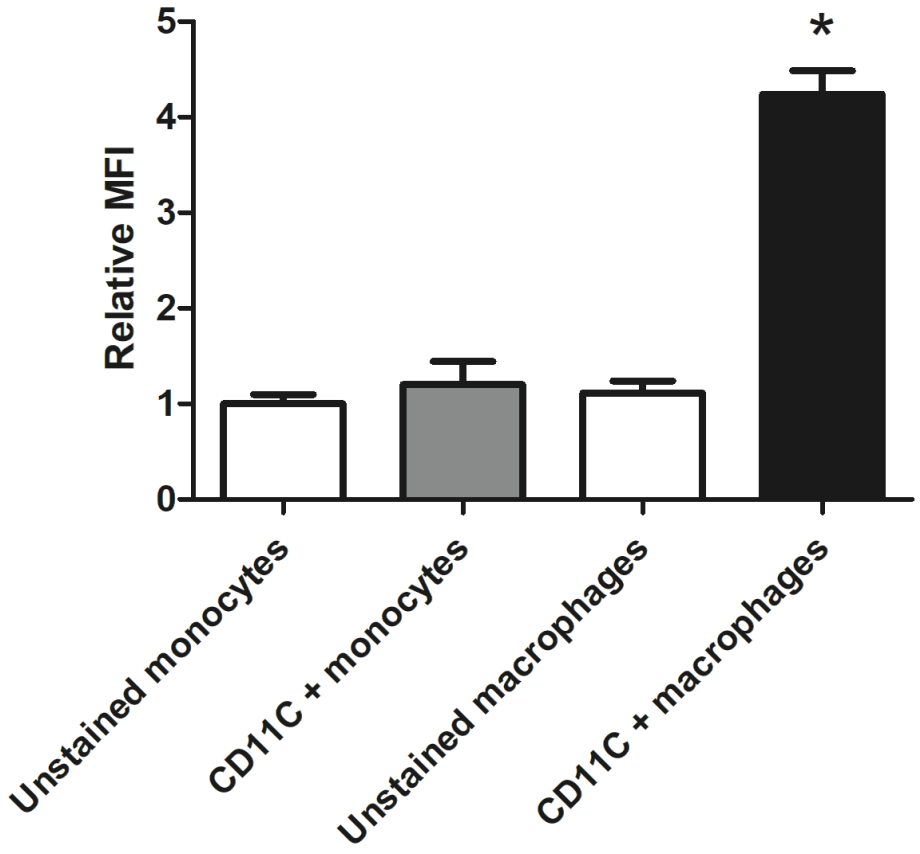

*Figure 4: CD11c analysis of U937 monocytes and macrophages using flow cytometry. Error bars represent the standard error of the mean (SEM). * indicates significant difference in relative MFI (P<0.05). n = 3 in all groups.*

There was significantly (P<0.05) higher expression of CD11c (relative MFI) in macrophages stained for CD11c (mean MIF = 4.2, 98.4% CD11c+) in comparison to U937 monocytes (mean MIF = 1.2, 1.2% CD11c+). The expression of CD11c in the unstained monocytes/macrophages (0.2% and 0.3%, respectively) was negligible (Figure 5). This upregulation of the CD11c marker confirmed the differentiation of U937 monocytes to macrophage-like cells following PMA treatment.

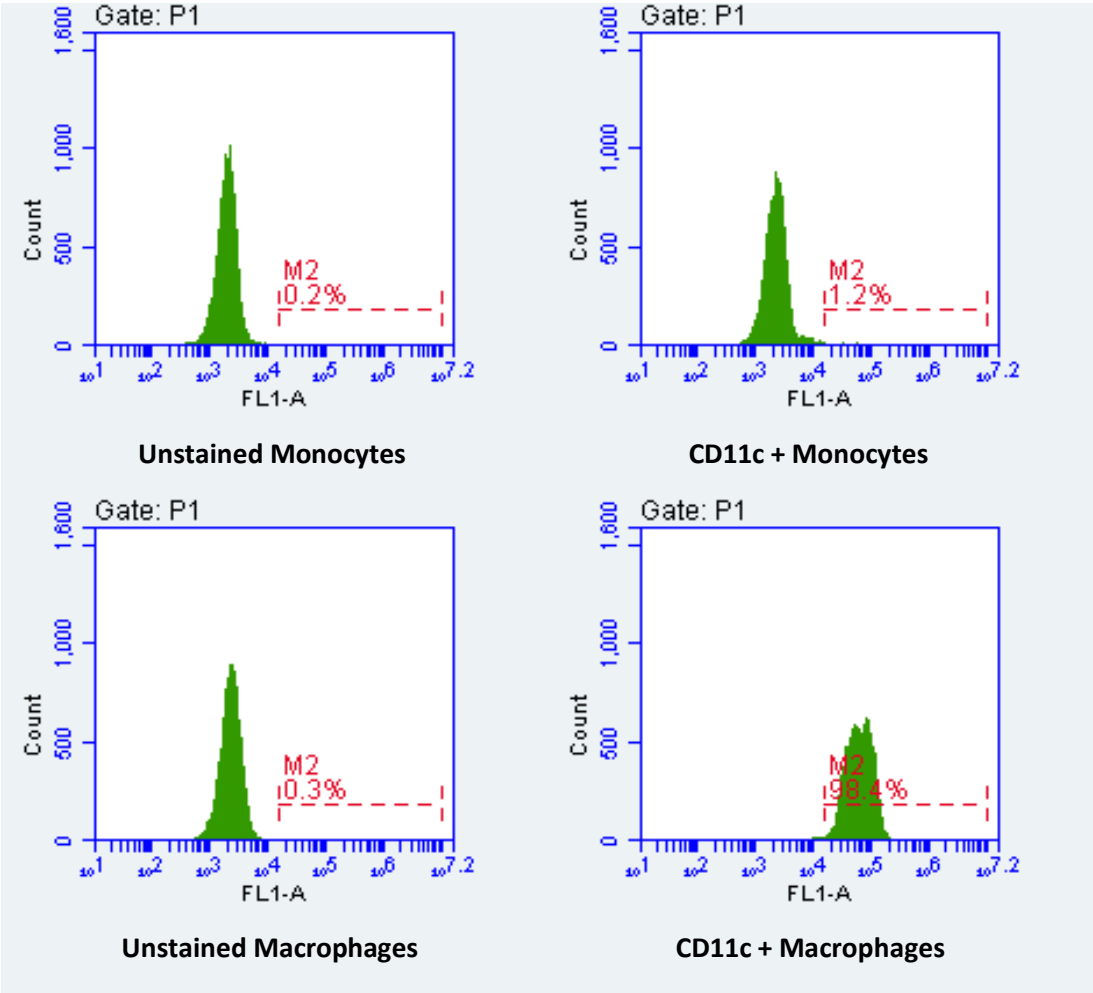

Figure 5: CD11c analysis of U937 monocytes and macrophages using flow cytometry. Graphs showing percentage of CD11c expression across all samples.

