Unraveling the Anti-inflammatory Mechanisms of Dietary Fatty Acids

A thesis submitted for the degree of Ph.D.

By

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Declaration

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Acknowledgements

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ABBREVIATIONS

AA	Arachidonic acid
AP-1	Activator protein-1
APC	Antigen presenting cell
BSA	Bovine serum albumin
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Crohn's disease
CD14	Cluster of differentiation 14
CFP	Cyan fluorescent protein
CHO	Chinese Hamster Ovary
CLA	Conjugated linoleic acid
CNS	Central Nervous System
COPD	Chronic obstructive pulmonary disease
COX	Cyclo-oxygenase
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DHA	Docosahexanoic acid
DMSO	Dimethyl sulphoxide
DSS	Dextran sodium sulphate
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EEA1	Early endosomal antigen 1
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-related kinase
FCS	Foetal calf serum
GMCSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GPI	Glycosylphosphatidylinositol
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IFN	Inteferon
IKKα/β	IkB kinase α/β
IL	Interleukin
IL-1Ra	IL-1 receptor anatgonist
IRF	Interferon regulatory factor
ISRE	IFN-stimulated response element
IκB	Inhibitor of NκB
Jak	Janus kinases
JNK	Jun amino-terminal kinase
LA	Lauric acid

LAT	Linker for activation of T cells
LBP	Lipid binding protein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LTB	Leukotriene
Mal	MyD88 adaptor like
MAPK	Mitogen-activated protein kinase
mCD14	Membrane CD14
MCP	Macrophage chemoattractant protein
MD-2	Myeloid differentiation protein -2
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mLDL	Modified low density lipoprotein
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor-κB
NK	Natural killer
NLR	NOD like receptor
NSB	Non-specific binding
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral Blood Mononuclear Cell
PG	Prostaglandin
PI3K/Akt	Phosphoinostide 3-kinase/Akt protein family
PPAR	Peroxisome proliferator-activated receptor
PRR	Pathogen recognition receptor
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
RLR	RIG like receptor
sCD14	Soluble CD14
SDS	Sodium dodecylsulphate
SLE	Systemic lupus erythematosus
SR-A	Scavenger receptor-A
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
T_H	T-helper
Tirap	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TMB	3,3'5,5'-tetramethyl-benzidine
TNF	Tumour necrosis factor
TRAM	Trif related adaptor molecule
T_{reg}	T regulatory

Trif	TIR-domain-containing adaptor inducing interferon-β
TXB	Thromboxane
UC	Ulcerative colitis
YFP	Yellow fluorescent protein

PRESENTATIONS AT CONFERENCES/MEETINGS

2005

Immunology Masterclass 2005 – National University of Ireland, Maynooth.

Modulation of Toll-like receptor 4 (TLR4) and associated molecules by polyunsaturated fatty acids

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Modulation of Toll-like receptor 4 and associated molecules by polyunsaturated fatty acids

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Modulation of Toll-like receptor 4 and associated molecules by polyunsaturated fatty acids

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Irish Society Immunology Conference, Dublin, DCU, September 2007.

Upregulation of scavenger receptor-A by polyunsaturated fatty acids through a MAPK-dependent pathway.

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Irish Society Immunology Conference, Dublin, RDS, September 2008.

Polyunsaturated fatty acids selectively suppress transcription factor activation downstream of TLR4.

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Surface layer proteins isolated from *Clostridium difficile* activate macrophages for bacterial clearance.

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Ulster Immunology Group Meeting, Belfast, 2009

Conjugated linoleic acid decreases IRF3 activation downstream of TLR4 via down-regulation of CD14.

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PUBLICATIONS

Manuscripts under review

Surface layer proteins isolated from *Clostridium difficile* activate innate and adaptive immunity via TLR4.

Ryan A, McCoy CE, Dowling JK, Draper E, O'Reilly V, Smith SM, O'Brien J, Ní Eidhin D, O'Neill LA, Kelleher D and Loscher CE.

Gastroenterology. Manuscript number GASTRO-D-08-01721. Resubmitted following revisions May 2009.

Manuscripts in preparation

Polyunsaturated fatty acids differentially modulate macrophage activation following TLR4 ligation by LPS.

Dowling JK., Perez M. and Loscher CE.

'Conjugated linoleic acid suppresses IRF3 activation via suppression of CD14 and its micrdomain localisation'

Dowling JK, McCoy C, Doyle S, O'Neill LA and Loscher CE.

Surface layer proteins isolated from *Clostridium difficile* activate macrophages for bacterial clearance.

Collins L, Raham A, Dowling JK, Ryan AJ, Perez M, Ni Eidhin D, Kelleher D and Loscher CE.

ABSTRACT

Polyunsaturated fatty acids (PUFA) have been shown to modulate immune responses and have therapeutic effects in inflammatory disorders. The specific mechanisms of their actions have yet to be defined. The objective of this work was to elucidate such mechanisms. Macrophages are a key component of the innate response, which express toll-like receptors (TLRs). Ligation of TLR4, by its ligand lipopolysaccharide (LPS), results in macrophage activation. This study demonstrates that the n-6 derivative, conjugated linoleic acid (CLA) and n-3 PUFA, DHA and EPA differentially modulate the response of macrophages to LPS. Specifically, phagocytosis was enhanced by CLA and suppressed by n-3 PUFA and these PUFA suppressed TNFα, IL-6 and enhanced IL-10 production, rendering the macrophage less inflammatory. PUFA also suppressed macrophage migration in response to LPS and inhibited production of chemokines. Furthermore, CLA inhibited activation of the TLR4 downstream transcription factors NF-κB and IRF3, while n-3 PUFA, DHA and EPA solely inhibited NF-κB.

Further investigation revealed that PUFA selectively regulate the expression of TLR4 and its associated molecule CD14 in response to LPS, but had no effect on LPS binding to TLR4. The exact mechanism of the effects of PUFA on CD14 was elucidated by examining lipid raft 'microdomains', the location where the receptor complex clusters upon activation. We found that treatment of macrophages with CLA reduced the incorporation of CD14 into lipid rafts following activation with LPS. We then examined endocytosis of TLR4 given the role of CD14 in this process, and we found that it was suppressed by CLA. This study therefore reveals a novel mechanism whereby CLA exerts its anti-inflammatory effects. This involves suppression of CD14, the subsequent suppression of TLR4 endocytosis culminating in decreased IRF3 activation.

CHAPTER 1

GENERAL INTRODUCTION

1 THE IMMUNE SYSTEM

The immune system is a complex network of lymphoid organs, cells and humoral factors. Collectively, they provide defences of increasing specificity by means of innate ‘early’ and adaptive ‘delayed’ immunity. Although considered separate, both responses work closely to initiate and maintain protection within the body.

1.1 OVERVIEW OF INNATE IMMUNITY

Innate immunity represents a non-specific first line of defence to the body. Metazoans have survived millions of years with innate defence alone. Only vertebrates have developed alternative modes of protection and pathogen elimination, collectively known as ‘adaptive immunity’ [see section 1.2]. Primary components of innate defence include physical and chemical barriers to infection. Upon breach of these barriers a combination of cellular and humoral elements are at the face of innate immunity (Beutler 2004). These elements are involved in both the sensing or ‘activation’ and action or ‘effector mechanisms’ of fighting infection. Present since birth, the innate network operates non-specifically and immediately against practically any pathogen/molecule to threaten the body.

1.1.1 CELLULAR AND HUMORAL ACTIVATION

Cellular activation is mainly dependant on myeloid cells that engulf and destroy pathogens, including macrophage (MØ) and neutrophils. Additionally, mast cells and polymorphonuclear phagocytes such as eosinophils and basophils are crucial to the containment of infection as reviewed by (Beutler 2004). Pathogens that overcome the epithelial barrier are first encountered by MØ in residing tissues and secondly by neutrophils and monocytes recruited to the site of infection. As specialised phagocytes, macrophage and neutrophils are equipped with pathogen recognition receptors (PRRs) that bind recognisable structures or components of the invading microbe [see sections 1.6 – 1.8] (Janeway et al. 2008). Such components are shared by many types of prokaryotic organisms and are not found on eukaryotic cells allowing for discrimination between self and non-self [see section 1.10] (Janeway et al. 2008). They also express surface receptors that recognize the Fc portion of antibodies. Binding of these receptors induces the engulfing of microbes into a membrane-bound vesicle or 'phagosome' (Beutler 2004). Concurrently, binding of PRRs triggers cell signaling, producing soluble mediators such as cytokines, chemokines, acute phase proteins and complement initiating an inflammatory response.

Neutrophils are released daily into the blood stream in high numbers and recruited to sites of infection in response to cytokines and complement activation. They express some but not all PRRs and have a half-life of several hours. Importantly, they express other cell associated molecules that have been recognised as microbial sensors. For example, f-methionyl-leucyl-phenylalanyl (fMLP) receptor is an important inducer of chemotaxis during infection (Gao, Lee and Murphy 1999).

The innate system also has ‘humoral’ means of detecting microbial pathogens and activating a response. Extracellular proteins such as mannose binding lectin (MBL) of the collectin family (Super and Ezekowitz 1992) recognise terminal mannosyl residues on the surface of microbes and activate the complement (C') cascade (Janeway et al. 2008). Similarly, CD14 in its shed form (a co-receptor for lipopolysaccharide (LPS)), [see section 1.9.1] aids the cellular activation of innate cells in response to gram-negative bacteria (Frey et al. 1992). Collectively, cellular and humoral activation initiate the inflammatory response leading to an influx of effector cells and an accumulation of plasma proteins at the site of infection.

1.1.2 CELLULAR AND HUMORAL EFFECTOR MECHANISMS

Effector cells include macrophage, dendritic cells (DC), neutrophils and natural killer (NK) cells. Monocytes and neutrophils migrate to the site of infection, engulf and destroy invading pathogens. Monocytes, which mature to macrophage at the site of infection, are long lived and play a broad role in the clearance of invading pathogens, apoptotic cells and necrotic cells by phagocytosis [see section 1.3] (Gordon and Taylor 2005). Within the phagosomes of neutrophils and macrophage, reactive oxygen species (ROS) act as powerful microbicidal molecules destroying and often eliminating infection. ROS are produced in a series of enzymatic reactions initiated by NADPH oxidase (HIRSCH and COHN 1960) and are reviewed by (Hampton, Kettle and Winterbourn 1998).

DC, like macrophage, reside in connective tissues and are antigen presenting cells (APC), meaning they have the ability to present antigen and up-regulate cell surface markers required for the activation and co-stimulation of T-cells in adaptive immunity. This includes up-regulation of major histocompatability complex II (MHCII) (antigen presentation) and CD40, CD86 (co-stimulation). (Janeway et al.

2008). Up-regulation of such molecules is highly efficacious and was described as accounting for the adjuvant effects of microbial endotoxin or LPS 50 years ago (CONDIE, ZAK and GOOD 1955).

Influx of effector cells is critical to immune function and accomplished in three stages that include adhesion, diapedesis and chemotaxis [see figure 1.1.2]. Cell-adhesion molecules and soluble mediators in the immediate inflammatory milieu control interactions between leukocytes and endothelial cells allowing this process to occur (Janeway et al. 2008). Homeostatically, leukocytes flow in the centre of the blood vessel and some roll slowly along the vascular endothelium making contact via intergrin receptors on their surface and selectins expressed on the endothelium. However, under inflammatory conditions autocoid substances (i.e histamine, bradykinin) cause local vasodilation, slowing blood flow and allowing many leukocytes to interact with the vascular endothelium (Beutler 2004). Secretion of the cytokine TNFα by macrophage activates the endothelium increasing expression of selectin molecules (i.e P-selectin (minutes), E-selectin (~2 h)). Selectins interact with sulfated-sialyl-Lewis^x (s-Le^x) on leukocytes. This interaction increases the percentage of leukocytes rolling slowly and adhering to the endothelium. Concurently, TNFα upregulates intracellular adhesion molecules (i.e ICAM-1) on the inflamed endothelium. ICAMs in turn bind to integrin LFA-1 on leukocytes in the later and stronger stages of adhesion (Beutler 2004). Chemokines such as IL-8 stimulate leukocyte diapedesis, the process by which leukocytes cross the vasculature. They convert leukocyte-endothelium adhesion into stable binding by inducing conformational changes to integrins (i.e. LFA-1). In turn, leukocytes cross the blood vessel walls squeezing between endothelial cells. In the final step chemokines direct the migration or ‘chemotaxis’ of leukocytes along a concentration gradient increasing towards the site of infection (Janeway et al.

2008). The entire process is reversible, facilitating the trafficking of antigen to and from local lymph nodes [see section 1.2].

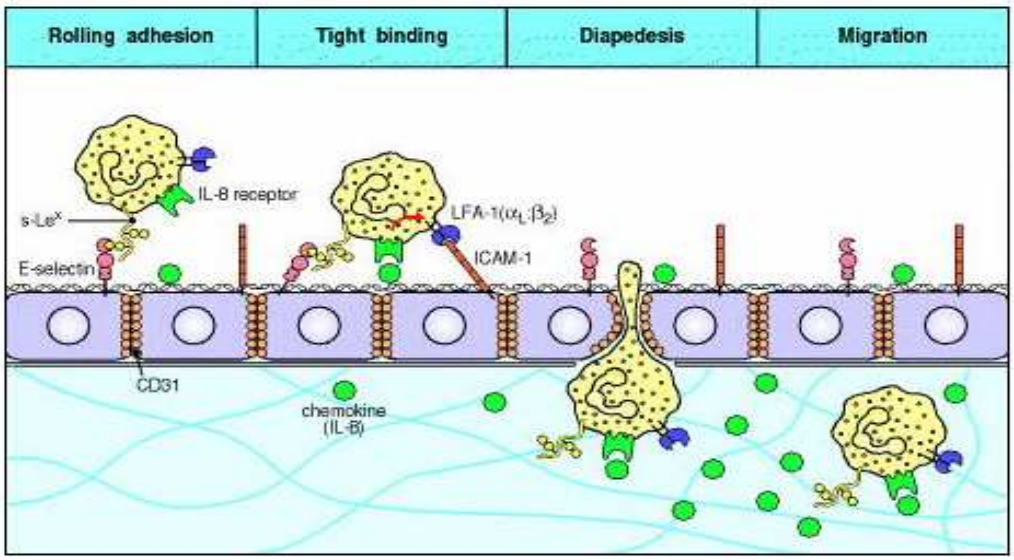


Figure 1.1.2: *Diagrammatic representation of leukocyte adhesion, diapedesis and chemotaxis at the inflammed endothelium. Taken from* (Janeway et al. 2008).

Humoral effector mechanisms include the actions of enzymes and proteins particularly those of the acute phase response (APR) and complement system (C') (Beutler 2004, Janeway et al. 2008, Wood 2006). Continued stimulation of MØ in the presence of infection leads to increased production of the pro-inflammatory cytokines TNFα, IL-1 and IL-6, (common nomenclature, interleukin, IL). These cytokines affect other organs such as the brain and liver which are far removed from the site of infection. IL-1 acts on the brain inducing fever, lethargy and loss of appetite (Wood 2006). While IL-6 potently activates hepatocytes to secrete a group of proteins termed 'acute phase proteins' (APPs) (Janeway et al. 2008). Like antibodies, APPs have the ability to bind and opsonize bacterium (the process by which pathogens are targeted for phagocytosis). However, unlike antibodies APPs have a broad specificity and depend solely on the presence of cytokines for their production (Wood 2006).

Acute phase proteins include fibrinogen, mannose binding lectin (MBL), C-reactive protein (CRP), serum amyloid A (SAA) and pulmonary surfactants A and D (Janeway et al. 2008, Wood 2006) to name a few. APPs play a direct role in the killing of microbes by activating the complement system/cascade (C'). C' is involved in the destruction of microbes and consists of a group of inactive precursor proteins that upon activation are converted to their proteolytic form. Activation of one is followed by the sequential activation of the next protein in the cascade (Janeway et al. 2008). C' can be activated in one of three ways: 1. The Classical Pathway, 2. The Lectin Binding Pathway and 3. The Alternative Pathway. All three pathways culminate in the activation of the enzymatic cascade commonly referred to as the complement cascade, C' (Beutler 2004, Janeway et al. 2008, Castellano et al. 2004).

Enzymes such as lysozyme and lactoferrin also destroy bacteria. Lysozyme destroys the cell walls of gram-negative and gram-positive bacteria (Beutler 2004). Lactoferrin alters bacterial motility and was particularly found to inhibit biofilm formation in certain bacteria (Singh et al. 2002).

1.2 OVERVIEW OF ADAPTIVE IMMUNITY

Infectious agents must overcome the innate host defenses to establish a focus of infection. In such circumstances the innate system sets the scene for the induction of an adaptive response. Adaptive immunity utilizes antigen-specific receptors found on T and B lymphocytes to direct effector responses. T and B lymphocytes originate in the bone marrow from progenitor cells. T cells migrate and mature in the thymus whereas B cells remain in the bone marrow undergoing further development (Janeway et al. 2008). Antigen specificity is attributed to membrane-bound antibody (Ab) and a T cell receptor (TCR) found on B cells and T cells, respectively. In both cases specificity is due to the random rearrangements of the genes encoding Ab and TCR. This results in the expression of a huge repertoire of receptors (Nemazee 2006). Naïve lymphocytes enter peripheral lymphoid organs where the majority of immune responses occur. Each lymphocyte cell bears surface receptors for a single antigen. However, high specificity combined with the huge repertoire of lymphocytes means only a small number of lymphocytes are able to recognise a given antigen (Janeway et al. 2008).

Naïve lymphocytes are activated in the presence of three signals: 1) TCR stimulation 2) co-stimulatory molecule ligation and 3) stimulation by polarising cytokines. This results in the activation and clonal expansion of specific lymphocytes over several days. Naïve T cells proliferate and differentiate into effector T cells (Bono et al. 2007). An effective immune response upon re-exposure to antigen or ‘immunological memory’ is achieved by means of a number of antigen-specific B and T lymphocytes that persist for some time after removal of the initiating antigen (Janeway et al. 2008).

Signal 1 is the antigen-specific signal via TCR ligation with MHC class-II-peptide complexes found on APCs. APCs include DC, MØ and B cells, however, DC are viewed as the most potent APC. DC act by ingesting antigen at sites of infection and then travelling to local lymph nodes where they mature to present antigen and activate T cells (Janeway et al. 2008). Conversely, to enhance opportunities of encountering antigen, lymphocytes continually circulate between the blood and peripheral lymph nodes. This migration is aided by the production of chemokines during an inflammatory response (Ebert, Schaerli and Moser 2005), as discussed [see section 1.1.2]. Signal 2 is the co-stimulatory molecule ligation mainly mediated by the triggering of CD28 on naïve T cells by CD80 and CD86 on an APC. Finally, signal 3 is a polarizing signal mediated by various soluble or membrane-bound factors, such as cytokines. This last signal will depend on the response of APCs to a particular pathogen via their PRRs (Janeway et al. 2008).

Naïve T cells are divided into $CD4^+$ and $CD8^+$ populations. $CD8^+$ T cells, also known as cytotoxic lymphocytes (CTL), kill their target cells and are important in defence against intracellular pathogens such as viruses. Viral antigens are displayed to $CD8^+$ T cells complexed with MHCI molecules on the surface of APCs. $CD4^+$ T cells have the ability to differentiate into subsets of effector cells mainly T helper types (T_H), T_H1, T_H2, T_H17 and regulatory T cells (T_{reg}). T_H1 differentiation is regulated by IL-12, IL-27, type I IFNs, and IFN-γ; T_H2 differentiation is influenced by IL-4 (Agnello et al. 2003) and T_H17 are induced by TGFβ and IL-6 (Janeway et al. 2008). T_H subtypes are regulated by a heterogenous group of effector cells, T_{reg}. T_{reg} are characterised by an ability to suppress adaptive T cell responses in order to prevent autoimmunity. In general, T_{reg} can target effector T cells, compete with pathogenic T cells for access to APCs, or directly target APCs, reviewed by (Mills 2004).

T helper cells also vary in terms of their cytokine profiles and the type of pathogen they target. T_H1 cells produce IL-2, TNF-β, and IFN-γ, which activate macrophage, NK cells, and CTL, primarily targeting intracellular pathogens (Trinchieri 2003a). T_H2 cells produce IL-4, which stimulates IgE production, IL-5, an eosinophil activating factor, and IL-10 and IL-13, which in combination with IL-4 can suppress cell-mediated immunity and inflammation (Gouwy et al. 2005). Extracellular pathogens tend to induce differentiation towards a T_H2 subtype. Additionally, IL-27 secreted by APCs inhibits various immune responses and negatively regulates T_H1 and T_H2 cells (Stumhofer and Hunter 2008). T_H17 cells produce a distinct set of cytokines, including IL-17 and IL-6. This subtype are believed to target pathogens distinct from those targeted by T_H1 and T_H2 cells (Weaver et al. 2006).

Cellular communication is of paramount importance during an immune response, particularly between T lymphocytes and APCs. MHCII is found mainly on MØ and DC and it is long established that MØ are required for an adaptive immune response (Beutler 2004). Expression of this marker and other co-stimulatory molecules is pivotal in supporting bidirectional stimulatory signals between MØ and T cells. In fact, such signals not only make T cell activation possible but also act in the regulation and activation of MØ. For example, MØ-T cell contact via T cell-associated CD40 ligand (CD40L) with CD40 on monocytes is required for the production of IFNγ and IL-12 by MØ (Shu et al. 1995). Indeed, $CD4^+$ T cells can activate MØ and therefore have a critical role in defence against pathogens that resist killing after being engulfed by MØ (Janeway et al. 2008).

Interferon-γ (IFNγ) produced by T_H1 cells or CTLs, along with TNFα from APCs gives rise to classically activated macrophages, [see section 1.3.2.1]. These MØ secrete cytokines IL-1, IL-6 and IL-23 which give rise to T_H17 cells. In addition, classically activated MØ produce IL-12 promoting a T_H1 phenotype. IL-10 produced by T_{reg} cells can give rise to a population of regulatory MØ. Regulatory MØ produce IL-10 and can induce T_H2 phenotype (Mosser and Edwards 2008). Recent evidence also indicates that alternatively activated MØ [see section 1.3.2.2] actively induce T_{reg} in the periphery of the body indicating that T_{reg} development is not solely dependent on the thymus. Conversely, groups have shown that T_{reg} are able to gain influence on the innate immune system by interacting with MØ (Mahnke et al. 2008). The relationship between cytokine production and signaling between T cells and MØ is represented in *figure 1.2.1*.

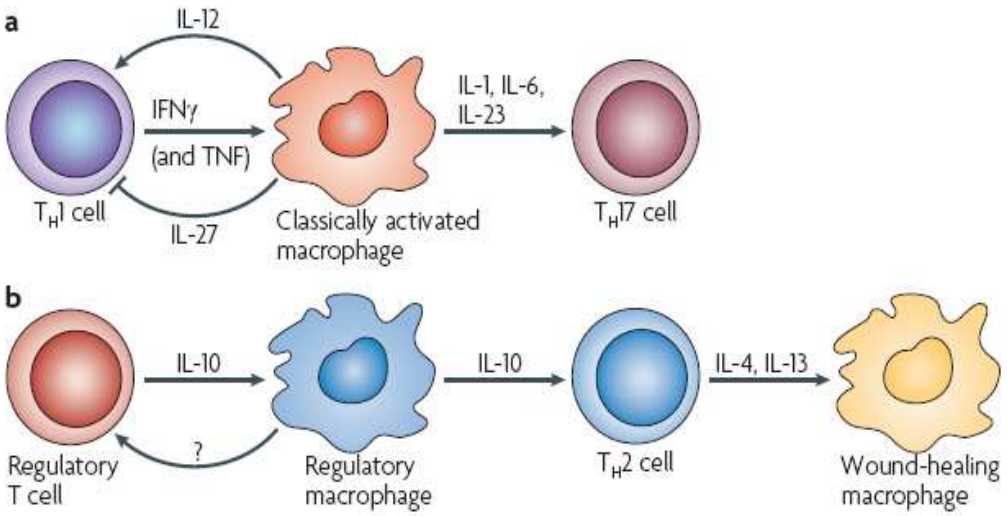


Figure 1.2.1: *Taken from* (Mosser 2003). *Diagrammatic representation of the interaction between macrophage and T cells.*

1.2.1 LINKING INNATE AND ADAPTIVE IMMUNITY

Co-ordination of innate and adaptive responses is supported by signalling between different PRRs [see section 1.5]. Without vital actions of innate 'effector' cells such as antigen presentation and cytokine production, adaptive responses are ineffectual. At best, innate mechanisms can prevent infection being established. While crucially important in this role, innate mechanisms do not lead to immunological memory (Janeway et al. 2008). It is only the adaptive response that provides specifically enhanced and lasting protection against pathogens. In essence the innate system can more often play a delaying function, holding an infection at bay while a more specific adaptive response is mounted.

1.2 MACROPHAGE

MØ are immune effector cells with well established roles in host defence, wound healing and immune regulation. First recognised by Elie Metchnikoff in 1905 as important phagocytes (Metchnikoff 1905), MØ have since been a constant source of investigation for immunologists. MØ develop from common myeloid progenitor cells (hematopoietic stem cells) in the bone marrow, precursors for many different cell types, for example the closely related DC (Janeway et al. 2008). These precursor cells undergo differentiation until committing to the monocyte lineage upon which they are released from the bone marrow into the blood stream (Gordon and Taylor 2005). These monocytes add integrity to endothelial-cell lining of blood vessels (Auffray et al. 2007). Furthermore, under steady state conditions they migrate and mature to become tissue resident MØ and during infection and injury are the main source of inflammatory MØ (Gordon and Taylor 2005).

MØ are distributed widely throughout body tissue (Janeway et al. 2008). Monocytes mature and replenish tissue resident MØ continually to make up MØ of the bone (osteoclasts), central nervous system (migroglial cells), alveoli, liver (Kupffer cells), spleen, peritoneum, connective tissue (histocytes) and gastrointestinal tract (Gordon and Taylor 2005). As a cell type they are also morphologically diverse including, the ‘spindle-shaped’ tissue histocyte, the flattened Kuppfer cell and the stellate microglial cell (Beutler 2004).

1.3.1 MACROPHAGE FUNCTION

MØ have important roles within immunity and homeostasis. As specialised phagocytes they have the ability to recognise, engulf and destroy invading pathogens. Their involvement in the immune response can be divided into innate and adaptive components. Recognition of invading microbes is performed by means of PRRs they express such as toll-like receptors (TLRs) recognising specific pathogen associated molecular patterns or PAMPs. Recognition of PAMPs by MØ initiates signalling pathways culminating in the production of immune and inflammatory genes (Janeway et al. 2008). As previously discussed, MØ are capable of initiating an adaptive immune response by presenting antigen to $CD4^+$ T cells via MHC II molecules (Janeway et al. 2008) [see section 1.2]. Indeed, this is accompanied by the production of soluble mediators such as cytokines and chemokines [see section 1.4 – 1.5] that act by recruiting immune cells to the site of injury or infection thereby amplifying the immune response (Beutler 2004).

