The Effects of Dietary Fibres on Inflammatory Processes under Hyperglycaemic Conditions

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The Effects of Dietary Fibres on Inflammatory Processes under Hyperglycaemic Conditions

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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List of Abbreviations

AX	Arabinoxylan
AB – free	Antibiotic free Media
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
CD11c	Cluster of Differentiation 11c
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CFU	Colony Forming Unit
CFU/ml	Colony Forming Unit per millilitre
CM	Complete Media
CVD	Cardiovascular disease
DCs	Dendritic cells
DFUs	Diabetic foot ulcers
DM	Diabetes Mellitus
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediamine tetra-acetic acid
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FL1 – A	Fluorescence Parameter 1
FSC	Forward scattered light

g	G force during centrifugation
GALT	Gut Associated Lymphoid Tissue
HbA1C	Baseline glycated haemoglobin A1C
HDL	High Density Lipoprotein
HSCs	Haemopoietic stem cells
IL – 6	Interleukin - 6
IL – 18	Interleukin – 18
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharides
M cells	Microfold cells
MFI	Mean Fluorescence Intensity
mM	Millimolar
MRF	Mean Relative Fluorescence
MRSA	Methicillin Resistant *Staphylococcus aureus*
MTF	Mean Total Fluorescence
M0	Macrophage
M1	Pro-inflammatory macrophage
M2	Anti-inflammatory macrophage
NA	Nutrient Agar
NB	Nutrient Broth
NC	Negative Control

ng/mL	Nanogram per millilitre
NK	Natural killer cell
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PMA	Phorbol 12-Myristate 13-Acetate
PRRs	Pathogen recognition receptors
RPMI	Roswell Park Memorial Institute
SEM	Standard Error of the Mean
TLRs	Toll-like receptors
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF α	Tissue Necrosis Factor Alpha
µl	Microlitre
µm	Micrometre
°C	degree Celsius

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Acknowledgments

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Declaration

I can confirm that all of the work submitted in this thesis is my own and has not been submitted as part of any other degree or qualification.

Signed: …… (Candidate)

Date: ……..

Abstract

Diabetes Mellitus (DM) has been recognised as one of the most common chronic condition worldwide with a rise in number of young adults and children developing the condition. The common symptoms seen in DM are chronic inflammation and infections (e.g. diabetic foot ulcers (DFUs)). This is thought to be due to defects in the immune response. An alternative or possibly complementary strategy to treat infections is to develop novel therapies that stimulate the body's own natural innate immune system. Dietary fibres such as MGN3 may help to increase the clearance of bacteria in DFUs whilst at the same time reducing inflammation. This study investigated the effect of MGN3 on the phagocytosis of MRSA by U937 macrophages and CD14 expression in U937 monocytes/macrophages under hyperglycaemic conditions.

An *in vitro* host-pathogen assay (n=12) was carried out to test the effectiveness of MGN3 (2mg/ml) on bacterial (MRSA) clearance by U937 macrophages at different levels of glucose (11, 15, 20 and 30mM). CD14 protein expression in U937 monocytes/macrophages was visualised by confocal microscopy and determined by flow cytometry following exposure to glucose (11 or 30mM) with/without MGN3 (2mg/ml). The study showed MGN3 increases bacterial clearance with increasing periods (2 to 5 hours) of host-interaction. The phagocytosis of MRSA became increasingly impaired with rising glucose levels but this detrimental effect on U937 macrophages could be significantly ($P < 0.05$) reversed in the presence of MGN3. MGN3-treated macrophages increased overall bacterial clearance under hyperglycaemic conditions, even at high (30mM) glucose levels. Lipopolysaccharide (LPS) significantly stimulated CD14 protein expression in U937 monocytes/macrophages cultured in high (30mM) glucose. Moreover, CD14 analysis indicated there was competition taking place between LPS and MGN3, with a significant ($P < 0.05$) decrease in mean relative fluorescence (relative CD14 protein levels) after combined treatment of U937 monocytes/macrophages with both LPS and MGN3 compared to just LPS treatment alone.

In conclusion, this study indicated that MGN3 can reverse some detrimental effects of hyperglycaemia on monocyte/macrophage function, by inhibiting glucose-mediated elevation of CD14 and reversing glucose-mediated inhibition of MRSA clearance. These findings can have a major impact for diabetic patients since MGN3 may be a potential therapeutic strategy to dampen inflammation, stimulate healing and promote bacterial clearance in diabetic patients with infected wounds.

1. Introduction

1.1 Diabetes Mellitus

Diabetes mellitus (DM) refers to a group of diseases resulting from high levels of blood glucose (hyperglycaemia) (Chait and Bornfeldt, 2009) and is a common chronic disease recognised globally (Shaw *et al,* 2010). This is different to diabetes insipidus, which is caused by complications to the hypothalamo-neurohypophysial system, which could lead to defects in the synthesis of the vasopressin hormone (Di Iorgi *et al,* 2012). There are two types of DM; type 1 DM and type 2 DM. This distinction was first made in 1936 (Olokoba *et al,* 2012).

Type 1 DM usually starts in childhood or early adolescence, and may be caused by an autoimmune response in which pancreatic β cells are destroyed by self-antibodies (Ozougwu *et al,* 2013). This results in very low levels of insulin or no insulin produced, and thus fluctuating glucose levels. As a result, chronic hyperglycaemia can cause cognitive dysfunction and visual impairments from an early age (McCrimmon *et al,* 2012). Type 2 DM occurs in adults when insulin becomes ineffective or is produced at very low levels. This disease is linked with other diseases, mainly obesity (McCrimmon *et al,* 2012). One of the characteristics of type 2 DM is elevated levels of free fatty acids (FFA). FFAs are normally stored in adipose tissue in the form of triglycerides and can be used as an alternative source of energy to glucose. Insulin is involved in regulating this lipolysis process, resulting in the release of these triglycerides as energy (Hussain *et al,* 2010). Type 2 DM can be managed by a closely monitored diet and pharmacological interventions are used to control glucose levels, including sulfonylureas and thiazolidinediones that stimulate insulin secretion or in some cases direct insulin replacement (Olokoba *et al,* 2012).

Globally diabetes is recognised as one of the most common chronic diseases, with type 2 DM being the most common (Shaw *et al,* 2010; Olokoba *et al,* 2012). Around 300 million people were living with diabetes worldwide a decade ago (Shaw *et al,* 2010). This had risen to 360 million by 2011, with incidence rates being higher in poor countries compared to wealthy countries (Olakoba *et al,* 2012). It is estimated that

by 2030 numbers will rise to 440 million, with more children and younger adults becoming affected. These increases have been linked to factors such as increased physical inactivity, imbalanced diets and increased alcohol consumption that lead to higher obesity rates and thus higher incidence rates in type 2 DM (Olokoba *et al*, 2012; Shaw *et al,* 2010).

1.1.1 Health Risks Associated with Diabetes Mellitus

There are many health risks associated with DM, with diabetic patients most likely to be susceptible to cancers as well as suffer from a number of psychiatric disorders. The risk of cancer is higher in diabetic patients and could be due to the link between obesity and hyperglycaemia (Suh and Kim, 2011). Diabetic patients indeed have a higher mortality rate of pancreatic, liver or kidney cancer (Harding *et al,* 2015). Patients suffering from type 1 DM have a higher tendency to have suicidal thoughts (de Ornelas Maia *et al,* 2012).

It is widely agreed that type 2 DM is linked to obesity, which in turn is associated with chronic inflammation (McCrimmon *et al,* 2012; Dandona *et al,* 2005). This chronic inflammation leads to pronounced and excessive synthesis of pro-inflammatory cytokines and destructive mediators (Idriss and Naismith, 2000). DM also impairs immunological functions such as clearance of pathogens and wound healing (Anas *et al,* 2010).

1.1.2 Effect of Diabetes Mellitus on Inflammation

Immune cells are recruited to sites of infection or injury (Grivennikov *et al,* 2012). Acute inflammation involves a limited and controlled influx of leukocytes from the peripheral blood to the site of infection/injury to clear pathogens and cellular debris (Buckley *et al,* 2001). Acute inflammation resolves once the infection and dead tissues are removed, thus preventing the inflammatory response becoming excessive and prolonged (Buckley *et al,* 2001).

Immune cells are derived from the haemopoietic stem cells (HSCs). HSCs can differentiate into myeloid progenitor cells before turning into granulocyte monocyte

progenitor cells, and then becoming monocytes, dendritic cells (DCs) and macrophages (Höchst *et al,* 2013; Zimmermann *et al,* 2010). Monocytes are leukocytes that circulate in the blood (Geissmann *et al,* 2003). Monocytes display cell surface markers that can assist in their identification, including CD14 and CD16. In some cases, monocytes can display equal levels of CD14 and CD16, whereas in other situations CD14 levels are high and CD16 levels are low (Geissman *et al,* 2003). CD14 are proteins which act as endotoxin receptors on most cells which express TLR4 (e.g. Monocytes and macrophages) and they regulate TLR4 endocytosis. CD14 is specific to LPS and is an example of a lipopolysaccharide binding protein (Zanoni *et al,* 2011; Zweigner *et al,* 2006). Monocytes differentiate into tissue macrophages (M0) that express CD11c and M0 can become polarised into either pro-inflammatory (M1) or anti-inflammatory (M2) macrophages (Savina and Amigorena, 2007; Raggi *et al,* 2017). M1 are classically activated by microbial factors and pro-inflammatory cytokines, whilst M2 are alternatively activated by anti-inflammatory cytokines (Raggi *et al,* 2017; Espinoza-Jimenez *et al,* 2012).

Chronic inflammation fails resolve, leading to an unabated influx of leukocytes to the site of infection/trauma, and subsequent tissue damage (Buckley *et al,* 2001). Chronic inflammation due to infection or autoimmune diseases has been linked to the growth of carcinogenic tumours (Grivennikov *et al,* 2010).

The immune response is amplified in DM through elevated inflammatory cell expression of CD14 (Fernandez-Real *et al,* 2011; Anas *et al,* 2010; Sahay *et al,* 2009). Studies have shown that during prolonged periods of chronic inflammation, macrophages release large amounts of CD14 which results in elevated levels of proinflammatory markers (Fernandez-Real *et al,* 2011; Anas *et al,* 2010; Sahay *et al,* 2009). In a murine study, chronic inflammation in adipose tissue following insulin resistance has been linked to obesity (Xu *et al,* 2003). In type 2 diabetes, chronic inflammation can occur due to increased apoptosis and tissue damage (Donath and Shoelson, 2011).

1.1.3 Diabetic Foot Ulcers

Diabetic patients have been shown to have defects in wound healing (Daniel *et al,* 2012). The peripheral neuropathy and ischemia associated with DM can lead to a type of chronic wound, called a diabetic foot ulcer (DFU). DFUs can develop as a result of diabetic neuropathy, which has been linked to chronic inflammation (Doupis *et al,* 2009) or in some cases ischaemia. Diabetic patients may not feel the initial trauma which would result in continuous stress being applied to the wound. If the wound is left untreated it could become infected (Cavanagh *et al,* 2005). Chronic inflammation is a key histological feature of DFUs, yet the impaired inflammatory cell function in patients with DM increases the risk of infection (Leung, 2007) and developing other complications such as osteomyelitis (Dinh *et al,* 2008).

1.1.4 The Effect of Diabetes Mellitus on Innate Immunity

The role of the immune system is pivotal in providing protection and maintenance of the human body. The immune system defends against invading pathogens through a process called phagocytosis (Hooper *et al,* 2012). Hyperglycaemic conditions found in DM cause polymorphonuclear leukocytes to become less responsive (Daniel *et al,* 2012; Lin *et al,* 2006), suggesting DM is likely to impair phagocytosis and increase in the likelihood of infection. A murine study performed by Khanna *et al* (2010) concluded that hyperglycaemic conditions impair phagocytic function, resulting in insufficient clearance of apoptotic cells and causing elevated release of inflammatory cytokines.

When a pathogen is first detected it triggers the non-specific (innate) response (Kumar *et al,* 2011). Invading pathogens present molecules, called pathogen-associated molecular patterns (PAMPs), that are detected by the host through pathogen recognition receptors (PRRs) (Akira *et al,* 2006). There are multiple types of PRRs, one group being toll like receptors (TLRs). TLRs are membrane glycoproteins found on immune cells that are involved in gene expression within the innate immune system (Takeda and Akira, 2005; Akira *et al,* 2006). TLRs can recognise specific groups of pathogens; for example, LPS released from Gram negative bacteria is recognised by TLR4 whereas peptidoglycan from Gram positive bacteria is detected by TLR2

(Kumar *et al,* 2009; Takeda and Akira, 2005; Akira *et al,* 2006). Lipopolysaccharides (LPS) are endotoxins found on the outer cell membrane of gram negative (e.g. MRSA). These endotoxins can be secreted by the bacteria trigger an immune response. LPS attaches CD14 receptors and causes the production of cytokines (e.g. Tissue necrosis factor alpha (TNFα) and interleukin (IL) 6) (Meng and Lowell, 1997; Zweigner *et al,* 2006; Komatsuzawa *et al,* 2006). Phagocytosis is carried out by macrophages, neutrophils and in some cases DCs (Savina and Amigorena, 2007). PAMPs released by the bacteria attach to PRRs on phagocytes (Kumar *et al,* 2011), leading to the pathogen being engulfed within a phagocytic vesicle called a phagosome (Greenberg and Grinstein, 2002) that binds to a lysosome within the phagocyte to form a phagolysosome. The lysosome contains hydrolytic enzymes that break down the pathogen, and debris is released via exocytosis from the phagosome (Greenberg and Grinstein, 2002; Aderem and Underhill, 1999).

1.1.5 Methicillin Resistant *Staphylococcus aureus* (MRSA) Infection in Diabetic Patients

DFUs can become infected by one or more bacteria, with methicillin-resistant *Staphylococcus aureus* (MRSA) being a common opportunistic pathogen in both hospital and community-acquired infections (Stanaway *et al,* 2007; Tentolouris *et al,* 2005; Yates *et al,* 2009). *Staphylococcus aureus* is a gram-positive bacterium found as part of the natural, commensal microflora but it can become an opportunistic pathogen (Chambers, 2001). If MRSA infections are left untreated, they can cause bacteraemia with is often fatal (Pastagia *et al,* 2012; Kempker *et al,* 2010). MRSA has become resistant to many antibiotics from the penicillin and β lactam groups (Turos *et al,* 2007; Fisher *et al,* 2005) including methicillin, making it difficult to treat. Vancomycin has been shown to be the most effective antibiotic treatment for MRSA infections, but even some cases of vancomycin resistance have now been reported (Pastagia *et al,* 2012).