3.2 Internalisation of MGN-3

MGN-3 internalisation was measured by two different methods, the phloroglucinol colorimetric assay and confocal microscopy.

3.2.1 Phloroglucinol colorimetric assay to measure uptake of MGN-3 by U937 macrophages

Xylose concentration was measured at 0.002 mg/ml per million cells inside MGN-3 treated macrophages. In contrast, xylose was undetected inside LPS treated macrophages and the negative control. The percentage uptake of the total available xylose/MGN-3 by U937 macrophages was estimated to be 29%.

3.2.2 Confocal microscopy to visualise the uptake of MGN-3 & LPS by U937 macrophages.

Untreated (negative control) U937 macrophages (Figure 6; Panels 1A and 1B) showed negligible fluorescence of internalised AX. Similarly, no fluorescent signal was detected in MGN-3 treated macrophage in the absence of primary and secondary antibody, confirming neither the FITC-labelled AX primary antibody nor the secondary antibody were binding in a non-specific manner.

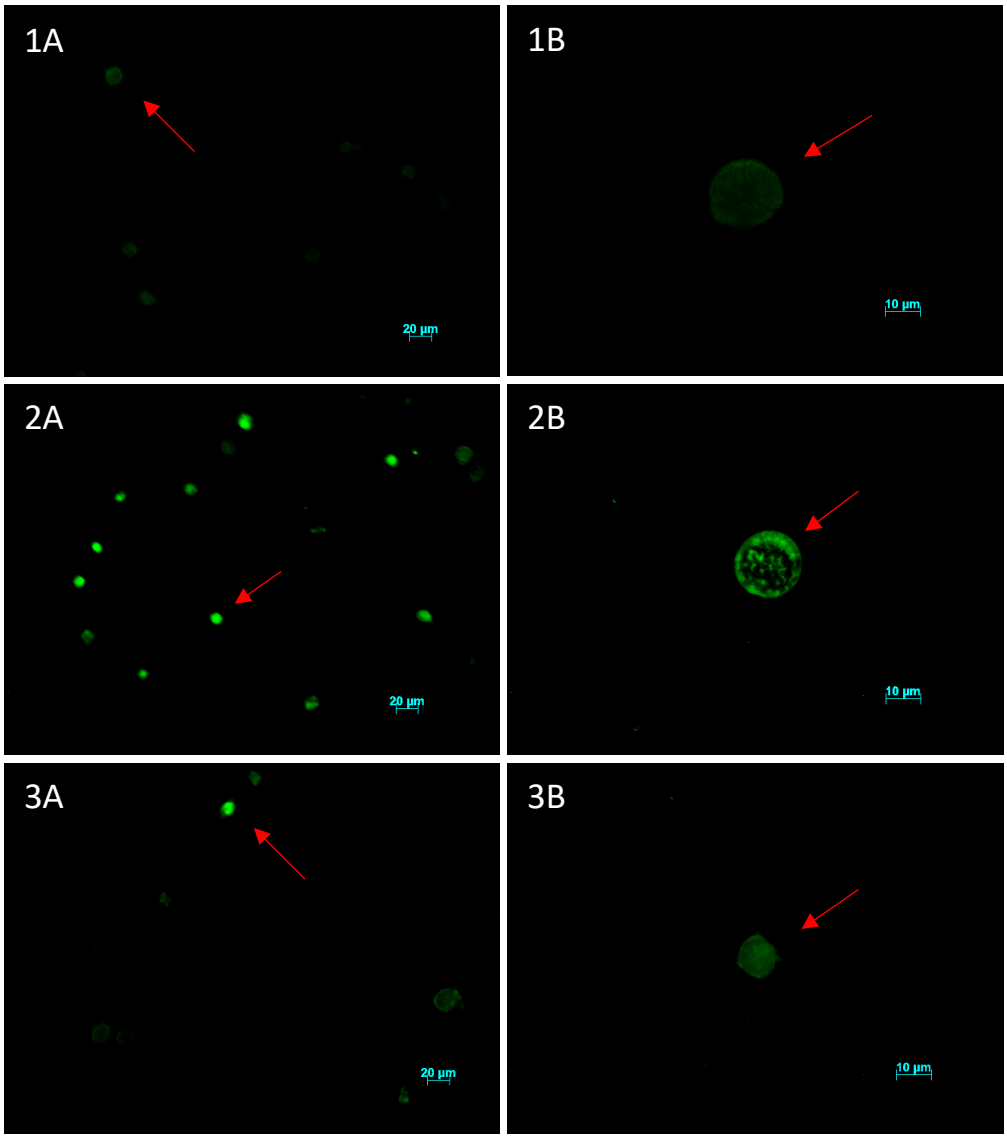

Figure 6: Detection of AX internalisation by fluorescence microscopy was negligible in untreated (negative control) U937 macrophages (Panels 1A and 1B). However, there was substantial detection of AX in MGN-3 treated macrophages in the presence of the AX-specific primary antibody and the appropriate secondary antibody (Panels 2A and 2B). In contrast, fluorescence was low in LPS-treated macrophages (Panels 3A and 3B), confirming the primary antibody was not binding to non-specific polysaccharides. Images were captured at magnifications of 20x (A) and 63x (B), with scale bars of 20 and 10 µm respectively. Red arrow shows a single macrophage and green fluorescence indicates localisation of macrophage.

U937 macrophages treated with 2.0 mg/ml MGN-3 (Figure 6: Panels 2A and 2B) showed substantial uptake of AX with substantial green fluorescence compared to the untreated negative control cells. U937 macrophages treated with LPS (Figure 6: Panels 3A and 3B) showed small amounts fluorescence.

3.3 Effect of MGN-3 and LPS on phagocytosis of MRSA

Cultured U937 macrophages were treated with MGN-3 (0.5, 1.0 and 2.0 mg/ml) and/or LPS (5.0 μg/ml) alone or in combination (Figure 7). A one-way ANOVA confirmed statistical differences P<0.001 between treatment groups.