As exceptional phagocytic cells they are involved in the clearance of debris from wound healing and apoptosis. Receptors that mediate this homeostatic clearance include phosphatidyl serine receptors, thrombospondin receptor, integrins, complement receptors and scavenger receptors (Erwig and Henson 2007b). This is a vital role without which the host would not survive. Importantly, the removal of apoptotic debris does not induce the production of inflammatory mediators in unstimulated MØ (Erwig and Henson 2007a). However, phagocytosis of necrotic debris initiates a striking change in their physiology. Necrosis is a form of cell death resulting from states of hypoxia, stress or toxic injury (Mosser and Edwards 2008). Often the self antigens released in this form of cell death such as heat shock proteins (hsp), histones and deoxyribonucleic acids (DNA) are immunogenic (Zhang and Mosser 2008). The profound physiological change that occurs within

any MØ population includes the up-regulation of various surface markers and the production of inflammatory cytokines (Erwig and Henson 2007b).

1.3.2 MACROPHAGE ACTIVATION

A variety of stimuli can trigger MØ activation. Thus far categories of MØ activation have included classically and alternatively activated populations (Lewis 1999, Ma et al. 2003). However, due to the remarkable plasticity displayed by macrophage these categories are under constant review and investigation. Currently, classification has shifted to incorporate not only immune functions but the crucial homeostatic roles of macrophage. Subsets include classically activated, alternatively activated (wound healing) and regulatory MØ (Mosser and Edwards 2008). The work within this study has focused on the functional status of classically activated macrophage.

1.3.2.1 Classical Activation

MØ activated during cell-mediated immune responses are commonly referred to as 'classically activated'. Classical activation involves two signals. Firstly, MØ are primed by cytokine IFNγ, produced by cells of the adaptive response and acting as the most important priming stimulus. IFNγ is produced by both T helper (T_H1) cells or $CD8^+$ T cells (Mosser and Edwards 2008). The second is the pro-inflammatory cytokine TNFα (Lewis 1999, Ma et al. 2003). Typically TNFα is induced by the ligation of a TLR to its respective ligand, activating the MyD88-dependant signalling pathway [see sections 1.9.4]. MØ are also classically activated by innate stimuli but in a transient manner (Mosser and Edwards 2008). Natural Killer (NK) cells respond to stress and infection by producing IFNγ and prime the population see figure 1.3.1. Crucially, the combination of these signals enhances the overall microbicidal and tumoricidal activity of the MØ (O'Shea and Murray 2008). IFNγ

primes MØ to secrete the pro-inflammatory cytokine IL-12 (Trinchieri 2003b) and nitrogen radicals (MacMicking, Xie and Nathan 1997) enhancing their ability to kill microbes. These cells also produce moderate amounts of the anti-inflammatory cytokine, IL-10 (Mosser and Edwards 2008). IL-10 inhibits T_H1 cell responses by reducing the capacity of macrophage to produce IL-12. Classical activation is also associated with increased MHC II (antigen presentation) and CD86 (co-stimulatory molecule) expression along with enhanced production of chemokines, MIP-1α/CCL3, IP-10/CXCL10 and MCP-1 (Mosser 2003). IFNγ induces several transcription factors such as interferon regulatory factors (IRF1 to IRF 9) (Ma et al. 2003). Particularly, certain TLR agonists activate the MyD88-independent pathway leading to production of IFNβ (Yamamoto et al. 2003). Therefore, in response to certain TLR ligands, endogenously produced IFNβ can replace IFNγ produced by NK and T cells to classically activate MØ. The role of classically activated macrophages in the defence of intracellular pathogens is reviewed extensively by (Gordon 2007). However, classically activated MØ must be tightly regulated. Exacerbation of their actions can lead to extensive tissue damage associated with autoimmune diseases such as rheumatoid arthritis (Szekanecz and Koch 2007) and inflammatory bowel disease (Zhang and Mosser 2008).

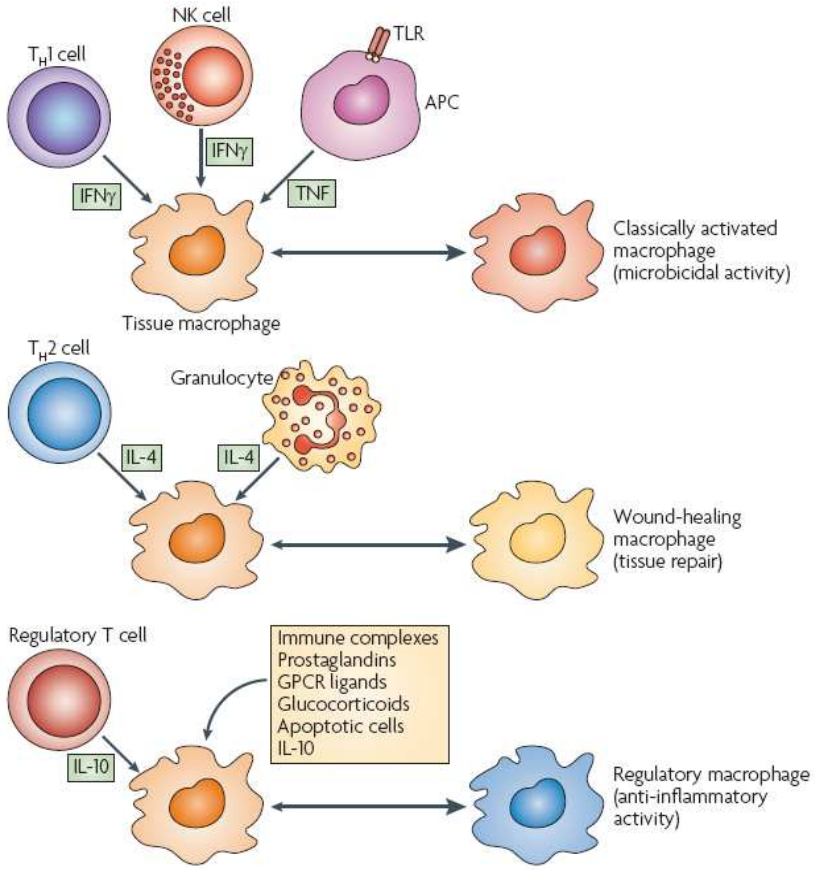


Figure 1.3.1: *Taken from* (Mosser 2003). *Diagrammatic representation of cytokines produced by immune cells and their effect on macrophage physiology.*

1.3.2.2 Alternative Activation

MØ are said to undergo 'alternative activation' in the presence of T_H2 cytokines, IL-4 and IL-13 (Martinez, Helming and Gordon 2008). The original classification of 'alternative' was assigned as IL-4 stimulated MØ displayed up-regulation of mannose receptor (Stein et al. 1992) compared to classically activated MØ (Kreider et al. 2007). However, alternatively activated MØ are now commonly referred to as wound healing MØ. Similar to classically activated MØ they can develop in response to innate and adaptive signals. Basophils, mast cells and other granulocytes provide an early source of innate IL-4 (Brandt et al. 2000), see *figure 1.1.1*. Granulocytes are activated in response to injury and also chitin, a structural component of fungi and parasites (Reese et al. 2007). Adaptive responses to disturbances in mucosal surfaces induces a T_H2 cell phenotype (Reese et al. 2007).

This phenotype is also found in non-mucosal tissues in response to helminth infections (Wilson et al. 2007). IL-4 secreted by these cells rapidly converts MØ to wound healing populations by stimulating arginase. Arginase activity leads to cell growth, division and collagen formation. In this way MØ are directed to contribute to repair and production of the extracellular matrix (Kreider et al. 2007) and thus the term wound healing MØ. Additionally, several groups have shown this MØ population aids clearance of helminth and nematodes (Anthony et al. 2006, Zhao et al. 2008). However, this is overwhelmed by the evidence pointing towards their main function as wound healers. The polyamines and chitinase molecules produced by arginase are vital to matrix reorganisation and wound healing (Kzhyshkowska et al. 2006, Zhu et al. 2004). Similar to classically activated MØ, dysregulated activation of this population can have deleterious effects. For example, uncontrolled activation of wound healing MØ has been attributed with tissue fibrosis that occurs in schistosmiasis (Hesse et al. 2001).

1.3.2.3 Regulatory Macrophage

Regulatory MØ are activated by innate and adaptive responses and other varied stimuli. These include prostaglandins (Strassmann et al. 1994), ligands of G-protein coupled receptors (GPCR) (Hasko et al. 2002), glucocorticoids (Sternberg 2006), apoptotic cells (Erwig and Henson 2007b), immune complexes (Gerber and Mosser 2001) or IL-10 (Mosser and Edwards 2008) [see figure 1.3.1]. This population also requires two stimuli to induce their anti-inflammatory phenotype. The first signal is provided by stimuli mentioned above and the second signal from the ligation of a TLR ligand. Both signals direct the MØ to produce IL-10, the most potent characteristic of regulatory MØ (Edwards et al. 2006). Regulatory MØ also down-regulate IL-12 (Gerber and Mosser 2001), express high levels of CD80 and CD86 (required for co-stimulation) and have the ability to present antigen to T cells

(Edwards et al. 2006). They do not produce any soluble mediators involved in production of extracellular matrix and they secrete high levels of the chemokine, CCL1/TCA3 (Mosser and Edwards 2008). Significantly, phagocytosis is a fundamental role of all MØ populations. Stimulation by pathogens, particularly through TLRs enhances the phagocytic activity of MØ and promotes phagosome maturation, allowing sufficient capture and destruction of microbes. (Blander and Medzhitov 2004). As iterated, MØ activation must be tightly regulated to avoid deleterious effects. While all phenotypes have important roles required for host defence and repair the right balance must be maintained between populations for adequate healing and resolution.

Macrophage execute many roles including, yet not limited to, those within innate immunity. As described, they exhibit remarkable plasticity allowing them to change phenotype in response to various stimuli, a topic reviewed extensively by (Mosser and Edwards 2008, Mosser 2003, Martinez, Helming and Gordon 2008). The immense plasticity of MØ makes it difficult to assign specific biochemical markers to each population. It also remains elusive as to whether discrete monocyte populations give rise to specific MØ in tissues and whether this may be used in disease diagnosis (Mosser and Edwards 2008). A basic understanding of the molecular mechanisms involved in MØ activation should provide a foundation for novel drug development aimed at modulating MØ activity. Also, the potential of utilising MØ subsets as biomarkers for disease is immense and it remains for further studies to be carried out.

1.3 CYTOKINES

Cellular communication within the immune system is largely mediated by a group of low molecular weight polypeptides or glycoproteins known as cytokines (Parkin and Cohen 2001). Cytokines act by binding their respective receptors and are grouped on the basis of their structural features (Pugh-Humphreys and Thompson 1998). Signalling is dependant on families of tyrosine kinases and the signalling mechanism of many cytokines is mediated through Jak (Janus-kinase)-STAT (signal transducer and activator of transcription) pathways (O'Shea, Ma and Lipsky 2002, Fujii 2007).

Unlike hormones, cytokines are produced by virtually all immune cells and act in an autocrine and paracrine manner (Zidek, Anzenbacher and Kmonickova 2009). They have multiple regulatory functions (pleiotropy) and often the effects of different cytokines overlap. In general terms they influence cellular division, apoptosis, activation or recruitment (Parkin and Cohen 2001). Modes of action depend on the cytokine itself and cell types in the local environment. In addition, cytokine functions are tightly regulated by feedback mechanisms that influence their production both synergistically and antagonistically (Zidek, Anzenbacher and Kmonickova 2009). Cytokines are separated according to their biological function, generally those promoting inflammation are said to be pro-inflammatory such as IFN-γ, TNF-α, IL-17, IL-12, and IL-1β (Gay and Gangloff 2008, Vilcek 2003). These cytokines are commonly produced during T_H1/T_H17 responses. On the other hand those generated during a T_H2 or regulatory response such as IL-4, IL-10, and TGF-β are considered anti-inflammatory (Hill and Sarvetnick 2002). Representative cytokines, their modes of action and the cells that produce them are listed in table 1.2.

1.4.1 CHEMOKINES

Immune cells are recruited to sites of infection by a class of cytokines with chemoattractant properties known as chemokines (Zidek, Anzenbacher and Kmonickova 2009). Chemokines are produced by and act on the majority of cells. They are the only members of the cytokine family not to signal through cytokine receptors and signal through the G-protein coupled receptor (GPCR) superfamily (Lodowski and Palczewski 2009, Goncharova and Tarakanov 2008). Chemokines are classified into four subfamilies based on the position of the first N-terminal cysteine residue in a conserved cysteine motif. In the CC family (β-chemokines) the first two cysteines are adjacent, in the CXC family (α-chemokines), cysteines are intervened by one amino acid. For the third group the CX3C family (δ-chemokines) the first two cysteines are separated by three amino acids. Finally, the fourth group the C family (γ-chemokines) have only two of the four conserved cysteines (Zlotnik and Yoshie 2000). Chemokines are secreted in response to T_H1 cytokines IFNγ and IL-2 and other pro-inflammatory cytokines such as IL-1 and TNFα. In contrast, the T_H2 cytokine IL-4 and T_{reg} cytokines, IL-10 and TGFβ are known to down-regulate secretion of chemokines (Adams and Lloyd 1997). Interestingly, imidazoquinoline derivatives, inducers of interferons, also activates secretion of IL-1β, IL-6, TNFα and IL-12p40 along with chemokines IL-8/CXCL8, MIP-1α/CCL3 and MCP-1/CCL2 (Gupta, Cherman and Tyring 2004, Thomsen et al. 2004). Representative chemokines, their modes of action and the cells that produce them are listed in table 1.2.

1.4.2 CYTOKINES AND CHEMOKINES IN DISEASE

As with many components of the immune system, ()dysregulation among cytokines and their receptors can have direct implications in the development of disease. Tissue destruction in autoimmune disease is associated with elevated levels of cytokines IFNγ, TNFα and IL-1 (Rabinovitch 1994, La Cava and Sarvetnick 1999). Particularly, high levels of TNFα are evident in the pathophysiology of rheumatoid arthritis (RA) (Furst 2008). The recruitment of leukocytes in response to chemokines is paramount in aiding clearance of infection, however elevated levels of chemokines are also implicated in inflammatory disease. Depleted levels of MIP-2 and MCP-1 are associated with impaired clearance of *Klebsiella pneumon* and *Cryptococcus neoforms* (Strieter et al. 1996), respectively. Similarly, a role for MIP-1α and RANTES in eosinophil recruitment during the pathogenesis of allergic airway inflammation has been established (Lukacs et al. 1996). Additionally, studies have shown a role for MCP-1 in macrophage rich areas of atherosclerotic plaques (Nelken et al. 1991).

As a result considerable effort has been placed on developing therapeutic targets that can modulate the activities of chemokines and cytokines. Development of an anti-TNFα therapy has been the most successful therapy in treatment of RA to date (Furst 2008). Also of particular interest is the development of genetically modified chemokines to abborogate excessive chemokine expression in disease. A recombinant MIP-1α has been developed that retains its receptor binding properties yet looses its functional activity and ability to recruit monocytes (Graham et al. 1994). Chemokine receptors are also implicated in several disease states including psoriasis, atherosclerosis, and malaria (Patel, McInnes and Graham 2009, Plant et al. 2006, de Groot et al. 2007, Kershaw et al. 2009). However, comprehensive experimental and clinical evidence remains inconclusive as to whether possible

treatments targeting chemokines and cytokines can lead to resolution of established inflammation. As such further studies are warranted in this area.

Cytokine	Source	Mode of Action	Inflammatory Status
IL-1β	Monocytes, MØ, B cells, DC	Co-stimulates T cells, enhances NK cell activity, chemoattractant	Pro-inflammatory
IL-4	T_H2 cells, NK cells, Basophils	T_H2 polarising cytokine, MØ activation	Anti-inflammatory (T_H2)
IL-6	Monocytes, MØ, DC, T_H17 cells	Activates T and B cells, T_H17 cell differentiation	Pro-inflammatory
IL-10	T_{reg}, DC, Monocytes, MØ, B cells	Immunosuppressive, inhibits T_H1 response and cytokine production	Anti-inflammatory (T_{reg})
IL-12	MØ, monocytes, DC, neutrophils	Directs T_H1 cell development, stimulates APC, NK cells, and $CD8^+$ T cells	Pro-inflammatory
IL-13	T_H2 cells, NK cells, Basophils	MØ activation, induces B cell isotype switching	Anti-inflammatory (T_H2)
IL-17	T_H17 cells, Neutrophils, $CD8^+$ T cells	Induces cytokine and chemokine production, DC maturation	Pro-inflammatory (T_H17)
IL-23	Monocytes, MØ & DC	Expansion and survival of T_H17 cells, induces pro-inflammatory cytokine production	Pro-inflammatory
IFN-γ	T_H2, $CD8^+$ T cells, NK cells, MØ, B cells	T_H1 expansion, induces cytokine & chemokine secretion, enhances NK cell function	Pro-inflammatory (T_H1)
TNF-α	MØ, DC, T cells, NK cells	Amplifies inflammation & induces cytokine release	Pro-inflammatory
TGF-β	T_H3, MØ, Neutrophils, many non-lymphoid cells	Immunosuppressive but involved in T_H17 cell differentiation	Anti-inflammatory but multi-faceted (mainly T_{reg})
Chemokines	**Source**	**Mode of Action**	**Inflammatory Status**
MIP-1 α	MØ, DC, T cells, NK cells, stem cells	T cell proliferation, enhanced $CD8^+$ cytotoxity, cell recruitment, Wound Healing	Pro-inflammatory
MIP-2	MØ, Monocytes, neutophils	Recruitment of effector cells, mucosal lymphocyte migration	Pro-inflammatory
MCP-1	Monocytes, T cells, Basophils, stem cells	Inflammatory, Wound Healing	Pro-Inflammatory
MCP-2	Monocytes, T cells, Eosinophils, Mast cells	Recruitment of pro-inflammatory cells	Pro-inflammatory

TABLE 1.2 *Representative cytokines produced by different cells and their mode of action. Main reference sources (Janeway et al. 2008, Zidek, Anzenbacher and Kmonickova 2009 and, Adams and Lloyd 1997).*

1.6 INNATE IMMUNE RECEPTORS

The general strategy of innate immune detection relies on the recognition of microbial molecules with conserved molecular patterns known as ‘pathogen associated molecular patterns’ (PAMPs). As highly conserved components of microbes, PAMPs are not readily altered by mutation or selection and provide ideal targets for innate recognition. PAMPs are sensed by a limited number of specialised receptors commonly referred to as ‘pattern recognition receptors’ (PRRs). PRRs within innate immunity are reviewed by (Beutler 2004) .

1.6.1 PATTERN RECOGNITION RECEPTORS (PRRs)

PRRs include TLRs, Scavenger receptors (SRs), NOD-like receptors (NLRs) and RIG-I-like proteins, named the RIG-like receptors (RLRs) [see sections 1.9]. All play critical roles in early defence against invading pathogens. PRRs can recognise and respond to molecules derived from bacterial, fungal and viral pathogens. The response involves the maturation of immune cells and activation of intracellular pathways leading to the influx of inflammatory cytokines and other soluble factors (Creagh and O'Neill 2006, Ishii et al. 2008, Kumagai, Takeuchi and Akira 2008).

1.7 TOLL-LIKE RECEPTORS

TLRs were discovered with the identification of Toll, a receptor expressed by *Drosophilia melanogaster*. Originally, Toll was found to be essential for development of dorsoventral polarity during embryogenesis (Hashimoto, Hudson and Anderson 1988). Further studies identified a role for Toll in the innate response of *Drosophilia* to fungal infection (Lemaitre et al. 1996). Subsequently, it was found that the cytoplasmic region of Toll was analogous to that of the type I receptor (IL-1RI) in mammals and several homologues were identified in humans. This resulted in the classification of 10 human TLRs (Takeda and Akira 2005) and the designation of the Toll/Interleukin-1 (TIR) superfamily of receptors. Importantly, the first human Toll, TLR4 was classified in 1998 (Poltorak et al. 1998). TLR structure, activation and signalling are discussed further below.

Activation of the innate system is prerequisite for induction of an adaptive response. TLRs play a pivotal role in this process. TLRs are expressed on many cells of the innate system including; MØ, DC, fibroblasts, neutrophils, mast cells, endothelial and mucosal epithelial cells (Andreakos, Foxwell and Feldmann 2004, Medzhitov 2001). TLRs are type I transmembrane receptors and form part of the Toll/interleukin-1 (TIR) superfamily that includes the IL-1 receptors (IL-1Rs) because of the shared homology of their cytoplasmic regions (Gay and Keith 1991). In contrast, their extracellular regions (ectodomains) are considerably different. TLR ectodomains contain tandem repeats of leucine rich regions referred to as leucine rich repeats (LRRs), while IL-1Rs have three immunoglobulin (Ig)-like domains see *figure 1.7.1*. The TIR family of receptors is reviewed by (Martin and Wesche 2002).

Each TLR has the ability to recognise a particular ligand or discrete set of ligands, as summarised in table 1.7.2. The arrangement of LRR side chains confers a unique combinatorial code to each TLR enabling it to bind a specific ligand. This was first found in the LRR detection of follicle stimulating hormone (FSH) through its G-protein-coupled receptor, FSHR(HB) (Fan and Hendrickson 2005). The particular ligand any TLR detects is dependant on the cellular location of the TLR and the unique code conferred to it by the arrangement of its LRRs (Brikos and O'Neill 2008, Gay, Gangloff and Weber 2006). In this way individual TLRs have the ability to interact with structurally unrelated ligands of endogenous and exogenous origin. The structure of TLRs and their respective ligand complexes are reviewed extensively (by Jin and Lee 2008).

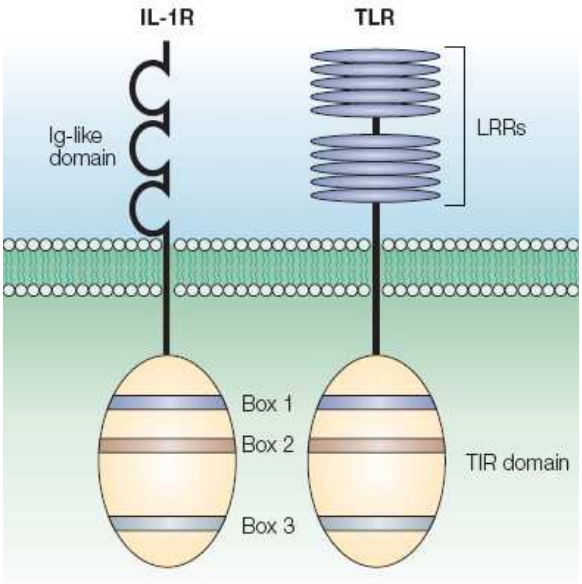


Figure 1.7.1: *Schematic of TLR versus IL-1 receptor structure taken from* (Akira and Takeda 2004).

1.7.2 TLR EXPRESSION AND LOCALISATION

TLRs are distinguished by ligand specificity, signal transduction, expression patterns and cellular localisation, all of which are discussed here. As mentioned, TLRs are expressed in cells at the front line of defence. However, innate immunity is not antigen specific and varied expression of TLRs between cell types facilitates the direction of an immune response to a particular pathogen (Andreakos, Foxwell and Feldmann 2004, Medzhitov 2001). TLR1 is found ubiquitously while TLR2-10 display more limited expression patterns see table 1.7.1 (McGettrick and O'Neill 2004). The exact expression of TLRs in cell types remains conflicting as studies demonstrate differences between mRNA levels and the responsiveness of a given cell to TLR agonists (Iwasaki and Medzhitov 2004). For example, highly purified human peripheral blood mononuclear cells (PBMCs) express TLR1, TLR2 and TLR4 and low levels of TLR9 yet they do not respond to unmethylated CpG (Hornung et al. 2002). Similarly, eosinophils express TLR1, TLR4, TLR7, TLR9 and TLR10 mRNA and only respond to stimulation with a TLR7 agonist (Nagase et al. 2003). Ultimately, the diversity of an immune response lies greatly on the set of TLRs expressed by cells and the locations those cells reside in (Iwasaki and Medzhitov 2004). The location of any given TLR is related to the origin of ligand it recognises. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the plasma membrane and are largely involved in the detection of bacterial products in the extracellular space. On the other hand TLR3, TLR7, TLR8, and TLR9 are located within endocytic compartments that present nucleic acids of viral origin to these TLRs (Akira and Takeda 2004, Boehme and Compton 2004).

Toll-Like Receptor (TLR)	Expression
TLR1	Ubiquitous
TLR2	Monocytes, DC
TLR3	DC and NK cells
TLR4	MØ, DC, Endothelial cells
TLR5	Monocytes, NK cells, immature DC
TLR6	Monocytes, B and NK cells
TLR7	Plasmacytoid precursor DC, B cells
TLR8	Monocytes, NK and T cells
TLR9	Plasmacytoid precursor DC, MØ, microglial cells, B and NK cells
TLR10	Plasmacytoid precursor DC and B cells

Table 1.7.1: *Differential expression patterns of TLRs taken from* (McGettrick and O'Neill 2004)

TLR4 recognises lipopolysaccharide (LPS) from gram-negative bacteria [see section 1.6.1] (Poltorak et al. 1998, Hoshino et al. 1999). Recognition of LPS by TLR4 leads to the activation of transcription factors NF-κB and IRF3 [see section 1.9.4]. Signalling by all TLRs culminates in NF-κB activation and up-regulation of pro-inflammatory cytokines (see figure 1.7.2) (McGettrick and O'Neill 2004, Boehme and Compton 2004). TLR signalling, relevant adaptor molecules and inducible genes are listed in table 1.7.2. TLR2 recognises peptidoglycan (PG) from gram-positive bacteria (Takeuchi et al. 1999). In certain cases TLRs form dimers broadening the range of ligands they can detect. TLR2/1 and TLR2/6 dimers recognise triacylated and diacylated bacterial lipoproteins (BLP), respectively (Takeuchi et al. 2002, Takeuchi et al. 2001, Shimazu et al. 1999). TLR5 recognises bacterial flagellin, a principal component of bacterial flagella, from both gram-positive and gram-negative bacteria (Hayashi et al. 2001). TLR3 is activated by double-stranded RNA (dsRNA), a molecular pattern associated with viral infection (Alexopoulou et al. 2001). TLR7 and TLR8 both detect viral single-stranded RNA (ssRNA) (Diebold et al. 2004, Heil et al. 2004). In addition TLR7 and to a lesser extent TLR8 are activated by small synthetic molecules, the imidazoquinolines (Hemmi et al. 2002). TLR9 recognises unmethylated CpG (CpG) motifs from bacterial and viral DNA (Wagner 2002).