1.2 Dietary Fibres and Innate Immunity

An alternative or complimentary strategy to using antibiotics for the treatment of DFU infections is to develop novel therapies that stimulate the body's own natural innate immune system. Factors such as malnutrition and a poor diet can impair immune function (Gleeson, 2005; Plat and Mensik, 2005; Marketon and Glaser, 2008). An effective way to prevent immune functions from declining may include the use of dietary supplements and some evidence suggests they can reduce the risk of infection and/or cancer (Kaminogawa and Nanno, 2004; Meoni *et al,* 2013). Moreover, dietary fibres have been shown to reduce inflammation in chronic diseases such as cardiovascular disease (CVD), kidney disease and diabetes (King, 2005; Krishnamurthy *et al,* 2012).

Fibre-derived substances such as β-glucan have been shown to stimulate immune activity in both animal models and humans when consumed in the diet (Tzianabos, 2000; Volman *et al,* 2008). Dietary fibres are organic components of foods that cannot be digested by humans in the small intestine (Mudgil and Barak, 2013). However, consumption of dietary fibres has shown to reduce the risk of developing coronary heart disease, hypertension and stroke (Yan *et al,* 2015), alter gut microbiota to control the symptoms of type 2 diabetes (Zhao *et al,* 2018) and lower cholesterol (Kristensen *et al,* 2012). Dietary fibres are found in many different plant-derived foods, such as vegetables, cereals and nuts (Dhingra *et al,* 2012), as well as crustaceans and some fibre-enriched foodstuffs that contain synthetically-made dietary fibres (Fuentes-Zargoza *et al,* 2010). Dietary fibres can be put into two subtypes based on their solubility; soluble and insoluble. This can be confirmed using a solution containing human digestive enzymes (Tosh and Yada, 2010; Tungland and Meyer, 2002). Dietary fibres are absorbed in different ways. Dietary fibres have the ability to resist hydrolysis (Lockyer and Nugent, 2017) due to the body's inability to break down the glycosidic bonds within the fibres, allowing them to pass through the intestine undigested (Palafox-Carlos *et al,* 2011).

Dietary fibres are typically plant-derived carbohydrate polymers, either oligosaccharides or polysaccharides. Examples of these include cellulose, pectin

substances and resistant starch (Elleuch *et al,* 2011). Celluloses are polysaccharides typically containing thousands of glucose molecules, whereas pectins are made up sugars and galacturonic acid and resistant starches are made up of linear alpha-glucan chains (Fuentes-Zargoza *et al*, 2010). They have also been used as functional ingredients in meat-derived products to enhance their nutritional value (Biswas *et al,* 2011).

Plant cell walls contain polysaccharides known as hemicelluloses. This fibre group includes xyloglucans and xylans (Scheller and Ulvskov, 2010). Xylans can sometimes have arabinose sugars attached to their beta-glucose backbone, changing them to arabinoxylans (Scheller and Ulvskov, 2010; Tan *et al,* 2013). Arabinoxylans (AXs) derived from cereal hemicelluloses have been shown to modulate both innate and adaptive immune responses in animal models (Zhang *et al,* 2015). In mice, AXs have shown to induce macrophage activation and phagocytosis (Zhou *et al,* 2010; Kim *et al,* 2005). The arabinoxylan known as MGN3 or Biobran, has the ability to activate immune cells, including T cells and monocytes (Ghoneum and Agrawal, 2011). MGN3 has shown to increase dendritic cell activation, making it a potential strategy to fight infections and possibly cancer (Ghoneum and Agrawal, 2011). MGN3 has also shown it can reduce the effects of immunosenescence in natural killer cells and possibly lowering the incidence rate of fatal diseases such as cancer in the elderly (Elsaid *et al,* 2018). MGN3 was named using the surnames of the scientists who developed it in 1992 (Maeda, Ghoneum and Ninomiya) with 3 indicating it is a third-generation product (Masood *et al,* 2013).

1.2.1 Effects of Dietary Fibres on Type 2 Diabetes Mellitus

Studies have shown that dietary fibres have the potential to reduce the effects of DM, possibly by targeting the high glucose levels or reducing its effects, or by mediating other related chronic illnesses such as obesity and CVD. It has been reported that consuming cereal fibre, wholegrain foods and bran products lowers the risk of CVD, obesity and type 2 DM (Cho *et al,* 2013).

It has been previously stated that diabetic patients have defects in collagen production, resulting in impaired wound healing (Daniel *et al,* 2012). Interestingly, studies have shown that the consumption of dietary fibres such as glucomannans, can help to promote collagen production, in addition to stimulating keratinocytes to move the site of trauma (Al-Ghazzewi *et al,* 2015). As well as reducing the number of inflammatory markers (Weickert and Pfeiffer, 2008), there is evidence to suggest dietary fibres can enhance gut associated lymphoid tissue (GALT), increasing the rate of phagocytosis (Schley and Field, 2002). This evidence suggests dietary fibres could be beneficial in assisting in the healing process of DFUs. Dietary fibres could either be ingested and/or applied directly to wounds via dressings, which has not yet been proposed to date.

A randomized trial was performed in patients with type 2 DM, to compare diets consisting of low glycaemic index foods with a high cereal fibre diet. The results showed that both diets reduced the mean baseline glycated haemoglobin A1c (HbA1c) concentration but the effect was more prominent in the diet containing foods that have a low glycaemic index (Jenkins *et al,* 2008). A study has been done to test the effectiveness of diet and the need of supplementary insulin in type 2 DM patients. Patients were given diets consisting of low glycaemic foods, with or without wheat bran. The results showed that 80% of patients who consumed wheat bran needed insulin supplementation whereas only 40% of patients who consumed wheat bran needed supplementary insulin (Afaghi *et al,* 2013).

1.3 Uptake of Dietary Fibres

Research on the dietary uptake of β-glucans and arabinoxylans, such as MGN3, has confirmed that ingested fibres are transported throughout the body, including the spleen and bone marrow (Hong *et al,* 2004). Studies have shown that oral intake of dietary fibres has an influence on the mucosal membranes of the intestine, in particular the Peyer's patches and also the intestinal intraepithelial lymphocytes in mice (Suzuki *et al,* 1990; Tsukada *et al,* 2003). Fluorescently labelled β glucan taken orally and later isolated from the intestinal epithelium and Peyer's patch were found to be transported from the intestine to specialised lymphoid tissue by Microfold (M)

cells (Rice *et al,* 2005). DCs within the mucosal membrane are also responsible for taking up polysaccharides, including dietary fibres (Sandvik *et al,* 2007). It has been shown that DCs project their dendrites through the epithelium lining and sample the gut contents by endocytosis (Rescigno *et al,* 2001). Hong *et al,* (2004) have shown that dietary fibres, administrated orally at 400μg/day, are transported to multiple tissues including the spleen, lymph and bone marrow by gastrointestinal macrophages.

The intestine is covered with long finger-like structures called villi containing a large network of blood cells and epithelial cells with microvilli. These work together to increase the intestinal surface area by 60 - 120 times to maximise the absorbtion of nutrients (Helander and Fändriks, 2014). The lymph fluid, containing immune cells from the Peyer's patches, filters into the mesenteric lymph node (Tamoutounour *et al,* 2012; Schenk and Mueller, 2008). Within the villi there are areas of connective tissue called lamina propria that separate epithelial cells from smooth muscle layers (Schenk and Mueller, 2008). The intestinal epithelium has a specialised lymphoid layer, known as the GALT, which contains up to 70% of the body's immune cells (Jung *et al,* 2010). Abnormal immune responses within the GI tract can lead to chronic inflammatory diseases. This could be due pathogenic bacteria, e.g. *Escherichia coli* seen in Crohn's disease (Chassaing *et al,* 2011; Gullberg and Söderholm, 2006) or protein intolerances as seen in coeliac disease (Dewar *et al,* 2003). Changes in the intestinal microbiota are detected by macrophages and DCs, as well as other lymphocytes (Kumar *et al,* 2011; Akira *et al,* 2006).

It is known that dietary fibres, including β-glucans and arabinoxylans, taken orally come in contact with the mucosal immune system (Volman *et al,* 2008). Within the intestinal mucosa there are dome shaped lymphoid structures called Peyer's patches, named after the scientist who discovered them in 1677 (Jung *et al,* 2010). The Peyer's patch is covered in lymphoid tissue that is involved in immune responses (Parsons *et al,* 1991). This lymphoid tissue is known as the follicle associated epithelium (FAE) which is where the microfold (M) cells are located (Jung *et al,* 2010; Chassaing *et al,* 2011; Gullberg and Söderholm 2006; Kanaya *et al,* 2018). The role of M cells is to

sample intestinal antigens (Kanaya *et al,* 2018; Schenk and Mueller, 2008). M cells transport antigens across the FAE into the Peyer's patch via a process called transcytosis (Hase *et al,* 2009). DCs derived from monocytes are able to extend and sample the antigens from M cells and present them to lymphocytes for phagocytosis (Schenck and Mueller, 2008; Salim *et al,* 2009). The dendritic cells move to regions of the Peyer's patch containing T cells, where they convert the T cells in immunomodulatory cells called regulatory T cells (T_{reg}) that move to the lamina propria and secrete interleukins to signal an immune response (Tamoutounour *et al,* 2012; Gullberg and Söderholm 2006).

In summary, the key principal mechanisms for dietary fibre uptake and transportation across the mucosal epithelial membrane are by gastrointestinal M cells and DCs. After transportation to the spleen, lymphatics and bone marrow, AXs (e.g. MGN3) can modulate inflammatory responses in non-intestinal/peripheral tissues by interacting with inflammatory cells (such as macrophages).

1.4 Hypothesis

An alternative or complimentary strategy to using antibiotics for the treatment of DFU infections is to develop novel therapies that stimulate the body's own natural innate immune system. In theory, dietary fibres such as MGN3 may help to increase the clearance of bacteria in DFUs whilst at the same time reducing inflammation. This study investigated the effect of MGN3 on the phagocytosis of MRSA by U937 macrophages and CD14 expression in U937 monocytes/macrophages under hyperglycaemic conditions.

Alternative Hypothesis – MGN3 significantly reduces the CD14 marker expression of inflammation in U937 monocytes/macrophages but promotes the phagocytosis of MRSA by U937 macrophages under hyperglycaemic conditions.

Null Hypothesis – MGN3 has no significant effect on the CD14 marker of inflammation in U937 monocytes/macrophages but promotes phagocytosis of MRSA by U937 macrophages under hyperglycaemic conditions.

1.5 Aim

To investigate the effect of MGN3 on the CD14 marker expression of inflammation in U937 monocytes/macrophages and the phagocytosis of MRSA by U937 macrophages under hyperglycaemic conditions.

1.6 Objectives

1) To confirm the differentiation of U937 monocytic cells into U937 macrophages via detection and quantification of CD11c levels by flow cytometry.
2) To evaluate the effect of LPS and MGN3 on the clearance of MRSA during phagocytosis by U937 macrophages under hyperglycaemic conditions.
3) To measure the CD14 marker expression by flow cytometry in U937 monocytes/macrophages under hyperglycaemic conditions.
4) To confirm CD14 detection by fluorescence microscopy in U937 monocytes/macrophages following treatment with LPS or MGN3 under hyperglycaemic conditions.

2. Methodology

2.1 Materials

U937 Human Monocyte cell line – sample isolated from a male patient (37 years old), histiocytic lymphoma (*Health Protection Agency Culture Collections, Salisbury*)

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

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

Penicillin-Streptomycin (5,000 U/mL) (*Thermo Fisher Scientific, Loughborough)*

Phorbol12-Myristate 13-Acetate [PMA] (*Applichem, Darmstadt)*

Trypan Blue (*Sigma-Aldrich, Dorset)*

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

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

Nutrient Broth (*Oxoid, Basingstoke)*

Nutrient Agar (*Oxoid, Basingstoke)*

Saline (*Sigma-Aldrich, Dorset)*

Methicillin Resistant *Staphylococcus aureus* [MRSA] Strain II – Patient specimen (*Withington Hospital, Manchester*)

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

Trypsin EDTA (*Thermo Fisher Scientific, Loughborough)*

Bovine Serum Albumin [BSA] (*Sigma-Aldrich, Dorset)*

Ethanol (70%) (*Sigma-Aldrich, Dorset)*

Dulbecco's phosphate-buffered saline [DPBS] (*Thermo Fisher Scientific, Loughborough*)

Silicon wafers (*Sigma-Aldrich, Dorset)*

Paraformaldehyde (*Sigma-Aldrich, Dorset)*

Triton X-100 (*Sigma-Aldrich, Dorset)*

FITC-conjugated anti-human CD11c antibody (*Abcam, Cambridge)*

FITC-conjugated anti-human CD14 antibody (*Abcam, Cambridge)*

Unconjugated anti-human CD14 antibody (*Abcam, Cambridge)*

2.2 Ethics

An ethical application (Review reference number: 2019-3255-4589) was submitted for this project. No ethical issues were raised in relation to the project and ethical approval was granted.

2.3 Media

Complete medium (CM): RPMI-1640 media with L-Glutamine, 11mM glucose and 25mM HEPES, supplemented with 10% FBS and 2% (v/v) penicillin-streptomycin.

Antibiotic-free (AB-free) medium: RPMI-1640 media with L-Glutamine, 11mM glucose and 25mM HEPES, supplemented with 10% FBS.

Glucose-supplemented media: D-(+)-glucose dissolved in CM or AB-free medium to give a final concentration of 30mM glucose and then used to prepare additional glucose-supplemented media with final glucose concentrations of 15, 20 or 25mM by serial dilution.

MGN-3 supplemented medium: MGN-3 dissolved in CM or AB-free medium to a concentration of 2mg/ml.

All media were sterile filtered (0.5 μm) prior to use in cell culture experiments.