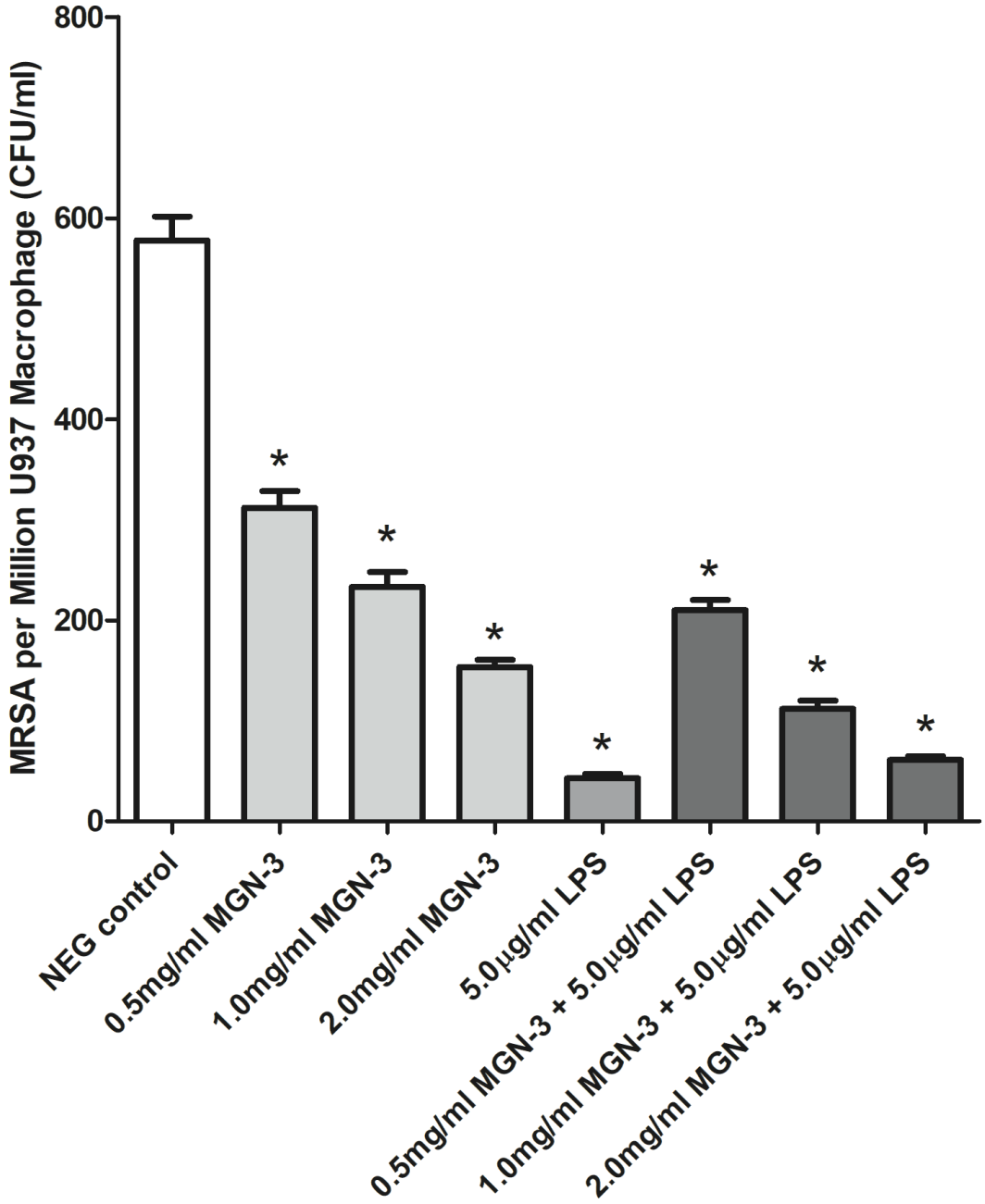

*Figure 7: Mean MRSA recovery per million viable macrophages following treatment with MGN-3 and/or LPS. Error bars represent the standard error mean (SEM). * represents significant differences compared to the NEG control (P<0.001). n = 24 for all treatments.*

The phagocytic ability of U937 macrophages were significantly (*, P<0.001) enhanced by treatment with all concentrations (0.5, 1.0 and 2.0 mg/ml) of MGN-3 (MRSA recovery = 316, 240 and 185 CFU/ml, respectively) and even more so by 5 μg/ml LPS (MRSA recovery = 42 CFU/ml) compared to the negative control (MRSA recovery = 576 CFU/ml). Moreover, MGN-3 treatment increased phagocytosis in a dose dependant manner.

The combination of MGN-3 and LPS resulted in an intermediate phagocytic ability compared to MGN-3 or LPS treatment alone. For example, 0.5 mg/ml MGN-3 combined with 5.0 μg/ml LPS resulted in a mean MRSA recovery of 210 CFU/ml. Similarly, MGN-3 at 1.0 and 2.0 mg/ml showed a bacterial count of 114 and 61 CFU/ml respectively in the presence of LPS. The combined MGN-3/LPS treatments significantly (P<0.001) increased phagocytosis compared to MGN-3 alone (at the same concentration) or the negative control, although not as effectively as LPS 5.0 μg/ml alone.

3.4 Effect of toll like receptor 4 (TLR-4) inhibition on MGN-3/LPS-mediated phagocytosis of MRSA

Cultured U937 macrophages were treated with MGN-3 or LPS (as the positive control) in the presence/absence of the TL4-inhibitor, TLR4-IN-C34 (Figure 8). A one-way ANOVA confirmed statistical differences P<0.001 between treatment groups.