TLR location is also important for the discrimination between ‘self’ and ‘non-self’. In contrast to most TLR ligands nucleic acids can be of self and foreign origin. A study by (Barton, Kagan and Medzhitov 2006) demonstrates that a chimeric TLR9 consisting of a transmembrane and cytoplasmic domain of other TLRs is localised to the plasma membrane. Here it is able to detect and respond to mammalian DNA yet remain unresponsive to viral nucleic acids, highlighting the importance of TLR location. Expression of the chimeric TLR9 on the cell surface exposes the receptor

to mammalian DNA. Endogenous TLR9 is not exposed to mammalian DNA and can only be activated by viral DNA ingested and acidified within endosomes. Additionally, (Barton, Kagan and Medzhitov 2006) demonstrate the ability of transmembrane and cytoplasmic regions to dictate TLR localisation.

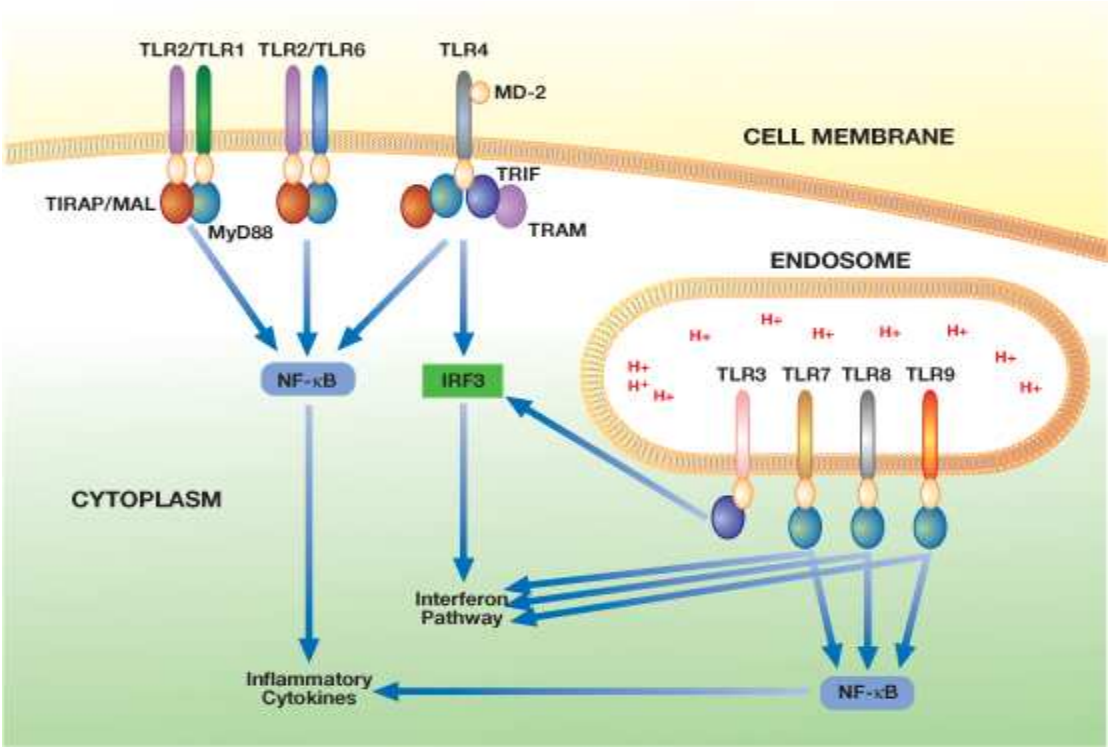


Figure 1.7.2: *Schematic of TLR localisation and signalling pathways taken from (Boehme and Compton 2004). TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the plasma membrane while TLR3, TLR7, TLR8 and TLR9 typically localize within endocytic compartments. TLRs make use of adaptor molecules Mal, MyD88, Trif and TRAM to activate signaling pathways. Like TLR4, TLR2 in combination with TLR1 or TLR6 utilizes MyD88 and Mal as primary adaptors to activate NF-κB and inflammatory cytokine secretion. TLR4 can also make use of Trif and TRAM to activate IRF3 and the IFN pathway. TLR3 utilizes Trif but not TRAM to activate IRF, while TLR7, TLR8 and TLR9 trigger inflammatory cytokine secretion and the IFN pathway through MyD88. Other than MyD88, the signalling components used by TLR7, TLR8 and TLR9 to activate IFN responses remain undefined.*

1.7.2 TLR ACTIVATION AND SIGNALLING

Recognition of PAMPs by TLRs results in the activation of signalling pathways that induce the up-regulation of cytokines, chemokines and co-stimulatory molecules. The initial step in signal transduction of class I transmembrane receptors involves the binding of ligand resulting in dimerization of two receptor chains. In the case of TLR4 this homodimer is induced by the binding of MD-2 to the lipid A moiety of LPS (Saitoh et al. 2004). Conformational changes in the receptor then leads to the association of two receptor TIR domains (Gay, Gangloff and Weber 2006, Gay, Gangloff and Weber 2006, Gangloff, Weber and Gay 2005). It is believed the overall structure of the TLR ectodomain, transmembrane and cytoplasmic regions in turn constitute a molecular switch 'turned-on' by a sequence of stimulus (ligand binding) induced conformational changes. To date several crystal structures of TLR dimers have been elucidated and dimers of TLR3, TLR2/1 and TLR4 taken from (Jin and Lee 2008) are shown in figure 1.8.2. Ultimately, association of TIR domains provides a new scaffold for the recruitment of specific adaptor molecules which also contain a TIR domain. The result is a post-receptor signalling complex associating relevant adaptor molecules to active TIR domains of TLR dimers.

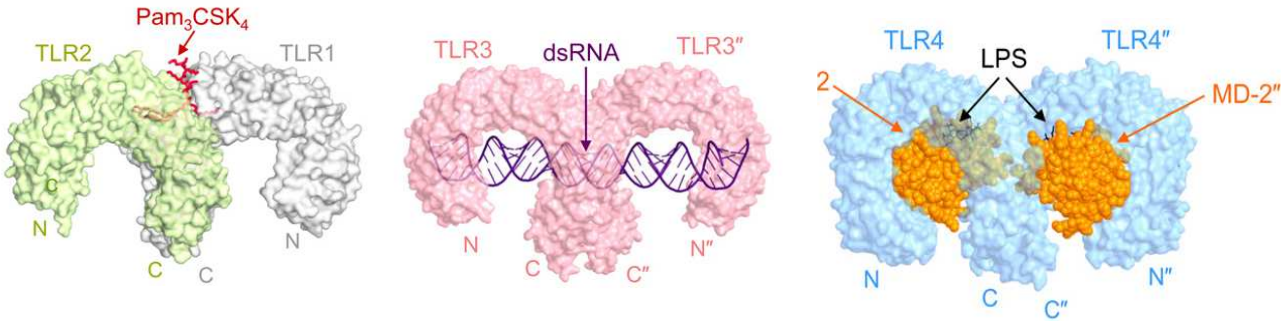


Figure 1.7.3: *The crystal structures of 'm' shaped TLR dimers induced by ligand binding are shown taken from* (Jin and Lee 2008) *(A) TLR1-TLR2-Pam3CSK4, (B) TLR3-dsRNA and (C) a model of TLR4-MD-2-Eritorian complex. Double apostrophes mark the second TLR or associated molecule (MD-2) in the receptor complex.*

Alternatively, it is also considered that TIR dimers may exist in an ‘inactive’ form within the cell and upon ligand binding reorientation of TIR domains may occur to facilitate adaptor recruitment. This has recently been demonstrated by (Latz et al. 2007) who show that an ‘inactive’ TLR9 dimer exists in a steady state prior to ligand binding. Binding of CpG resulted in close apposition of the cytoplasmic TIR-domains required for the recruitment of adaptor molecules. TLRs, relative adaptor molecules, signalling pathways and inducible genes are listed in table 1.7.2.

Toll-like receptor	Ligand	Origin of Ligand	Adaptor/Pathway	Transcription Factor/ Inducible genes
TLR1/2	Lipopeptide (BLP)	Bacteria and mycobacteria	Mal, MyD88	NF-κB*
TLR2/6	Peptidoglycan (PG), BLP	Gram +/-bacteria	Mal, MyD88	NF-κB
TLR3	dsRNA	Viruses	Trif	IRF3/Type I IFN α/β, NF-κB
TLR4	Lipopolysaccharide (LPS), hsp60, F protein	Gram +bacteria	Mal, MyD88 Trif, TRAM	NF-κB IRF3
TLR5	Flagellin	Gram +/-bacteria	MyD88	NF-κB, TNFα, IL-6
TLR6	Triacylated BLP, Zymosan	Bacteria, Yeast	MyD88	NF-κB
TLR7	ssDNA	Viruses	MyD88	NF-κB
TLR8	ssDNA	Viruses	MyD88	NF-κB
TLR9	CpG DNA, Hemazoin	Bacteria and Viruses	MyD88	NF-κB, IRF-3, IRF-7/IFN-α
TLR10	Orphan [+]	-	-	NF-κB
TLR11	Uropathogenic bacteria	*Toxoplasma gondii*	-	NF-κB

Table 1.7.2: *Toll-like receptors 1-11 and their corresponding ligands, adaptor molecules and inducible genes. Main reference sources listed in text. * All TLRs activate NF-κB. [+]Orphan: denotes a receptor of similar structure to other identified receptors but no endogenous ligand has been identified.*

There are five adaptor molecules known to transduce signals through TLRs via their TIR-domain and include; myeloid differentiating protein 88 (MyD88) (Lord, Hoffman-Liebermann and Liebermann 1990), MyD88 adaptor like/TIR domain-containing adaptor protein (Mal/TIRAP) (Fitzgerald et al. 2001), TIR-domain-containing adaptor inducing interferon-β/TIR-containing adaptor molecule-1 (Trif/TICAM-1) (Yamamoto et al. 2002b), Trif-related adaptor molecule/TIR-containing adaptor molecule-2 (TRAM/TICAM-2) (Bin, Xu and Shu 2003), and sterile alpha (SAM), HEAT/Armidillo motif and TIR-containing adaptor protein (SARM) (Carty et al. 2006). The proximal events of ligand binding and adaptor recruitment to the active TIR-domains of TLRs can result in the activation of two major signalling cascades, namely the MyD88-dependant and MyD88-independent pathways (Sharma et al. 2003, Kaisho and Akira 2006).

The MyD88 pathway leads to the activation of transcription factor NFκB and the expression of pro-inflammatory cytokines [see section 1.10.1]. As the name implies this pathway involves the recruitment of MyD88. The death domain of MyD88 interacts with the IL-1 receptor associated kinase (IRAK) family. Firstly, phosphorylation of IRAK-1 leads to the association of TNF-receptor associated factor 6 (TRAF6). TRAF6 is subsequently ubiquitinated via a TAK1-TAB-1-TAB2 kinase complex. This activates inhibitor of NFκB protein (IκB) kinase (IKK), IKKα and IKKβ. IKKs in turn phosphorylate IκB leading to its degradation. Degradation of IκB means NFκB is then free to translocate to the nucleus and initiate transcription of genes with a κB promoter element. In addition to NFκB, MyD88-dependant signalling also gives rise to the activation of mitogen activated protein

kinases (MAPK) such as p38 and JNK, which also result in the expression of inflammatory cytokines (Kaisho and Akira 2006, Dunne and O'Neill 2005).

MyD88-independent signalling activates the transcription factor interferon (IFN) regulatory factor 3, (IRF3). IRF3 activation leads to the expression of type I interferons, IFNα and IFNβ and other IFN-inducible genes. The MyD88-independent pathway requires the recruitment of adaptor Trif. The N-terminus of Trif has a binding site for tank binding kinase-1 (TBK-1) and IKKi. These kinases phosphorylate IRF3 resulting in its dimerisation and translocation to the nucleus. Upon translocation IRF3 binds to interferon stimulated response element (ISRE) in the promoters of IFN-inducible genes (Fitzgerald et al. 2003). As an example, schematic representation of TLR4 activation of MyD88 dependant and independent pathways is outlined in figure 1.7.4.

All TLRs with the exception of TLR3 are known to recruit MyD88 and activate the MyD88-dependent pathway activating MAPK and NFκB [see section 1.8.1] (Boehme and Compton 2004). In addition to MyD88, TLR2 and TLR4 require Mal/TIRAP to activate the MyD88-dependant pathway (Yamamoto et al. 2002a). TLR3 typically activates IRF and expression of interferons (IFN) from its endocytic compartments via Trif (Takeda and Akira 2004). TLR4 is unique in that it utilises both MyD88 and Mal to activate NFκB and Trif and TRAM to activate IRF3 [see section 1.9.4]. Of particular interest is that TLR4 signalling via Trif and TRAM also induces a late phase of NFκB activation (Kawai et al. 1999). TLR7, TLR8 and TLR9 act through MyD88 to induce pro-inflammatory cytokine secretion and the IFNs.

Other than MyD88, the signalling proteins employed by TLR7-9 to activate IFN remain unidentified.

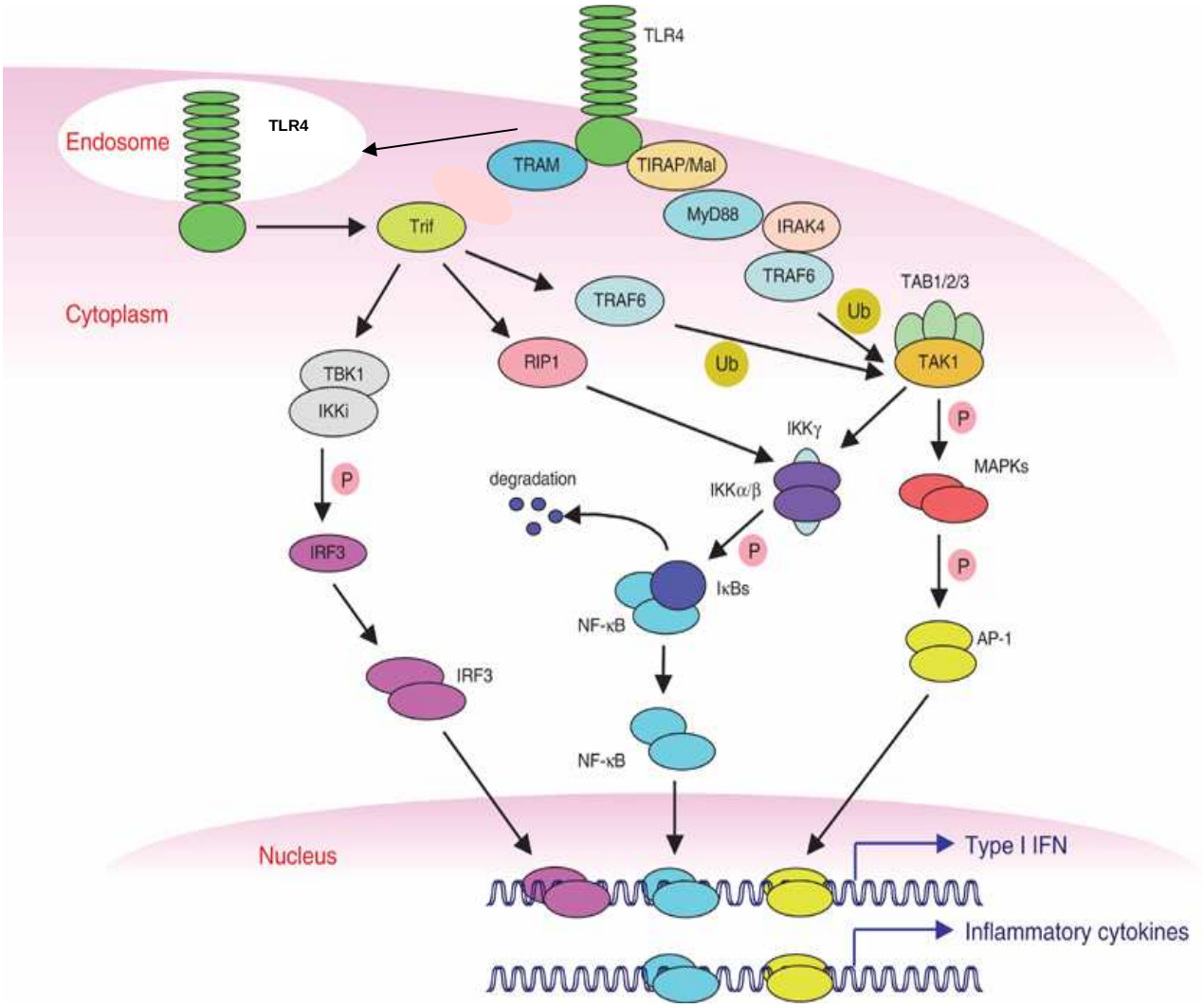


Figure 1.7.4: *TLR activation of MyD88 dependant and independent pathways. Adapted from* (Kawai and Akira 2006).

TLRs have the ability to harness great immunostimulatory signals and are therefore tightly regulated and activated. Incorrect and/or over activation of these pathways can lead to autoimmune disease (Reindl et al. 2003) and fatal sepsis (Karima et al. 1999). While many questions surround the exact interaction of TLRs and their

ligands, the complexities remain under constant investigation. TLR signalling and the responses they control continue to challenge thinking regarding the pathogenesis and treatment of cancers (O'Neill 2003), immune and infectious diseases as discussed in section 1.7.3.

1.7.3 TOLL-LIKE RECEPTORS: DISEASE AND THERAPEUTIC TARGETS

Over the past 30 years a great deal has been learned about innate immune activation on a molecular level. The TLR family represents just one of four important signalling systems involved in this activation. As such, the role of TLRs in the pathogenesis of infection and inflammatory disease is under constant investigation. Roles for TLRs have emerged in sepsis (Karima et al. 1999, BRAUDE, JONES and DOUGLAS 1963, Lorenz et al. 2002), rheumatoid arthritis (RA) (Seibl et al. 2003, Lee et al. 2006), inflammatory bowel disease (IBD) (Himmel et al. 2008), cancer (Eder et al. 2004, Smit et al. 2009), atherosclerosis (Kiechl et al. 2002, Liu et al. 2008, Schoneveld et al. 2008), asthma (Eder et al. 2004, Smit et al. 2009) and multiple sclerosis (Reindl et al. 2003) to name a few. Also a role for TLRs has been implicated in autoimmune disease with TLR4 (Ohashi et al. 2000, Okamura et al. 2001) and TLR9 (Leadbetter et al. 2002) responding to endogenous ligands. As a result, specific TLRs and their downstream signalling components present important targets for drug development in a wide range of inflammatory diseases and cancer.

The potential for TLRs and their signalling components in this regard lies in the ability of TLRs to ‘tailor’ signalling events in response to specific ligands. For example, while all TLRs are known to induce a common set of genes, individual TLRs use combinations of adaptor molecules to activate different signalling

pathways, inducing specific 'sets' of genes optimising host responses to a particular infection. Consequently, several approaches are taken to the development of therapies. Limiting TLR function may limit disease pathogenesis whilst in contrast stimulating TLRs may have adjuvant effects. Table 1.7.3 summarises the links between specific TLRs and disease.

TLR	Target Disease
TLR1/2	Bacterial/Fungal Diseases, Gram-positive sepsis
TLR3	Viral Diseases
TLR4	Bacterial diseases, Gram-negative sepsis, Chronic inflammation, Autoimmune diseases, Vaccines, Cancer, Atherosclerosis
TLR5	Bacterial diseases
TLR6/2	Mycobacterial diseases
TLR7	Viral diseases
TLR8	Viral diseases
TLR9	Bacterial and viral diseases, Autoimmune diseases, Vaccines, Cancer

Table 1.7.3: *Taken from* (O'Neill 2003).

As pointed out, TLR signalling can be inhibited in an attempt to limit exacerbated inflammation in inflammatory and autoimmune disease. Blocking of TLR signalling by means of neutralising antibodies is a popular and effective approach. TLR4, the receptor for LPS implicated in gram-negative sepsis, is by far the most studied. TLR4 is notably activated in response to endogenous factors such as heat shock

protein 60 (hsp60) (Ohashi et al. 2000) and fibronectin (Okamura et al. 2001) released during tissue injury. This and other work suggests an involvement of TLR4 in 'sterile' or non-infectious inflammation of autoimmune disease (Kerfoot et al. 2004, Racke, Hu and Lovett-Racke 2005).

Most recently TLR4 antibodies have been generated that have been shown to protecting mice from lethal endotoxic shock and *E. coli* sepsis (Roger et al. 2009, Daubeuf et al. 2007), demonstrating the potential of TLR4 targeted therapy in the treatment of gram-negative sepsis. Also a model employing *Malassezia furfur*, implicated in the development of scalp lesions of psoriasis, was used to demonstrate that a TLR2 antibody could successfully inhibit *M. furfur*-induced IL-8 in human keratinocytes (Baroni et al. 2006). Uniquely, in attempts to evade the immune system, vaccinia virus expresses the proteins A46R and A52R that limit host defence (Bowie et al. 2000). Importantly, A52R specifically inhibits TLR3 activation (Harte et al. 2003) and it is envisaged that inhibitors of TLR signalling may be developed based on viral proteins such as those found in vaccinia.

TLR antagonists have also been widely investigated and several attempts have been made to develop a TLR4 antagonist. The latest of which includes a phosphatidyl ethanolamine which blocks LPS activation of TLR4 by binding its co-receptor CD14 (Lee et al. 2009) and a chemically developed inhibitory peptide (Slivka et al. 2009). Plant sterol and phytochemical glugglsterone is a potent antagonist of TLR4 and TLR3 (Youn, Ahn and Lee 2009) while oligonucleotides with unmethlyated motifs antagonise TLR7 and TLR9 (Robbins et al. 2007). Another approach is to inhibit common signalling components or specific adaptors of TLR pathways leading to

decreased inflammatory gene expression. Glugglsterone suppresses NFκB activation by inhibiting the activity of inhibitor-κB kinase (IκB) (Youn, Ahn and Lee 2009). In terms of targeting specific adaptors the exact function of several of them remains to be elucidated.

Activation of TLRs can have adjuvant effects, particularly in relation to anti-tumor and anti-viral immunotherapies. Currently, aluminium hydroxide (alum) is the only approved human adjuvant in many countries. While extremely effective at boosting antibody responses, repeated administration is necessary and responses tend to be anti-parasitic T helper 2 (T_H2) rather than anti-viral and anti-bacterial T helper 1(T_H1), as reviewed by (Petrovsky and Aguilar 2004). Two clinically relevant TLR ligands have been investigated in this regard, CpG oligonucleotides (Lubaroff and Karan 2009) and a low toxicity derivative of LPS, monophosphoryl lipid A (MPLA) (Mata-Haro et al. 2007). CpG oligonucleotides induce type I interferon in a MyD88-dependent manner (Honda et al. 2005) while MPLA has been shown to induce type I interferon in a TRIF-dependant manner (Mata-Haro et al. 2007). This demonstrates the ability of different TLR ligands to reach the same endpoint using different adaptor molecules. With this understanding improvements can be made to possible TLR-dependent adjuvants. In addition, TLRs detecting viral components such as TLR3, 7 and 8 are potent activators of anti-viral responses. It is predicted that stimulating TLR3, 7 and 8 would have adjuvant effects in immunotherapies (O'Neill 2003). TLR7 and TLR8 are important inducers of type-I interferon's in response to viral infection. A group of small molecules such as the imiquimods are potent ligands for TLR7 and TLR8 and are currently in trial for treatment of genital herpes (Jurk et al. 2002, Syed et al. 1998). Adjuvant effects of TLR ligands is reviewed

extensively by (Warshakoon et al. 2009). Similarly, enhancing TLR activity is favoured in developing cancer treatment. However, this is suggested to have the most effect when used in combination with anti-cancer agents. This is reviewed extensively in the Oncogene issue comprising ten reviews on the topic (Journal Issue 2008).

Importantly, blocking TLR-mediated responses can lead to inappropriate allergic T_H2 responses or tolerance (Ishii, Uematsu and Akira 2006). It is also strongly desired that TLR antagonists present minimal toxicity *in vivo*. Furthermore, blocking TLR responses may also inhibit the inherent anti-inflammatory signalling of pathways essential in resolution (Serhan et al. 2007). It is crucial that mechanisms by which these anti-inflammatory pathways can be enhanced are investigated in order to promote resolution of inflammation. Current thinking has begun to focus on this aspect of TLR signalling in the development of therapies (Serhan et al. 2007). It is in no doubt that the risks and advantages involved in altering TLR signalling need to be balanced and investigated further.

1.8 SCAVENGER RECEPTORS

Scavenger receptors (SRs), as the name implies were first described for their ability to bind, internalize or 'scavange' modified low-density lipoproteins (mLDL) (Goldstein et al. 1979, Brown and Goldstein 1983). SRs are expressed by macrophage, dendritic cells and certain endothelial cells. By binding and internalising mLDL, they contribute to the onset of atherogenesis (Kunjathoor et al. 2002, Manning-Tobin et al. 2009). As PRRs they facilitate the receptor mediated endocytosis of microbes, and components of gram-negative (Amiel et al. 2007) and gram-positive bacteria (Peiser, Mukhopadhyay and Gordon 2002) including LPS and lipotechnoic acid (LTA), respectively. Additional functions include the clearance of apoptotic debris (Todt, Hu and Curtis 2008) and tissue homeostasis. The eight classes of the SR family (A-H) are structurally different with varying combinations of collagenous, cysteine-rich and/or C-type-lectin domains. Yet as a family of receptors they recognise and bind a range of common lipid and lipo-protein based ligands along with mLDL (Murphy et al. 2005). Although initial research focused on the role of SRs in atherogenesis, their role in defence is increasingly under examination. Scavenger receptor signalling has implications for cell morphology as well as cytokine and survival responses. Several *in vivo* studies demonstrate mice lacking SR-A have altered responses to LPS (Kobayashi et al. 2000, Fulton et al. 2006). Overexpression of class E scavenger receptor (LOX-1) has been demonstrated in rat zymosan-induced arthritis models (Nakagawa et al. 2002). The gene for human LOX-1 is located on a cluster within chromosome 12 linked to NK cell function and transcription of lectin proteins implicated in immune function (Sobanov et al. 2001). Furthermore, mice deficient in the scavenger receptor,

MARCO, display reduced bacterial clearance from lung tissue indicating an important role in host defence against airborne pathogens (Arredouani et al. 2004). The biological function and structure of the SR family is reviewed extensively (by Murphy et al. 2005, Adachi and Tsujimoto 2006).