2.4 Cell Culture

U937 monocytes were cultured at $0.5x10^6$ viable cells/ml in CM at 37°C and 5% CO_2, with medium changes and cell viability checks performed every 2 days on a Biorad

TC10 automated cell counter using the trypan blue staining method (Tran *et al*, 2011). In this method trypan blue enters dead (non-viable) cells via gated cell membrane channels due to its negative charge. Cell viability was maintained at 85% or greater for experimental assays.

To model prolonged hyperglycaemia the glucose concentration was increased in an incremental (step-wise) manner from 11mM to 15, 20, 25 or 30mM in subsets of U937 cells every 7 days using glucose-supplemented media until the highest glucose concentration (30mM) was reached. This process generated five sets of U937 cells (each with viability at 85% or higher) growing under different glucose concentrations (11, 15, 20, 25 or 30mM). Glucose-supplemented U937 cells were utilised in experiments after growing for 3 weeks at the appropriate elevated glucose concentration.

2.5 U937 Monocyte Differentiation into Macrophages

U937 monocytes were re-suspended at $1x10^6$ cells/ml in CM or glucose-supplemented (11, 15, 20 or 30mM glucose) medium. PMA (50ng/mL) was added to differentiate the U937 monocytes into adherent U937 macrophages following incubation at 37°C and 5% CO_2 for 72 hours. PMA induces differentiation into macrophage-like cells by activating protein kinase receptors and altering cell gene expression of transcription factors such as activator protein-1 (Song *et al.,* 2015; Le *et al.,* 2015).

2.5.1 CD11c Analysis

In order to confirm that the U937 monocytes had been successfully differentiated into M0 macrophages, flow cytometry was carried out to assess the expression of the CD11c cell surface marker. The U937 cells were differentiated (Section 2.5) and compared to negative control (NC) U937 cells lacking PMA treatment. Once differentiated, cells were washed 3 times in DPBS and 50µl of trypsin EDTA was added to each well for 5 minutes at 37°C and 5% CO2. The trypsin was then neutralised by adding 50µl of RPMI media. The cells were then centrifuged at 500g for 5 minute and

the cell pellet washed 3 times in DPBS. After centrifugation, cells were fixed with 200µl of 4% paraformaldehyde at room temperature for 10 minutes. The cells were washed three times in DPBS before incubating in FITC-conjugated CD11c antibody diluted 1:5 with wash buffer [1:10 FBS in DPBS] at room temperature for 1 hour in the dark.

The cells were washed three times in DPBS and analysed on a BD Accuri C6F1 flow cytometer to determine the expression of the CD11c cell surface marker. The cytometer analysed 10,000 individual cell events using BD Accuri C6 Software in the FSC and FL1-A. The data collected were displayed as the mean fluorescence intensity (MFI) compared to the NC (undifferentiated U937 monocytes).

2.6 Effect of MGN3 on CD14 in U937 Monocytes and Macrophages under Hyperglycaemic Conditions

U937 monocytes were cultured as described in Section 2.4 without subsequent differentiation into the U937 macrophages. Half the monocytes were retained in CM and the other half in glucose-supplemented (30mM glucose) medium as appropriate throughout the experiment. Monocytes were resuspended at $1x10^6$ cells/ml in CM or glucose-supplemented media (30mM glucose), and then treated in plate format (n=4) for 24 hours with/without LPS (5µg/ml) in the presence or absence of MGN3 or BSA (2mg/ml). Following treatment, U937 monocytes were pelleted by centrifugation for 5 minutes at 500g and then washed 3 times in 200µl of DPBS. The localisation of CD14 in the U937 monocytes was investigated by confocal microscopy, whilst CD14 levels were determined by flow cytometry.

Another set of U937 monocytes was resuspended at $1x10^6$ cells/ml in CM or glucose-supplemented media (30mM glucose) at 37°C and 5% CO_2. Two sets of U937 macrophages were then generated from the U937 monocytes using PMA treatment as previously described (Section 2.5). One set was prepared on sterile silicon wafers and a second set in plate format lacking silicon wafers. Half the macrophages were retained in CM and the other half in glucose-supplemented (30mM glucose) medium as appropriate throughout the experiment. The macrophages were then treated for

24 hours with/without LPS (5μg/ml) in the presence or absence of MGN3 or BSA (2mg/ml). The localisation of CD14 in macrophages cultured on silicon wafers was investigated by confocal microscopy (n=4), whilst CD14 levels in macrophages prepared in plate format were determined by flow cytometry (n=4).

2.6.1 CD14 Localisation by Confocal Microscopy

Following treatments (Section 2.6) U937 monocytes were stained with FITC-conjugated anti-human CD14 antibody. After pelleting the cells by centrifugation at 500g for 5 minutes and washing 3 times in DPBS, cells were fixed with 200μl of 4% paraformaldehyde at room temperature for 10 minutes. The cells were washed 3 times in DPBS before adding 100μl 0.1% triton X-100 for 5 minutes. The cells were washed 3 times with DPBS before incubating in blocking buffer (3% BSA in DPBS) at room temperature for 1 hour. Cells were pelleted by centrifugation at 500g for 5 minutes before incubating in FITC-conjugated CD14 antibody diluted 1:5 with wash buffer [1:10 FBS in DPBS] at 4°C overnight in the dark. A small 20μl aliquot of each sample was placed on a microscope slide, covered with a coverslip and observed under a Nikon E600 epifluorescence microscope at 100X magnification and analysed using AxioVision 2.0 software.

U937 macrophages adhered to silicon wafers were treated as described in Section 2.6 before being fixed and stained with FITC-conjugated anti-human CD14 antibody using a method similar to that described for monocytes but without requiring centrifugation between sequential steps during the staining process. Silicon wafers were washed 3 times in DPBS and cells were then fixed with 200μl of 4% paraformaldehyde at room temperature for 10 minutes. Silicon wafers were washed 3 times in DPBS before incubating in 100μl 0.1% triton X-100 for 5 minutes. Silicon wafers were washed 3 times with DPBS before incubating in blocking buffer (3% BSA in DPBS) at room temperature for 1 hour. Silicon wafers were then incubated in FITC-conjugated CD14 antibody diluted 1:5 with wash buffer [1:10 FBS in DPBS] at 4°C overnight in the dark. The silicon wafers were placed onto microscope slides and observed by confocal microscopy.

2.6.2 CD14 Analysis by Flow Cytometry

Following treatments (Section 2.6), U937 monocytes or adherent U937 macrophages in plate format were retrieved for CD14 analysis by flow cytometry. For U937 macrophages, 50µl of trypsin EDTA was added to all of the wells for 5 minutes to detach the cells. After 5 minutes, the trypsin EDTA was neutralised by adding 50µl of CM or glucose-supplemented (30mM glucose) medium. The detached U937 macrophages cells were then centrifuged at 500g for 5 minutes and the cell pellet washed 3 times in DPBS. For non-adherent U937 monocytes, cells were pelleted by centrifugation for 5 minutes at 500g and then washed 3 times in DPBS.

Macrophage or monocyte pellets were then fixed using 200µl 4% paraformaldehyde in DPBS before being washed 3 times in DPBS. The cells were then centrifuged at 500g for 5 minutes and the cell pellet incubated in 100µl 0.1% triton X-100 added for 5 minutes. The cells were then centrifuged and washed 3 times with DPBS. Cell pellets were then incubated in blocking buffer (3% BSA in DPBS) at room temperature for 1 hour. The blocking buffer was removed and cells were incubated at 4°C in the dark with FITC-conjugated anti-human CD14 antibody diluted 1:5 with wash buffer [1:10 FBS in DPBS] or wash buffer (unstained control).

Samples were centrifuged, washed 3 times with DPBS and CD14 assessed by flow cytometry on a BD Accuri C6FI cytometer. The cytometer analysed 10,000 events using BD Accuri C6 Software in the FSC FL1-A. The data collected were displayed as the total and MFI compared to the untreated negative control (NC).

2.7 Effect of MGN-3 on Bacterial Clearance under Hyperglycaemic Conditions

2.7.1 Bacteria preparation

Methicillin resistant *Staphylococcus aureus* (MRSA) strain II was grown on nutrient agar (NA) via the plate streak method to obtain single MRSA colonies. The plate was incubated at 37°C for 24 hours. After incubation one single bacterial colony was selected and added to nutrient broth (NB) which was incubated at 37°C on a shaker for 24 hours.

2.7.2 Serial dilutions

The serial dilution technique is often used to calculate the number of bacterial colonies, measured in colony forming units (CFUs) (Brugger *et al,* 2012). Ten-fold serial dilutions of the cultured broth were made using sterile saline down to 10^{-8}. The serial dilutions were plated out onto duplicate NA plates and incubated at 37°C for 24 hours. Plate counts were used to determine the concentration of MRSA in the original broth solution.

2.7.3 MGN3/LPS treatments

U937 monocytes were cultured in CM (11mM glucose) or glucose-supplemented CM medium at various glucose concentrations (15, 20, 25 or 30mM) as previously described (Section 2.4). A 24-well plate (apart from one row of controls) containing adherent U937 macrophage was created from U937 monocytes using PMA (Section 2.5). Treatments were applied with/without LPS (5μg/ml) in the presence or absence of MGN3 or BSA (2mg/ml) to each row using AB-free medium containing the appropriate glucose concentration. The plate was incubated for 24 hours at 37°C and 5% CO_2. The supernatants were discarded and $1x10^4$ CFU of MRSA in AB-free medium at the appropriate glucose concentration were added to each well on the plate. The plate was incubated for 2, 3, 4 or 5 hours at 37°C and 5% CO_2 to enable phagocytosis to take place.

The supernatant was removed and retained before adding 450μl of trypsin to each well for 5 minutes followed by 450μl of AB free media. The appropriate supernatant was added back to each well and mixed thoroughly before spreading 100μl onto duplicate nutrient gar plates and incubating for 24 hours at 37°C. MRSA recovery was then determined using the bacterial counts on the agar plates.

2.7.4 Direct effect of MGN3 on MRSA

MRSA ($1x10^4$ CFU) were prepared in AB-free medium and incubated at 37°C for 3 and 5 hours in a 24 well-plate following treatment with/without 2mg/ml MGN3. The supernatant was removed and retained before adding 450μl of trypsin to each well

for 5 minutes followed by 450µl of AB free media. The appropriate supernatant was added back to each well and mixed thoroughly before spreading 100µl onto duplicate nutrient agar plates and incubating for 24 hours at 37°C. MRSA recovery was then determined using the bacterial counts of the incubated agar plates.

2.8 Statistical analysis

Data was analysed using IBM SPSS Statistics (version 25) software. The data sets were compared between each other using one-way analysis of variance (ANOVA) followed by either Tukey *Post Hoc* comparison tests or student's t-tests. Significant differences were indicated by a probability (*P* value) < 0.05 in all cases.

3. Results

3.1 Differentiation of U937 Monocytes

U937 monocytes were cultured and differentiated into macrophages (M0) using PMA (50ng/mL). The differentiation of monocytes into M0 was confirmed by detection of CD11c by flow cytometry (Figure 1 and 2).

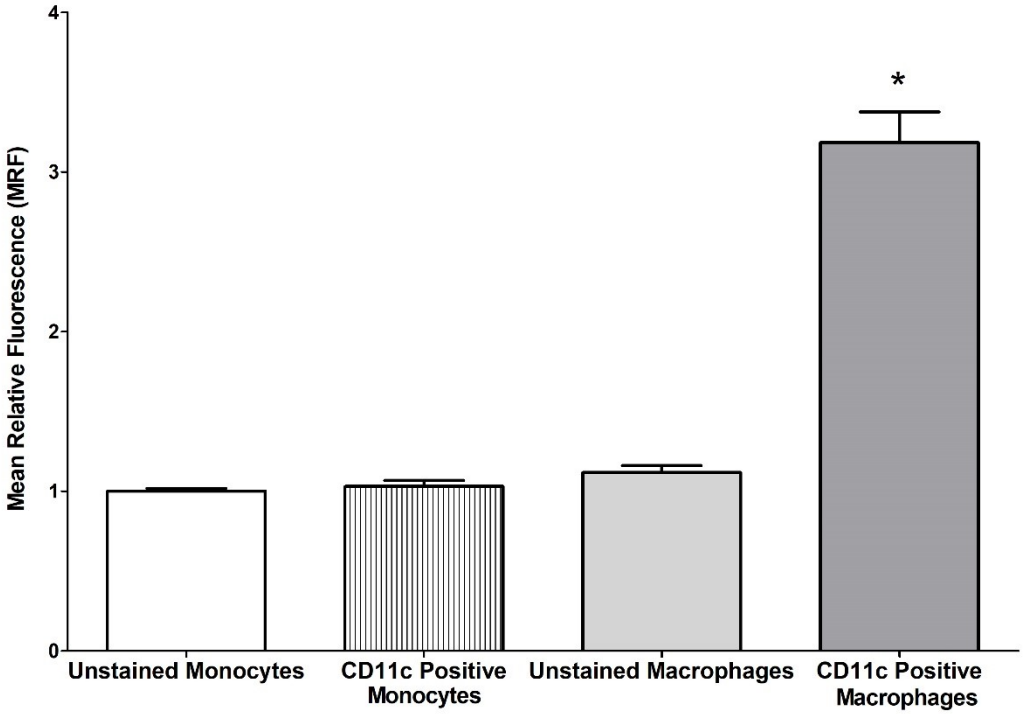
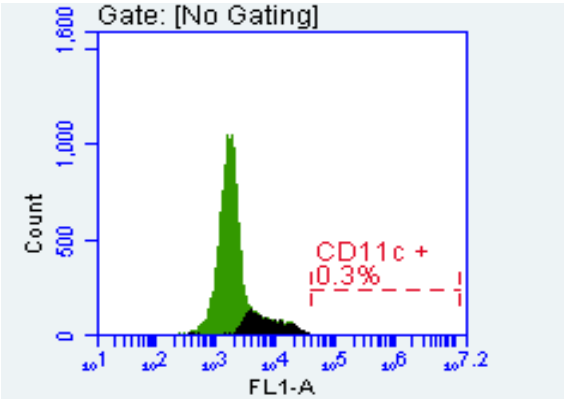
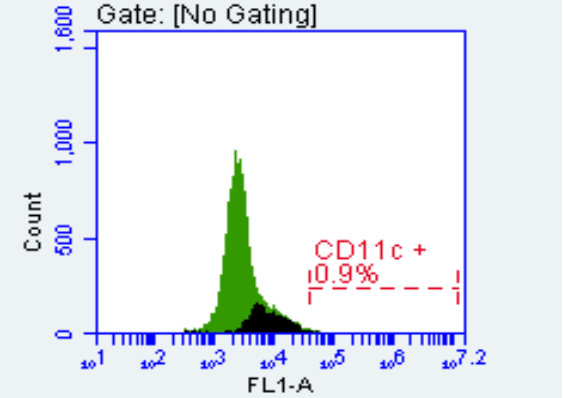
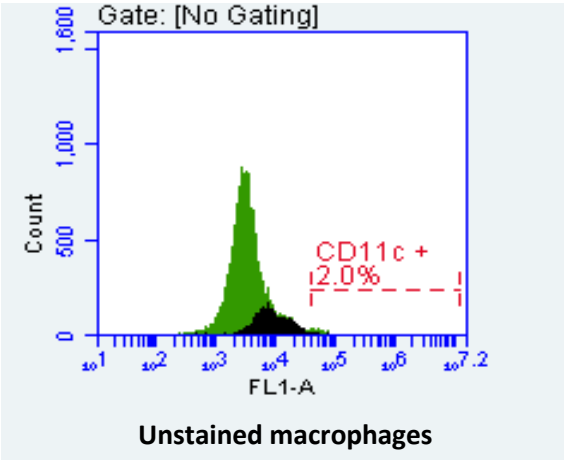