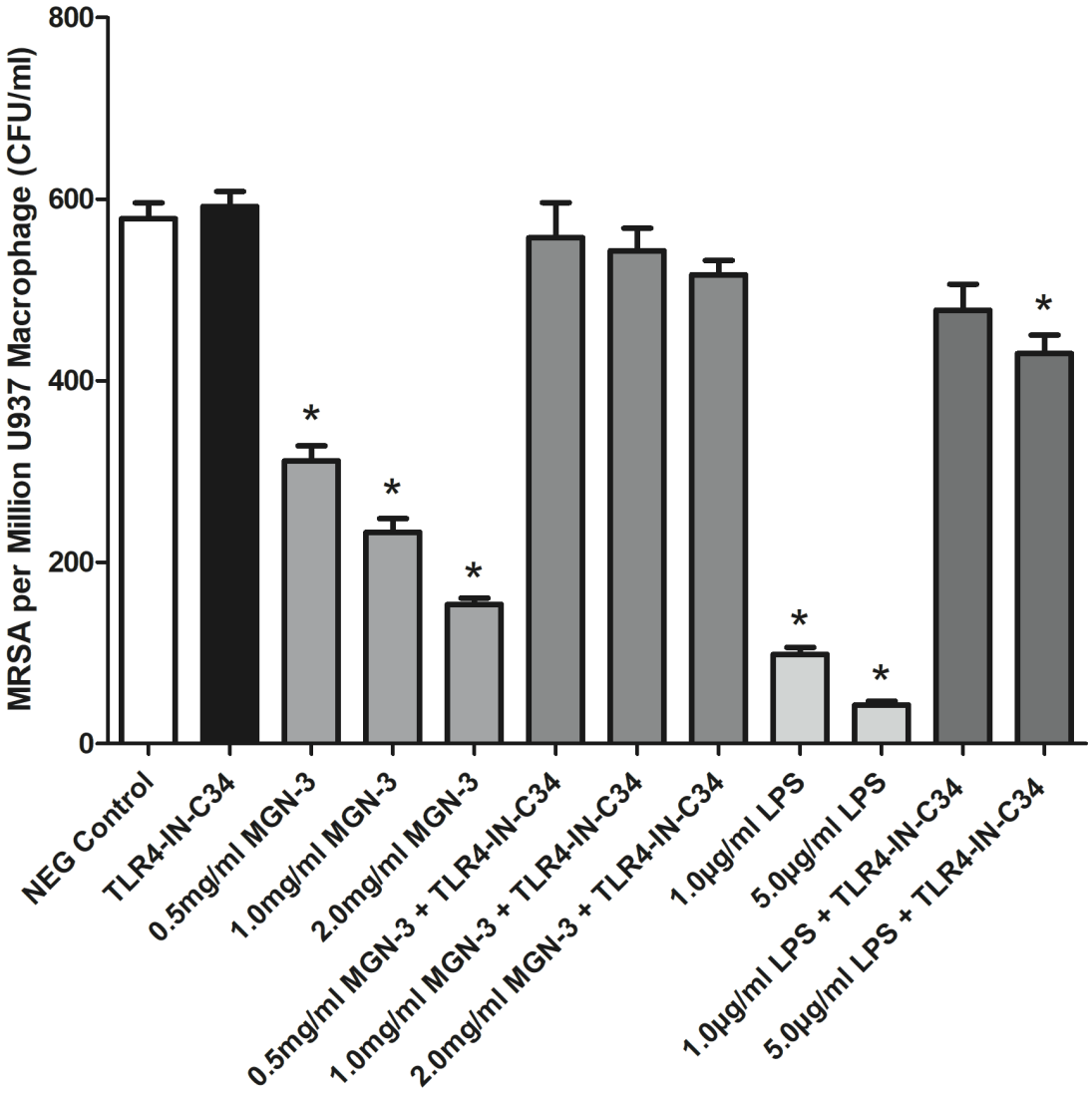

*Figure 8: Mean MRSA recovery per million viable U937 macrophages following treatment with MGN-3 or LPS in the absence/presence of TLR-4 inhibitor. Error bars represent the standard error mean (SEM). * represents a significant difference compared to the NEG control (P<0.001). n = 24 for all treatments.*

As reported in section 3.3, treatment of U937 macrophages with MGN-3 resulted in significantly (P<0.001) increased levels of phagocytosis when compared to the negative control (untreated macrophages). The level of phagocytosis increased in a dose

dependant manner, with MGN-3 2.0 mg/ml inducing the most phagocytosis compared to the negative control. Treatment of U937 macrophages with LPS was more effective at inducing phagocytosis than the MGN-3 treatments at 0.5 and 1.0 mg/ml ($P<0.001$), with phagocytosis increasing in a dose dependant manner compared to the negative control ($P<0.001$).

The addition of the TLR-4 inhibitor (TLR4-IN-C34) significantly ($P<0.001$) inhibited the clearance of MRSA induced by MGN-3 or LPS treatments. The mean MRSA recovery per million viable macrophages for 0.5, 1.0 and 2.0 mg/ml MGN-3 increased from 312, 233 and 154 CFU/ml in the absence of TLR-4 inhibitor to 558, 543 and 517 CFU/ml in the presence of TLR-4 inhibitor respectively. Similarly, mean MRSA recovery per million viable macrophages for 1.0 and 5.0 μg/ml LPS increased substantially from 92 and 41 CFU/ml in the absence of TRL-4 inhibitor to 478 and 430 CFU/ml in the presence of TLR-4 inhibitor respectively.

No significant difference ($P=1.000$) was found between treatment of U937 macrophages with the TLR-4 inhibitor alone and the negative control, confirming the TLR-4 inhibitor had no inherent/direct effect on phagocytosis in the absence of appropriate receptor activators such as LPS (which is not a component of MRSA).

3.5 Effect of dectin-1 inhibition on MGN-3/LPS-mediated phagocytosis of MRSA

Cultured U937 macrophages were treated with MGN-3 or LPS (as the positive control) in the presence/absence of the dectin-1 inhibitor, WGPS (Figure 9). A known dectin-1 receptor activator (WGPD) was used as an internal positive control to confirm the selective inhibition of dectin-1 by WGPS. A one-way ANOVA confirmed statistical differences $P<0.001$ between treatment groups.