1.8.1 SCAVENGER RECEPTOR A (SR-A)

Class A scavenger receptors (SR-A) were first among eight classes of the SR family (A-H) to be cloned (Kodama et al. 1990, Rohrer et al. 1990). They are type II trimeric transmembrane glycoproteins initially described for their ability to bind (mLDL) (Goldstein et al. 1979) although they are now known to bind many polyanionic ligands (Platt and Gordon 2001). The group is comprised of five polypeptides encoded by three related genes and includes: macrophage receptor with collagenous structure (MARCO), scavenger receptor with C-type lectin (SRCL), SR-AI, SRAII and SR-AIII (Kangas et al. 1999, Nakamura et al. 2001, and Freeman et al. 1990). The later three of the group are naturally occurring isoforms and alternative splice variants of the same gene (Gough, Greaves and Gordon 1998). Both SRA-I and SRA-II isoforms bind mLDL, bacterial components, polynucleic acids and some carbohydrate based ligands (Dhaliwal and Steinbrecher 1999). SR-AIII has no known ligand binding activity and is trapped in the endoplasmic reticulum (Gough, Greaves and Gordon 1998). These SR-A isoforms are largely expressed on macrophage but can also be found in smooth muscle and endothelial tissues (Murphy et al. 2005). All three isoforms are collectively referred to as SR-A (Mukhopadhyay and Gordon 2004). SR-A is structurally made of six domains: a transmembrane domain, α-helical coiled-coil domain, N-terminal cytoplasmic region, a spacer, a collagenous domain and a C-terminal domain see figure 1.8.1 (Kodama et

al. 1990, Rohrer et al. 1990). Several point mutation studies have indicated that the characteristic collagenous domain of each isoform confers ligand binding properties to the receptors (Doi et al. 1993).

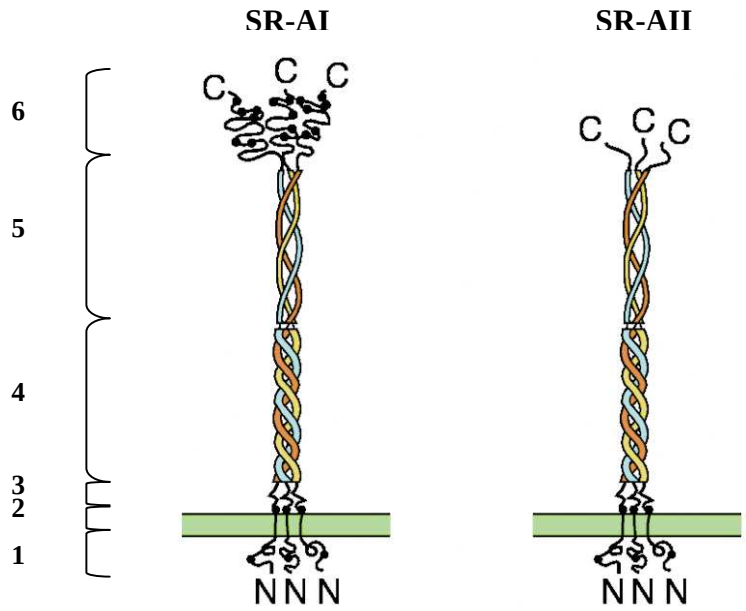


Figure 1.8.1: *Domain organization of SRAI and SRAII adapted from* (Murphy et al. 2005). *Each structure is made up of domains 1-6.* ***1****. N-terminal cytoplasmic region,* ***2.*** *transmembrane domain,* ***3.*** *a spacer* ***4.*** *α-helical coiled-coil domain,* ***5****. collagenous domain and* ***6****. a C-terminal domain.*

Research has focused on the ability of SR-A to bind mLDL contributing to atherogenesis by foam cell formation (Dhaliwal and Steinbrecher 1999, de Winther et al. 2000). However, their biological complexity and ability to bind a range of ligands corresponds to a much broader role for the receptors. Indeed, SR-A is known to be multifunctional having definitive roles in innate immunity, apoptotic cell clearance, tissue homeostasis and the pathogenesis of other inflammatory diseases such as Alzheimers, reviewed extensively by (Murphy et al. 2005, Mukhopadhyay and Gordon 2004, Peiser and Gordon 2001).

Studies involving SR-A$^{-/-}$ mice indicate a clear role for SR-A in innate immunity. SR-A$^{-/-}$ mice are more susceptible to gram-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) compared to wild-type mice (Kodama et al. 1990, Suzuki et al. 1997, Thomas et al. 2000). SR-A mediated phagocytosis of gram-negative and gram-positive bacteria has also been demonstrated (Thomas et al. 2000, Peiser et al. 2000). In addition, SR-A is a critical phagocytic receptor in bone marrow derived dendritic cells (BMDCs) for internalisation of gram-negative bacteria (Amiel et al. 2007) all of which indicate a vital role of SR-A in the clearance of bacteria. Importantly, SR-A mediated phagocytosis does not activate macrophage or induce pro-inflammatory cytokines such as TNFα, crucial to maintaining homeostasis (Platt and Gordon 2001, Peiser and Gordon 2001) .

Particularly, *in vitro* studies have demonstrated the ability of SR-A to bind LPS suggesting a possible role for SR-A in LPS clearance (Hampton et al. 1991). (Haworth et al. 1997) demonstrate that SR-A$^{-/-}$ mice (Haworth et al. 1997)are more susceptible to septic shock than wild type mice. The exact mechanism by which SR-A confers protection remains unclear. However, it is believed it is involved in the removal of excess LPS from the site of inflammation making it less available for CD14 (another LPS receptor) [see section 1.9.2]. SR-As are a prime example of how PRRs play critical roles within innate immunity and may also contribute to disease processes under certain pathological conditions.

1.9 OTHER PATHOGEN RECOGNITION RECEPTORS

TLRs occupy both the membrane and endosomes, giving them the ability to detect pathogens at the cell surface and within the cytosol. Together with TLRs, NLRs and RLRs exist as cytosolic sensors and facilitate the detection of intracellular PAMPs. TLRs recognize bacteria, fungi, protozoa and viruses; NLRs have been found to recognize bacteria and RLRs detect viral components. Nods are key regulators of apoptosis and NFκB activation in mammals in response to invading pathogens. Nod1 and Nod2 play important roles in innate and acquired immunity as sensors of bacterial components and their activation induce the production of pro-inflammatory mediators. Specifically, Nod1 confers recognition of bacterial lipopolysaccharide (LPS) and NFκB activation in a TLR4-independent manner (Inohara et al. 2001). Mutations within the *Nod2* gene, primarily expressed in monocytes have been widely implicated in susceptibility to Crohn's disease (Ogura et al. 2001) and linked to several immune diseases including psoriatic arthritis (Rahman et al. 2003) and allergic diseases (Kabesch et al. 2003).

The RLR family of receptors detect viral DNA are widely expressed and are encoded by 3 genes in the human and mouse genomes (Yoneyama et al. 2005). Like anti-viral TLRs (TLR3, 7 and 9) [see section 1.7.1 – 1.7.2], RLR activation leads to NFκB and IRF3 induction and expression of type I IFNs. In contrast, recent studies indicate RLRs protect all virally infected cells unlike TLRs, known to mainly detect viral infections in plasmacytoid dendritic cells (pDC). The activation and roles of NLRs and RLRs within immunity are extensively reviewed by (Creagh and O'Neill 2006, Yoneyama et al. 2005 and Inohara et al. 2005).

In certain cases it has been established that different PRRs co-operate. TLRs and certain NLRs are known to interact and mediate the induction of pro-inflammatory cytokine IL-1β. TLRs induce pro-IL-1β production and prime 'inflammasomes' or NLR-containing multi-protein complexes which respond to bacterial products and products of damaged cells activating caspase-1, which leads to the processing of pro-IL-1β to its active form (Netea et al. 2008). Similar interactions are believed to occur between TLRs and RLRs and further studies have to be carried out. Importantly, the interactions of various PRRs act synergistically or co-operatively to direct the overall innate response and provide ubiquitous protection.

1.10 TLR4 RECEPTOR COMPLEX

By far, the most widely recognised and characterised microbial activator of an innate immune response is lipopolysaccharide (LPS), also referred to as bacterial endotoxin (Janeway et al. 2008). The potent role of LPS in the pathogenesis of sepsis was first recognised in the 1960s (BRAUDE, JONES and DOUGLAS 1963). LPS is an important component of the membrane of gram-negative bacteria. LPS activates monocytes, MØ and other leukocytes promoting secretion of pro-inflammatory cytokines, TNFα, IL-12, IL-1, IL-6 and IL-8 (Cohen 2002) causing fever, hypotension, inadequate tissue perfusion, metabolic acidosis and organ failure (Beutler 2001). Due to the intensity with which LPS can induce inflammation and the pathogenesis of sepsis, many efforts were made to identify its receptor. In 1998 a group using the C3H/HeJ mice strain known to be hypo-responsive to LPS, demonstrated that TLR4 was the receptor for LPS (Poltorak et al. 1998). To date, TLR4 has been the most studied TLR in terms of it biological complexity, functioning in association with two accessory proteins, cluster of differentiation 14 (CD14) and myeloid differentiation-2 (MD-2) (Shimazu et al. 1999, Kirkland et al. 1993).

In its most effective form a receptor complex of TLR4, CD14 and MD-2 is activated by LPS. However, LPS can induce minimal signalling through TLR4 and MD-2 alone (Shimazu et al. 1999, Visintin et al. 2001). TLR4 is the signalling subunit directing signals intracellularly via the recruitment of adaptor molecules [see section 1.7.2]. CD14 is a glycosylphosphatidylinositol (GPI) anchored protein devoid of signalling capacity acting as the major recognition receptor of LPS (Kirkland et al.

1993). A lipid binding protein, (LBP) acts as a lipid transferase aiding LPS recognition by CD14 (Schumann et al. 1990). While MD-2, a secreted protein retained at the cell surface by TLR4, is indispensable in the cellular recognition and signalling of LPS (Shimazu et al. 1999). A schematic of the TLR4 receptor complex is represented in figure 1.10.1. Activation of TLR4 is mediated by the ligation of LPS and results in signalling via the MyD88-dependent (principal adaptor – Mal) and independent (principal adaptor TRAM) pathways activating NFκB and IRF, respectively (Kaisho and Akira 2006, Takeda and Akira 2004).

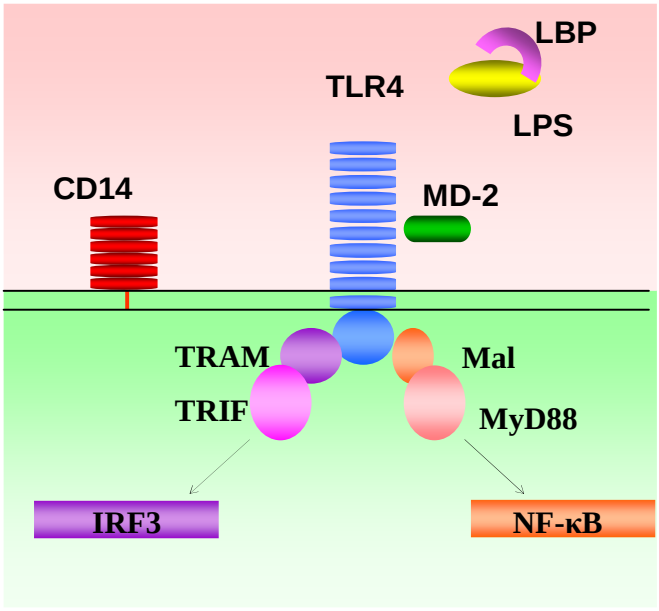


Figure 1.10.1: *Illustration of TLR4 receptor complex comprising the four cellular adaptor proteins known to participate in LPS recognition: Toll-like receptor 4 (TLR4), cluster of differentiation 14 (CD14), myeloid differentiation protein 2 (MD-2) and lipid binding protein (LBP).*

1.10.1 TLR4

TLR4 is an atypical TLR with extracellular leucine rich repeat (LRR), transmembrane and toll/interleukin-1 receptor (TIR) like cytoplasmic domains [see section 1.6.11]. (Espevik et al. 2003) reports two major localisation sites of TLR4; namely the plasma membrane and the Golgi apparatus. Furthermore, this group report localisation of TLR4 on the membrane of early endosomes. The exact mechanisms regulating TLR4 localisation remain controversial. (Visintin et al. 2006) report accessory molecule MD-2 is not required for surface expression of TLR4, while other reports conflict with this (Nagai et al. 2002, Ohnishi, Muroi and Tanamoto 2003). However, it is recognised that TLR4 rapidly traffics between the Golgi and plasma membrane, suggesting it is a highly mobile protein (Espevik et al. 2003, Latz et al. 2002). While TLR4 recognises a wide range of molecules including heat shock proteins, fibrinogen and taxol, LPS remains its most potent ligand (Gay and Gangloff 2007).

1.10.2 CD14

CD14 is a 56kDa, GPI-anchored protein lacking a transmembrane and intracellular domain. CD14 acts as an accessory molecule for both TLR4 and TLR2 signalling (Wright et al. 1990, Jiang et al. 2005). TLR2 employs CD14 for the recognition of lipopeptide, lipoteichoic acid and zymosan as studies indicate that CD14 mutant mice display impaired responses to these TLR2 ligands (Jiang et al. 2005). LPS has poor immuno-stimulating activity while it is part of the bacterial membrane. To become potent, it must be extracted and presented to the TLR4-MD-2 receptor. LBP, a serum glycoprotein and lipid transferase, catalyzes the transfer of LPS from the bacterial outer membrane to membrane-bound (mCD14) or soluble CD14 (sCD14)

(Schumann et al. 1990, Miyake 2006a). In turn, CD14 facilitates physical proximity and binding of LPS completing the TLR4 ‘activation cluster’. Indeed, CD14 plays a crucial role in LPS signalling as CD14-deficient mice are highly resistant to LPS induced septic shock (Haziot et al. 1996).

1.10.3 MD-2

MD-2 is a small glycosylated protein, physically associated with TLR4 on the surface of cells and confers LPS responsiveness to the receptor (Shimazu et al. 1999, Gangloff and Gay 2004, Nu-A et al. 2009). Indeed, the absolute requirement of MD-2 for maximal responsiveness of TLR4 signalling was demonstrated when disruption of the MD-2 gene in mice completely abrogated their responses to LPS (Schromm et al. 2001). Furthermore, (Visintin et al. 2001) show that soluble MD-2 alone had great capacity to restore LPS responsiveness to reporter cells that expressed TLR4 but not MD-2, demonstrating the high affinity interaction between the two. In contrast to TLR4, MD-2 binds LPS directly and does not require LBP or CD14 (da Silva Correia and Ulevitch 2002). A hydrophobic core and cationic lipid binding motif are structural features of the protein that make this binding possible (Gangloff and Gay 2004). A schematic of the crystal structure of TLR4-MD-2 binding LPS is depicted in section 1.7.2, figure 1.7.3.

The exclusive interactions of the accessory molecules CD14 and MD-2 with LPS have been studied and reviewed extensively (Kirkland et al. 1993, Miyake 2006a, Gangloff and Gay 2004and, Nu-A et al. 2009, Kim et al. 2005, Visintin et al. 2003).

1.10.4 TLR4 SIGNALLING

As discussed, the specific bacterial product, LPS from gram-negative bacteria predominantly drives TLR4 signalling. As this can mediate an overactivation of the immune response leading to chronic sepsis and organ failure, TLR4 signalling is finely tuned. Binding of agonistic ligand, LPS, to the TLR4 receptor complex causes dimerisation of the extracellular domain of two TLR4 receptors constituting a molecular 'switch'. This induces conformational changes to the cytoplasmic domain of the TLR dimer producing two symmetrically related adaptor binding sites (Nunez Miguel et al. 2007). Subsequently, signalling cascades are activated via recruitment of adaptors MyD88, Mal, Trif and TRAM (Brikos and O'Neill 2008, Akira, Yamamoto and Takeda 2003, Lu, Yeh and Ohashi 2008). Signalling via TLR4 is unique in that it activates both the MyD88-dependant pathway via MyD88 and Mal and the MyD88-independent pathway via TRAM and Trif. It remains unknown as to whether an activated TLR4 dimer can stimulate Mal and TRAM directed pathways simultaneously or whether the engagement of each adaptor is mutually exclusive (Nunez Miguel et al. 2007). The MyD88-dependent pathway results in nuclear translocation of NFκB and induction of pro-inflammatory cytokines, while the MyD88-independent pathway mediates induction of Type I interferons and IFN-inducible genes (e.g. IRF3) (Lu, Yeh and Ohashi 2008).

It is well accepted that cellular activation of the MyD88-dependent pathway via TLR4 is an event occurring at the plasma membrane. Indeed, (Latz et al. 2002) demonstrate recruitment of MyD88 to the cell surface upon LPS exposure and antibody-induced signalling and aggregation of surface TLR4. Mal acts as a sorting adaptor and recruits MyD88 to TLR4 through its ability to interact with

phosphatidylinositol 4, 5-bisphosphate (PIP2) (Kagan and Medzhitov 2006). Furthermore, by analogy the same group postulated that TRAM may also function as a sorting adaptor recruiting Trif to TLR4. Indeed, (Kagan et al. 2008) demonstrate that TRAM does act in this context. Surprisingly however, the group demonstrate that TRAM couples the endocytosis of TLR4 to the induction of MyD88-independent signalling (Kagan et al. 2008). This was further supported by a study at the time showing that TLR4 activates Trif-signalling via endosomes after its relocation from the cell surface (Tanimura et al. 2008). In addition, prior to the above (Jiang et al. 2005) demonstrate the absolute requirement of CD14 for LPS induced activation of the MyD88-independent pathway, while (Shuto et al. 2005) elucidate the requirement facilitates LPS-induced endocytosis and down-regulation of surface TLR4 expression in CHO cells.(Lee et al. 2001, Lee and Hwang 2006 and Lee et al. 2003) As a result CD14 plays a fundamental role in the regulation of TLR4 signalling. An outline of TLR4 activation via the MyD88 dependant and independent pathways is illustrated in figure 1.10.2.

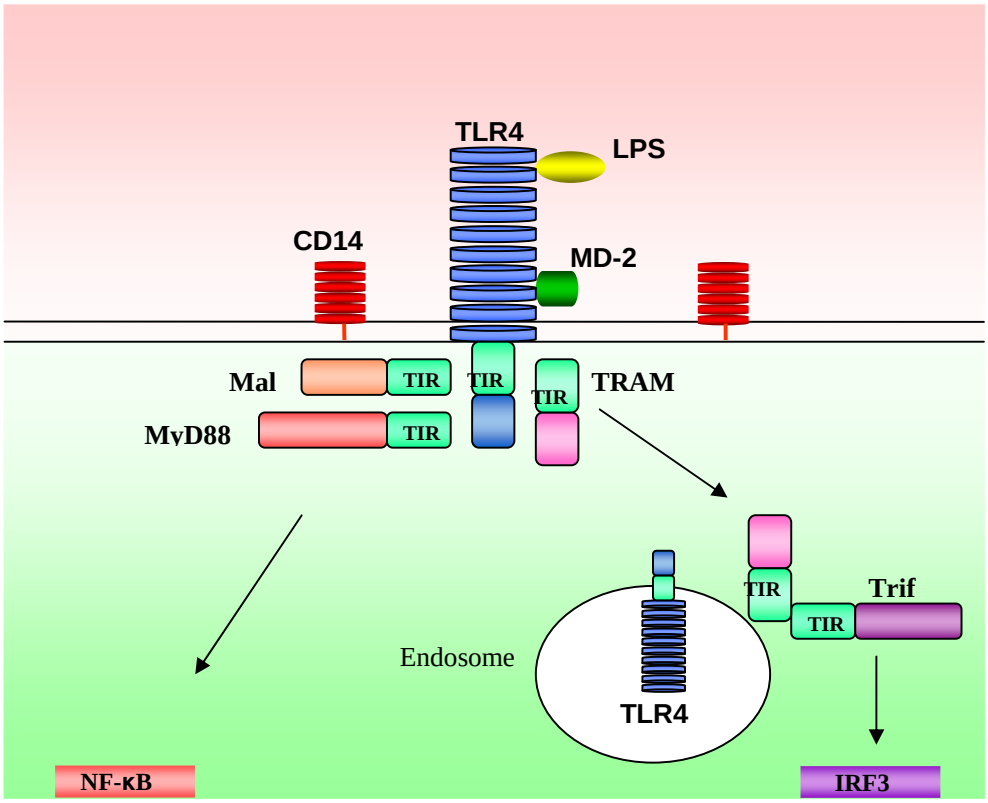


Figure 1.10.2: *Illustration of TLR4 activation of MyD88 dependant and independent pathways. TLR4 activation of MyD88 signalling occurs at the membrane. Activation of the MyD88-independent pathway is mediated by endocytosis of TLR4 and TRAM in the presence of CD14 and subsequent interaction with Trif in early endosomes.*

1.11 INTRACELLULAR SIGNALLING

Intracellular signalling is the ultimate mechanism by which cellular activation occurs. PAMPS, cytokines and other mediators act through distinct signalling pathways to activate protein kinases, initiating phosphorylation and ubiqitination events. At the level of signalling kinases a data compression occurs, channelling all signals towards activation of specific transcription factors controlling gene expression. The complexity of cellular networks means they commonly overlap and cross regulate each other.

1.11.1 NUCLEAR FACTOR (NF-) κB

Transcription factors such as NFκB play a vital role in inflammation, controlling genes that encode pro-inflammatory cytokines, chemokines, immune receptors and cell surface adhesion molecules (Li and Stark 2002). NFκB transcription factors include proteins with a highly conserved DNA-binding and dimerisation region known as the Rel homology (RH) domain (Hoffmann et al. 1999). Proteins of this family are divided into two groups based on their function, structure and mode of synthesis. Class I includes NFκB1 (p50), NFκB2 (p52) and class II includes RelA (p65), RelB and c-Rel. Members of both groups have the ability to form homodimers or heterodimers. Importantly, NFκB1 (p50) and NFκB2 (p52) function as transcription factors only upon forming dimers with a member of the second group (Baeuerle and Baltimore 1996).

NFκB proteins exist in resting cells as homo/heterodimers sequestered in the cytoplasm due to their association with an IκB inhibitory protein. IκB disguises the

sequence of the NFκB protein important for nuclear localization and DNA-binding (Chen et al. 1998, Ghosh et al. 1995). Multiple innate stimuli can lead to the activation of NFκB (i.e. LPS via the MyD88 dependent pathway via TLR4 activation). Ultimately, these stimuli lead to the activation of IκB kinase, IKK and subsequent phosphorylation of IκB proteins. Phosphorylated IκB proteins are targeted for degradation in the proteosome by ubiquitination. Degradation of IκB uncovers the nuclear localization sequence on NFκB. Translocation of NFκB to the nucleus facilitates the induction of pro-inflammatory genes that contain a κB-binding motif in their promoter regions (Hanada and Yoshimura 2002). NFκB signalling can occur through either the classical (canonical) or alternative (non-canonical) pathway. The classical pathway is activated by members of IL-1R/TLR superfamily and utilises IKKβ and IKKγ. The alternative pathway is initiated by members of the TNF superfamily (i.e. CD40L) and is IKKα dependent (Bonizzi and Karin 2004). More than 150 extracellular signals can lead to the activation of NFκB and many of the possible signalling pathways involved in the induction of this transcription factor are reviewed extensively by (Li and Stark 2002).

NFκB plays a critical role in both innate and adaptive immunity. It is associated with the transcription of many genes essential for lymphocyte survival and activation and is heavily involved in the induction of pro-inflammatory cytokines and chemokines (i.e TNF, IL-1, IL-6) (Li and Verma 2002). Constitutive activation of NFκB is commonly associated with inflammatory diseases including; RA, IBD and MS (Sun and Zhang 2007). Interestingly, NFκB activation is also involved in resolution of inflammation and is associated with the expression of anti-inflammatory genes. Indeed, inhibiting NFκB during resolution stages was found to prolong the

inflammatory response and prevent apoptosis (Hanada and Yoshimura 2002). While various stimuli lead to the induction of NFκB, many are largely unknown. Currently research in the field supports identifying these stimuli in order to elucidate the signalling pathways they induce. Hopefully this will aid the development of small molecule drugs for the treatment of inflammatory disease.

1.11.2 INTERFERON REGULATORY FACTOR 3 (IRF3)

Transcription factors such as the family of interferon regulatory factors (IRF) play critical roles in anti-viral responses and are responsible for the induction of Type I Interferons (IFN) (Paun and Pitha 2007). Type I interferons include IFNα and IFNβ, two important anti-viral cytokines involved in preventing the replication and spread of invading viral pathogens (Janeway et al. 2008). The expression of IFNα/β genes is tightly regulated and the search for an IRF that could activate both, led to the discovery of IRF3 and IRF7 (Au et al. 1995, Marie, Durbin and Levy 1998). Particularly, the discovery of IRF3 had a major impact on understanding the molecular mechanisms in anti-viral innate responses. IRF3 is ubiquitously expressed and typically activated by viral double stranded RNA (dsRNA) through TLR3 (Au et al. 1995). Recognition of bacterial components such as LPS also activate IRF3 (Navarro and David 1999, Sakaguchi et al. 2003). TLR3 initiates this activation from early endosomes within the cell, while recognition of LPS by TLR4 induces endocytosis of TLR4 and TRAM which also traffic to early endosomes to activate IRF3 (Kagan et al. 2008). Indeed, in response to these stimuli, IRF3 becomes phosphorylated by two non-canonical IκB kinases; TBK-1 and IKKε (Fitzgerald et al. 2003). This leads to a conformational change in IRF3, facilitating subsequent homodimerization or heterodimerization with IRF7 and translocation to the nucleus (Lin et al. 1998). Here dimers associate with co-factor cAMP-response binding protein, CREB. Furthermore, entry of IRF3 into the nucleus facilitates binding to a consensus DNA sequence known as the interferon-stimulated-response-element (ISRE) and the induction of IFN-inducible genes including; IFNα, IFNβ, and RANTES (Paun and Pitha 2007, Schafer et al. 1998, Lin et al. 1999).

There are well established links emphasising the importance of IRF3 induction and anti-viral responses. Indeed, many viruses target IRF3 functionally to inhibit Type I IFN genes (Weber, Kochs and Haller 2004). Furthermore, ubiquitous expression of IRF3 facilitates induction of IFNβ in the majority of virally infected cells (Sato et al. 2000). Conversely, a distinct role for the transcription factor in anti-bacterial innate immune responses continues to emerge (Honda and Taniguchi 2006). Indeed, IFNβ is required for the induced expression of co-stimulatory molecules CD80 and CD86 on dendritic cells (Hoebe et al. 2003). In addition, the production of IFNβ is essential for induction of endotoxic shock and IRF3 deficient mice are resistant to endotoxin shock (Sakaguchi et al. 2003). Furthermore, the absence of IRF3 affects the expression profile of other cytokines as even small amounts of paracrine or autocrine IFNβ stimulates IRF7 activation and amplifies the response (Sato et al. 1998). Overwhelmingly, studies point towards the global effects of IRF3 in the immune response. Future investigations will prove useful in the development of possible immunomodulators and anti-viral drugs.

1.12 LIPID RAFTS

In general terms, lipid rafts are tightly packed, liquid ordered plasma membrane microdomains enriched in cholesterol, sphingolmyelin and glycolipids (Dykstra et al. 2003). These cellular domains are considered evolutionary structures that play a role in a number of cell signalling processes and have received widespread interest and investigation regarding their possible biological functions. Indeed, their very discovery has turned on its head the historic fluid-mosaic model of the plasma membrane described by Singer and Nicolson in 1972 (Singer and Nicolson 1972). (Schnitzer et al. 1995) were first to indicate the existence of structurally distinct subsets of rafts. This is now a broadly accepted concept and two types of rafts, caveolae and flat lipid rafts are considered common features of the plasma membrane. Caveolae exist as small invaginations of the plasma membrane containing caveolin. This protein is responsible for the flask-shaped structure of these lipid rafts and lines the invagination (Anderson 1998). Furthermore, flat lipid rafts do not contain caveolin and therefore are level or 'flat' on the membrane and hence the name. Typically during subcellullar fractionation of rafts these two subsets are isolated together. As such, the term lipid raft is used generically to refer to both.