Figure 1: Generation of CD11c-positive U937 Macrophages. The mean relative fluorescence (MRF) of CD11c was significantly (: P<0.05; n = 3) elevated in CD11c-poitive U937-derived macrophages (M0) compared to non-differentiated U937 monocytes that were CD11c-negative. The MRF values are relative to levels detected in unstained monocytes (MRF =1). Columns and error bars indicate the MRF ± standard error of the mean (SEM) in all cases.*

Flow cytometry showed successful differentiation of U937 monocytes into U937-derived macrophages (M0) following treatment with PMA. Flow cytometry demonstrated a significant (*: P<0.0001) increase in MRF of CD11c (Figure 1) in PMA-differentiated U937 cells (MRF = 3.20) compared to untreated U937 monocytes (MRF = 1.00). This upregulation of the CD11c marker (Figure 2), reflected by the percentage of CD11c positive cells increasing in PMA-differentiated macrophages (76.0%) compared to monocytes (0.9%), confirmed the differentiation of U937 monocytes to macrophage-like cells following PMA treatment.







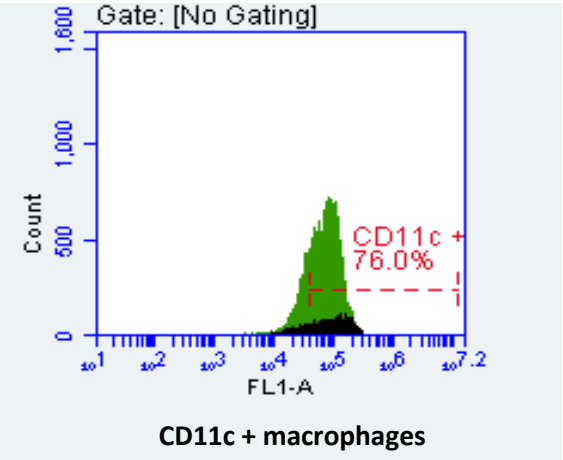


Figure 2: CD11c protein expression in U937 monocytes and macrophages (M0). Flow cytometry charts showed the percentage of CD11c positive cells increases in PMA-differentiated macrophages (76.0%) compared to monocytes (0.9%).

3.2 Effect of MGN3 on the Phagocytosis of MRSA

U937 macrophages were cultured in glucose (11mM) prior and during treatment with/without BSA or MGN3 (2mg/mL) for 24 hours, and then incubated with MRSA for different periods (2, 3, 4 or 5hrs) of host-pathogen interaction (Figure 3).

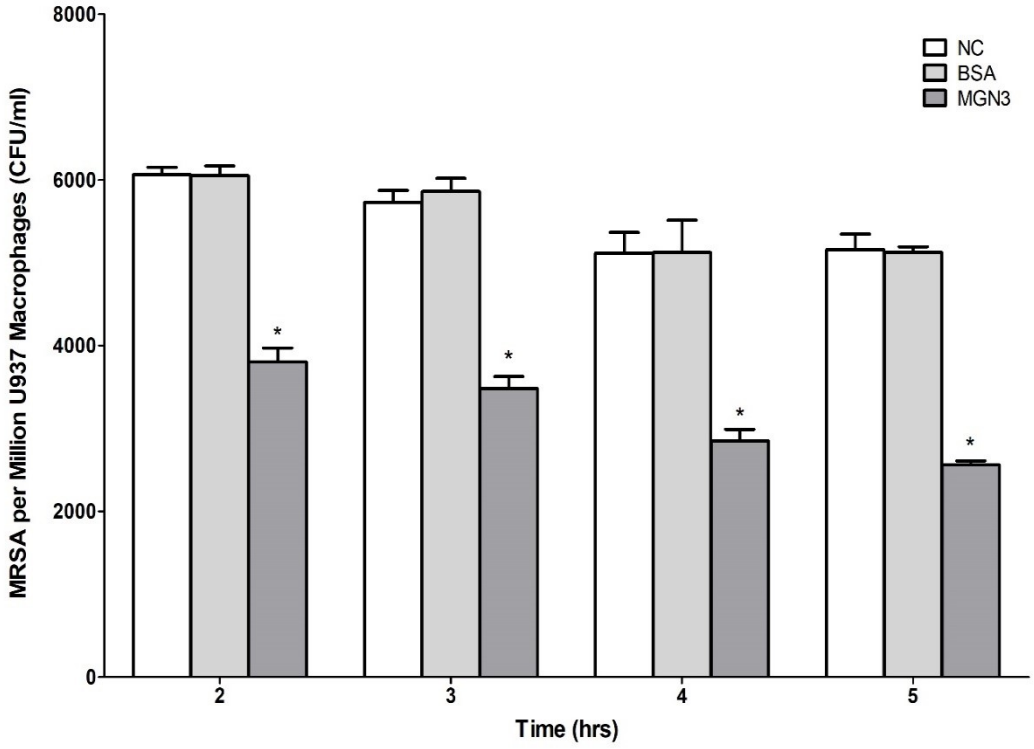


Figure 3 Effect of MGN3 on the Phagocytosis of MRSA. Mean MRSA recovery (CFU/ml) per million viable macrophages cultured in 11mM glucose was determined following a period (2, 3, 4 and 5 hours) of host-pathogen interaction with/without prior treatment of macrophages with MGN3. Significant (: P < 0.05; n = 12) changes in MRSA recovery (CFU/ml) per million viable macrophages compared to the untreated negative control (NC) were observed following treatment with MGN3 but not BSA. Columns and error bars indicate the mean MRSA recovery (CFU/ml) per million viable macrophages ± the standard error of the mean (SEM) in all cases.*

MRSA recovery (CFU/ml) per million viable macrophages progressively decreased with increasing periods of host-pathogen interaction, indicative of macrophages carrying out successful bacterial clearance. Statistical analysis confirmed a significant decrease (P = 0.0001; n = 12) in MRSA recovery (CFU/ml) per million viable macrophages compared to the corresponding negative control (NC) following treatment of macrophages with MGN3 (but not the BSA control), regardless of the period of host-pathogen interaction. This was understandable as the BSA treatment was a control molecule not expected to have any activity on bacterial clearance. These findings demonstrated MGN3 enhances the phagocytosis of MRSA by U937

macrophages. Moreover, the absolute difference in bacterial clearance between the corresponding NC and MGN3 treated macrophages was maintained as the host-pathogen incubation period increased from 2 to 5 hours.

3.3 Direct Effect of MGN3 on MRSA Growth

MRSA was grown in 11mM glucose with/without direct treatment with MGN3 (2mg/mL) to span similar periods (3 or 5hrs) of host-pathogen interaction outlined in Section 3.2. There was no significant difference (P = 0.981, 3hrs and P = 0.87, 5 hrs; n = 12) noted between MGN3-treated bacteria and corresponding negative controls consisting of MRSA grown in the absence of MGN3 (Figure 4).

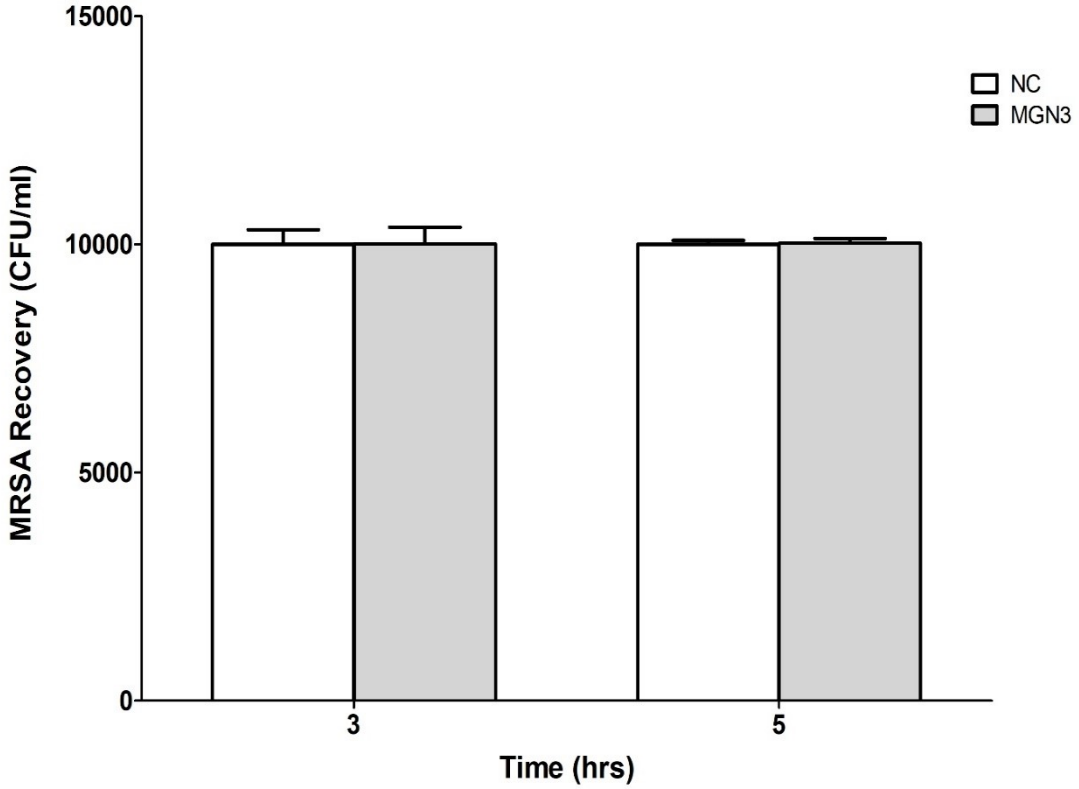


Figure 4: Direct Effect of MGN3 on MRSA Growth. Mean MRSA recovery (CFU/ml) after incubation in 11mM glucose with/without MGN3 treatment for 3 or 5 hours. Columns and error bars indicate the mean MRSA recovery (CFU/ml) ± the standard error of the mean (SEM) in all cases. No significant differences (P > 0.05; n = 15) were detected between MRSA growth during MGN3 treatment and the corresponding negative control (NC) consisting of MRSA grown in the absence of MGN3.

These findings suggest that MGN3 has no direct effect on MRSA growth and confirm the enhanced bacterial clearance observed in Figure 2 was due to the effect of MGN3 on macrophage (host) phagocytic activity rather than trace amounts of MGN3 having any direct inhibitory effect on bacterial growth.

3.4 Effect of Glucose Concentration on MGN3-Induced Phagocytosis

U937 macrophages were cultured in different glucose concentrations (11, 15, 20, 30mM) and treated with or without MGN3 (2mg/mL) for 24 hours prior to a 3-hour period of host-pathogen interaction (Figure 5). Statistical analysis using a one-way ANOVA confirmed a significant difference ($P < 0.0001$) between the treatment groups.

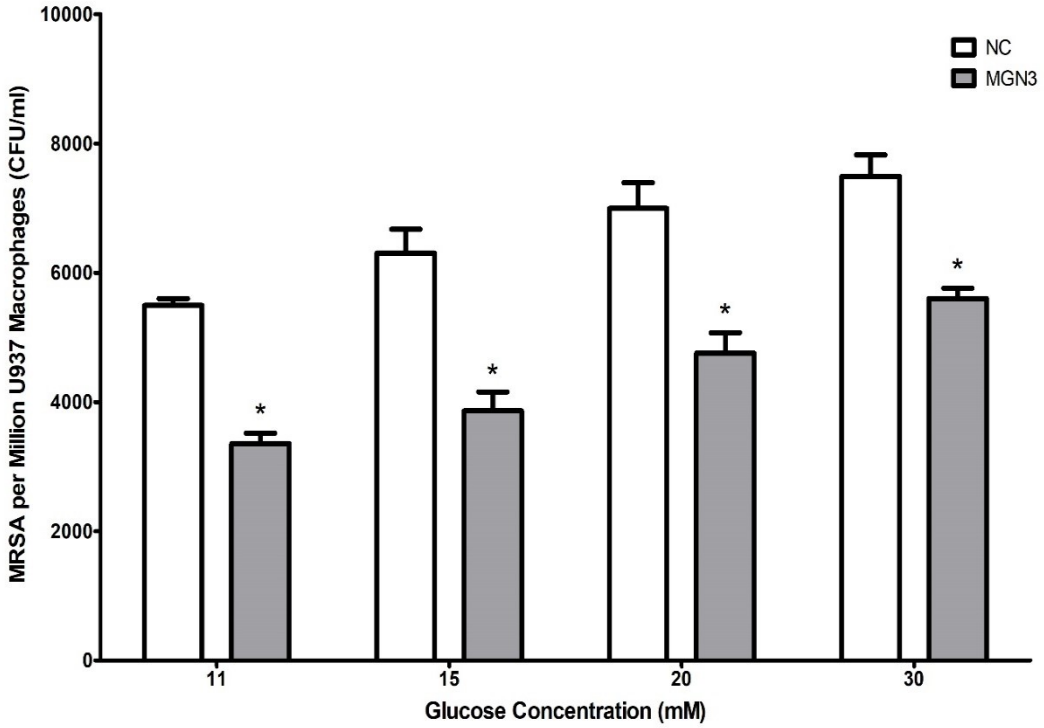


Figure 5: Mean MRSA recovery (CFU/ml) per million viable macrophages cultured in progressively increasing glucose concentrations (11, 15, 20, 30mM) was determined following treatment with or without MGN3 and a 3-hour period of host-pathogen interaction. Bacterial clearance was significantly (P<0.05; n = 15) impaired by increasing glucose concentration in both the negative control (NC) and MGN3-treated macrophages. However, MRSA recovery (CFU/ml) per million viable macrophages was significantly (: P < 0.05; n = 15) reduced following treatment with MGN3 compared to the corresponding NC, regardless of the glucose concentration. Columns and error bars indicate the mean MRSA recovery (CFU/ml) per million viable macrophages ± the standard error of the mean (SEM) in all cases.*

The phagocytic ability of U937 macrophages was significantly ($P < 0.0001$) impaired by increasing glucose concentration in both the negative control (NC) and MGN3-treated macrophages, indicated by increased MRSA recovery following the 3-hour host-pathogen interaction. However, MGN3 significantly increased phagocytosis compared to the corresponding NC, regardless of the glucose concentration. This suggests that MGN3 can negate the detrimental effects of glucose on macrophage-mediated bacterial clearance, even at very high glucose concentrations of 30mM.