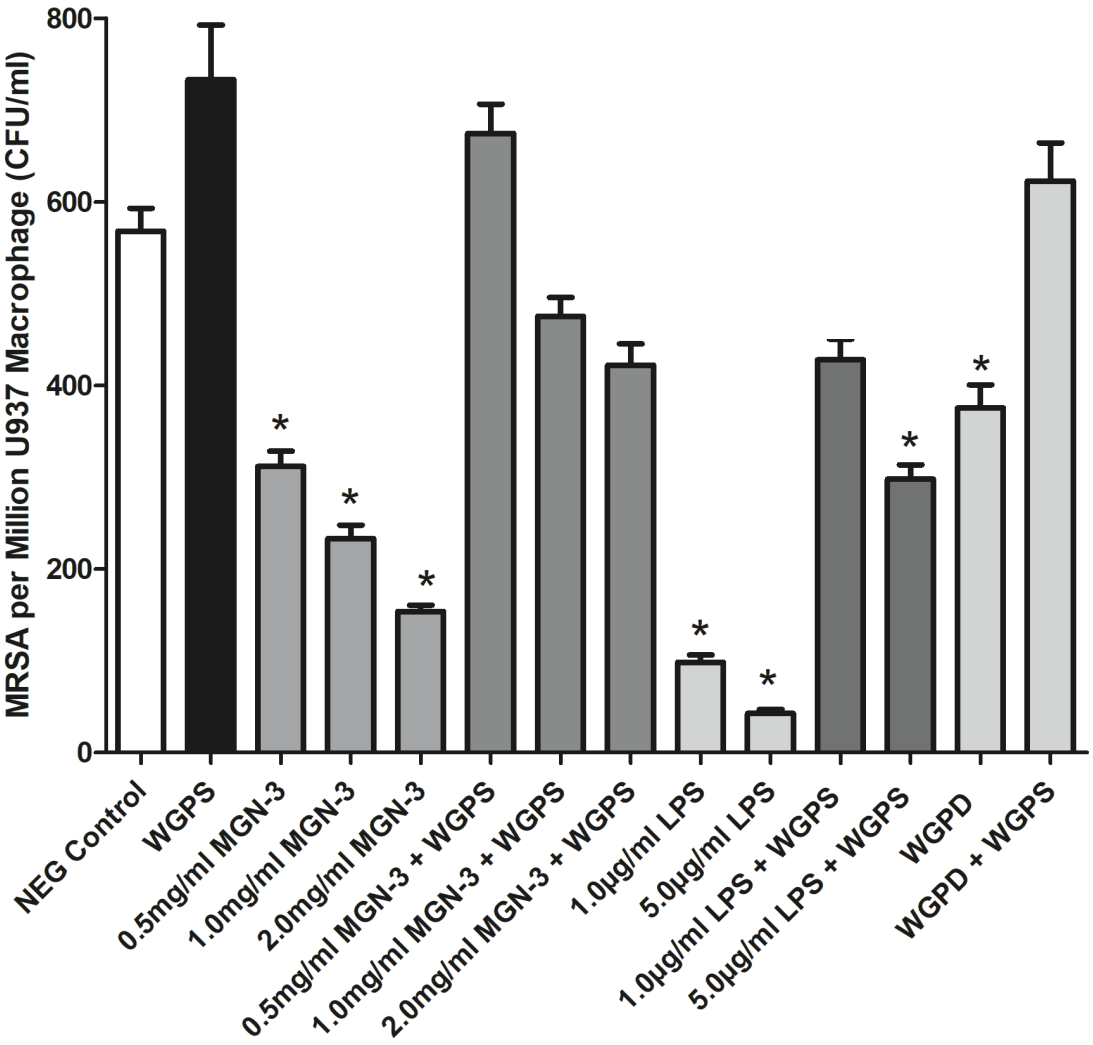

*Figure 9: Mean MRSA recovery per million viable U937 macrophages following treatment with MGN-3 or LPS in the absence/presence of the dectin-1 inhibitor WGPS. Error bars represent the standard error of the mean (SEM). * represents significant difference compared to the NEG control (P<0.001). n = 24 across for all treatments.*

As highlighted in section 3.3, MRSA recovery significantly ($P<0.001$) decreased with following treatment of U937 macrophages with MGN-3 or LPS.

The addition of the dectin-1 inhibitor (WGPS) significantly ($P<0.001$) inhibited the clearance of MRSA induced by MGN-3 or LPS. The mean MRSA recovery per million viable macrophages for 0.5, 1.0 and 2.0 mg/ml MGN-3 increased from 312, 233 and 154 CFU/ml in the absence of WGPS to 675, 475 and 422 CFU/ml in the presence of WGPS respectively. Similarly, mean MRSA recovery per million viable macrophages for 1.0 and 5.0 μg/ml LPS increased substantially from 92 and 41 CFU/ml in the absence of WGPS to 428 and 298 CFU/ml in the presence of WGPS respectively.

Treatment of U937 macrophages with WGPS alone (733 CFU/ml) resulted in a non-significant ($P>0.05$) decrease in phagocytic function compared to the NEG control (576 CFU/ml), confirming the significant reversal of MGN-3/LPS-induced phagocytosis by WGPS were real effects and not just an artefact of WGPS on macrophages. Moreover, WGPS significantly ($P<0.001$) reversed the effects of WGPD, a known dectin-1 receptor activator, increasing MRSA recovery from 376 CFU/ml (WGPD) to 635 CFU/ml (WGPD + WGPS).

3.6 Scanning electron microscopy (SEM) to visualise the effect of MGN-3 & LPS on phagocytosis of MRSA

Host-pathogen interaction using untreated macrophages (Figure 10; Panels 1A and 1B) showed MRSA as small (≤1 µm) spherical cells engaging with lamellipodia-producing macrophages, indicative of phagocytosis.