1.12.1 LIPID RAFT STRUCTURE

The outer leaflet of lipid rafts is composed of organised interactions between sphingolipids and cholesterol. The highly saturated acyl chains of sphingolipids pack tightly into microdomains. Any voids between sphingolipids are filled with cholesterol which acts as a spacer. Binding of sphingolipids and cholesterol promotes formation of a tight liquid 'ordered' phase. The remainder of the

membrane exists as a liquid 'disordered' phase due to the unsaturated, kinked acyl chains of the glycerophospholipid bilayer (Dykstra et al. 2003). Proteins are also associated with the outer leaflet of lipid rafts, particularly, through glycosyphoshatidylinositol, GPI-linkage. In this case, the saturated lipid tail of the GPI-anchored protein preferentially partitions into lipid rafts. Examples include, raft associated immune receptor, CD14, an Fc receptor, CD16 and adhesion and costimulatory molecules CD48 and CD58 (Dykstra et al. 2003). While a schematic of lipid raft structure is presented in figure 1.12.1, the biochemistry of lipid rafts is extensively reviewed by (Pike 2003, Pike 2003, Brown and London 2000, van der Goot and Harder 2001).

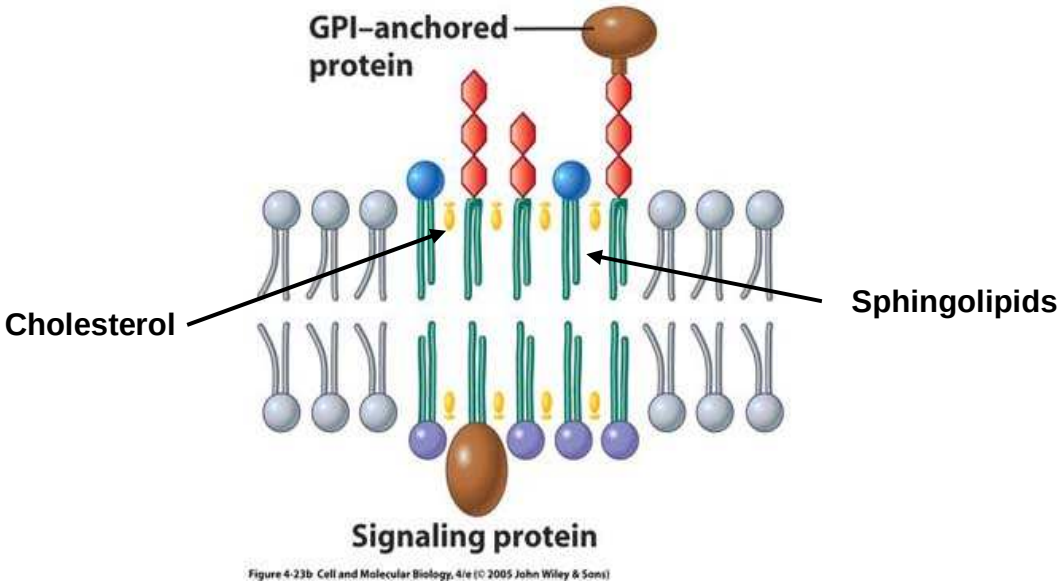


Figure 1.12.1: *Schematic representation of lipid raft microdomains comprising packed cholesterol, sphingolipids, GPI-anchored proteins and signalling proteins. Adapted from* (Karp and van der Geer 2005).

1.12.2 LIPID RAFT SIGNALLING

A central feature of lipid rafts owed to their biochemistry and physical structure is their ability to allow lateral segregation of proteins within the plasma membrane, e.g. GPI-anchored proteins, as described (Simons and Toomre 2000). Furthermore, rafts have been largely implicated in signal transduction, inducing close proximity of signalling receptors by specifically recruiting complexes to raft domains upon activation (Webb, Hermida-Matsumoto and Resh 2000, Liang et al. 2001).

Indeed, certain integral proteins reside constitutively at the border of lipid rafts and translocate to rafts following activation. This is particularly the case for multichain immune recognition receptors including, TCR and BCR, T cell and B cell receptors, respectively. In resting T cells, TCR is excluded from lipid rafts that actually contain several signalling components of the TCR signalling pathway including, LAT (linker for activation in T cells). Furthermore, upon engagement of TCR with antigen presenting cells, TCR associates with rafts (Montixi et al. 1998, Xavier et al. 1998). Similarly, BCR is found to be excluded from rafts in resting cells, however, following cross linking with Ig-specific antibodies or antigen, BCR and a number of components of the BCR signalling pathway are recruited to rafts (Petrie et al. 2000). Studies are limited with regard to additional immune receptors and lipid raft involvement. Most significantly, (Triantafilou et al. 2004) demonstrate recruitment of TLR4 to raft domains following stimulation with LPS. Characteristics that allow the translocation of proteins to raft domains are not understood, however evidence suggests transmembrane domains are critical (Scheiffele, Roth and Simons 1997, Field, Holowka and Baird 1999).

The exact biological activities and functions of lipid rafts remains unclear and under constant investigation. However, what is clear; is the mounting evidence to suggest a role of lipid rafts and their modulation in health and disease. Roles for lipid rafts are emerging in cancer (Li et al. 2006), insulin resistance (Fruhbeck, Lopez and Dieguez 2007) and microbial pathogenesis (van der Goot and Harder 2001). Until recently the biological features of the plasma membrane with regard to lipid rafts has been under-appreciated. While there is particular challenges to be faced in raft research with regard their isolation and exact structure, their mounting biological importance warrants further investigation.

1.13 POLYUNSATURATED FATTY ACIDS

It has long been accepted that nutrition has a predominant role in health. However, only at the beginning of the 20th century were fatty acids linoleic acid and α-linolenic acid recognised as essential components of the diet. Furthermore, the anti-inflammatory potential of elongated and desaturated derivatives of both these molecules became evident only thirty years ago (Wan, Haw and Blackburn 1989). The presence of more than one double bond denotes that a fatty acid is unsaturated. In the absence of double bonds the fatty acid is said to be saturated. Derivatives of both linoleic acid and α-linolenic acid have more than one double bond and are commonly referred to as polyunsaturated fatty acids, (PUFA). Classical nomenclature of PUFA is based on the number of carbon atoms in the fatty acid, the number of double bonds and the position of the first double bond (Canalejo et al. 1996). Derivatives of linoleic acid include n-3 PUFAs, n-3 denoting the first double bond is on the third carbon. The n-3 PUFAs, EPA or eicosapentaenoic acid (20:5) and DHA or docosahexaenoic acid (22:6) are commonly found in high fat fish and marine mammals (Marszalek and Lodish 2005). On the other hand, conjugated linoleic acids (CLAs), exist as positional and stereoisomers of conjugated dienoic derivatives of linoleic acid, specifically, dienoic octadecadienoate (18:2). The c9,t11 (*cis-9,trans-11*) CLA isomer predominantly found in foods derived from ruminants such as meat and dairy is used throughout this study (Belury 2002).

Ingestion of PUFA leads to their incorporation into virtually all cells in the body (Kew et al. 2004, Fritsche 2006). Recognition of the anti-inflammatory properties of PUFA has led to several nutritional studies investigating their health benefits

including; protection against the onset and incidence of coronary heart disease (Hamer and Steptoe 2006) and diabetes (Suresh and Das 2003). The impact of PUFA in immune-mediated disease in humans is also widely reported including; rheumatoid arthritis (RA), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE) and asthma (Fritsche 2006, MacLean et al. 2004, Schachter et al. 2004and).

Promising results from various animal models investigating the beneficial effects of PUFA has encouraged an emerging number of clinical trials and nutritional studies in the field. (Leslie et al. 1985) demonstrated very early in the 1980's that n-3 PUFA delayed and reduced incidence of type II collagen induced arthritis in mice. Similarly, EPA and DHA have been shown to reduce streptococcal cell wall induced arthritis in rats (Volker, FitzGerald and Garg 2000). (Bassaganya-Riera et al. 2004) found that feeding animals CLA ameliorated the severity of dextran sodium sulphate (DSS) induced colitis by reducing weight loss and lessening disease activity. In addition, mounting evidence from *in vitro* studies report the ability of PUFA to dampen production of inflammatory mediators such as cytokines and alter infiltration of immune cells such as lymphocytes and macrophages which has spurred clinical interest.

Due to the vast number of clinical trials performed results are routinely collated and reviewed extensively by many to assess progress in the field (Fritsche 2006, Calder 2006, Calder 2008). (Fritsche 2006) reviews multiple randomized control trials (RCTs) assessing the effects of n-3 PUFA in various disease states. RCTs regarding n-3 PUFA efficacy in RA, involve clinical assessment measuring parameters such as

pain, swollen joints and disease activity. Here for example several groups report n-3 PUFA reduced pain over placebo while others also report reduced severity of swollen joints in comparison to baseline. Similarly, (MacLean et al. 2004) reviewed extensively multiple trials focused on the role of n-3 PUFA in the prevention or treatment of diseases including arthritis and asthma. In a qualitative analysis of seven studies that assessed the effect of n-3 PUFA on anti-inflammatory drug or corticosteriod requirement among arthritis patients, six demonstrated reduced requirement for these drugs. Encouraging results have also been reported by others, Belluzzi et al. showed that supplementation with 2.7 g of n-3 PUFA preparation daily reduced the rate of relapse of patients with Crohns disease in remission (Belluzzi et al. 1996). However, following a systematic review of 26 studies regarding the beneficial effects of n-3 PUFA in asthma Fritsche and collegues also report a clinical outcome comparable to baseline with inconsistency being a major issue.

As such, while many trials look promising with regard to the beneficial effects of PUFA among immune mediated disease the general consensus in the field is for the strengthening of controls. Testing the effects relative to a control substance is of paramount importance. Also, many clinical studies cannot account for the background diets of their subjects unlike in the majority if not all animal studies were the diet is completely controlled therefore including a baseline assessment of dietary n-3 and n-6 fatty acid intake should be accounted for.

Ultimately researchers endeavour to elucidate the mechanisms through which PUFA exert their effects and there are namely three categories under which PUFA exert their modulatory actions, none of which are mutually exclusive. They include altered membrane composition and function, modified eicosanoid production and modifications in gene expression, as reviewed by (Lee and Hwang 2006, Fritsche 2006, Li et al. 2006, Li et al. 2005, Sampath and Ntambi 2005) and represented in figure 1.13.1.

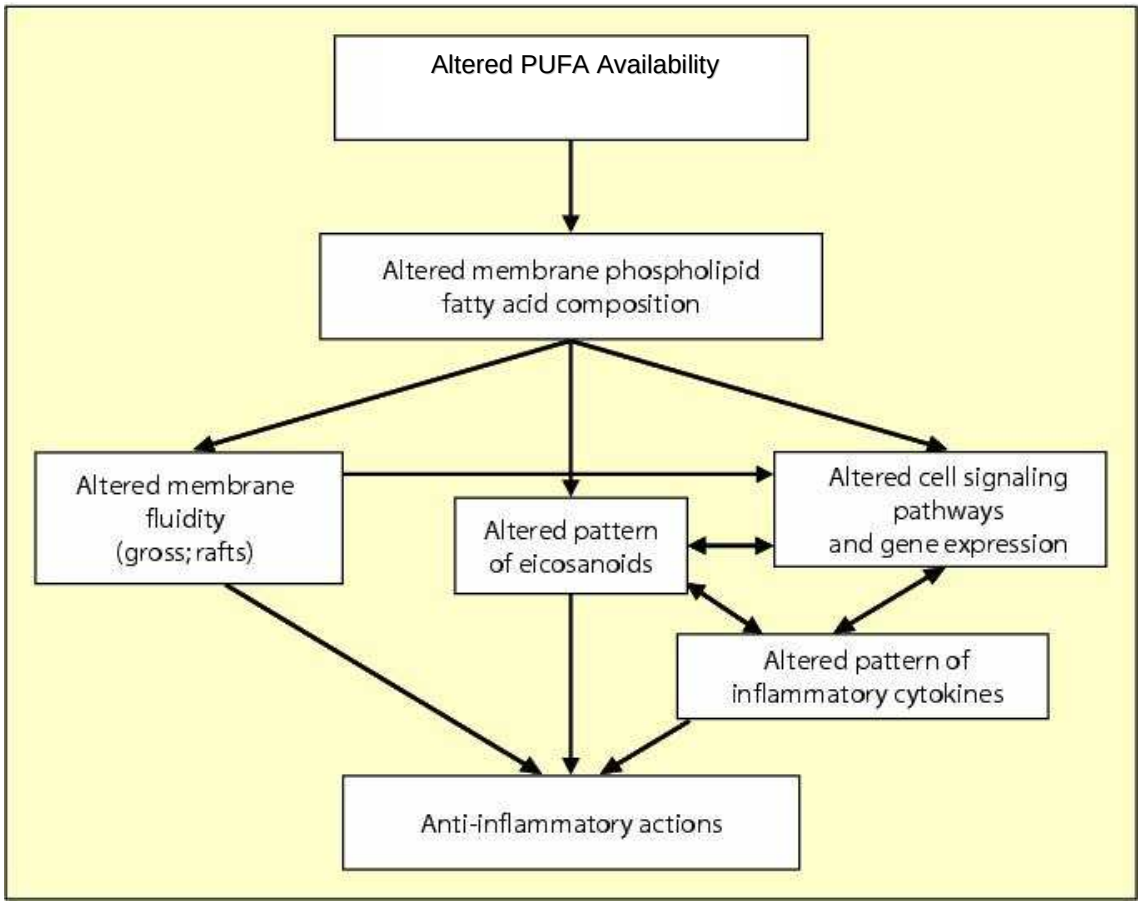


Figure 1.13.1: *Potential mechanisms by which polyunsaturated fatty acids (PUFA) can affect inflammatory cell function. Adapted from* (Calder 2004).

During inflammation, arachidonic acid ((AA), [20:4n-6]) is released from membrane phospholipids of immune cells and metabolised to eicosanoids, which have potent inflammatory properties. Increased incorporation of PUFA in cell membranes is partly at the expense of arachidonic acid and results in decreased production of eicosanoid products (Albers et al. 2002, Mantzioris et al. 2000). The cyclooxygenase (COX) pathway gives rise to prostaglandins and thromboxanes (TXB) and the lipoxygenase (LOX) pathway produces leukotrienes (LTB) (Luster, Alon and von Andrian 2005). Alterations in eicosanoid production as a result of altered PUFA intake is relatively well understood (Calder 2006, Hwang 2000). Particularly, EPA and DHA can act as substrates for COX and LOX enzymes giving rise to less potent inflammatory mediators known as resolvins and lipoxins (Serhan 2007).

PUFA can affect gene expression through several mechanisms including, but not limited to, changes in calcium levels, membrane composition and indeed eicosanoid production (Sampath and Ntambi 2005). Particularly, there is evidence to suggest that PUFA exert their effects by altering NFκB activation. (Weldon et al. 2007) demonstrate that EPA and DHA down-regulate NFκBp65 and increase cytosolic IκBα levels in a human macrophage cell line. Additionally, we have previously demonstrated CLA to suppress NFκBp65 activation in dendritic cells (Loscher et al. 2005a). Furthermore, cell specific metabolism and fatty-acid regulated transcription factors such as peroxisomal proliferator-activated receptors (PPARs) are likely to play a role in determining cell responses to altered PUFA compostion (Jump 2002a).

PUFA mediated modulation of gene expression and nuclear receptor activation are reviewed extensively by (Sampath and Ntambi 2005, Bordoni et al. 2006).

The fatty acid composition of cell membranes influences their fluidity. Increased saturated fatty acids and cholesterol increase rigidity, while unsaturated fatty acids make membranes more fluid (Brenner 1984, Stubbs and Smith 1984, Bruno, Koeppe and Andersen 2007). A change in membrane fluidity modifies membrane function and membrane bound receptors, enzymes and proteins. PUFA incorporation was reported to enhance the phagocytic activity of murine macrophage (Lokesh and Wrann 1984, Calder et al. 1990). In addition, membrane fluidity has been shown to upregulate insulin receptors on the membrane and as a result lower insulin resistance (Das 2005).

Specifically, much research has focused on PUFA modulation of immune function by means of 'lipid raft' microdomain alterations (Li et al. 2006, Li et al. 2005, Stulnig and Zeyda 2004). Current evidence suggests that translocation of immune receptors into lipid rafts regulates immune activation. PUFA treatment has been shown to alter lipid raft composition and displace signalling molecules leading to impaired signalling (Stulnig et al. 1998). The Src kinase family of protein tyrosine kinases and transmembrane adaptor, LAT (linker for activated T cell), play a vital role in T cell activation (Bijlmakers 2009). Lck and Fyn two Src kinases and LAT are activated following T cell stimulation and are constitutively expressed in rafts. (Stulnig et al. 1998and, Stulnig et al. 2001) show that treatment of Jurkat T cells with EPA displaced Lck, Fyn and LAT from lipid rafts. This and other studies suggest that lipid raft alterations are an underlying inhibitory effect of PUFA on T

cell signalling. Furthermore, research in the area of PUFA-mediated raft modifications has focused on the immunological synapse. As such, it remains for further studies to examine the effects of PUFA on other important immune receptor complexes. Taken together the potential mechanisms by which PUFA can affect cell function warrants further investigation.

Much research has focused on the modualtory actions of PUFA among immune cell types including lymphocytes, dendritic cells and macrophage.Particularly, multiple *in vitro* studies demonstrate the dampening of pro-inflammatory cytokine production in PUFA treated cells. CLA has previously been shown to suppress potent inflammatory cytokine, IL-12 in murine DC and a caco-2 cell model of human intestinal epithelium (Loscher et al. 2005a, Reynolds et al. 2008). Similarly, EPA-mediated inhibition of IL-12 has been reported in murine dendritic cells (DC) (Wang et al. 2007). Of particular interest to the macrophage model employed in the work presented here, several studies report suppression of inflammatory cytokines in PUFA treated macrophage. (Babcock et al. 2002) demonstrate that EPA can suppress TNFα production by murine macrophage. In addition, studies employing human macrophage models have reported that EPA and DHA inhibit TNFα, IL-6 and IL-1β production (Goua et al. 2008, Chu et al. 1999). While cytokine profiling is an aspect of macrophage functional status widely investigated in terms of PUFA mediated effects, phagocytosis has also been largely examined. Indeed, (Lokesh and Wrann 1984, Calder et al. 1990, Mahoney et al. 1977) demonstrated that altering the fatty acid composition of murine macrophages *in vitro* with PUFA made macrophage more phagocytically active. However, discrepencies do exist, (Kew et al. 2003) report no change in monocyte or neutrophil phagocytic activity in a murine

PUFA feeding study. Similarly, EPA and DHA were described as having no effect on the ability of alveolar macrophage to phagocytose (D'Ambola et al. 1991).

As a result of several other dispcrepencies in PUFA studies it has become largely accepted that inhibition of pro-inflammatory cytokines by PUFA may partly explain their overall anti-inflammatory effects. As such, it remains for more complete and thorough investigations to be carried out, specifically, looking at the complete spectrum representing functional status of macrophage. Indeed, this is infact true for all cell types as most recent literature suggests a clear dependency on cell type for PUFA exerted effects.

AIMS AND OBJECTIVES

While polyunsaturated fatty acids (PUFA) have been shown to modulate immune responses and have therapeutic effects in inflammatory disorders, the specific mechanisms of their actions remain unknown. Specifically this work aims to elucidate mechanisms regarding the effects of the n-6 derivative, conjugated linoleic acid (CLA) and n-3 PUFA, DHA and EPA on macrophages responses to LPS. Herein a tailored approach is taken to examine possible mechanisms of PUFA mediated modulation in macrophage by looking at their effects on cellular function, membrane markers and downstream signalling; as outlined below.

- The effects of PUFA on macrophage function are investigated by examining maturation, cytokine and chemokine production, migration and phagocytosis.

- The possible mechanisms used by PUFA to elicit their anti-inflammatory effects in macrophage are deduced by looking at alterations in NFκB and IRF-3 downstream of TLR4.

- The possibility of a PUFA modulated interaction of LPS with the TLR4 receptor complex is investigated by examining LPS-binding, surface marker expression over time, lipid raft localization and endocytosis of the receptor complex.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

TISSUE CULTURE MATERIALS/REAGENTS

Materials	Source
Tissue culture flasks T-75 cm^2	Nunc™
6.5mm Transwell® plate (8.0 μm pore)	Corning Inc.
Sterile Petri Dishes	Nunc™
6, 24, 96-well tissue culture plates	Nunc™
96 round bottom plates	Sarstedt
Dimethyl sulphoxide (DMSO)	Sigma®
Recombinant IL-2	BD Pharmingen
GM-CSF	R&D Systems®
Trypan blue (0.4% v/v)	Sigma®
G418 Geneticin	Sigma®
CellTiter 96® Aqueous One Solution	Pierce
RPMI-1640	Invitrogen™
Foetal Calf Serum (FCS)	Invitrogen™
Penicillin Streptomycin	Invitrogen™
DMEM	Invitrogen™
LPS (*E.Coli* serotype R515)	Alexis Biochemicals
Hygrogold	Invitrogen™
Blasticidin	Invitrogen™
DPBS	Invitrogen™

TABLE 2.1: *All tissue culture materials/reagents and corresponding sources.*

PROTEIN MANIPULATION

Materials	Source
Chloroform > 99.99%	Sigma®
Methanol	Sigma®
BCA Protein Assay	Pierce
Triton X-100	Sigma®
Potassium Chloride (KCl)	Sigma®
Sodium phosphate dibasic (Na_2PHO_4)	Sigma®
Dithiothreitol (DTT)	Sigma®
Potassium phosphate (KH_2PO_4)	Sigma®
Glycerol 99.99 %	Sigma®
Trizma Base	Sigma®
Sodium dodecylsulphate (SDS)	Sigma®
Tween® 20	Sigma®
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma®
Sodium Azide	Sigma®
Ammonium persulphate (APS)	Sigma®
Fuji SuperRX film	FujiFilm Ireland Ltd.
Precision Plus Protein™ Dual Color Standard	Bio-Rad
Propan-2-ol (isopropanol)	VWR International Ltd.
30 % Acrylamide/Bis solution	Bio-Rad
Sodium Orthovanidate	Sigma®
Leupeptin	Sigma®
Aprotinin	Sigma®
Iodoacetamide	Sigma®
Immobilon Western HRP Substrate	Millipore
Re-Blot Plus Solution (10 X)	Millipore
Ponceau S Solution	Sigma®
Phenylmethanesulfonyl fluoride (PMSF)	Sigma®

TABLE 2.2: *All reagents/materials used for protein purification, quantification, western blotting, and lysis buffers.*

FATTY ACIDS

Materials	Source
Eicosapentaenoic Acid (EPA)	Sigma®
Docosahexaenoic Acid (DHA)	Sigma®
Conjugated linoleic acid cis-9, trans-11 (CLA)	Cayman Chemical
Lauric Acid (LA) – Dodecanoic acid	Sigma®

TABLE 2.3: *All polyunsaturated fatty acids and saturated fat lauric acid with corresponding sources.*

ELISAs

Materials	Source
96-well microtitre plate	Nunc™
3,3',5,5'-tetramethyl-benzidine (TMB)	Sigma®
Tween® 20	Sigma®
Bovine serum albumin (BSA)	Sigma®
DuoSet ELISA kits	R&D Systems®
IL-23 (p19/p40) "Ready-SET-Go!" ELISA kit 5x assay diluent and TMB also provided	eBioscience

TABLE 2.4: *All ELISA materials/reagents and corresponding sources.*

LIPID RAFT ISOLATION

Materials	Source
Triton® X-100	Sigma®
MES	Sigma®
Mineral Oil	Sigma®
25G 1″ needle	BD Microlance™
Dounce Homogeniser set	Sigma®
Sucrose	Sigma®
Iodoacetamide	Sigma®
p-Nitrophenyl phosphate (pNPP) tablets	Sigma®

TABLE 2.5: *Reagents/materials used for isolation of lipid rafts.*

WESTERN BLOTTING ANTIBODIES

Antibody	Source
Anti-CD14	AbCam®
Anti-Flottilin-1	BD Transduction Laboritories
Anti-β-actin	Sigma®
Anti-mouse IgG peroxidase	Sigma®
Anti-rabbit IgG peroxidase	Sigma®

TABLE 2.6: *All antibodies used for western blotting analysis.*

FLOW CYTOMETRY

Antibody	Fluorochrome	Source	Isotype Control	Concentration/ 10^6 cells
TLR4-MD-2	PE	BD	RatIgG2a	0.5 µg
CD14	FITC	eBiosciences	Rat IgG2a	0.5 µg
CD204 (SR-A)	FITC	Acris Antibodies	Rat IgG2b	0.5 µg
CD40	FITC	BD	Ham IgM	0.5 µg
CD80	PE	BD	Ham IgG	0.5 µg
CD86	FITC	BD	Rat IgG2a	0.5 µg
MHCII	FITC	BD	Rat IgG2a	0.5 µg

TABLE 2.7: *Antibodies used for FACs analysis of cell surface markers; suppliers and concentrations used.*

FACS MACHINE/PREPARATION

Materials	Source
FACS Flow	BD
FACSRinse	BD
FACSClean	BD
37% (v/v) paraformaldehyde	Sigma®

TABLE 2.8: Materials/reagents used for flow cytometry and FACS preparation.

LPS BINDING

Materials	Source
Free FITC	Sigma®
Biotin-LPS	InvivoGen
Avidin, Alexa Flour® 488 conjugate	Invitrogen™
Biotin	Sigma®
LPS (*E.Coli* serotype R515)	Alexis Biochemicals
Biotin-labelling Kit	Pierce

TABLE 2.9: *Materials used for LPS-binding assay by Flow Cytometry.*

DNA MANIPULATION AND LUCIFERASE ASSAYS

Materials	Source
geneJuice® Transfection Reagent	Novagen®
QIAprep Spin Maxiprep kit	QIAgen©
10 X Passive Lysis buffer	Promega

TABLE 2.9: *Materials used for manipulation of DNA plasmids in transient transfections.*

CONFOCAL

Materials	Supplier
Poly-L-lysine 0.1% (w/v) in H_2O	Sigma®
Coverslips	Sigma®
Glass slides	Sigma®

TABLE 2.10: *Materials used for preparation of cell monolayer slides for confocal microscopy.*

BRUSH BORDER MEMBRANE VESICLE PREPARATION

For brush border membrane vesicle (BBMV) isolation, buffers A and B were pre-prepared and stored at 4 ºC. On the day of isolation all materials denoted * and protease inhibitor mix, C were freshly prepared and added just before use.