3.5 Effect of Glucose on Monocyte/Macrophage CD14 Protein Levels

3.5.1 Monocyte CD14 Protein Levels

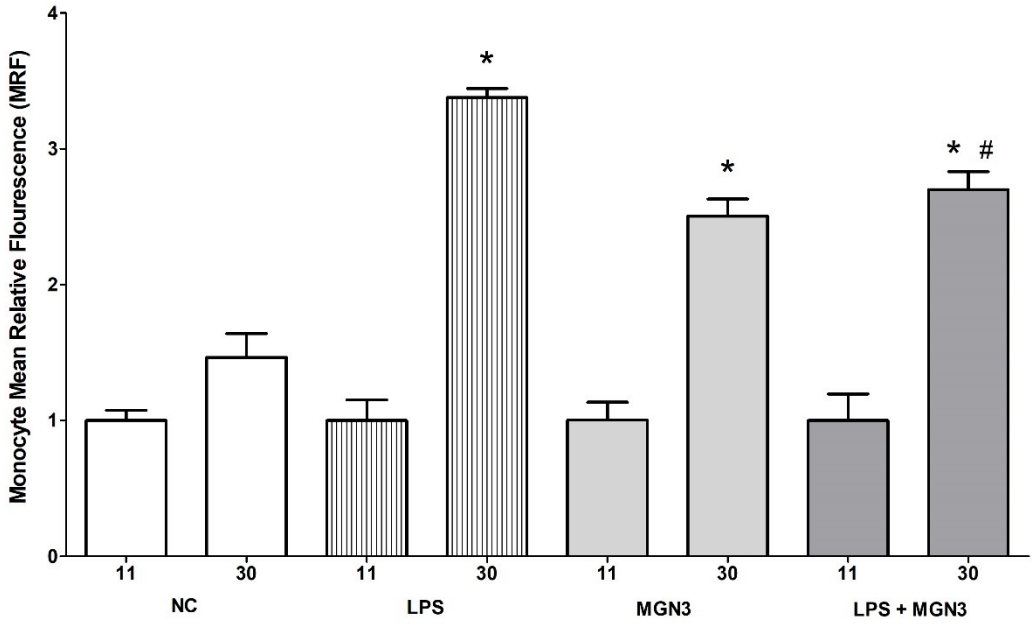


Figure 6: Effect of High Glucose on CD14 Levels in U937 Monocytes. MGN3 and particularly LPS-activation of U937 monocytes significantly (:P < 0.0001; n = 4) increased levels of CD14, measured as mean relative fluorescence (MRF) by flow cytometry, following exposure to high (30mM) glucose levels compared to corresponding lower (11mM) glucose conditions. MGN3 was able to significantly (#: P < 0.0001; n = 4) reverse the effects of high glucose on LPS-mediated induction of CD14 protein expression. Columns and error bars indicate the MRF ± the standard error of the mean (SEM) in all cases.*

The effect of glucose on CD14 protein levels in U937 monocytes was assessed by flow cytometry (Figure 6). Findings showed high (30mM) glucose only significantly (*: $P < 0.0001$, $n = 4$) increased CD14 levels in U937 monocytes following treatment with

MGN3 or LPS, when comparing to corresponding CD14 levels found at 11mM glucose. In contrast, high (30mM) glucose had no significant (P>0.05) effect on CD14 levels (mean relative fluorescence (MRF) = 1.47) in inactivated negative control (NC) monocytes compared to CD14 levels found at 11mM glucose (MRF = 1.00) in NC monocytes. Although both MGN3- and LPS-activation appeared to mediate the response of monocytes to high (30mM) glucose concentration, the induction of CD14 protein levels by MGN3 (MRF = 2.51) was relatively modest compared to levels induced by LPS (MRF = 3.38). Moreover, MGN3 was able to significantly (#: P = 0.005) reverse the effects of high (30mM) glucose on LPS-mediated CD14 levels, reducing the MRF by 20.1% (from 3.38 to 2.70).

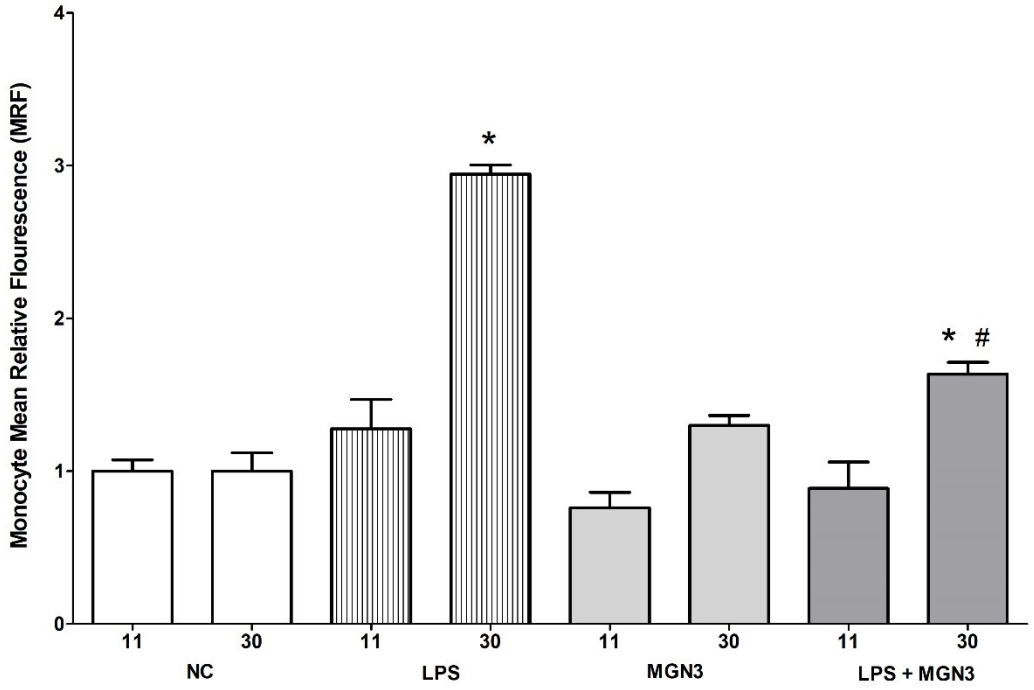


Figure 7: Effect of High Glucose on CD14 Levels in U937 Monocytes. MGN3 and particularly LPS-activation of U937 monocytes significantly (:P < 0.0001; n = 4) increased levels of CD14, measured as mean relative fluorescence (MRF) by flow cytometry, following exposure to high (30mM) glucose levels compared to corresponding lower (11mM) glucose conditions. MGN3 was able to significantly (#: P < 0.0001; n = 4) reverse the effects of high glucose on LPS-mediated induction of CD14 protein expression. Columns and error bars indicate the MRF ± the standard error of the mean (SEM) in all cases.*

By determining the mean relative fluorescence (MRF) compared to the appropriate glucose-matched negative control (NC), CD14 flow cytometry data (Figure 7) showed that at lower glucose conditions (11mM), neither LPS (MRF = 1.28) or MGN3 (MRF = 0.76) had significant effects on CD14 levels relative to the glucose-matched NC (MRF = 1.00). Similarly, at high glucose (30mM), MGN3 had no significant effect on CD14 levels (MRF = 1.30) compared to the glucose-matched NC (MRF = 1.00). In stark contrast, CD14 levels were significantly (*: $P < 0.0001$; $n = 4$) stimulated by LPS (MRF = 2.95) at high glucose (30mM) conditions compared to the glucose-matched NC (MRF = 1.00). However, concomitant treatment of MGN3 with LPS (MGN3+LPS) could significantly (#: $P < 0.0001$; $n = 4$) dampen the elevation of CD14 levels (MRF = 1.63) induced by LPS activation (MRF = 2.95).

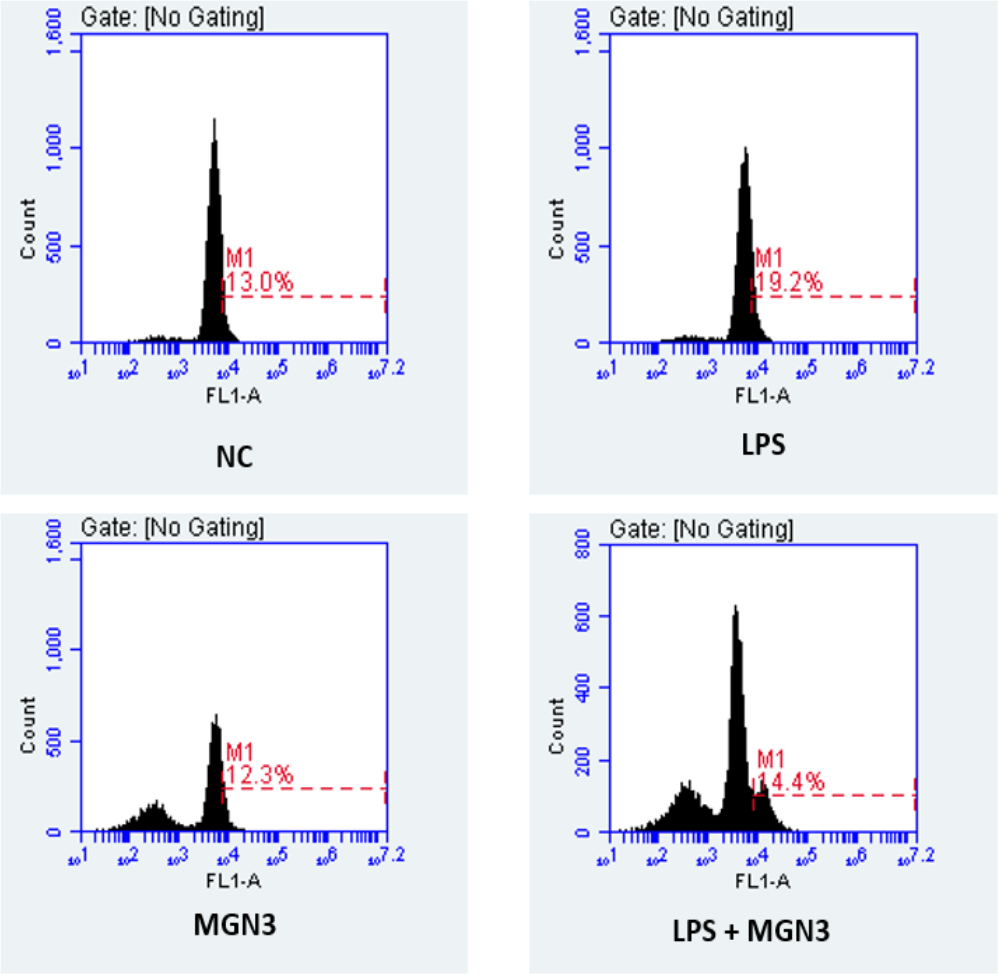


Figure 8: Expression of CD14 in monocytes. Example of flow cytometry charts showing percentage of CD14-positive cells increased in LPS-treated monocytes after exposure to high (30mM) glucose compared to the negative control (NC). MGN3 inhibited the effect of LPS on CD14, with combined (LPS+MGN3) treatments resulting in intermediate levels of CD14 in monocytes.

It was noted that there was an increase in percentage of CD14-positive U937 monocytes in all treatment groups after exposure to high (30mM) glucose (Figure 8). However, MGN3 was seen to reduce the effect of LPS on CD14, bringing CD14 down to an intermediate level with the combined (LPS+MGN3) treatments.

3.5.2 Macrophage CD14 Protein Levels

The effect of glucose on CD14 protein levels in U937 macrophages was assessed by flow cytometry (Figure 9). Compared to corresponding CD14 levels at low (11mM) glucose conditions, only LPS-activation significantly (*: $P = 0.043$; $n = 4$) increased CD14 levels in U937 macrophages cultured under high (30mM) glucose conditions, increasing MRF from 1.0 to 1.4. All other treatments and the negative control (NC) showed no significant difference ($P > 0.05$) between CD14 protein levels at high (30mM) glucose compared to corresponding low (11mM) glucose conditions. Similarly, to findings found with U937 monocytes, MGN3 significantly (#: $P = 0.03$; $n = 4$) reversed LPS-mediated elevation of CD14 levels in macrophages at high (30mM) glucose, reducing MRF by 17.9% (from 1.40 following LPS-activation to 1.15 in macrophages receiving combined LPS and MGN3 treatment).