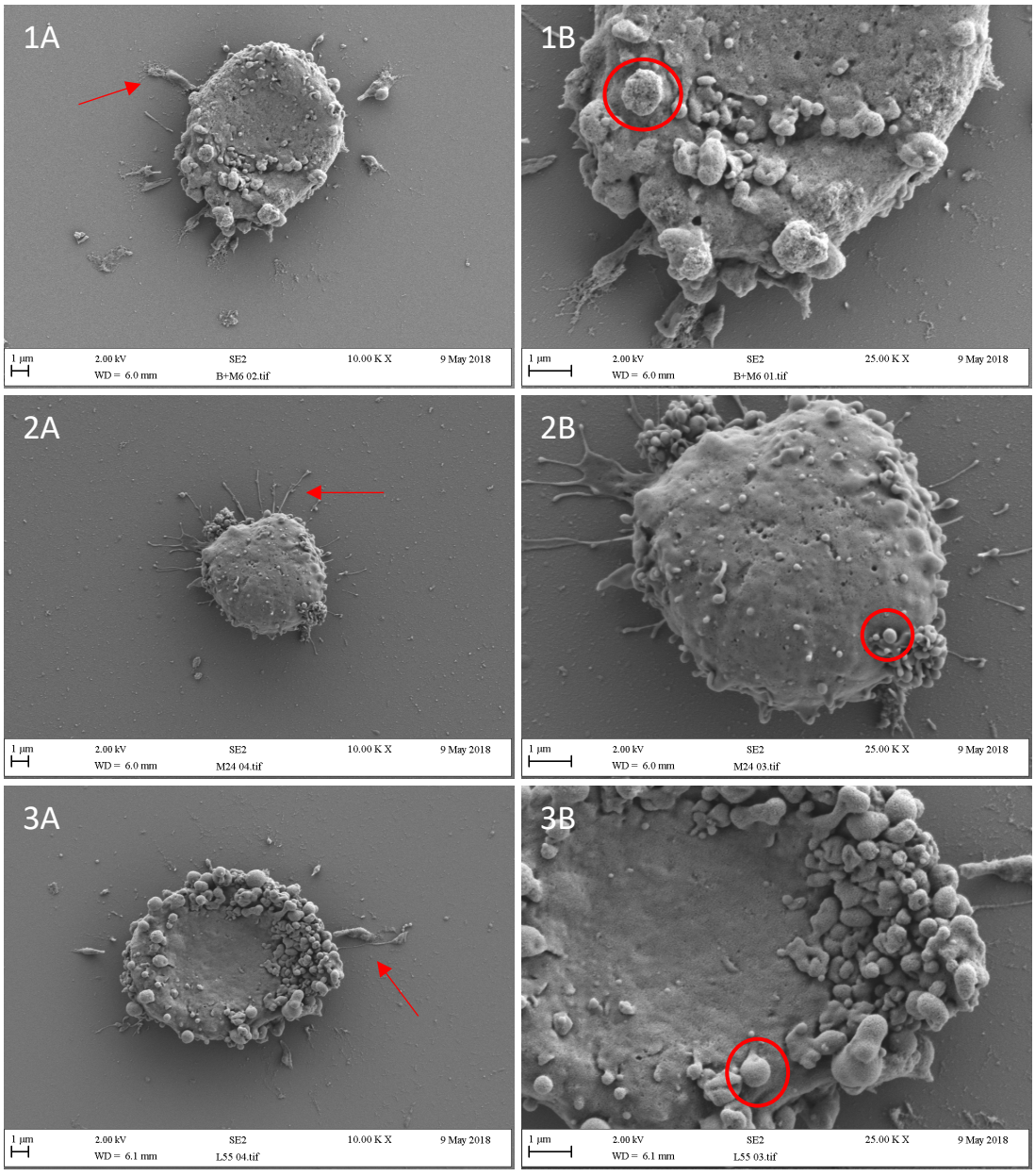

Figure 10: SEM of host-pathogen interactions of untreated U937 macrophages with MRSA at a magnification of 10,000x (1A) and 25,000x (1B). Host-pathogen interactions of MGN-3 treated U937 macrophages with MRSA were captured at magnifications of 10,000x (2A) and 25,000x (2B). Host-pathogen interactions of LPS-treated U937 macrophages with MRSA were captured at magnifications of 10,000x (3A) and25,000x (3B). Scale bars indicate 1 µm. The red circles show a single MRSA bacterium engaging with the U937 macrophage. The red arrow indicates the formation of a lamellipodium.

The U937 macrophages treated with 2.0 mg/ml MGN-3 (Figure 10; Panels 2A and 2B) showed increased lamellipodia formation and interaction with MRSA compared to the untreated macrophages. LPS-treated macrophages (Figure 10; Panels 3A and 3B) resulted in visibly more engagement with MRSA in comparison to the untreated and MGN-3 treated macrophages, however it shows a decrease in lamellipodia formation compared to MGN-3 treated cells.

4. Discussion

This study determined the effect of MGN-3 on the clearance of MRSA by human U937 macrophages, and possible mechanisms of action of MGN-3 on phagocytes. The internalisation of MGN-3 was assessed by a colorimetric assay and then visually confirmed by confocal microscopy, using a fluorescent (GFP-tagged) antibody specific for AX. To study the potential mechanism of action of MGN-3, the involvement of specific receptors (TLR-4 and dectin-1) was interrogated by pharmacological receptor inhibition. Finally, to visualise the findings, SEM was used to show host-pathogen interaction.

4.1 MGN-3 internalisation

The internalisation of MGN-3 by U937 macrophages was confirmed via two independent assays (Section 3.2); the phloroglucinol colorimetric assay (Douglas, 1981) to detect intracellular xylose, and by intracellular detection of AX by confocal microscopy using an AX specific fluorescently-labelled antibody. This independent confirmation of MGN-3 uptake by phagocytes such as macrophages, provides a mechanistic route by which MGN-3 may induce phagocytosis.

Receptor-mediated endocytosis and pinocytosis (both of which are actin polymerisation independent mechanisms), together with phagocytosis are some of the cellular processes that are likely to lead to MGN-3 internalisation (McCann et al., 2005). However, a study on the internalisation of β-glucan by RAW murine macrophages indicated that internalisation is not essential for inducing inflammatory responses (McCann et al., 2005), suggesting only interaction with surface receptors is necessary. Consequently, further research is required to understand whether MGN-3 internalisation is essential to illicit phagocytosis, or whether simply activation of cell surface receptors alone is sufficient to stimulate MGN-3-induced phagocytosis.

Previous murine studies have shown that GI macrophages can take up and transport orally administered β-glucan (400 μg/day) systemically to the lymph, spleen and bone marrow (Hong et al., 2004). The uptake of MGN-3 shown in the current study may

suggest that MGN-3 could be transported through the intestinal lumen by GI macrophages, to modulate immune responses both locally and systemically.

The data in section 3.2.1 showed the percentage uptake of MGN-3 by U937 macrophages to be approximately 29%. This is suggestive that the majority of the total available MGN-3 may act on extracellular macrophage receptors to illicit immune responses.

4.2 MGN-3 and its effect on phagocytosis of MRSA

The flow cytometry results (Figures 4, 5) confirmed the generation of CD11c positive macrophages in concordance with the findings of Sproston et al. (2018).

MGN-3 at several concentrations (0.5, 1.0 and 2.0 mg/ml) was used to determine its effect on macrophage-mediated phagocytosis of MRSA. The results (Figure 7) showed significant dose-dependent clearance of MRSA in the presence of MGN-3, indicating MGN-3 is effective stimulator of macrophage phagocytosis. This suggests MGN-3 may be used to stimulate innate host (human) responses during MRSA infection, and potentially reduce the over-reliance on antibiotics in the treatment of MRSA infections in patients.

Host-pathogen interaction with MRSA was confirmed by SEM (Figure 10), which showed that MGN-3 and LPS induced the formation of lamellipodia and increased macrophage bacterial interaction. Lamellipodia are critically involved in the process of bacterial engulfment and phagocytosis (Veale et al., 2011). This further suggests that MGN-3 can enhance inflammatory processes in response to MRSA infection.

The results from this study mirror previous studies showing MGN-3 augments phagocytic ability *in vitro* (Ghoneum and Matsuura, 2004), with MGN-3 enhancing phagocytosis in a dose dependant manner. For example, significant NK cell activation was detected following treatment with MGN-3 at concentrations $\geq$ 100 μg/ml (Ghoneum and Matsuura, 2004).

Similar studies have investigated the enhancement of phagocytosis by natural agents. A study published in 2010 suggested that green tea polyphenols (pyrogallol-type) enhance

the phagocytic activity of 1,25-dihydroxyvitamin D3 (VD-3) differentiated HL60 cells through caspase signalling (Monobe et al., 2010). An *ex vivo* study using female *Balb/c* mice indicated that garlic extract has the ability to activate macrophages, and augment phagocytosis in response to parasitic infection (Ghazanfari et al., 2006). Finally, macrophage activation is shown to be modulated by *Panax ginseng* polysaccharide against *S. aureus* induced sepsis (Lim et al., 2002).