A

BUFFER A	**To Make 500 ml**
10 mM Immidazole	0.340 g
5 mM EDTA	0.730 g
1 mM EGTA	0.190 g
	To Make 25 ml
0.2 mM DTT*	5 µl of 1M stock
200 µg/ml Pefabloc*	5 ml of 1 mg/ml stock
BUFFER A + Protease Inhibitor (PI) Mix	
1 µl PI mix added per ml of BUFFER A	

B

BUFFER B	**To Make 500 ml**
75 mM KCL	2.790 g
5 mM $MgCl_2$	0.238 g
1 mM EGTA	0.190 g
10 mM Imidazole	0.340 g
	To Make 25 ml
0.2 mM DTT*	5 µl of 1M stock
BUFFER B + Protease Inhibitor (PI) Mix	
1 µl PI mix added per ml of BUFFER B	

C

Protease Inhibitor (PI) Mix	Stock	To Make 10 ml
1 µg/ml pepstatin	1 mg/ml	10 µl
1 µg/ml α_2-macroglobulin (papaine)	1 mg/ml	10 µl
15 µg/ml benzamidine	2.5 mg/ml	6 µl

BRUSH BORDER MEMBRANE VESICLE ANALYSIS

Materials	Source
CHAPS	Sigma®
Biacore L1 sensor chip	GE Healthcare
HEPES	Sigma®
Purified anti-mouse CD66a	eBioscience
Anti-mouse FITC conjugate	eBioscience
Carboxymethyl-dextran (CM-dextran)	Sigma®
Bovine Serum Albumin (BSA)	Sigma®

TABLE 2.11: *Materials used for BBMV analysis by flow cytomtry and Biacore3000™.*

Running Buffer pH 7.4	**To make 100 ml**
10 mM HEPES	0.238 g
150 mM NaCl	0.876 g
3 mM EDTA	0.087 g
Conditioning Buffer	**To make 100 ml**
20 mM CHAPS	1.229 g
Activation/Regeneration Buffer	**To make 100 ml**
40 mM CHAPS	2.459 g

TABLE 2.12: *Buffers used for BBMV analysis on L1 chip using Biacore3000™.*

2.2 METHODS

2.2.1 PREPARATION AND HANDLING OF REAGENTS

2.2.1.1 BUFFERS

The most commonly used buffers are listed in **Table 2.13** and any others are described within the text and detailed within the **Appendix**. EDTA (ethylene diamine tetraacetic acid) is used in the isolation of BBMV [see section 2.9.1] to chelate magnesium ions thus removing the essential cofactor required by protein kinases. Similarly, EGTA (ethylene glycol tetraacetic acid) is also used for BBMV isolation however; EGTA has a much higher affinity for calcium than magnesium ions. Both EDTA and EGTA are important for making buffers representative of the environment within living cells. Various protease inhibitors have been used for protein manipulations. Sodium orthovanidate is used to inhibit protein tyrosine phosphatases. Iodoacetamide is an alkylating reagent for cysteine and histidine residues in proteins and acts as an irreversible enzyme inhibitor. Aprotinin is a competitive serine protease inhibitor while leupeptin and phenylmethanesulfonyl fluoride (PMSF) inhibit serine and cysteine proteases. The combination of inhibitors used ensured that after lysis that proteolysis and protein kinase activities were negligible. As a result proteins under investigation were fixed at the levels *in vitro* at the time of cell lysis.

BUFFER	COMPOSTITON
Membrane Extraction Buffer (MEB)	20 mM MES, 150 Mm NaCl, pH 6.5
Membrane Fractionation Buffer (MFB)	20 mM Tris, pH 7.5/10 mM $MgCl_2$/1 mM EDTA/250 µM sucrose/200 µM PMSF
10 X Phosphate Buffered Saline (10 X PBS)	8 mM Na_2HPO_4, 1.5 M KH_2PO_4, 137 Mm NaCl, 2.7 mM KCL, pH 7.4
PBS-Tween (PBS-T)	1 X PBS with 0.05% Tween® 20
10 X Tris Buffered Saline (10 X TBS)	20 mM Trizma, 150 mM NaCl pH 7.2 – 7.4
TBS-Tween (TBS-T)	1 X TBS with 0.05% Tween® 20

TABLE 2.13: *Composition of most commonly used buffers.*

2.2.1.2 FATTY ACIDS

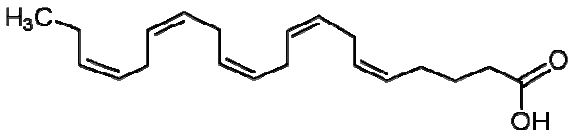
Fatty acids were dissolved in sterile filtered DMSO to a stock concentration of 100 mM, and sterile filtered again. Aliquots of 10 µl were kept at -20 °C and were thawed once and discarded after use to prevent oxidation. Fatty acids were purchased from suppliers and were tested for purity by HPLC prior to purchase. All newly purchased fatty acids were further tested for reliability; new stocks of PUFA were tested for their known inhibitory effect on pro-inflammatory cytokine production.

1. **Eicosapentaenoic acid (EPA) 20:5n-3**

   $C_{20}H_{30}O_2$

   M.W. 302.45

   cis-5,8,11,14,17- Eicosapentaenoic acid

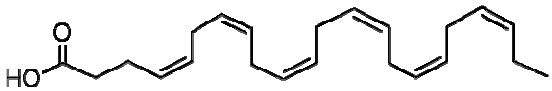


2. **Docosahexaenoic acid (DHA) 22:6n-3**

   $C_{22}H_{32}O_2$

   MW 328.49

   cis-4,7,10,13,16,19-Docosahexaenoic acid



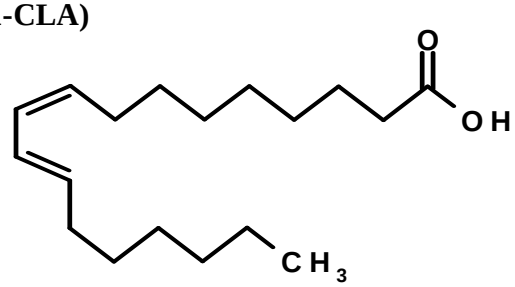
3. ***cis*-9, *trans*-11-conjugated linoleic acid (c9, t11-CLA)**

   (c9, t11-CLA)

   $C_{18}H_{32}O_2$

   MW 280.5

   (9Z,11E)-octadeca-9,11-dienoic acid



4. **Lauric acid (LA)** - SATURATED

   12:0 $CH_3(CH_2)_{10}COOH$

   MW 200.32

   Dodecanoic acid

2.3 CELL CULTURE

All tissue culture was carried out using aseptic technique in a class II laminar airflow unit (Holten 2010 – ThermoElectron Corporation, OH, USA). Cell cultures were maintained in a 37 °C incubator with 5 % CO_2 and 95 % humidified air (Model 381-Thermo Electron Corporation OH USA). Cells were grown in complete RPMI-1640 or DMEM medium as indicated**.** All media compositions and supplements are given in the **Appendix**. FCS was heat inactivated (56 °C for 30 min) to inactivate complement and aliquoted for storage at -20 °C. Supplemented medium was stored at 4 °C.

2.3.1 MURINE MACROPHAGE CELL LINE J774

The murine macrophage cell line J774A.1 was used extensively in this study and is referred to solely as J774 throughout. The J774 cell line was purchased from the ECACC. J774A.1 cells were maintained in complete RPMI-1640 **[see Appendix]** in 75 cm^2 flasks**.** Cell monolayers were passaged at a confluency of 80 % (every 3 to 4 days). Cells were detached by gentle tapping and transferred to a 50 ml falcon. Cells were spun at 1200 rpm for 5 min and supernatant discarded. Cells were resuspended in 10 ml of complete RPMI-1640. Each 75 cm^2 flask yielded approx. 20 x 10^6 cells. For subculture, cells were split 1 in 20 into 25 ml complete RPMI-1640 in a fresh 75 cm^2 flask. For experiments cells were counted as described [see section 2.3.4].

2.3.2 HUMAN EMBRYONIC KIDNEY CELL LINES HEK293

Human embryonic kidney cell line HEK293 and those stably transfected with TLR4, (HEK293-TLR4), TLR4, CD14 and MD-2, (HEK293-MTC) were a kind gift from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. All HEK293 cell lines were cultured with appropriately supplemented complete DMEM media **[see Appendix]**. Cells were cultured in 75 cm^2 flasks as follows; HEK293: complete DMEM, HEK293-TLR4: complete DMEM supplemented with 500 µg/ml Geneticin (G418) to maintain TLR4 expression and HEK-MTC: complete DMEM supplemented with 50 µg/ml Hygrogold and 1 µg/ml Blasticidin to maintain expression of TLR4, CD14 and MD-2. Cells were passaged every 3 to 4 days based on confluency. For subculture, the media was removed from flasks and cells washed twice with 5 ml ice cold sterile PBS (Invitrogen™). Following this cells were detached from flasks by incubating for 5 min at 37 °C with 1 ml 1 X Trypsin solution (Sigma®). 4 ml of appropriate media was used to resuspend cells and cells were spun at 1200 rpm for 5 min. Finally cells were resuspended in media, subcultured or counted for experiments.

2.3.3 HUMAN ASTROCYTOMA CELL LINE U373

In addition, human astrocytoma cell line U373 and those stably expressing CD14 (U373-CD14) were another kind gift from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. Cells were cultured in 75 cm^2 flasks as follows; U373: complete DMEM, U373:CD14: complete DMEM supplemented with 500 µg/ml G418 to maintain CD14 expression. Similarly to section 2.3.2, cells were passaged based on confluency, subcultured or counted for experiments.

2.3.4 CELL ENUMERATION AND VIALBILITY ASSESMENT

Cell viability was assessed using the trypan blue dye exclusion test. This test is based on the ability of viable cells to actively exclude dye as a result of having an intact cell membrane. Dead cells are unable to exclude the dye and appear blue when viewed under a microscope. 100 µl of cell suspension was mixed with 150 µl PBS and 250 µl of trypan blue solution (0.4% (v/v)). After ~2 min cells were applied to a brightline haemocytometer (Sigma®) and examined under high-power magnification (× 40) using an inverted microscope (Olympus CKX31, Olympus Corporation, Tokyo, Japan). An average count of four 'Grids' was taken **[Figure 2.1]**.

A viable cell count was determined using the following formula:

$$\text{Cell/ml} = N \times 5 \times 10^4$$

Where, N = average cell number counted, 5 = dilution factor, and 10^4 = constant.

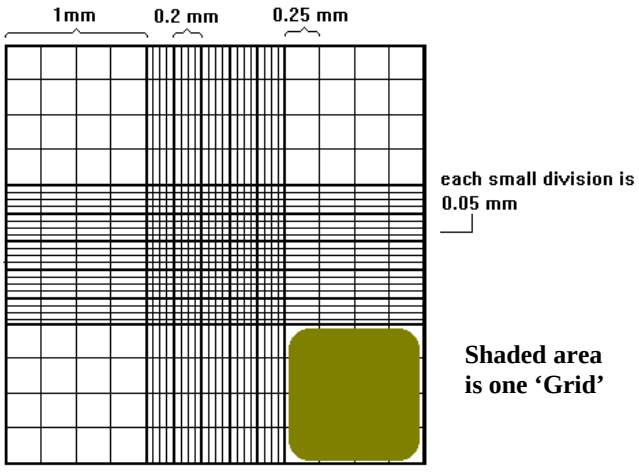


FIGURE 2.1 *Diagrammatic Representation of Haemocytometer used to count cells*

2.3.5 PREPARATION OF CELL STOCKS

Cell stocks were prepared from cultures that were approx. 70 – 80 % confluent. Cells were removed from culture as appropriate and resuspended in 1 ml cryoprotectant (10 % (v/v) dimethylsulphoxide (DMSO), 40 % (v/v) FCS and 50% RPMI) and transferred to labelled and dated cryovials (Nalgene®, Cryoware). Cryovials were placed in a Nalgene® Mr. Frosty freezing container. The Mr. Frosty container was filled with isopropanol and placed in a – 80 °C freezer. Mr. Frosty freezing container provided slow preservation of cells at a rate of 1 °C/min. After 2 h vials were transferred to liquid nitrogen for long term storage.

2.3.6 REVIVAL OF FROZEN STOCKS

Cryovials were carefully removed from liquid nitrogen tank and quickly thawed in a 37 °C water bath. Thawed cells were transferred to 10 ml RPMI on ice and carefully resuspended. Cells were spun at 1200 rpm for 5 min to remove excess DMSO. Following this supernatant was discarded and cells resuspended in 10 ml of room temp RPMI. Cells were spun again and a third wash carried out using 37 °C RPMI. After the final wash cells were resuspended in 1 ml of the appropriate medium and transferred to a 75 cm^2 flask with appropriately supplemented media **[see Appendix]**.

2.3.7 ADDITION OF FATTY ACIDS

The vehicle control DMSO, and the fatty acids, CLA, and LA, were added to cells at a concentration of 50 µM. The n-3 PUFA, EPA and DHA were added at 25µM. In all experiments cell lines were cultured for 7 days in DMSO, CLA, EPA, DHA or LA at the indicated concentration.

2.3.8 STIMULATION WITH LPS

Cells were activated with 100 ng/ml LPS *E.Coli* serotype R515 (Alexis Biochemicals) and incubated for 24 h, unless otherwise stated before being used in the relevant assays.

2.3.9 CYTOTOXICITY ASSAY FOR PUFA DOSE RESPONSE

The CellTiter 96® AQueous One Solution (Promega) is a colorimetric method for determining the number of viable cells in a sample. It contains an MTS tetrazolium compound (Owen's reagent) which is bioreduced by viable cells into a soluble coloured formazan product. The quantity of formazan product is measured at an absorbance reading of 490 nm and is directly proportional to the number of living cells in the culture medium. J774 macrophage were cultured for 7 days as described [see section 2.3.1]. Fatty acids were added at concentrations of 25, 50, or 100 µM on day 1 of the cell culture. On day 7 cells were counted and plated in a 96-well plate with 100 µl per well at 1 x 10^6 cell/ml left unstimulated for 24 h. After the 24 h, 20 µl of the CellTiter 96® AQueous One solution was added to each well. Plates were incubated for 2 h at 37 ºC in 5 % CO_2 and absorbance read at 490 nm. The cell viability of each sample was calculated by treating the absorbance of the vehicle

control, DMSO as 100 % and comparing remaining samples to this and expressing results as percentage viability.

2.4 FLOW CYTOMETRY

2.4.1 CELL SURFACE MARKER STAINING

J774 macrophage were cultured with fatty acids for 7 days as described [see section 2.3.7]. Cells were then plated at a concentration of 1 x 10^6 cell/ml in a 6-well plate (2 ml/well). To observe maturation of macrophage, cells were left unstimulated and stimulated with LPS (100 ng/ml) for 24 h. For kinetic analysis cells were stimulated with LPS for 0, 2, 4, 6, 12 and 24 h. Following LPS treatment cells were scraped using a pasteur pipette and collected in falcon tubes. An equal volume of FCS was added for 15 min to prevent non-specific binding. Tubes were spun at 1200 rpm for 5 min and cells resuspended in FACS buffer see **Table 2.13**. 200 µl of cells were added to a 96-well round bottom plate to give approximately 400,000 cell/well. One well per treatment group (i.e., each PUFA treatment +/- LPS) was allocated for each antibody group. Additionally, one well was plated for every corresponding isotype control group.

Plates were spun at 2000 rpm for 10 min and supernatant carefully removed from the wells. 100 µl of the correct antibody or isotype mixture was added to the appropriate wells. Plates were incubated in the dark at 4 ^{o}C for 30 min. Following incubation, plates were spun at 2000 rpm for 10 min at 4ºC. Cells were washed by resuspending in 200 µl FACS buffer twice. Cells were then fixed in 200 µl 4% (v/v) formaldehyde/PBS before being transferred to labelled FACS tubes. Samples were acquired immediately or left overnight in the dark at 4 ºC. 30,000 events were

acquired per sample using a 4-colour FACSCalibur (fluorescence activated cell sorter) Becton Dickinson (BD). Data was analysed using CellQuest™ software to generate histograms and fluorescence intensity values of surface marker staining.

Fluorochrome	Antibody Group 1	Antibody Group 2	Antibody Group 3	Antibody Group 4		
FITC	CD14	CD204	CD40	CD86		
PE		TLR4	CD80			

TABLE 2.13: *Antibody groups generally used for macrophage flow cytometry.*

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2.4.2 PHAGOCYTOSIS

J774 macrophage were cultured with fatty acids for 7 days as described [see section 2.3.7]. 5 x 10^5 cells were plated onto 6 well plates in a total volume of 2 ml/well and left to rest overnight at 37 ºC. On the next day, to investigate phagocytosis 2.5 µl of flourescently labelled latex beads were added at a concentration of 1 x 10^6 beads/µl for a period of 0, 2, 4, 6, 12 and 24 h. Following this media was removed and each well washed twice with ice cold PBS. Cells were then scraped and transferred to 15 ml falcons. Cells were spun at 1200 rpm for 5 min and resuspended in 200 µl 4% (v/v) paraformaldehyde/PBS. Phagocytosis of latex beads was assessed by flow cytometry on BD FACSCalibur™. For the analysis of phagocytosis after stimulation cells were treated with 100 ng/ml LPS for 24 h prior to the addition of latex beads. Note: latex beads were sourced from Sigma® and were 1 µm in diameter.

2.4.3 CHEMOTAXIS ASSAY

J774 macrophage were cultured with fatty acids for 7 days as described in [see section 2.3.7]. Cells were then plated at a concentration of 1 x 10^6 cell/ml in a 6-well plate (3 ml/well) and stimulated with LPS (100 ng/ml) for 24 h. Following incubation, cells were removed from wells using a transfer pipette and counted. Transwell® plates were used in accordance with the manufacturer's instructions; 3 x 10^5 cells were added to the insert well in 100 µl of media, and 600 µl of media supplemented with or without chemoattractants GM-CSF (10 ng/ml) and IL-2 (10 BRMP/ml; where 1 BRMP = 40 pg/ml) were added to the bottom chamber. Plates were incubated at 37 ºC for 5 h. Cells that had migrated to the bottom chamber were collected in eppendorf tubes, spun and resuspended in 4% (v/v) formaldehyde/PBS before being transferred to FACS tubes. Migrated cells were counted for 60 seconds on a BD FACSCalibur™.

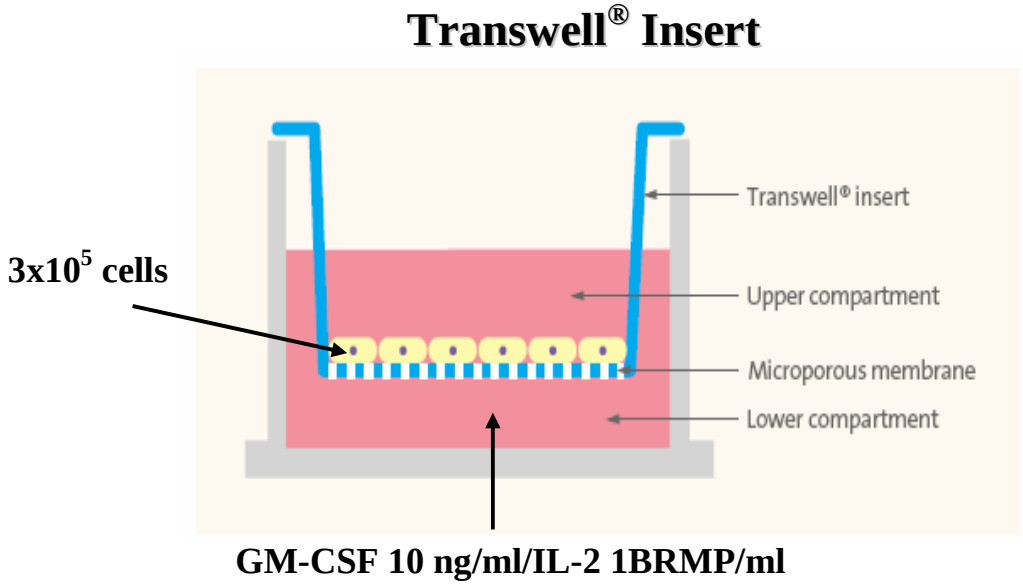


Figure 2.2: *Diagrammatic representation of Chemotaxis Assay Transwell™. Schematic taken from Transwell® Permeable Supports Selection and Use Guide, Corning.*

2.4.4 OPTIMISATION OF LPS BINDING IN HEK293 CELLS

HEK293 and HEK-MTC cells were cultured in the absence of fatty acids as described [see section 2.3.2]. On reaching confluency cell suspensions of 2 x 10^7 cells/ml were prepared. 50 µl of cells (1 x 10^6) were incubated with 50 µl of 5, 10 or 50 µg/ml LPS-Biotin (Invitrogen™), house LPS-Biotin (using LPS (*E.Coli* serotype R515), Alexis Biochemicals and Biotin-labelling kit, Pierce), free Biotin (Sigma®), or InlB-Biotin. Note: Internalin-B, (InlB) is found on the extracellular membrane of *Lysteria. monocytogenes* and known to be a TLR2 ligand. InlB-Biotin was provided by Dr. Elizabeth Tully of the Applied Biochemistry Group, Dublin City University. Incubation with the various biotinylated molecules was carried out overnight at 4 °C. On the following day cells were washed 3 times in ice cold PBS and incubated with avidin Alexa Flour® 488 conjugate (Invitrogen™) for 15 min on ice. Cells were then washed as before and resuspended in 1% (v/v) paraformaldehyde/PBS. Binding of LPS to cells was measured on a BD FACS Calibur™.

2.4.5 LPS BIINDING IN PUFA TREATED HEK293 CELLS

HEK-MTC cells were cultured with fatty acids for 7 days as described [see section 2.3.7]. Based on the optimisation of LPS binding in section 2.4.4, 5 µg/ml LPS-Biotin was used in this experiment. Intemal controls similar to those performed in section 2.4.4 were included.

2.5 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

The concentration of cytokines IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, TNF-α and chemokines MIP-1α, MIP-2 and MCP-1 in cell supernatants was determined using ELISA Duoset kits from R&D Systems in accordance with the manufacturers' instructions. A diagrammatic representation of the principles of a sandwich ELISA are shown in **Figure 2.3**

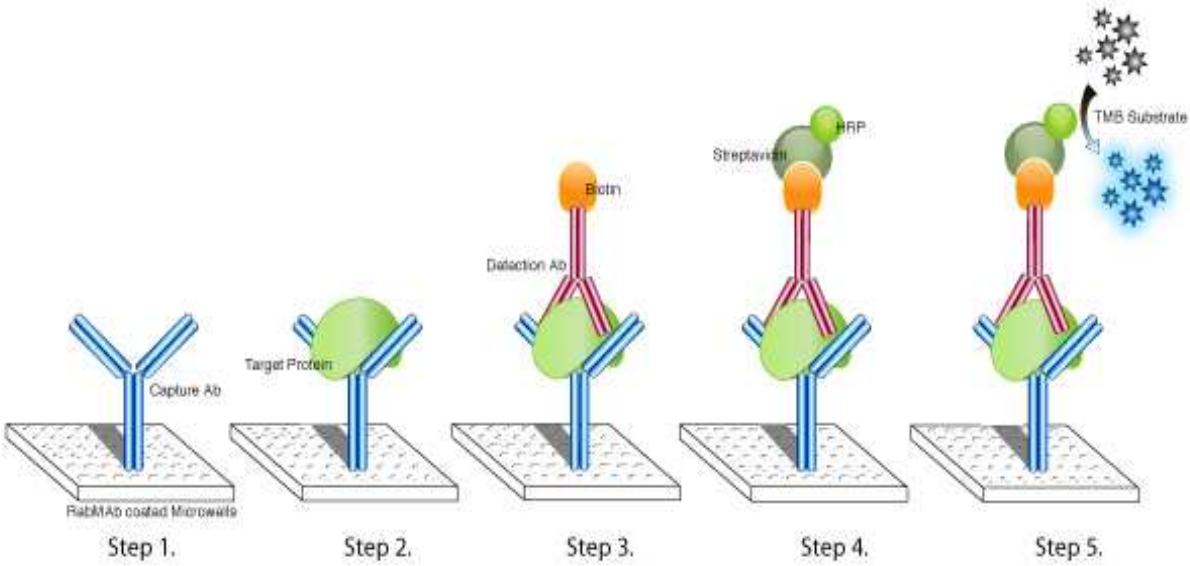


FIGURE 2.3: *Schematic representation of sandwhich ELISA. Schematic taken from ELISA Kit Technology Principals on www.epitomics.com.*

2.5.1 IL-10, IL-12p40, TNF-α, MCP-1, MIP-1α and MIP-2 ELISA

96-well Nunc™ microtitre plates were coated with 100 µl of the relevant capture antibody diluted to working concentration in PBS see **Table 2.13** and incubated overnight at room temperature. After washing plates x 3 with wash buffer (PBS/0.05% Tween® 20), wells were blocked with 300 µl of reagent diluent (1% w/v BSA/PBS) for at least 1 h at room temperature. After repeating the washing step, 50 µl of reagent diluent and 50 µl of supernatant or serially diluted standards (top standard serially diluted in reagent diluent – see **Table 2.13**) were added to wells in duplicate. Plates were incubated overnight at 4 ^{o}C. The following day plates were washed x 3 with wash buffer. 100 µl of the relevant biotinylated detection antibody, diluted in reagent diluent (1:180 dilution), was added to each well and plates were incubated for 2 h at room temperature. Plates were washed x 3 with wash buffer and 100 µl of streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well. Plates were incubated for 20 min in the dark at room temperature. Finally, wells were washed x 3 with wash buffer and 100µl of TMB was added to each well. Plates were incubated in the dark until colour developed. The reaction was stopped by adding 50 µl 2N H_2SO_4 per well. Optical densities were read immediately at 450 nm on VERSA Amax microplate reader (Molecular devices, CA, USA). Cytokine/chemokine concentrations in supernatants were determined from standard curves.

2.5.2 IL-1β

The method above was followed with two deviations:

Blocking buffer used was 1%BSA/PBS + 0.05% NaN_3 and the reagent diluent was 0.1% BSA/TBS + 0.05% Tween.

2.5.3 IL-6

Samples were diluted 1:10 in reagent diluent and 100µl of diluted samples and undiluted standards were added to plates in duplicate. Concentrations of samples were multiplied by the dilution factor once calculated from the standard curve.