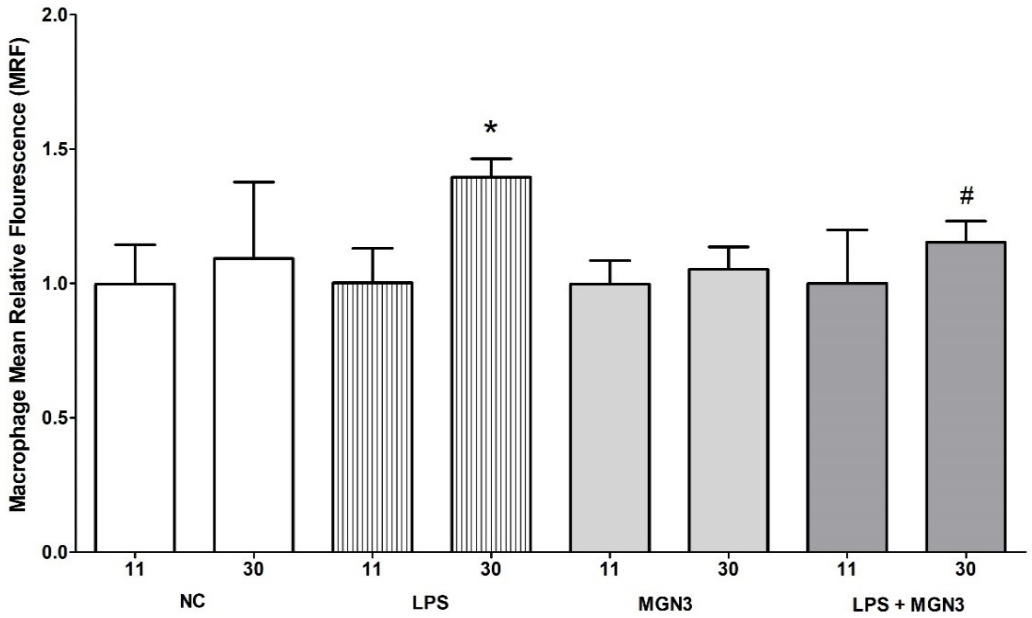


Figure 9: Effect of High Glucose on CD14 Levels in U937 Macrophages. LPS activation of U937 macrophages significantly (:P < 0.05; n = 4) increased levels of CD14, measured as mean relative fluorescence (MRF) by flow cytometry, following exposure to high (30mM) glucose compared to corresponding lower (11mM) glucose conditions. MGN3 was able to significantly (#: P < 0.05; n = 4) reverse the effects of high glucose on LPS-mediated CD14 protein expression. Columns and error bars indicate the MRF ± the standard error of the mean (SEM) in all cases.*

By determining the mean relative fluorescence (MRF) compared to the appropriate glucose-matched negative control (NC), CD14 flow cytometry data (Figure 10) showed that at lower (11mM) glucose conditions, neither LPS (MRF = 1.50) or MGN3 (MRF = 0.98) had significant (P > 0.05) effects on CD14 levels in U937 macrophages relative to the glucose-matched NC (MRF = 1.00). These findings in U937 macrophages mirrored those observed in U937 monocytes. Similar to U937 monocytes, MGN3 had no significant (P > 0.05) effect on CD14 levels at high (30mM) glucose (MRF = 1.02) compared to the glucose-matched NC (MRF = 1.00). In concordance with findings observed in U937 monocytes, CD14 levels were significantly (*: P = 0.001; n = 4) stimulated in U937 macrophages by LPS (MRF = 2.09) at high glucose (30mM) conditions when compared to the glucose-matched NC (MRF = 1.00). Furthermore, concomitant treatment of MGN3 with LPS (MGN3+LPS) could again significantly (#: P = 0.012; n = 4) dampen the elevation of CD14 levels induced by LPS activation (reducing MRF = 2.09 to 1.63).

CD14 levels for corresponding treatments closely mirrored each other in U937 monocytes and U937 macrophages, although the stimulation of CD14 in high (30mM) glucose conditions was moderately more pronounced in monocytes (MRF = 2.95) than macrophages (MRF = 2.09).

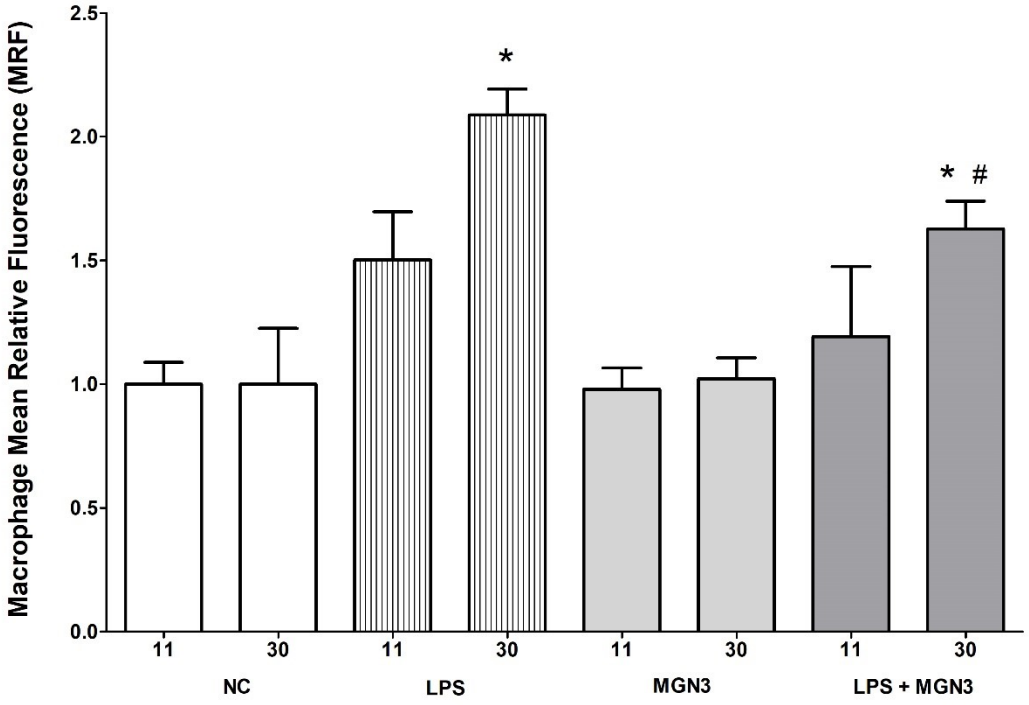


Figure 10: LPS-Activation of U937 Macrophages Mediates High Glucose Induction of CD14 Protein Expression. LPS-activation of U937 macrophages significantly (:P < 0.01; n = 4) increased mean levels of CD14 relative to the glucose-matched negative control (NC), measured as mean relative fluorescence (MRF) by flow cytometry, following exposure to high (30mM) glucose. MGN3 significantly (#: P < 0.05; n = 4) reversed the LPS-mediated induction of CD14 at high glucose (30mM) conditions. Columns and error bars indicate the MRF ± the standard error of the mean (SEM) in all cases.*

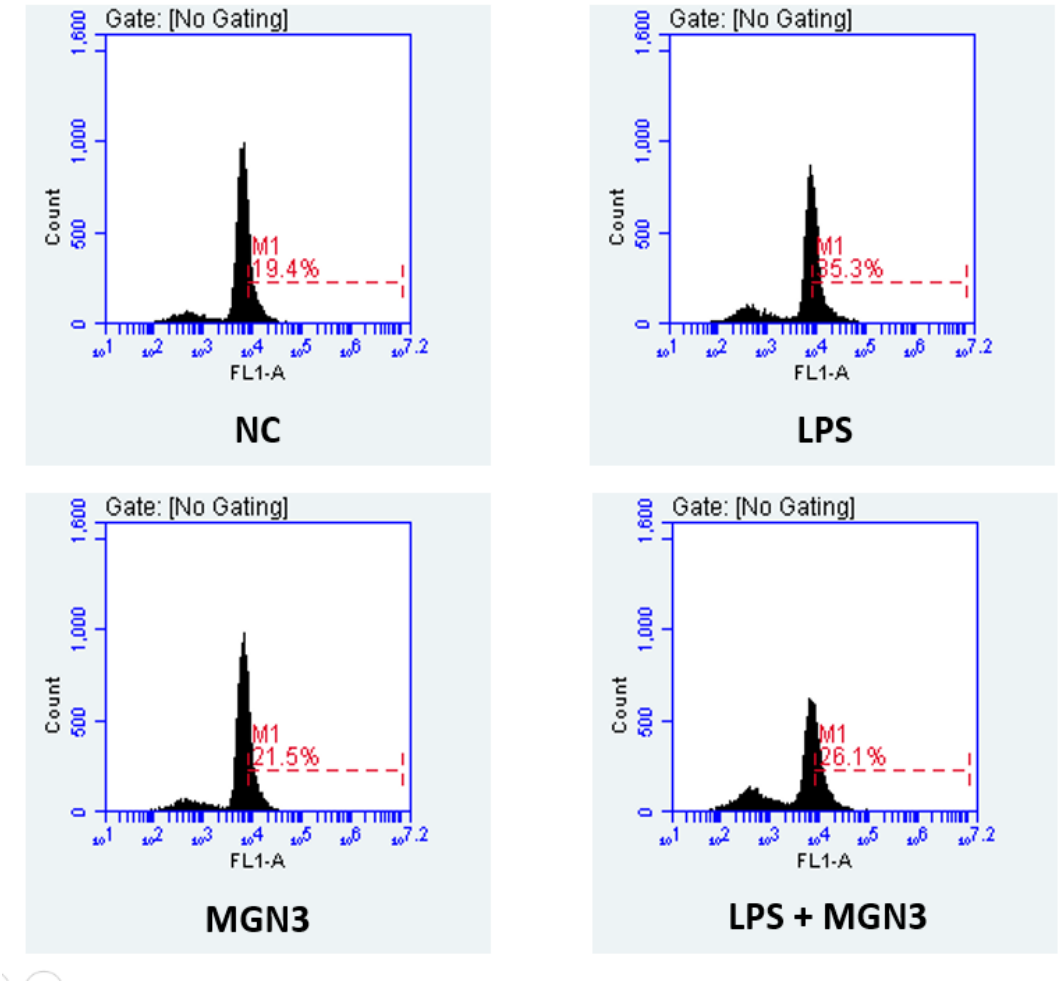


Figure 11: Expression of CD14 in U937-derived macrophages (M0). Example of flow cytometry charts showing CD14 increased in LPS-treated macrophages after exposure to high (30mM) glucose compared to the negative control (NC). MGN3 inhibited the effect of LPS on CD14, with combined (LPS+MGN3) treatments resulting in intermediate levels of CD14.

It was noted that there was an increase in CD14 expression by macrophages in all treatment groups after exposure to high (30mM) glucose (Section 3.5.2). MGN3 was seen to reduce the effect of LPS on CD14, reducing CD14 levels by 9.2% with the combined (LPS+MGN3) treatments (Figure 11).

3.6 Confocal Microscopy

3.6.1 Effect of Glucose on CD14 Levels in U937 Monocytes

U937 monocytes were cultured in 11mM or 30mM glucose and stained using a FITC-labelled anti-human CD14 antibody, following treatment with/without LPS and/or MGN3 (n = 4).

CD14 protein expression in U937 monocytes was very similar following all treatments (Figure 12) at lower (11mM) glucose conditions.

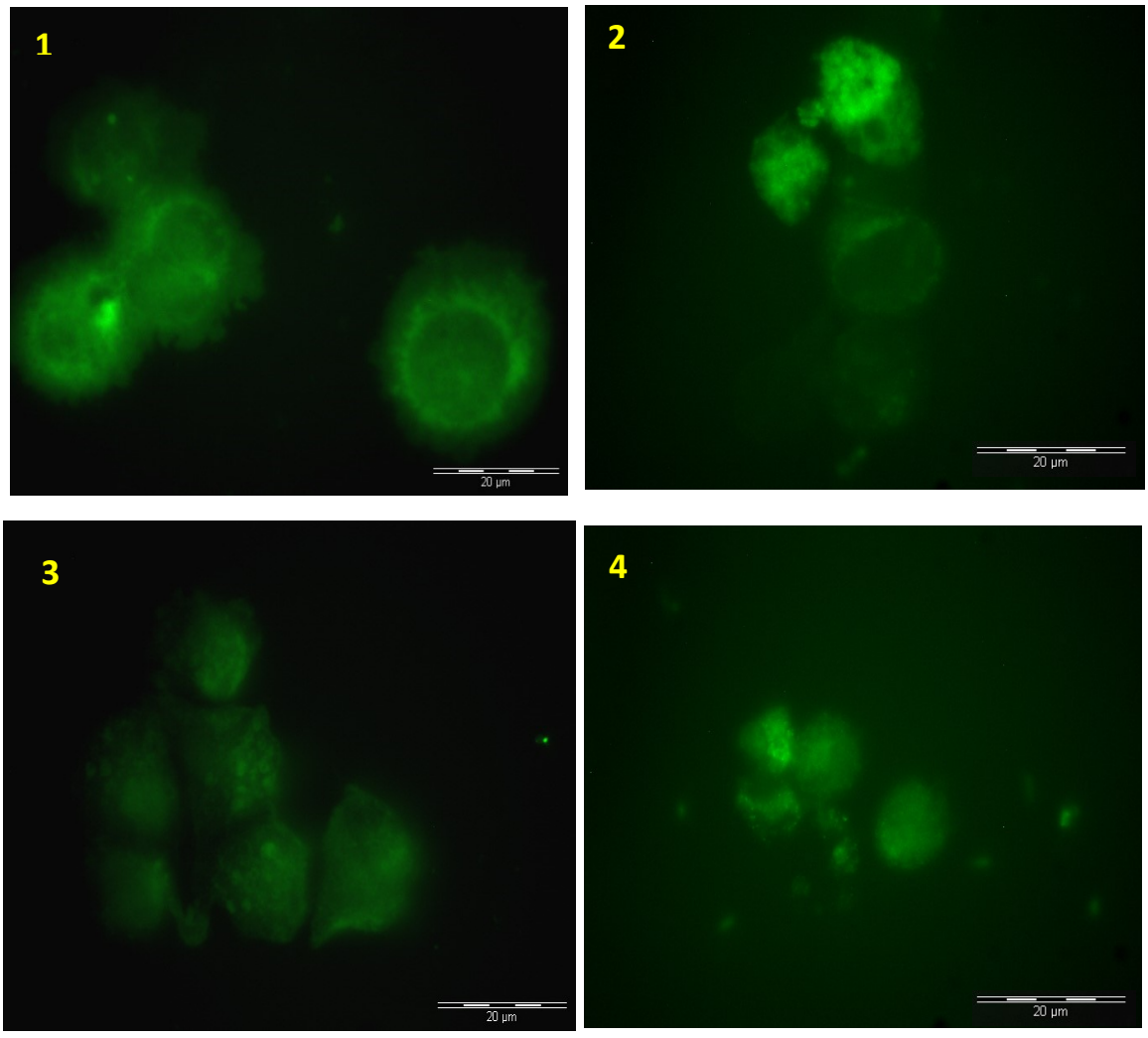


Figure 12: CD14 Protein Expression in U937 Monocytes Cultured in 11mM Glucose. CD14 Protein expression was detected by confocal microscopy using FITC-staining of CD14 protein expression, indicated by green fluorescence. Panel 1 = untreated negative control (NC) U937 monocytes, Panel 2 = LPS-activated monocytes, Panel 3 = MGN3-treated monocytes, Panel 4 = monocytes treated with both LPS and MGN3 (LPS + MGN3). Monocytes were cultured in 11mM glucose in all treatment groups. Images were captured at an objective magnification of 100x and scale bars represent 20µm.