4.3 MGN-3 and its mechanisms of action

The correlation between biological activities and polysaccharides have been reported (Shimada et al., 2012). LPS is a polysaccharide, made of bacterial cell surface components, known to stimulate biological activities (Lerouge and Vanderleyden, 2002). LPS and AX show similarities in terms of molecular weight (30 – 100 kDa) and general structure (Li et al., 2015). This suggests MGN-3, an arabinoxylane polysaccharide, could compete for similar receptors/mechanisms as LPS (Ghoneum et al., 2013). TLR-4 is a polysaccharide receptor, often associated with LPS, which could be a possible receptor for MGN-3 (de Lima et al., 2014; Zhang et al., 2016a).

MGN-3 and LPS receptor competition, highlighted in section 3.3 (Figure 7), examined this concept. The combination of MGN-3 and LPS resulted in an overall increased level of phagocytosis compared to the MGN-3 treatment alone, however, it was not as effective as LPS alone. The intermediate phagocytic response when MGN-3 was combined with LPS suggests they are potentially competing as ligands for the same receptor on the U937 macrophage. This indicates that during infection, MGN-3 could potentially act to modulate some of the more detrimental pro-inflammatory (non-phagocytic) responses caused by LPS, warranting further investigation in this area.

4.3.1 TLRs and their role in MGN-3-induced phagocytosis

TLR-4 is a known receptor for LPS (Fang et al., 2017). A receptor inhibitor was used in this study to uncover the involvement/contribution of TLR-4 to the activity of MGN-3. The results in section 3.4 (Figure 8) revealed a complete reversal of the effects of both MGN-3 and LPS with the addition of the TLR-4 inhibitor, which shows that both

molecules act on the same receptor. This suggests that TLR-4 may play a significant mechanistic role in the phagocytic effects of MGN-3. TLR-4 is known to recognise gram-negative bacterial LPS and DAMPs, leading to cytokine production and inflammation (Molteni et al., 2016; Fang et al., 2017). As suggested in section 1.6.1, MGN-3/TLR-4 engagement could activate the MyD88-dependant and/or the TRIF-dependant pathways, resulting in phagocytosis.

The TLR family is known for their high affinity for polysaccharides, suggesting other receptors from this family could be involved in MGN-3-mediated activity (Chen et al., 2009). For example, TLR-1, 2 and 6 recognise PAMPs such as mannan (Kawasaki and Kawai, 2014). Mannan is polysaccharide and a polymer of D-mannose which is a sugar, and like arabinoxylan, the sugars are linked together by β-1,4 glycosylic bonds (Moreira and Filho, 2008). These similarities in polysaccharide structure could potentially suggest that these three receptors could also recognise MGN-3 molecules.

4.3.2 Dectin-1 and its role in MGN-3 induced phagocytosis

Dectin-1 is a C-type lectin receptor, located on macrophages, neutrophils and dendritic cells that can initiate inflammatory responses such as phagocytosis and respiratory burst (Ma and Underhill, 2013). Dectin-1 has been identified as a PRR for β-glucan (Herre et al., 2004).

MGN-3 contains β-glucan, polysaccharides that are produced by fungi, yeast and many plants (H. S. Kim et al., 2011). The biological activities of β-glucan are dependent on its molecular weight, with inactivity frequently seen in smaller molecules (below 5,000 – 10,000 Da) (H. S. Kim et al., 2011). This suggests that the biological activity of MGN-3 could be down in part to the presence of β-glucan.

The results in section 3.5 (Figure 9) revealed that the phagocytosis induced by MGN-3 was significantly suppressed by the addition of a dectin-1 receptor inhibitor. This finding suggests that MGN-3 may also be acting in part through the dectin-1 receptor to promote phagocytosis. Dectin-1 is predominantly involved in antifungal immunity, along with other C-type lectin receptors, but is also involved in the recognition of bacteria and viruses (Drummond and Brown, 2011). Upon receptor engagement, spleen tyrosine

kinase (Syk) is activated leading ultimately to the initiation of NF-κB and MAPK via a signalling cascade (Kimura et al., 2014). Dectin-1 can initiate the production of lipid mediators and cytokines such as TNFα, IL-6 and IL-10 (Drummond and Brown, 2011). The dectin-1 receptor has been shown to be expressed on mast cells in humans and mice, which are related to allergic reactions and hypersensitivity (Kimura et al., 2014). This suggests that MGN-3 may also mediate inflammatory responses in cases of allergy and hypersensitivity.

4.3.3 Other mechanisms of action

In summary, the data from this study suggest MGN-3 may act on multiple receptors including TLR-4 and dectin-1 (Figure 11). However, other receptors may also recognise MGN-3. Another PRR receptor that may also be involved is complement receptor CR3, also known as CD11b (Chen et al., 2009). CD11b is a phagocytic receptor expressed on the surface of cells, including macrophages and dendritic cells (Kang et al., 2016). CD11b is a specific receptor for β-glucan in macrophages for opsonized polysaccharides (Chen et al., 2009) so may also contribute to the biological activity of MGN-3. The mannose receptor is known to recognise carbohydrate structures and is a PRR in the C-type lectin family, found on the surface of several cell types including macrophages and dendritic cells (Li et al., 2017). The mannose receptor plays an important role in macrophage phagocytic function, cytokine and ROS secretion (Li et al., 2017). It is unknown whether the mannose receptor could recognise the carbohydrate structures of MGN-3 and promote phagocytosis, thus warranting further investigations using a mannose receptor inhibitor.