2.5.4 IL-23p19

The IL-23 "Ready-SET-Go!" ELISA kit was purchased from eBioscience and IL-23 concentrations determined in supernatants according with the manufacturers' instructions. 96-well plates were coated with 100 µl of IL-23p19 capture antibody diluted 1:250 in PBS and plates were incubated overnight at 4 ^{o}C. After washing x 4 with wash buffer (PBS/0.05 % Tween® 20), wells were blocked with 300 µl of assay diluent for 1 h at room temperature. Assay diluent was provided in the kit at a 5 x stock and diluted in distilled water before use. After repeating the wash step, 50 µl of assay diluent, and 50 µl of supernatant or serially diluted standards (top standard serially diluted in assay diluent – see **Table 2.13**) were added to wells in duplicate, and plates were incubated overnight at 4 ^{o}C. The following day plates were washed x 4 with wash buffer. 100 µl of the biotinylated p40 detection antibody, diluted in assay diluent (1:500 dilution), was added to each well and plates were incubated for 1 h at room temperature. Plates were washed x 4 with wash buffer and 100µl of streptavidin-HRP (1:250 dilution in assay diluent) was added to each well. Plates were

incubated for 20 min at room temperature. Finally, wells were washed x 4 with wash buffer. 100 µl of TMB (eBioscience – supplied with kit) was added to each well and plates incubated in the dark. The reaction was stopped by adding 50 µl 2N H_2SO_4 per well once the colour had developed. Optical densities were read immediately at 450 nm on VERSA Amax microplate reader (Molecular devices, CA, USA). Cytokine concentrations in supernatants were determined from standard curve.

Cytokine	**Capture Antibody (µg/ml)**	**Top Standard (pg/ml)**	**Detection Antibody (ng/ml)**
IL-1β	4.0	1000	400
IL-6	2.0	1000	200
IL-10	4.0	2000	500
IL-12p40	4.0	2000	400
IL-23p19	(p19) 2	1000	(p40) 200
TNF-α	0.8	2000	75
Chemokine	**Capture Antibody (µg/ml)**	**Top Standard (pg/ml)**	**Detection Antibody (ng/ml)**
MCP	0.2	250	50
MIP-1α	0.4	1000	100
MIP-2	2.0	500	75

TABLE 2.13: *Concentration of standards, capture and detection antibodies used in sandwich ELISA assays.*

2.6 CELL MEMBRANE ANAYLSIS

2.6.1 MEMBRANE FRACTIONATION OF J774 MACROPHAGE

J774 macrophage were cultured with fatty acids for 7 days as described [see section 2.3.7]**.** Cells were seeded at 2 x 10^5 cell/ml in a 6-well plate (3 ml/well) and left to rest overnight. Cells were stimulated with LPS (100 ng/ml) for 0, 2, 4 and 6 h. Following stimulation cells were scraped into 300 µl membrane fractionation buffer (MFB) see **Table 2.13**. Protease inhibitors were added just before use as follows: 1 µg/ml aprotinin, 1 µg/ml leupeptin, 100 µM sodium orthovanidate and 0.5 M PMSF. Cells were lysed with 50 strokes of a dounce homogenizer (Sigma®) and spun in thick wall polycarbonate Beckkman tubes at 425,000 *g* for 1 h at 4 °C. The resulting supernatant (i.e., the cytosolic fraction) was removed to a fresh tube, and the pellet, (i.e. the membrane fraction) was resuspended in 60 µl of 5 X sample buffer **[see Appendix]**. Protein within the cytosolic fraction was concentrated by MeOH/Chloroform precipitation [see below section 2.6.2] and resuspended in 60 µl 5 X sample buffer. Equal volumes of samples were run on 10 % (v/v) SDS-PAGE gels **[see Appendix]**.

2.6.2 METHANOL (MeOH)/CHLOROFORM PROTEIN EXTRACTION

The entire protein extraction protocol was performed on ice. 200 µl of the relevant protein sample was placed in a sterile 2 ml centrifuge tube. 800 µl of ice cold MeOH was added. The sample was vortexed and pulsed for 30 sec in a benchtop centrifuge. 200 µl of ice cold chloroform was added in a fume hood, vortexed and pulsed for 30 sec. 600 µl of ice cold H_20 was added and the sample was vortexed and spun at 6000 g for 5 min. At this point the upper phase was removed as protein was concentrated at the interphase. 600 µl of ice cold MeOH was added and the sample was vortexed and spun at 14000 g for 10 min. The supernatant was aspirated off quantitatively and the protein pellet dried under nitrogen gas for 20 min. Finally, the protein pellet was resuspended in 60 µl of 5 X sample loading buffer **[see Appendix].** Samples were vortexed vigorously to ensure full resuspension of the protein pellet.

2.6.3 LIPID RAFT ISOLATION AND OPTIMISATION

2.6.3.1 MEMBRANE PREPARATION

J744 macrophage were cultured in fatty acids for 7 days as described [see section 2.3.7]. 100 x 10^6 cells are required for sufficient protein levels for lipid raft analysis. As such, with an 80 % 75 cm^2 flask yielding approx. 20 x 10^6 cells, 5 x 75 cm^2 flasks were cultured per sample. Cell monolayers were detached by gentle tapping and spun out of culture at 1200 rpm for 5 min. Cell pellets were washed three times in ice cold sterile PBS (Invitrogen™). Following this cell pellets were resuspended in 2 ml membrane extraction buffer (MEB) [see **Table 2.13**] containing 5 mM iodoacetamide, 1 mM PMSF, 1ug/ml aprotinin and 1 ug/ml leupeptin and left on ice for 30 min. For mechanical cell disruption lysates were freeze thawed in liquid nitrogen three times. This was followed by 40 strokes with a dounce homogeniser and passage through a 25G 1" syringe (BD Microlance™) ten times. Lysates were spun at 100,000 g for 1 h at 4 ºC. The cytolsolic fraction was washed away and the membrane pellet resuspended in 500 µl MEB containing 0.5 % (v/v) Triton X-100 and stored at – 80 ^oC before application to sucrose density gradient [see section 2.6.3.2]. Note: For the optimisation of lipid raft isolation different concentrations of Triton X-100 were employed and are indicated in the text. In addition, for the isolation of lipid raft in stimulated cells, J774 were treated with 100 ng/ml LPS for 30 min prior to membrane preparation.

2.6.3.2 SUCROSE DENSITY GRADIENT

For the isolation of lipid rafts membrane preparations [see section 2.3.6.1] were mixed with an equal volume (500 µl) 90 % (w/v) sucrose/MEB (with protease inhibitors as described section 2.3.6.1). This 1 ml sample was transferred to the bottom of a beckman polycarbonate tube and overlaid with 5.5 ml 30% (w/v) sucrose/MEB (with protease inhibitors) followed by 4.5 ml 5% (w/v) sucrose/MEB (with protease inhibitors). Tubes were filled to the top with mineral oil (Sigma®) and spun for 18 h at 175,000 g in a TH-641 swinging bucket rotor in a Sorvall WX ultracentrifuge. After spinning 1 ml fractions were carefully collected form the top of the gradient (designated fraction 1 – 11) and stored at – 80 ^{o}C for further analysis.

2.6.3.3 ALKALINE PHOSPHATASE ACTIVITY

The alkaline phosphatase activity of lipid raft fractions was determined using SIGMA*FAST*™ p-nitrophenyl phosphate (pNPP) tablets. pNPP was made according to manufacturers' instructions. 20 µl of each lipid raft fraction was added to a 96 well plate. Following this 200 µl of pNPP substrate was added to each well and the plate incubated in the dark for 30 min. Following incubation the plate was read at 405 nm on a VERSA Amax microplate reader.

2.6.3.4 ANALYSIS OF LIPID RAFT FRACTIONS

The flottilin-1, CD14 and β-actin content of lipid raft fractions was analysed by western blot [see section 2.7.3]. Protein from each fraction was precipitated as described [see section 2.6.2]. For qualitative comparison of protein, pellets form precipitations were resuspended in 60 µl 5 X sample loading buffer and equal volumes run on 10 % (v/v) SDS-PAGE gels **[see Appendix]**. However, for quantitative comparison of PUFA lipid raft fractions, protein pellets were resuspended in 60 µl MEB (with protein inhibitors) and protein levels normalised based on NanoDrop3300 quantification. Samples were normalised to 8 µg in a total volume of 50 µl with appropriate vol of 5 X loading buffer and run on a 10 % (v/v) SDS-PAGE gel. Antibodies used in western analysis are listed in **Table 2.6**.

2.7 PROTEIN ANALYSIS

2.7.1 PREPARATION OF SAMPLES FOR GEL ELECTROPHORESIS

All protein samples were prepared in 5 X sample buffer as described. Before loading on gels samples were boiled at 95 ^{o}C for 5 min to denature proteins.

2.7.2 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins were separated by SDS denaturing polyacrylamide gel electrophoresis (SDS- PAGE). Acrylamide gels (10 %) **[see Appendix]** were cast between two glass plates and affixed to the electrophoresis unit using spring clamps. Electrode running buffer **[see Appendix]** was added to the upper and lower reservoirs. 12µl of prepared samples were loaded into the wells and run at 30 mA per gel for approximately 45 minutes. Pre-stained protein molecular weight markers (Bio-Rad laboratories) ranging from 10 – 250 kDa were added to the first lane in each gel.

2.7.3 WESTERN BLOTTING

Proteins were quantitatively transferred to nitrocellulose membranes using the iBlot® Dry Blotting System (Invitrogen™). The iBlot® efficiently and reliably blots proteins from polyacrylamide gels in 7 min without the need for additional buffers or an external power supply in a self-contained unit. Following transfer, the nitrocellulose membrane was removed and processed for immunoblotting.

2.7.4 IMMUNODETECTION AND DEVELOPMENT

Following transfer, non-specific sites on the membrane were blocked with freshly prepared blocking buffer, 5 % (w/v) dried skimmed milk/TBS-T [see **Table 2.13**] for 1 h on a slow rocker at room temperature. Membranes were then washed x 3 with TBS-T (wash buffer) and incubated with appropriate primary antibodies. Incubation details including reagent diluent and the concentration of antibodies used are listed in **Table 2.14**. Membranes were gently agitated with the primary antibodies overnight at 4 ºC. Following overnight incubation, membranes were washed eight times for 2 min in wash buffer. Membranes were then incubated with the relevant secondary antibodies (horseradish peroxidase (HRP) conjugated secondary antibody) [see Table 2.14] and incubated with gentle agitation at room temperature for 1 h. Following incubation with secondary antibody, membranes were washed eight times for 2 min with washing buffer.

HRP-labelled antibody complexes were visualised using the enhanced chemiluminescence (ECL) method. Membranes were incubated for 5 minutes in 3 ml of Immobilon Western HRP Substrate (Millipore). Excess substrate was decanted and the membrane placed between acetate sheets and immediately exposed to FujiFilm SuperRX film in a dark room under red light. The film was developed using a film Hyperprocessor (Amersham Pharmacia Biotech). Exposure times varied depending on the concentration of protein used and the intensity of signals obtained. In general exposure times varied between 15 sec to 5 min. The density of resultant bands was calculated using the densitometry program on the Syngene gel analysis and documentation system (Syngene NJ USA).

2.7.5 STRIPPING AND RE-PROBING MEMBRANES

To reprobe membranes, antibody complexes were removed by incubating membranes in 10 ml 1 X Re-Blot Plus Solution (made according to manufacturers' instructions) for 15 min with gentle agitation. Following this membranes were washed in 5 ml of blocking buffer twice for 5 min to remove excess stripping solution. At this point membranes were either re-probed with antibodies or stored in TBS-T at 4 °C.

1° ANTIBODY AND DILUTION		2° ANTIBODY AND DILUTION	
CD14	1:5000	Anti-rabbit IgG peroxidase	1:2000
FL-1	1:250	Anti-mouse IgG peroxidase	1:2000
β-actin	1:10000	Anti-mouse IgG peroxidase	1:20000

TABLE 2.14: *Dilution of primary and secondary antibodies for western blotting. Both 1° and 2° antibody dilutions were made in 5 % (w/v) dry skimmed milk/TBS-T. All 1° antibody incubations were performed at 4 °C overnight and all 2° antibody incubations were performed for 1 h at room temperature.*

2.8 DNA MANIPULATION

2.8.1 DNA TRANSFORMATION INTO BACTERIA

Chemically competent DH5α *E. coli* cells and ligation DNA were generated by Kathy Banahan (Biochemistry, Trinity College Dublin). For transformation cells were thawed on ice after which, ligated DNA (5-10 µl) was added, mixed gently and incubated for 5 min on ice. The cells were heat shocked in a water bath at 42 ºC for 2 min and returned to ice for a further 2 min. As ligation DNA contained an ampicillin cassette for transformation, cells were plated onto selective LB agar plates containing 100 µg/ml ampicillin and grown overnight at 37 ºC.

2.8.2 PURIFICATION OF PLASMID DNA FROM BACTERIA

To prepare milligram quantities of plasmid DNA an individual quantity of plasmid transformed bacteria was inoculated into 4 ml LB broth for 6 h. This starter culture was then used to inoculate a 500 ml LB broth supplemented with 100 µg/ml ampicillin and grown overnight at 37 °C in a shaking incubator. Bacterial cells were harvested by centrifugation at 300 rpm for 15 min at 4 °C. Milligram quantities of plasmid DNA were purified using a QIAprep Spin Maxiprep kit according to manufacturers' instructions. The DNA was quantified using a NanoDrop3300. Stocks were stored at 4 ºC and used for transient transfection of HEK293 and U373 cell lines. A 500 ml culture typically yielded 1 – 3 mg/ml of plasmid DNA.

2.8.3 LUCIFERASE ASSAY BY TRANSIENT TRANSFECTION OF HEK293/U373 CELLS

GeneJuice® (Novagene) transfection reagent is a liposomal based transfection reagent. The ISRE luciferase plasmid, NFκB luciferase plasmid, *Renilla* luciferase plasmid and empty pcDNA3.1 vector (Invitrogen™) along with luciferase assay reagents were kind gifts from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. All transfections were performed in 24-well tissue culture plates with a total volume of 500 µl/well. Cells were seeded as follows; HEK293 and HEK-TLR4 cells at 2 x 10^5 cell/ml, HEK-MTC cells at 4 x 10^5 cell/ml and both U373 and U373-CD14 cells at 8 x 10^4 cell/ml. Cells were incubated overnight and transfected the following morning using geneJuice® transfection reagent according to the manufacturers' instructions. For ISRE/NFκB luciferase assays, 75 ng of ISRE/NFκB luciferase plasmid, 30 ng of *Renilla* luciferase, and 115 ng empty pcDNA3.1 vector made up to a total of 220 ng of DNA were transfected into each well of a 24-well plate. For both ISRE and NFκB luciferase assays cells were left to rest for 24 h after transfection before stimulating with 100 ng/ml LPS for 6 h. Following stimulation media was aspirated from each well and cells were lysed in 100 µl of 1 X passive lysis buffer (Promega, Southampton, UK) for 15 min. Firefly luciferase activity was assayed by the addition of 40 µl of luciferase assay mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 $MgSO_4$, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to 20 µl of the lysed sample. In addition, *Renilla* luciferase was read by the addition of 40 µl of a 1:1000 dilution of Coelentrazine (Argus Fine Chemicals) in PBS to 20 µl of lysed sample. Luminescence was read using a Reporter microplate luminometer

(Turner Designs). The *Renilla* luciferase plasmid was used to normalize for transfection efficiency in all experiments.

2.9 Confocal Microscopy

HEK-MTC cells were cultured for 7 days in fatty acids as described in section 2.3.7. Autoclaved glass coverslips were placed in 6 well cell culture grade plates. 500 µl of poly-L-lysine 0.1% (w/v) in H_2O was used to coat slides for 5 min in preparation for cell culture. Poly-L-lysine was removed and slides left to dry for 30 min. Cells were harvested from culture and counted. Cells were plated at 1.25 x 10^5 cell/ml (3 ml/well) and left to rest overnight. For the investigation of TLR4 and EEA1 localisation, TLR4-YFP construct was a kind gift from Douglas Gollenbock in the University of Massachusetts Medical School, Worcester, Massachusetts, USA and the EEA1-CFP construct was a gift from Terje Espeviks at the Norwegian University of Science and Technology, Institute of Cancer Research and Molecular Medicine, Trondheim, Norway. Yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) are spectral variants of green fluorescent protein (GFP) and make possible the visualisation of two or more different proteins. Cells were transfected with TLR4-YFP (0.75 µg) and EEA1-CFP (0.75 µg) using geneJuice® as described in section 2.8.3. Cells were left to rest for 24 h before removing media and supplying 3 ml fresh media to cells. On the 4th day cells were stimulated for 7.5 and 15 min with 250 ng/ml LPS. Following stimulation media was removed and cells washed on ice three time with ice cold PBS. Cells were fixed to glass coverslips by incubating with 2% (v/v) paraformaldehyde/PBS, pH 7.4 for 15 min at room temperature. Slides were washed a further three times in PBS and mounted onto glass slides using mounting medium (90% glycerol, 10% PBS) and sealed using varnish. Cell

preparations were analysed using an Olympus FluoView™ FV1000 and FV1000 Viewer Software version 1.7.

2.10 BRUSH BORDER MEMBRANE VESICLES

2.10.1 BBMV ISOLATION AND CHARACTERISATION

Brush border membrane vesicles, (BBMV) were prepared using an established method (Ferrary et al. 1999). Briefly, BBMVs were isolated from freshly harvested mucosa from the whole small intestine of a BALB/C mouse. The mucosa was diluted in 10 ml per mg mucosal tissue weight with Buffer A [see section 2.1] and stirred for 1 h at 4 °C. Mechanical cellular disruption was performed by 10 strokes in a glass dounce homogeniser (Sigma®). Following this the cellular suspension was spun at 1000 g for 10 min at 4 °C. Supernatant was discarded and the pellet washed again with 10 ml Buffer A and centrifuged at the same setting. Three washes were performed in total. After the final wash, BBMVs were isolated using a sucrose density gradient. The pellet was resuspended in 10 ml Buffer B [see section 2.1] and mixed with a 40 % (w/v) sucrose solution in Buffer B. This sample was overlaid onto an equal volume of a 65 % (w/v) sucrose solution in Buffer B. The sample was spun at 15000 g for 30 min at 4 °C. Purified BBMVs were isolated from the 40%:65% interphase of the sucrose gradient. BBMVs were characterised by flow cytometry on a BD FACSAria™ staining with a monoclonal antibody recognising CD66a, also known as 'CEACAM1' (carcinoembronic antigen-related cell adhesion molecule 1). CD66a is constitutively expressed on brush border membranes (Sundberg and Obrink 2002, Hansson, Blikstad and Obrink 1989).

2.10.2 STUDIES – BBMV IMMOBILISATION

Analyses were carried out on a Biacore 3000™ instrument using the L1 sensor chip. The L1 sensor chip has a surface matrix of carboxylated dextran to which lipophilic alkyl residues are covalently attached (Biacore 2003). This facilitates stable retention of lipid membranes on the surface at low flow rates. The running buffer for all Biacore experiments was detergent free HBS (HBS-DF), pH 7.4 containing 10 mM HEPES, 150 mM NaCl and 3 mM EDTA [see section 2.1]. Running buffer was freshly prepared, filtered (pore size of 0.22 μm) and degassed using a vacuum filtration apparatus (Millipore sintered glass filtration unit) before use. The L1 sensor chip was activated by injecting activation buffer (40 mM CHAPS) over the sensor chip for 3 min at a flowrate of 5 μl/min. A 1 in 5 or 1 in 10 dilution of purified BBMVs was injected over the activated chip surface for 3-5 min at a flowrate of 5 μl/min. In all experiments surface regeneration was mediated by a 45 sec pulse of 40 mM CHAPS.

2.10.3 BINDING OF LPS TO IMMOBLISED BBMV

Following the immobilisation of BBMV onto the L1 chip, LPS (10 μg/ml) was passed over the surface at a flowrate of 5 μl/min. LPS binding was reflected by the increase in sensorgram signal measured in arbitrary response units (RU).

2.10.4 ASSESSMENT OF NON SPECIFIC BINDING

In order to accurately qualify the LPS binding data it was important to determine the degree of interference from non-specific binding (NSB). A structured approach was taken to assess any NSB of LPS to the L1 chip. Initially, NSB to the unblocked L1 chip (without BBMVs) was determined. Subsequently, a 12 mg/ml solution of BSA and CM-dextran was incorporated as a surface blocking agent. Additionally, LPS was incubated with CM-dextran BSA before being passed over the surface. Finally, a liposome solution was used to pre-block the L1 chip following BBMV immobilisation and prior to injecting LPS. Liposome solution (30:30:10 % molar ratio DSPC:Cholesterol:DSPE-PEG) **see Appendix** was a kind gift from Paul Foran M.D. of the Applied Biochemistry Group, DCU.

2.11 Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance ($p<0.05$), post-hoc Student-Newmann-Keul test was used to determine which conditions were significantly different from each other. There was no significant difference between cells alone and DMSO (vehicle control) treated cells, therefore DMSO was used as the reference treatment. An unpaired t-test was performed to assess statistical differences between two treatment groups where indicated.

The confocal microscopy and luciferase experiments in chapter 5 represented by figure 5.9 – 5.10 and figures 5.11 – 5.14, respectively were carried out in conjunction with Dr. Sarah Doyle and Dr. Claire Mc Coy in the Biochemistry Group in TCD.

CHAPTER 3

THE EFFECTS OF PUFA ON MACROPHAGE FUNCTION

3.1 INTRODUCTION

There is widespread evidence that polyunsaturated fatty acids (PUFA) are beneficial within the immune system **[see section 1.11]** (Hwang 2000). PUFA are found naturally in the diet; n-3 PUFA, EPA and DHA, are commonly found in fish oils, and CLA, a derivative of n-6 PUFA, is present in meat and dairy products. Incorporation of PUFA within the diet is notably important in the prevention of autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus (SLE) and renal disease (Fernandes et al. 2008).

Increasingly, evidence is pointing towards the benefits of PUFA in the prevention and treatment of inflammatory disease. The n-3 fatty acids, EPA and DHA have been found to reduce cardiovascular mortality (Hamer and Steptoe 2006). While CLA is known to specifically reduce early atherosclerosis (Toomey et al. 2006). Contemporary treatments of inflammatory disease can have severe deleterious effects. As such, research has focused on the development of more natural therapeutic agents. While the exact mechanisms through which PUFA exert their effects remains elusive, our investigation and that of others have endeavoured to pinpoint their modes of action in order to elucidate their true therapeutic potential.

Macrophage, (MØ) execute many roles within innate immunity and their activation is vital to initial host defence and in directing subsequent immune responses [as discussed see section 1.3]. Concurrently, the role of MØ in various inflammatory disorders has been well documented including; inflammatory bowel disease (Zhang and Mosser 2008), schistosmiasis (Hesse et al. 2001) and atherosclerosis (Wilson, Barker and Erwig 2009). As a result, we hypothesized

that MØ may be one of the targets of PUFA. In this study we have addressed the effect of PUFA on MØ activation in a comparative assessment between EPA and DHA and CLA.

There are a number of parameters of MØ activation which PUFA may affect. Cellular activation signifies a change in the secretary profile and morphology of MØ. Activation signals the release of several pro-inflammatory cytokines including IL-1β, TNFα and IL-6 (Mosser 2003, Duffield 2003, Gordon 2003). On the other hand, production of anti-inflammatory or regulatory cytokines such as IL-10 act as potent deactivators of MØ pro-inflammatory cytokine synthesis (Clarke et al. 1998, Brandtzaeg et al. 1996). Differentiation of T cells into various subsets is partly determined by the cytokines MØ secrete. IL-10 inhibits T_H1 responses by reducing the capacity of MØ to produce IL-12, a potent inducer of a T_H1 phenotype. IL-23, IL-1 and IL-6 are involved in generating T_H17 cells and IL-10 secreted by regulatory MØ directs T_H2 phenotype (Mosser 2003). Therefore, the effect of PUFA on LPS-induced cytokine release from MØ was assessed to evaluate any beneficial changes that may occur.

In tandem, the activation of MØ leads to an upregulation of co-stimulatory molecules, pathogen recognition receptors (PRRs) and surface markers including CD40, CD80, CD86 and TLRs. Importantly, the expression of these markers is vital in providing bi-directional stimulatory signals between MØ and T cells (Janeway et al. 2008, Hoebe, Janssen and Beutler 2004). In fact, such signals make possible the interaction of MØ and T cells, supporting T cell activation and the regulation and activation of MØ. Therefore, changes to several surface makers in PUFA-treated MØ in response to LPS were assessed.

Activation also triggers the production of various chemokines acting as potent chemoattractants for immature DCs, neutrophils, NK cells and activated T cells. These include MIP-1α/CCLS, MIP-1β/CCL4 and MCP. Dysregulated expression of chemokines and their receptors has been implicated in the development of many human diseases including; allergy, psoriasis, atherosclerosis, and malaria (Murdoch and Finn 2000). Much effort has been placed on the development of therapeutic targets that modulate the activities of chemokines. As a result we have investigated the effect of PUFA on LPS-induced chemokine release from MØ to evaluate any advantageous changes. Furthermore, the response of cells to chemokines and subsequent recruitment to sites of inflammation remains crucial during an immune response. As such, we have also assessed the chemotaxis of PUFA-treated MØ in response to LPS.

Phagocytosis is a critical process in the clearance of infection. Throughout the body MØ are actively involved in this process. Phagocytosis of microbial products triggers the release of pro-inflammatory chemokines and cytokines and activates antigen processing and presentation by the enhancement of key surface markers MHCII, CD40 and CD80 (Kang et al. 2008). Subversively, it suppresses genes encoding molecules involved in bacterial recognition. TLR stimulation increases the phagocytic activity of MØ and promotes phagosome maturation, allowing sufficient capture and destruction of microbes (Blander and Medz 2004). As key phagocytes, the rate of phagocytosis by MØ in a PUFA environment in response to TLR4 ligand, LPS was also examined.

With an ever emergin role for MØ in the pathogenesis of various inflammatory disorders the effect of PUFA on these cells is the first focus of our investigations. By examining multiple parameters of macrophage function we aim to elucidate a collective view of the modulatory effects of PUFA on this cell type. The modulatory effect of PUFA on cytokine and chemokine production, surface marker expression, phagocytosis and migratory profile of macrophage in response to LPS are thoroughly examined.

3.2 RESULTS

3.2.1 THE CONCENTRATIONS OF PUFA USED HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY

The viability of cells following PUFA treatment was determined using CellTiter 96® AQueous One Solution (Promega) according to the manufacturer's instructions. The concentrations of fatty acids selected for use in future experiments did not have significant cytotoxic effects on J774 macrophage *in vitro*, i.e. 50 μM DMSO, 25 μM EPA, 25 μM DHA, 50 μM CLA and 50 μM LA **[Figure 3.1]**.

3.2.2 PUFA MODULATE *IN VITRO* LPS-INDUCED CYTOKINE PRODUCTION BY J774 MURINE MACROPHAGE

J774 macrophage were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM) or LA (50 μM). The resultant fatty acid-treated macrophage were harvested and plated at a concentration of 1 x 10^6 cell/ml before stimulating with 100 ng/ml LPS (*E.Coli* serotype R515). After 24 hours, supernatants were removed and assessed for levels of IL-12p40, IL-23p19, IL-1β, IL-10, TNF-α and IL-6 **[Figure 3.2]** using specific immunoassays.