Monocytes treated with MGN3 and cultured at high (30mM) glucose (Figure 13: Panel 3) showed a similar level of fluorescence to untreated (NC) monocytes (Figure 13: Panel 1). In contrast, monocytes treated with LPS (Figure 13: Panel 2) showed the highest CD14 protein expression compared to the NC, in agreement with flow cytometry data (Section 3.5). Moreover, intermediate fluorescence levels lying between those of the NC and LPS-treated cells were found (Figure 13: Panel 4) when U937 monocytes were treated with both LPS and MGN3 together (LPS + MGN3).

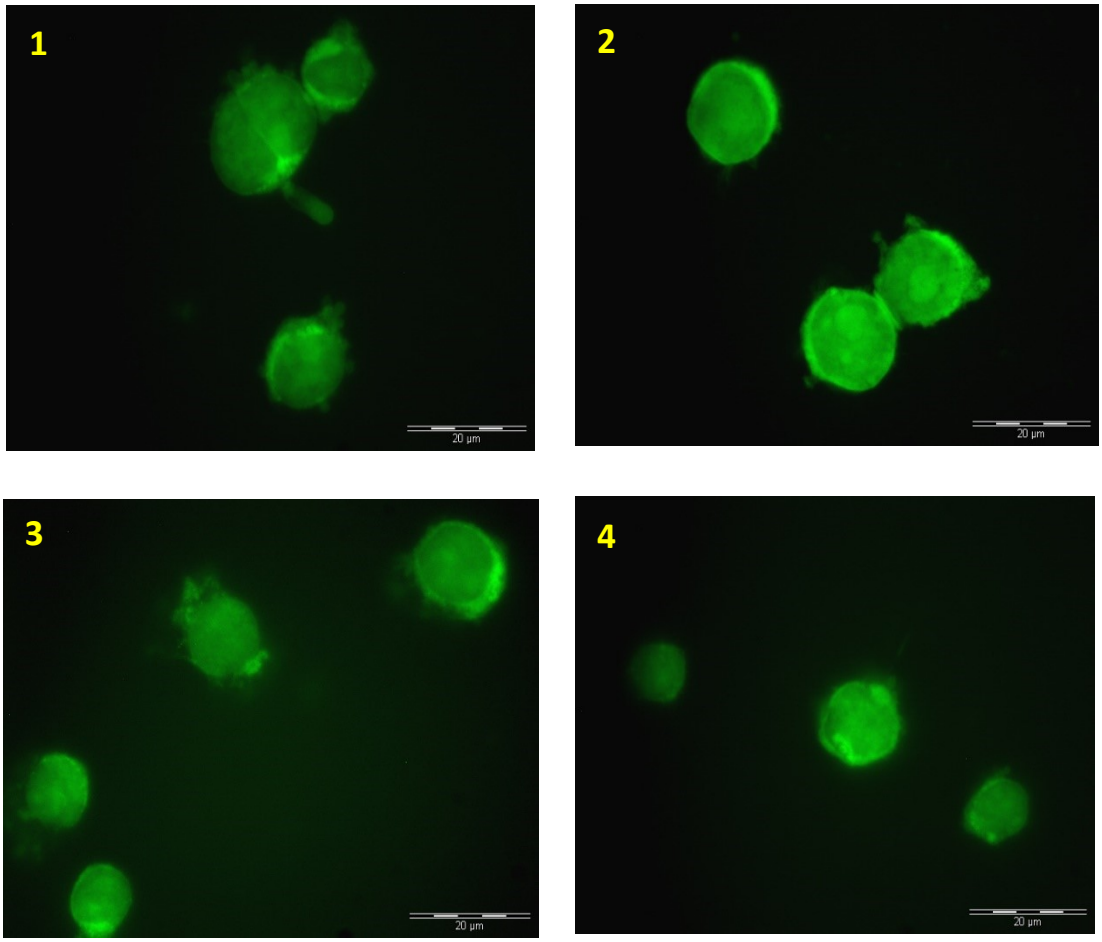


Figure 13: CD14 Protein Expression in U937 Monocytes Cultured in 30mM Glucose. CD14 Protein expression was detected by confocal microscopy using FITC-staining of CD14 protein expression, indicated by green fluorescence. Panel 1 = untreated negative control (NC) U937 monocytes, Panel 2 = LPS-activated monocytes, Panel 3 = MGN3-treated monocytes, Panel 4 = monocytes treated with both LPS and MGN3 (LPS + MGN3). Monocytes were cultured in 30mM glucose in all treatment groups. Images were captured at an objective magnification of 100x and scale bars represent 20µm.

3.6.2 Effect of Glucose on CD14 Levels in U937 Macrophages

U937 macrophages were cultured in 11mM or 30mM glucose and stained with FITC-labelled anti-human CD14 antibody, following treatment with/without LPS and/or MGN3 (n = 4).

CD14 protein expression in U937 macrophages was very similar following all treatments (Figure 14) at lower (11mM) glucose conditions.

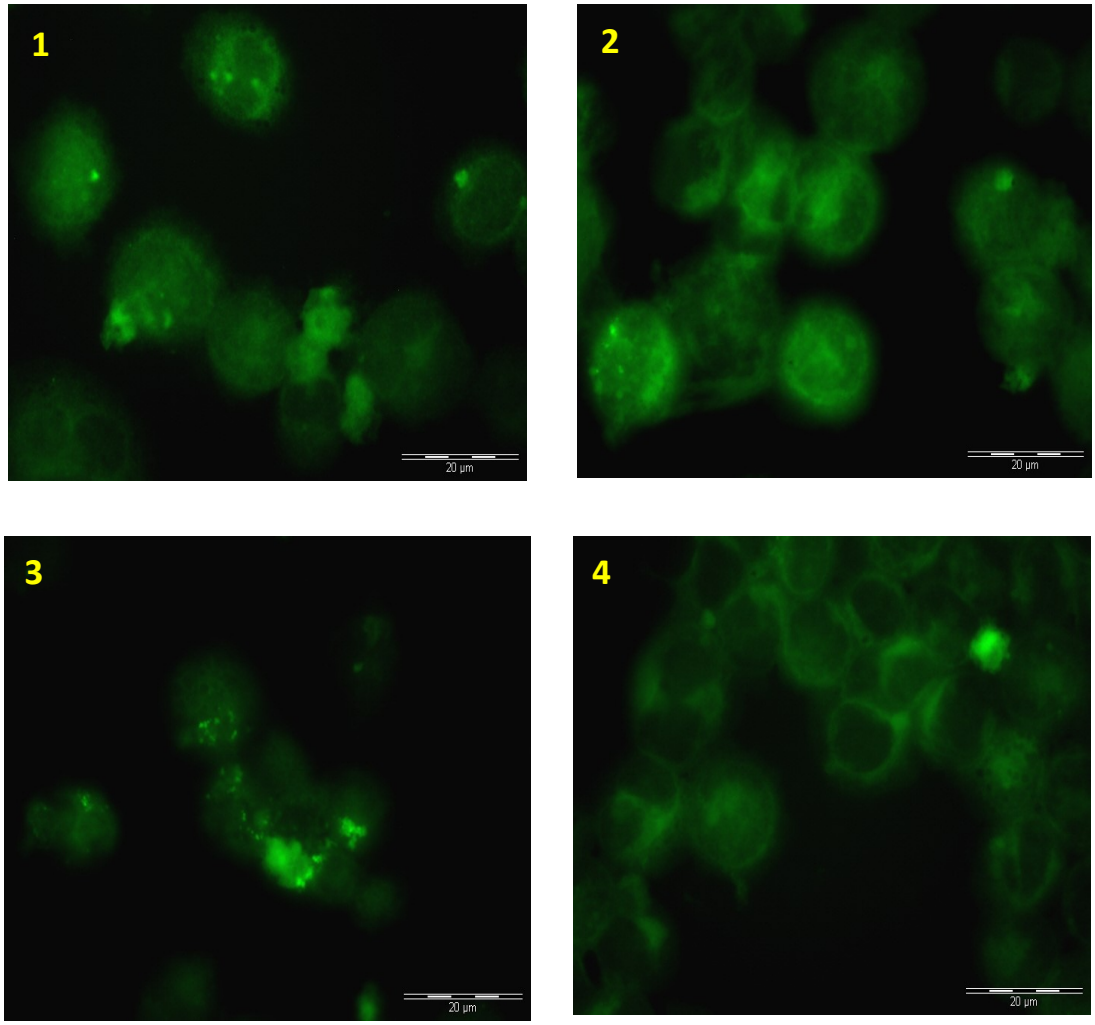


Figure 14: CD14 Protein Expression in U937 Macrophages Cultured in 11mM Glucose. CD14 Protein expression was detected by confocal microscopy using FITC-staining of CD14 protein expression, indicated by green fluorescence. Panel 1 = untreated negative control (NC) U937 macrophages, Panel 2 = LPS-activated macrophages, Panel 3 = MGN3-treated macrophages, Panel 4 = macrophages treated with both LPS and MGN3 (LPS + MGN3). Macrophages were cultured in 11mM glucose in all treatment groups. Images were captured at an objective magnification of 100x and scale bars represent 20µm.

Macrophages treated with MGN3 and cultured at high (30mM) glucose (Figure 15: Panel 3) showed a similar level of fluorescence to untreated (NC) monocytes (Figure 15: Panel 1). In contrast, macrophages treated with LPS (Figure 15: Panel 2) showed higher CD14 protein expression compared to the NC, in agreement with flow cytometry data (Section 3.5). Moreover, intermediate fluorescence levels lying between those of the NC and LPS-treated cells were found (Figure 15: Panel 4) when U937 macrophages were treated with both LPS and MGN3 together (LPS + MGN3).

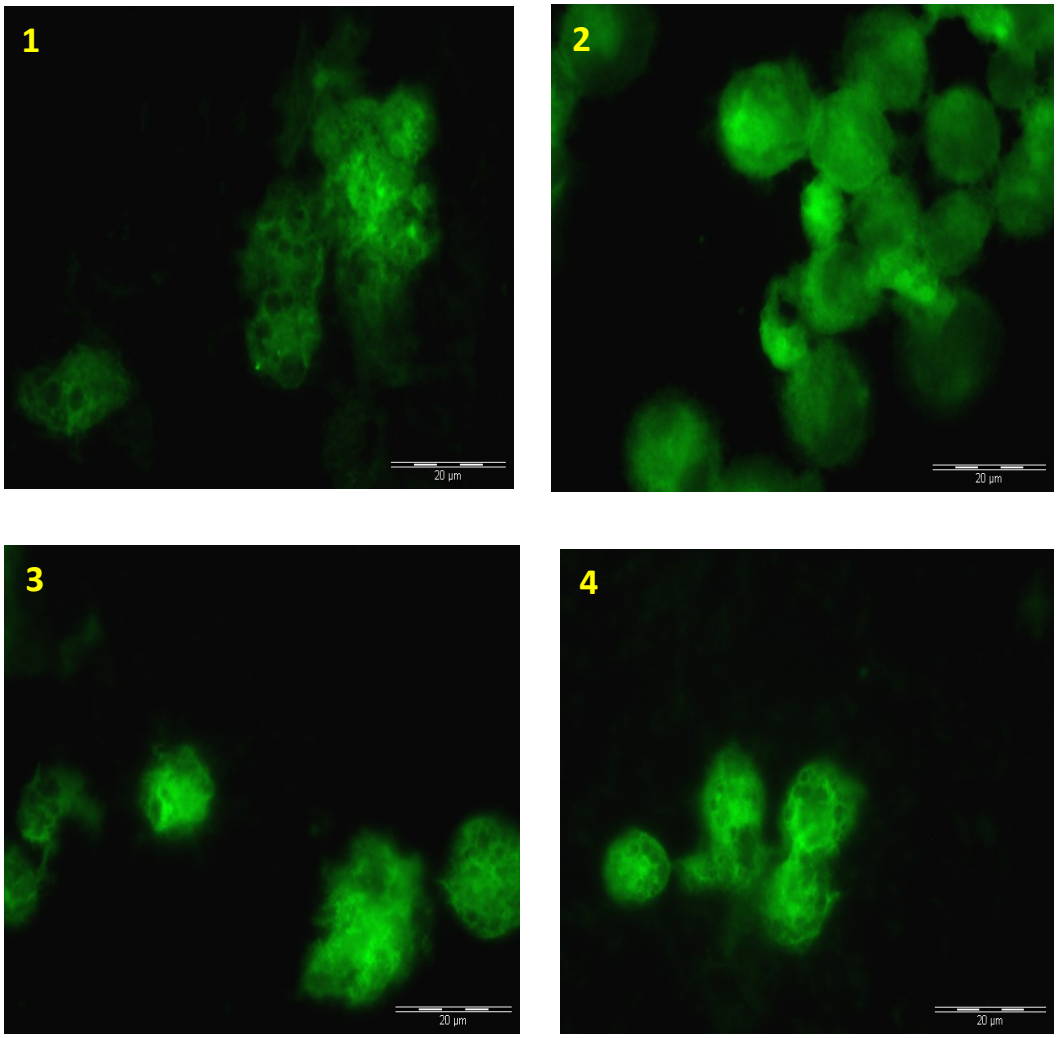


Figure 15: CD14 Protein Expression in U937 Macrophages Cultured in 30mM Glucose. CD14 Protein expression was detected by confocal microscopy using FITC-staining of CD14 protein expression, indicated by green fluorescence. Panel 1 = untreated negative control (NC) U937 macrophages, Panel 2 = LPS-activated macrophages, Panel 3 = MGN3-treated macrophages, Panel 4 = macrophages treated with both LPS and MGN3 (LPS + MGN3). Macrophages were cultured in 30mM glucose in all treatment groups. Images were captured at an objective magnification of 100x and scale bars represent 20µm.