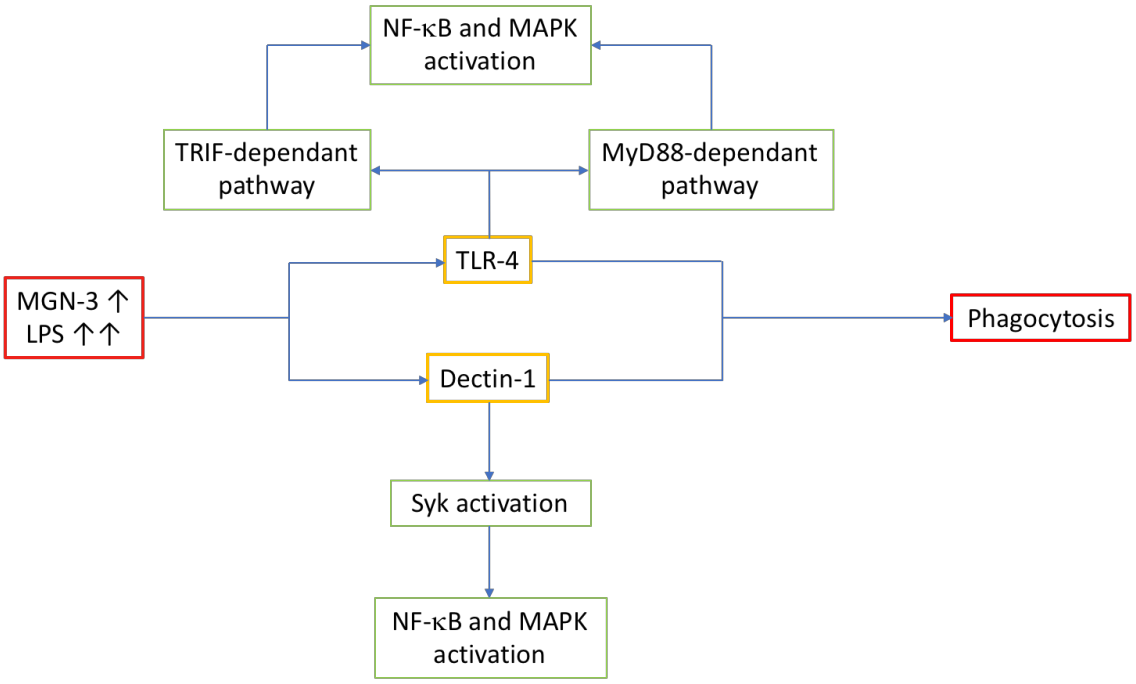

Figure 11: MGN-3 and LPS stimulates both TLR-4 and Dectin-1 promoting phagocytic clearance of MRSA. TLR-4 engagement activates the myeloid differentiation factor 88 (MyD88)-dependant pathway and the TIR domain-containing adaptor inducing interferon-beta (TRIF)-dependant pathway, leading to nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation. Dectin-1 engagement leads to spleen tyrosine kinase (Syk) activation which causes nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation. This all leads to phagocytosis.

4.4 Clinical impacts of MGN-3/AX

Dietary fibre consumption is associated with human health benefits, including the reduced risk of diabetes and cardiovascular disease development (Mendis et al., 2016). AX is a primary source of dietary fibre found in cereal grains such as corn, wheat and barley, whilst MGN-3 is a widely used nontoxic food supplement (Lattimer and Haub, 2010; Ooi et al., 2017). The data in this study suggest that increased consumption of these food groups/supplements may contribute to immune system enhancement leading to enhanced pathogen clearance. Previous studies have suggested that dietary fibre can enhance the protective potential of the GI mucosal barrier (Brownlee, 2011). The human intestine constantly encounters foreign antigens (Bain and Mowat, 2014), and MGN-3-induced enhancement of the mucosal barrier could inhibit systemic entry of possible pathogens. Studies have shown that AX such as MGN-3 can modulate systemic immune responses (section 1.5.1), not just local immune activity in the gut (Lissoni et al., 2008). This suggests that MGN-3 could act to enhance systemic phagocyte activity before and after infection presents.

Additional studies are needed to uncover further mechanisms of action of MGN-3 and its clinical impact. However, the current results suggest that MGN-3 may be used as a potential therapy for MRSA treatment, or in conjunction with antibiotics to promote a reduction in antibiotic usage.

4.5 Future work

Ex vivo testing on healthy human participants could be performed to confirm if oral consumption of MGN-3 can lead to detectable levels of circulating AX in the bloodstream. Moreover, its effect on cell activation, differential inflammatory cell responses and alteration of inflammatory marker profiles could be examined in conjunction with *ex vivo* phagocytosis assays. This could improve knowledge on the mechanisms of action of MGN-3 in humans.

Other phagocytes such as dendritic cells or macrophages derived from human peripheral blood monocytes (HPBM), could be tested to see if the findings can be reproduced in other cell types (including human primary macrophages) following MGN-3 stimulation. Confirmation of the findings in other cell types would provide a body of evidence to support the receptors involved in the phagocytic activity of MGN-3.

Further research to explore the precise mechanisms of action of MGN-3, including investigating additional cell surface receptors such as the CD11b complement receptor, is also required to highlight potential therapeutic strategies to promote the clearance of bacterial infections. This may be of importance in the search for new therapies for the treatment of MRSA.

Inhibiting macrophage uptake of MGN-3 by particle uptake antagonists such as cytochalasin D (McCann et al., 2005), can be used to gain further insight into the effects of MGN-3 internalisation.

Finally, other clinically relevant bacteria such as vancomycin-resistant *enterococci* and *Pseudomonas aeruginosa* could be tested to examine phagocytosis by MGN-3 stimulated macrophages.

5. Conclusion

In conclusion, this study established that MGN-3 significantly promotes phagocytosis of MRSA by U937 macrophages. In addition, TLR-4 and dectin-1 receptor inhibitors supressed the phagocytic actions of MGN-3, implicating both these receptors contribute at least in part to the mechanisms of action of MGN-3. Additional research is urgently needed to explore these receptor-mediated mechanisms in further detail and identify any further contributing mechanisms by which MGN-3 stimulates bacterial clearance. Such studies may ultimately lead to the development of targeted dietary supplements/treatments that promote innate immunity and combat bacterial infections, such as MRSA, without sole reliance on antibiotics. Such inexpensive and relatively safe dietary-based therapies would reduce the substantial costs incurred by healthcare providers to treat problematic bacterial infections such as MRSA. Moreover, reduced reliance on antibiotics would be a beneficial strategy to limit the spread of antibiotic resistance associated with pathogens such as MRSA.

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7. Appendix

7.1 Ethical approval memorandum

FACULTY OF SCIENCE AND ENGINEERING

Manchester Metropolitan University

MEMORANDUM

TO	Sally Tan
FROM	Karen Hartley
DATE	23rd April 2018
DATE OF EXPIRY:	30th September 2018
SUBJECT	Application for Ethical Approval (SE171862)

On the 23rd April 2018 the Head of Ethics for Science & Engineering considered your application for Ethical Approval (SE171862) entitled *"An investigation of the mechanisms of action and structure-function properties of dietary arabinoxylan on inflammatory processes"*. The application has been granted Favourable Opinion and you may now commence the project.

MMU requires that you report any Adverse Event during this study immediately to the Head of Ethics (Dr Gethin Evans) and the Research Degrees Administrator. Adverse Events are adverse reactions to any modality, drug or dietary supplement administered to subjects or any trauma resulting from procedures in the protocol of a study.

An Adverse Event may also be accidental loss of data or loss of sample, particularly human tissue. Loss of human tissue or cells must also be reported to the designated individual for the Human Tissue Authority licence. Please notify Professor Tristan McKay of any issues relating to this.

If you make any changes to the approved protocol these must be approved by the Faculty Head of Ethics. If amendments are required you should complete the MMU Request for Amendment form (found on the Graduate School website) and submit it to the Administrator.

Regards

Karen Hartley
Research Administrator
All Saints North

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