4. Discussion

Consumption of dietary fibres such as MGN3 is associated with several health benefits, including reduced risk of diabetes (Mendis et al., 2016). The aim of this study was to determine the effect of MGN3 on a key monocyte/macrophage-associated marker of inflammation (CD14) associated with diabetes and macrophage-mediated phagocytosis under hyperglycaemic conditions. Phagocytosis was assessed by testing the effect of MGN3 on the clearance of MRSA by LPS-activated and unactivated U937 macrophages cultured in *high* (30mM) and *low* (11mM) glucose. The inflammatory marker, CD14, was investigated in U937 monocytes/macrophages using a FITC-labelled anti-human CD14 antibody via flow cytometry and visualised using confocal microscopy.

4.1 U937 Monocyte Differentiation into Macrophages (M0)

The differentiation of U937 monocytes into macrophages was confirmed by detecting the expression of CD11c proteins (Figure 1). It is known that monocytes and other leukocytes can be identified by looking at their cell surface markers (Geissman *et al,* 2003). Macrophages display the cell surface marker CD11c (Li *et al,* 2015; Lumeng *et al,* 2007). Other literature has shown under inflammatory conditions monocytes can express low levels of CD11c, however large amounts are linked with monocyte derived macrophages (Geissmann *et al,* 2003; Arndt *et al,* 2007).

4.2 The effect of MGN3 on Immune Responses

4.2.1 The effect of MGN3 on the Phagocytosis

MGN3 significantly promoted phagocytosis of MRSA in a time-dependent manner during host pathogen interactions, with increasing phagocytosis occurring following increasing incubation times (2, 3, 4 or 5 hours) compared to the corresponding negative control (NC) or macrophages treated with a non-specific protein (BSA control). Published data has shown that MGN3 increases the rate of phagocytosis of yeast by murine macrophages treated with MGN3 (100 and 500μg/ml) for two days before incubation with yeast for 2 hours (Ghoneum and Matsuura, 2004).A later

study performed by Ghoneum *et al* (2008) showed MGN3 stimulates phagocytosis of *Escherichia coli* in human phagocytes (monocytes and neutrophils). Dietary fibre from *Gracilaria algae* is also effective at increasing the rate of phagocytosis in murine macrophages, as well as showing antioxidant properties (Ye *et al,* 2009). Another study showed extracted wheat bran has potent stimulatory effects on macrophage-mediated phagocytosis (Zhou *et al,* 2010).

However, to date the effect of MGN3 under hyperglycaemic conditions has not been investigated. Thus, the effect of MGN3 (2mg/mL) on the phagocytosis of MRSA by U937-derived macrophages under diabetic (hyperglycaemic) and non-diabetic conditions was investigated using host-pathogen assays with interaction periods of between 2-5 hours (Section 3.2 and 3.4: Figures 3 and 5). Macrophages were cultured throughout in different glucose concentrations (11 – 30mM) with/without the addition of MGN3.

In concordance with published research (Marhoffer *et al,* 1992; Yano *et al,* 2012; Peleg *et al,* 2007) the results showed MRSA clearance was significantly impaired by increasing glucose in a concentration-dependent manner. However, MGN3 treatment significantly stimulated the phagocytosis of MRSA by U937 macrophages at all glucose concentrations, despite the detrimental effect of increasing hyperglycaemic conditions on bacterial clearance in the presence and particularly the absence of MGN3. MGN3 significantly ($P < 0.05$) reduced MRSA recovery compared to the negative control (NC) under both 11mM and 30mM glucose conditions, thus indicating MGN3 stimulates U937 macrophage-mediated phagocytosis and can reverse the detrimental effects of high (30mM) glucose on bacterial clearance.

Moreover, MGN3 had no direct effect on MRSA growth, confirming MGN3 reduced MRSA recovery by enhancing the phagocytic function of macrophages. This finding is in agreement with the literature, with several other studies also showing MGN3 enhances phagocytosis in macrophages and NK cells without having any effect on bacterial growth (Ghoneum and Matsuura, 2004; Ghoneum and Gollapudi, 2005; Perez-Martinez *et al,* 2015).

4.2.2 Effect of MGN3 and LPS on CD14 Levels under Diabetic and Non-Diabetic Conditions

Monocyte and Macrophage CD14 expression was analysed using flow cytometry after activation with/without LPS and/or treatment with MGN3 (Section 3.5). When monocytes or macrophages were cultured in high (30mM) but not low (11mM) glucose, LPS-activation induced CD14 protein expression (Figure 6), in agreement with published findings that suggest this elevation in CD14 leads to induces inflammation and is mediated through increased nuclear factor kB and AP-1 activities (Nareika *et al* 2008). Bacterial toxins such as LPS are detected by a specific cell surface receptors called TLRs (such as TLR4 that detects LPS) and the lipopolysaccharide binding protein CD14 that acts as a endotoxin receptor on monocytes and macrophages (Takeda and Akira; Akira *et al,* 2006; Zanoni *et al,* 2011; Zweigner *et al,* 2006; Kumar *et al,* 2009). Indeed, CD14 appears to modulate adipose tissue inflammatory activity and inflammation-driven insulin resistance (Fernandez-Real *et al,* 2011). This finding also mirrors the activation of CD14+ monocytes that occurs in diabetic patients due to their hyperglycaemia (Cipolletta *et al,* 2005).

It is thought that arabinoxylans like MGN3 may compete with LPS for receptors such as TLR4 (Tan, 2018) and this potential mechanism of action explains the findings observed throughout Section 3.5 where combined treatment with LPS and MGN3 (LPS+MGN3) dampened LPS-induced CD14 levels (Figure 5). It has been shown by previous studies that LPS greatly increases CD14 levels (Fernandez-Real *et al,* 2011; Nareika *et al,* 2008). These findings indicate MGN3 can supress LPS-induced CD14 levels in monocytes and macrophages under hyperglycaemic conditions. Monocytes/macrophages that received combined treatment with LPS and MGN3 (Figure 6) had intermediate levels of CD14 compared to the corresponding negative control (NC) and LPS-activated cells, supporting growing evidence that LPS and MGN3 possibly compete for the same receptors (TLR4 and dectin-1 receptors) in monocytes/macrophages but MGN3 does not activate these receptors to the same level (Tan, 2018). This is probably due to the fact that LPS and arabinoxylans like MGN3 have many physical similarities including in their molecular weight and structure (Li *et al,* 2015; Ghoneum *et al,* 2013; Bowyer *et al,* 2010).

Murine studies (Zheng *et al,* 2012) have shown that sugars (e.g. D-galactosamine) increase TLR4 and CD14 expression whereas MGN3 inhibited mRNA expression of CD14. Another study (Son, 2014) showed after 4 weeks treatment of LPS, MGN3 was able reduce TLR4 expression in mice undergoing endurance exercise. A study done on chickens (Sato *et al,* 2012) showed that after 2hrs of endurance exercise followed by an injection of LPS, chickens given MGN3 supplementation had lower TLR4 and TLR7 expression than chickens which didn't receive MGN3 supplements.

MGN3 significantly ($P<0.0001$) increased CD14 levels in monocytes at high (30mM) glucose concentration when compared to 11mM glucose conditions. These findings support previous evidence that arabinoxylans such as MGN3 can stimulate cytokine and nitric oxide (NO) production in human monocytes in the absence of infection/LPS but this acute immuno-stimulatory effect is modest compared to the pro-inflammatory and often excessive response observed in the presence of infection/LPS (Zhang *et al,* 2016; Zhang *et al,* 2018). Studies have shown that MGN3 can also stimulate several other immune cells, including natural killer (NK), T and B lymphocytes (Ghoneum and Abedi, 2004; Ghoneum and Brown, 1999; Perez-Martinez *et al,* 2015). MGN3 has been shown to enhance the binding capacity of NK cells to tumours in aged mice (Ghoneum and Abedi, 2004), as well as increasing NK activity against neuroblastomas (Perez-Martinez *et al,* 2015).

However, several studies have shown MGN3 reduces inflammation. A murine study showed MGN3 reduces IL-18 protein expression in D-galactosamine-induced hepatitis (Zheng *et al,* 2012). A further murine study (Son *et al,* 2012) found that mice treated with rice bran for 4 weeks had significantly reduced TNFα levels compared to mice treated without rice bran. MGN3 has been shown to reduce pro-inflammatory cytokine production in elderly humans (Elsaid *et al,* 2019). Studies have shown rice bran has immunomodulatory effects on natural killer (NK) cells and reduces pro-inflammatory cytokine profiles (Ali *et al,* 2012; Lewis *et al,* 2018; Park *et al,* 2017).

4.3 Evaluation of MGN3 and Arabinoxylans for Diabetic Patients

A common symptom of DM is chronic inflammation (McCrimmon *et al,* 2012; Dandona *et al,* 2005). Chronic inflammation leads to an influx of leucocytes at sites of trauma or infection, resulting in an influx of CD14+ cells and pro-inflammatory cytokine production (Buckley *et al,* 2001; Fernandez-Real *et al,* 2011; Anas *et al,* 2010; Sahay *et al,* 2009). There is evidence to link constant pro-inflammatory cytokine production (e.g. IL-6 and TNF α) to insulin resistance, and this is especially observed in obese people (Zozulinska and Wierusz-Wysocka, 2006). Evidence suggests that dietary fibres like MGN3 may be able to reduce pro-inflammatory markers (Weickert and Pfeiffer, 2008), thereby dampening the effects of chronic inflammation.

MGN3 has shown been to be a beneficial supplement as part of a balanced diet helping to reduce chronic inflammation, aiding wound repair as well decreasing the likelihood of developing other illnesses such as obesity and CVD. In relation to diabetic patients, dietary fibres have been shown to counteract some of the complications of type 2 DM. This includes impaired wound healing (in the form of DFUs) (Al-Ghazzewi *et al,* 2015) as well as reducing the risk of other diseases linked to type 2 DM, including obesity and CVD (Cho *et al,* 2013; King, 2005). It has been shown that dietary fibres can promote collagen production, reduce inflammation and stimulate keratinocytes to proliferate (Al-Ghazzewi *et al,* 2015), which may aid the healing process of DFUs.

Type 2 DM and obesity have been linked together and dietary fibres could help to break this link. In terms of obesity, dietary fibres typically reduce hunger by having a long gut transit period (Kannan *et al,* 2012) and reduced food intake reduces blood glucose levels. Dietary fibres also reduce baseline glycated haemoglobin and high lipid profiles. A randomized trial assessing the effect of rice bran oil on hyperglycaemia and blood lipid profile showed that blood glucose levels significantly reduce in as little as 4 weeks when type 2 DM patients are treated with rice bran oil (Dervarjan *et al,* 2016). Soluble dietary fibres partially hydrolysed in guar gum as part of a balanced diet have been shown to reduce hyperglycaemia after meals (Kapoor *et al,* 2016). Dietary fibres extracted from mulberry leaves are effective at increasing

insulin levels and lowering high blood glucose levels (Jeszka-Skowron *et al,* 2014; Lown *et al,* 2017; Riche *et al,* 2017).

To add to the growing weight of evidence of health benefits of dietary fibres, the findings from this study suggests that MGN3 can dampen LPS-induced inflammation (elevated CD14 levels) in monocytes/macrophages under hyperglycaemic conditions, whilst simultaneously promoting the effectiveness of macrophages to carry out bacterial clearance. The potential impact of these findings are substantial, particularly in the context of treatment for DFUs that affect many diabetic patients. Successful evidence of efficacy and development of treatments involving MGN3 could promote the healing of DFUs, particularly those infected with bacteria. Moreover, DFUs infected with antibiotic-resistant bacteria may be susceptible to MGN3-mediated host clearance, thereby providing a strategy to overcome issues of antimicrobial resistance (AMR).

4.4 Future Work

MGN3 has been shown to stimulate phagocytosis in U937 macrophages and limit the detrimental effects of glucose on bacterial clearance. However, confirmation of these findings in ex vivo macrophages isolated from human peripheral blood and/or other phagocytic cells such as dendritic cells and neutrophils would provide further supporting evidence. Furthermore, studies should be continued to confirm whether MGN3 can stimulate bacterial clearance of a wider range of pathogens, including Gram negative bacteria. Moreover, the phagocytosis assay in this study assessed the effectiveness of MGN3 to promote macrophage-mediated eradication of planktonic bacteria but future work could consider biofilm arrangements that frequently colonise wounds and form environments that enable bacteria to evade host immune responses.

The downstream effects of MGN3 in reversing the detrimental effects infection (LPS-activation) under high glucose conditions in macrophages should be interrogated further using *in vitro* assays and animal models, considering CD14 and other inflammatory markers/pathways activated by hyperglycaemia to elucidate

mechanisms and key mediators by which MGN3 may dampen hyperglycaemic-induced inflammation.

Future clinical investigations following completion of in vitro and animal studies may ultimately determine whether arabinoxylans such as MGN3 could be applied with dressings directly to open wounds of diabetic patients (e.g. diabetic foot ulcers), instead of being taken as oral supplements. This local application of MGN3 could increase wound healing directly by stimulating phagocytes to clear bacteria whilst dampening excessive inflammation.

5. Conclusion

Overall, MGN3 reversed some detrimental effects of hyperglycaemia on monocyte /macrophage function, including inhibiting glucose-mediated elevation of CD14 in LPS-activated monocytes and macrophages, and reversing the glucose-mediated inhibition of bacterial (MRSA) clearance by macrophages. These findings have major potential impact for diabetic patients who typically have elevated inflammatory profiles and are at risk of developing diabetic foot ulcers that often become colonised (and in some cases infected) by bacteria. MGN3 may be a potential therapeutic strategy to concomitantly dampen inflammation, stimulate healing and promote bacterial clearance in diabetic patients with infected wounds. This strategy to mediate host immune responses could be utilised alone or in conjunction with antibiotics as a combination (dual) therapy option for infected diabetic ulcers. Moreover, future therapies could focus on use of arabinoxylans such as MGN3 in wound dressings that can be applied directly to (infected) wounds rather than through consumption of dietary fibres.

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