The production of the pro-inflammatory cytokine IL-12p40 **[Figure 3.2]** was inhibited significantly after culture with EPA ($p<0.001$). The saturated fatty acid, LA and PUFA, CLA and DHA had no effect on LPS induced IL-12p40 production. In contrast, both CLA ($p<0.001$) and EPA ($p<0.001$) suppressed the production of pro-inflammatory cytokine, IL-23p19, following LPS stimulation

[Figure 3.2]. In addition, CLA was found to enhance IL-1β in response to LPS **[Figure 3.2]**.

The production of IL-10, an anti-inflammatory or regulatory cytokine, was substantially increased in PUFA-treated macrophage following LPS stimulation **[Figure 3.2]**. CLA ($p<0.001$) had the most profound effect on IL-10 production enhancing secretion significantly both before and after LPS stimulation. EPA ($P<0.01$) also enhanced IL-10 but to a lesser extent. The saturated fatty acid control, LA, had no effect on IL-10 production following LPS stimulation. In contrast to the other cytokines, which were modulated significantly, levels of TNF-α **[Figure 3.2]** remained relatively unchanged regardless of fatty acid treatment. PUFA, EPA ($p<0.001$) and DHA ($p<0.01$) significantly reduced IL-6 **[Figure 3.2]** production in LPS stimulated macrophage, while CLA showed no effect. LA, had no effect on either of the cytokines.

3.2.3 PUFA MODULATE *IN VITRO* LPS-INDUCED CHEMOKINE PRODUCTION BY J774 MURINE MACROPHAGE

J774 macrophage were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM) or LA (50 μM). The resultant fatty acid-treated macrophage were harvested and plated at a concentration of 1×10^6 cell/ml before stimulating with 100 ng/ml LPS (*E.Coli* serotype R515). After 24 hours, supernatants were removed and assessed for levels of MIP-1α, MIP-2 and MCP **[Figure 3.3]** using specific immunoassays.

The production of pro-inflammatory chemokine, MIP-1α was not significantly affected by PUFA-treated macrophage following LPS stimulation **[Figure3.3]**.

Similarly, the saturated fatty acid control, LA had no effect. However, CLA significantly suppressed MIP-1α in unstimulated cells ($p<0.01$) **[Figure3.3]**.

CLA ($p<0.05$) and DHA ($p<0.01$) enhanced MIP-2 following LPS stimulation **[Figure 3.3]**, while EPA and LA exhibited no effect. CLA also enhanced MIP-2 secretion prior to treatment with LPS **[Figure 3.3]**. Neither PUFA nor LA had any effect on the production of MCP-1 following LPS stimulation **[Figure 3.3]** however, similar to MIP-1α and MIP-2, CLA enhanced secretion of this chemokine ($p<0.01$) in resting macrophage **[Figure 3.3]**.

3.2.4 PUFA MODULATE CELL SURFACE MARKER EXPRESSION IN J774 MACROPHAGE

J774 macrophage were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM) or LA (50 μM). The resultant fatty acid-treated macrophage were harvested and plated at a concentration of 1×10^6 cell/ml before stimulating with 100 ng/ml LPS (*E.Coli* serotype R515). Control and LPS-stimulated cells were subsequently stained with fluorochrome-labelled monoclonal antibodies for numerous cell surface markers (i.e., CD40, CD80, CD86, CD204 (SR-A), TLR4-MD-2 and CD14) [See **Table 2.7**].

PUFA modulated the levels of key surface markers in unstimulated and stimulated J774 macrophage. Expression of CD40 and CD86 was suppressed by all PUFA **[Figure 3.4]** and **[Figure 3.6]**. However, the effect of CLA on CD86 was more marked than with other PUFA treatments. The levels of CD80 expression in response to LPS were relatively unchanged with PUFA **[Figure**

3.5]. Treatment of macrophage with PUFA resulted in enhanced expression of SR-A which was not seen in the saturated, LA control **[Figure 3.7]**. Furthermore, expression of TLR4-MD-2 was markedly suppressed in PUFA-treated groups in response to LPS **[Figure 3.8]**. Again, no effect was observed in LA-treated cells. Expression of CD14 was modulated differently among PUFA-treated macrophage both before and after stimulation with LPS **[Figure 3.9]**. CLA significantly suppressed CD14 in both resting and stimulated macrophage while the n-3 fatty acid, EPA enhanced CD14. Levels of CD14 remained unchanged in both DHA- and LA-treated macrophage **[Figure 3.9]**.

3.2.5 THE RATE OF PHAGOCYTOSIS IN J774 MACROPHAGE INCREASES OVER TIME

Macrophage were cultured until reaching confluency and plated at 2.5 x 10^5 cell/well and left to rest overnight. On the next day, 2.5 x 10^6 fluorescently labelled latex beads (Sigma®) were added to each well for 0, 2, 4, 6, 12, 20 and 24 h. At each time point wells were washed to remove excess beads and cells scraped and fixed in 200 µl 4 % (v/v) paraformaldehyde/PBS. The rate of phagocytosis over time in macrophage was assessed using a BD FACSCalibur™, indicated by [$_{M2}$], **[Figure 3.10A]**. To assess the rate of phagocytosis in stimulated cells, J774 macrophage were treated with 100 ng/ml LPS 24 h prior to the addition of fluorescently labelled latex beads. The rate of phagocytosis also increased over time in LPS stimulated macrophage but to a greater extent **[Figure 3.10B]**.

3.2.6 PUFA MODULATE THE RATE OF PHAGOCYTOSIS IN MACROPAHGE *IN VITRO*

J774 macrophage were cultured for 7 days with either DMSO (50 µM, vehicle control), EPA (25 µM), DHA (25 µM), CLA (50 µM) or LA (50 µM). To assess the rate of phagocytosis in both resting and stimulated cells, PUFA treated cells were left treated or untreated with 100ng/ml LPS 24 h prior to the addition of fluorescently labelled latex beads. The phagocytosis assay was performed as described [see section 3.2.5]. Among unstimulated groups phagocytosis in DHA treated cells was enhanced robustly from 4 h onwards comparative to control (DMS0). Phagocytosis was enhanced in EPA treated cells at 6 and 24 h **[Figure 3.11]**. Graphical representation of phagocytosis in unstimulated PUFA treated cells by mean fluorescent intensity values (MFI) of [$_{M2}$] is shown in **[Figure 3.13]**. [$_{M2}$] MFI values are indicative of the rate of phagocytosis as described in **[Figure 3.10]**.

Among PUFA treated cells stimulated with LPS, CLA-treated cells displayed a small decrease in phagocytosis at 6 and 12 h **[Figure 3.12]**. EPA and DHA treated cells demonstrated a suppressed ability to phagocytose from 2 h onwards. While in LA-treated cells phagocytosis was unchanged comparative to control **[Figure 3.12]**. Graphical representation of phagocytosis in stimulated PUFA-treated cells by mean fluorescent intensity values (MFI) of [$_{M2}$] is shown in **[Figure 3.13]**. [$_{M2}$] MFI values are indicative of the rate of phagocytosis as described in **[Figure 3.10]**.

3.2.7 CLA ENHANCES J774 CHEMOTAXIS BEFORE AND AFTER STIMULATION WITH LPS

GM-CSF and IL-2 are commonly employed in macrophage chemotaxis studies and have also been used here (Stagg et al. 2004, Perri, Annabi and Galipeau 2007). IL-2 is a ligand for CXCR2, which is robustly expressed on macrophage and implicated in the recruitment of macrophage to sites of inflammation (Boisvert et al. 1998). GM-CSF (granulocyte-macrophage colony stimulating factor) is a cytokine that induces activation of monocytes and macrophages (Hasskamp, Elias and Zapas 2006). The chemotaxis of J774 macrophage following LPS activation towards chemokines IL-2 and GM-CSF was significantly enhanced by culturing with CLA ($p<0.01$) and DHA ($p<0.01$) **[Figure 3.14]**. The presence of EPA or LA had no effect on macrophage chemotaxis following LPS stimulation. Only CLA ($p>0.05$) rendered macrophage more responsive to IL-2 and GM-CSF prior to LPS stimulation **[Figure 3.14].**

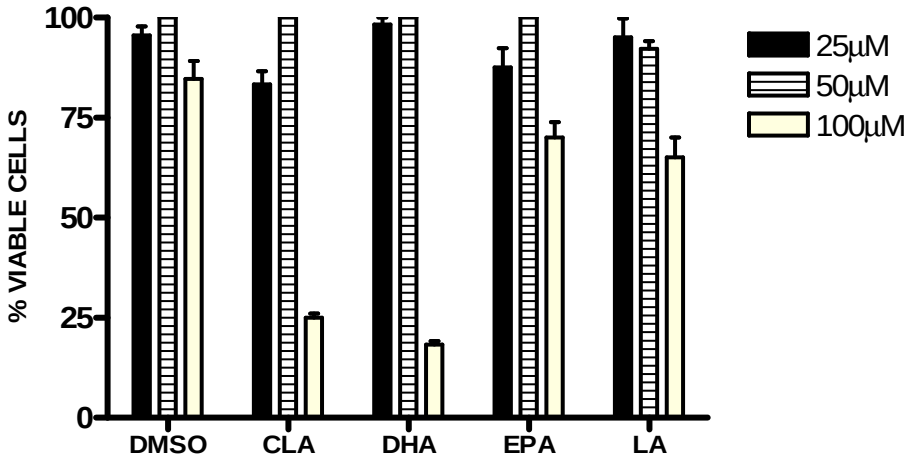


FIGURE 3.1: The concentrations of fatty acids used during the course of *in vitro* cell culture do not significantly affect the viability of J774 macrophage. A dose response assay was carried out in macrophage to assess the possible toxicity of increasing concentrations of fatty acids. Macrophage were cultured for 7 days with the specified concentrations (25, 50 and 100 µM) of either DMSO (vehicle control), EPA, DHA, CLA or LA. After 7 days cellular viability was assessed using an MTS assay (CellTiter 96® AQueous One Solution (Promega)). Results are expressed as a percentage of untreated cells.

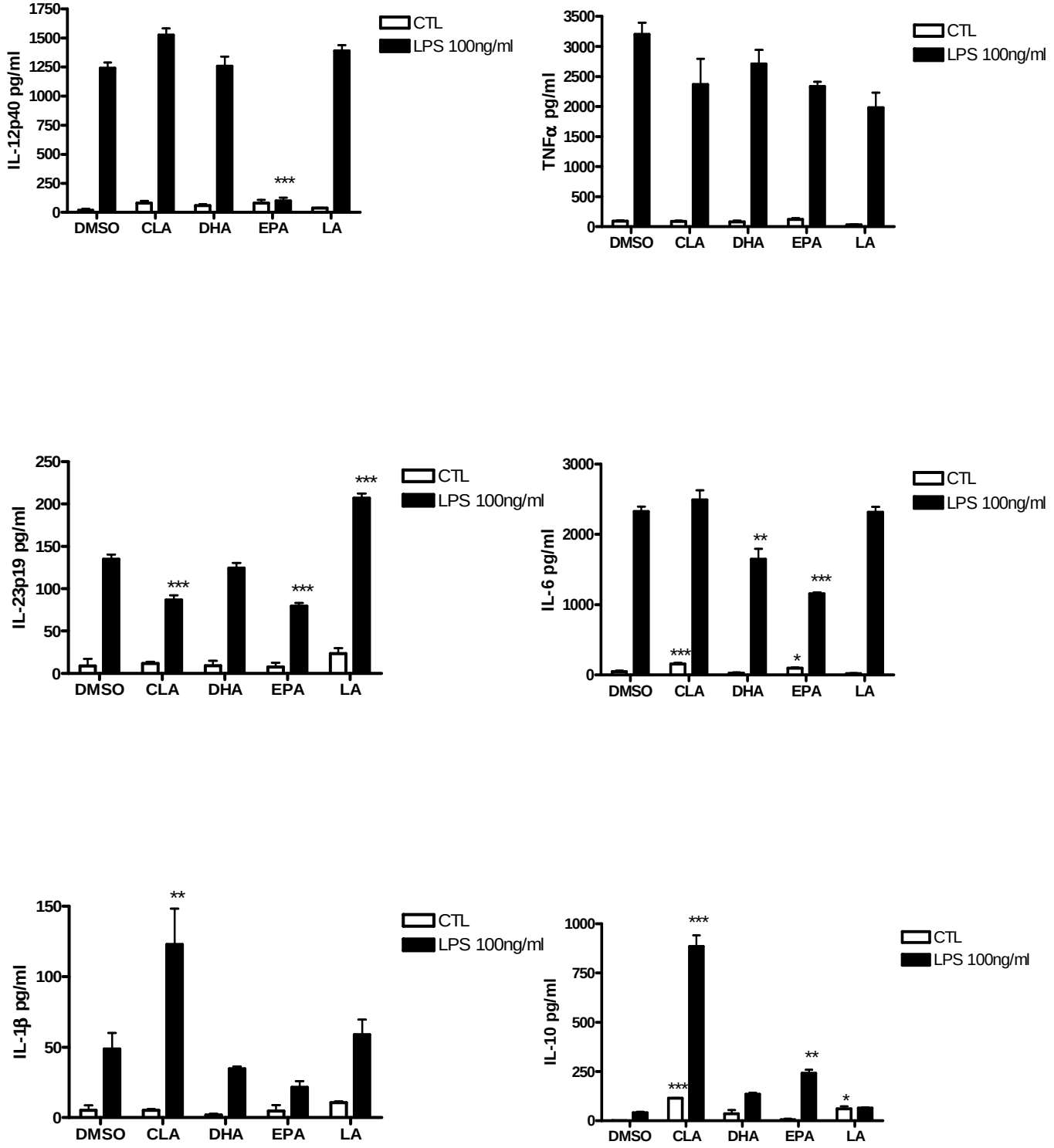


FIGURE 3.2: Pro-inflammatory (IL-12p40, IL-23p19, IL-1β, TNFα, IL-6) and anti-inflammatory (IL-10) cytokine production by fatty acid treated-J774 macrophage following LPS stimulation. Macrophage were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days. Subsequently, 1 x 10^6 cell/ml were stimulated *in vitro* with LPS (100ng/ml) and supernatants recovered after 24 hours. Levels of pro- and anti-inflammatory cytokines were measured using specific immunoassays.
Results are mean ± SEM of quadruplicate assays and represent three independent experiments.
***P<0.001, **P<0.01, *P<0.05 vs. DMSO vehicle control determined by one-way ANOVA test.

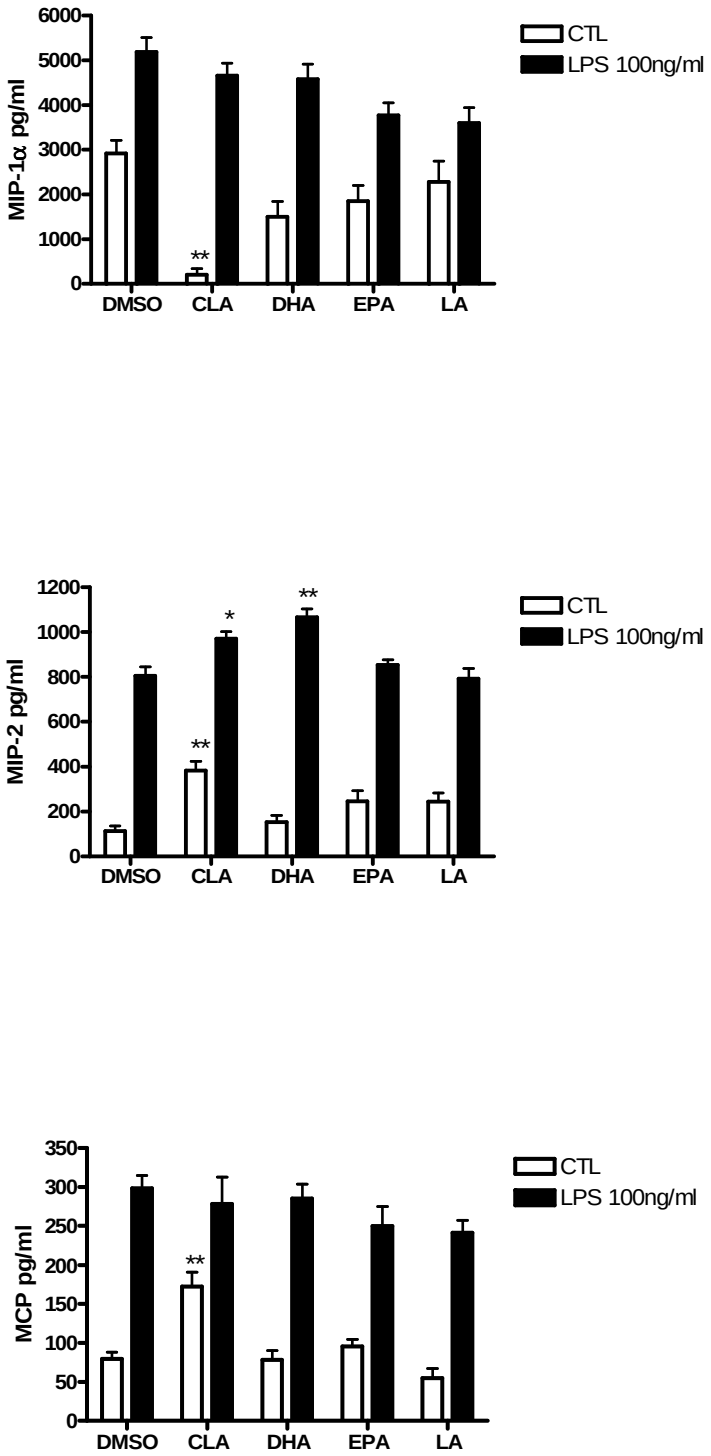


FIGURE 3.3: MCP production in PUFA-treated macrophage following LPS stimulation. Macrophage were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days. Subsequently, 1 x 10^6 cell/ml were stimulated *in vitro* with LPS (100 ng/ml) and supernatants recovered after 24 hours. Levels of MIP-1α **[A]** and MIP-2 **[B]** were measured using specific immunoassays.
Results are mean ± SEM of quadruplicate assays and represent three independent experiments.
***P<0.001, **P<0.01, *P<0.05 vs. DMSO vehicle control determined by one-way ANOVA test

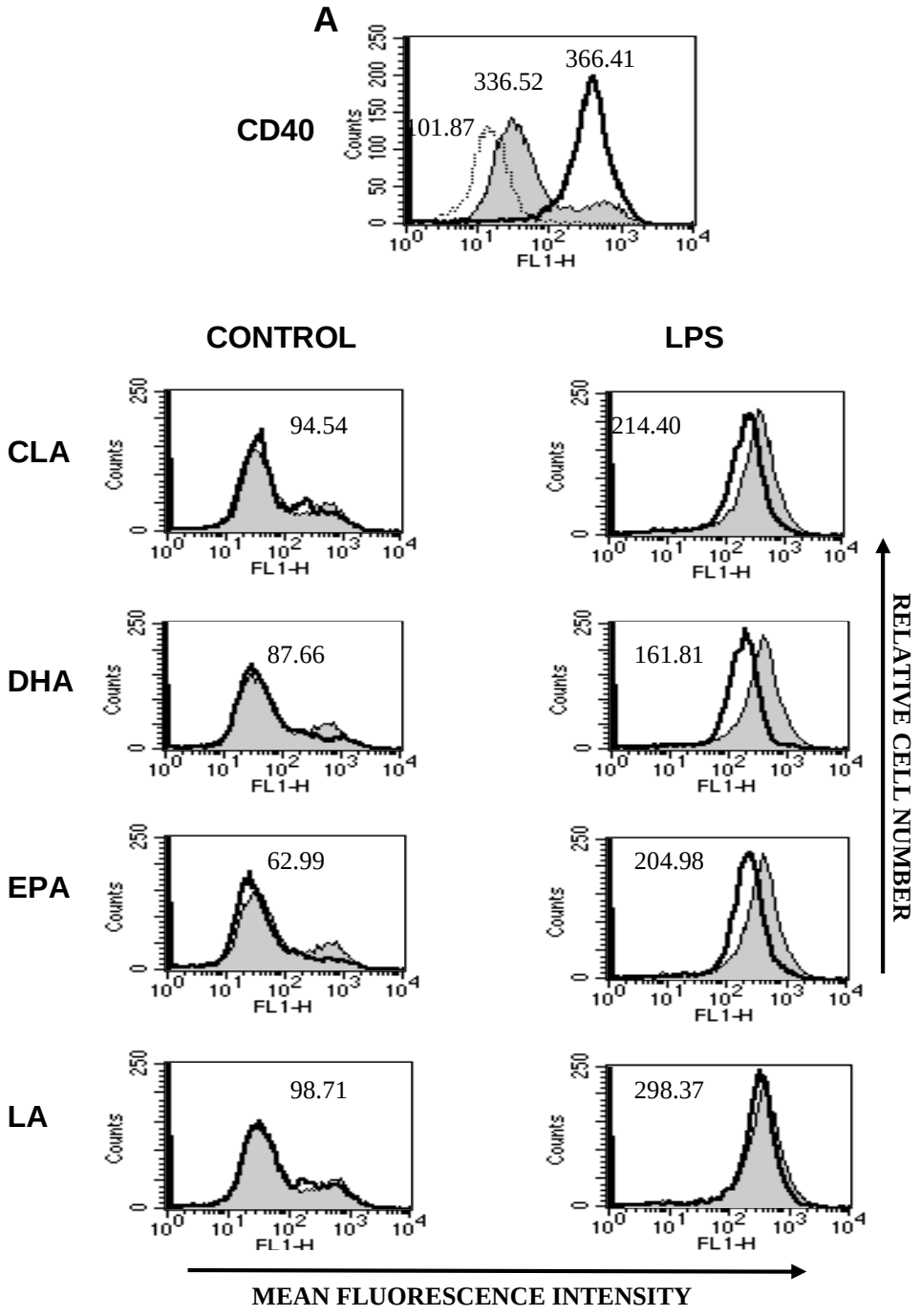


FIGURE 3.4: PUFA modulate the expression of CD40 on the surface of mature J774 macrophage. MØ were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml). Subsequently, cells were washed and stained with antibody specific for CD40 or with an isotype matched control. Results of flow cytometric analysis are shown for unstimulated DMSO-treated MØ (filled histogram), stimulated MØ (thin black line) and isotype control (dotted line) **[A]**. Results are also shown for DMSO-treated MØ (shaded histogram) vs. PUFA-treated MØ (black line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. Mean Flourescent Intensity (MFI) values are also presented for each histogram.
Profiles are shown for a single experiment and are representative of 3 experiments.

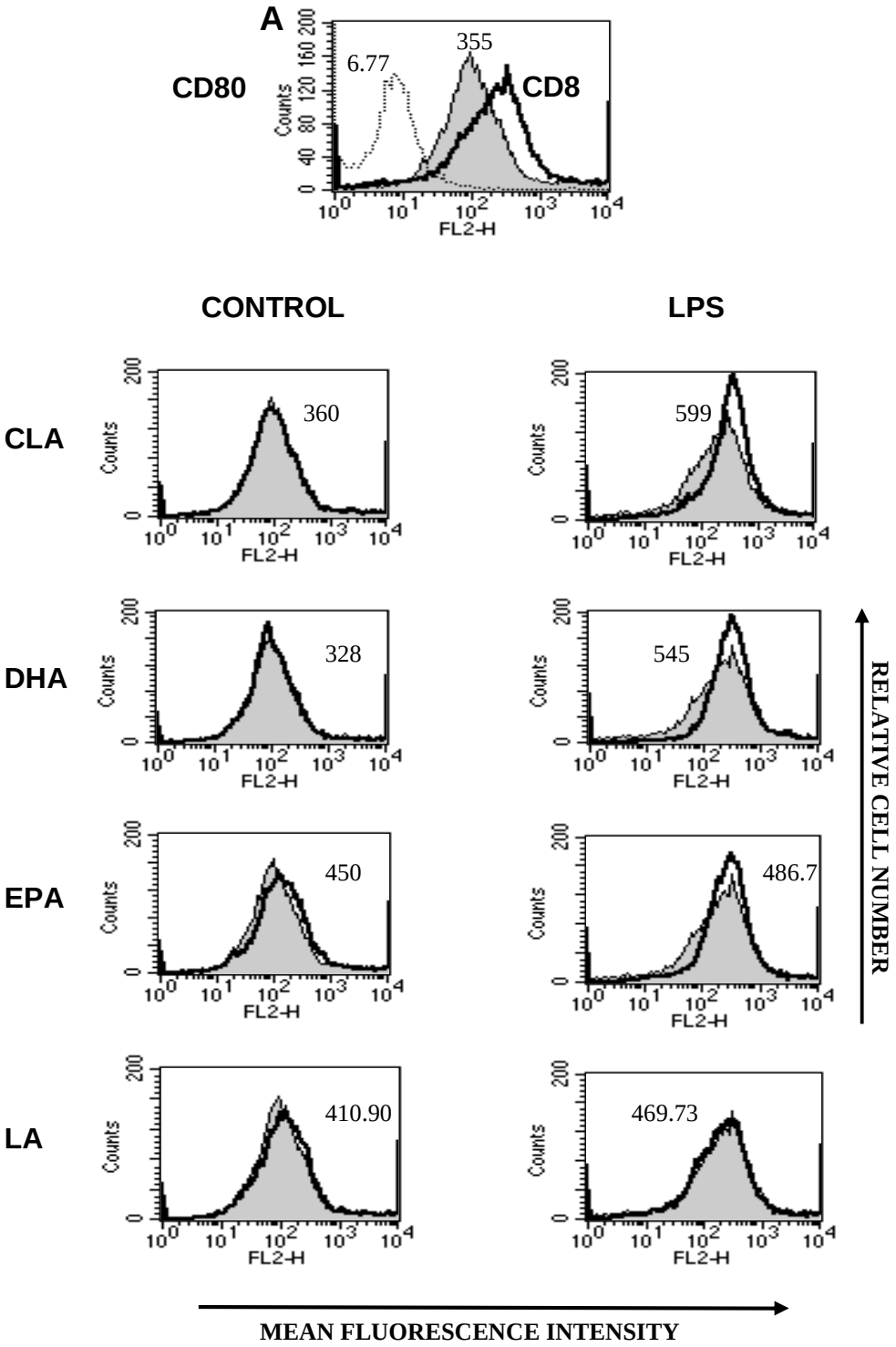


FIGURE 3.5: PUFA modulate the expression of CD80 on the surface of mature J774 macrophage. MØ were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml). Subsequently, cells were washed and stained with antibody specific for CD80 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown for unstimulated DMSO-treated MØ (filled histogram), stimulated MØ (thin black line) and isotype control (dotted line) **[A]**. Results are also shown for DMSO-treated MØ (shaded histogram) vs. PUFA-treated MØ (black line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for PUFA treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in **[A]**. Mean Flourescent Intensity (MFI) values are also presented for each histogram.
Profiles are shown for a single experiment and are representative of 3 experiments.

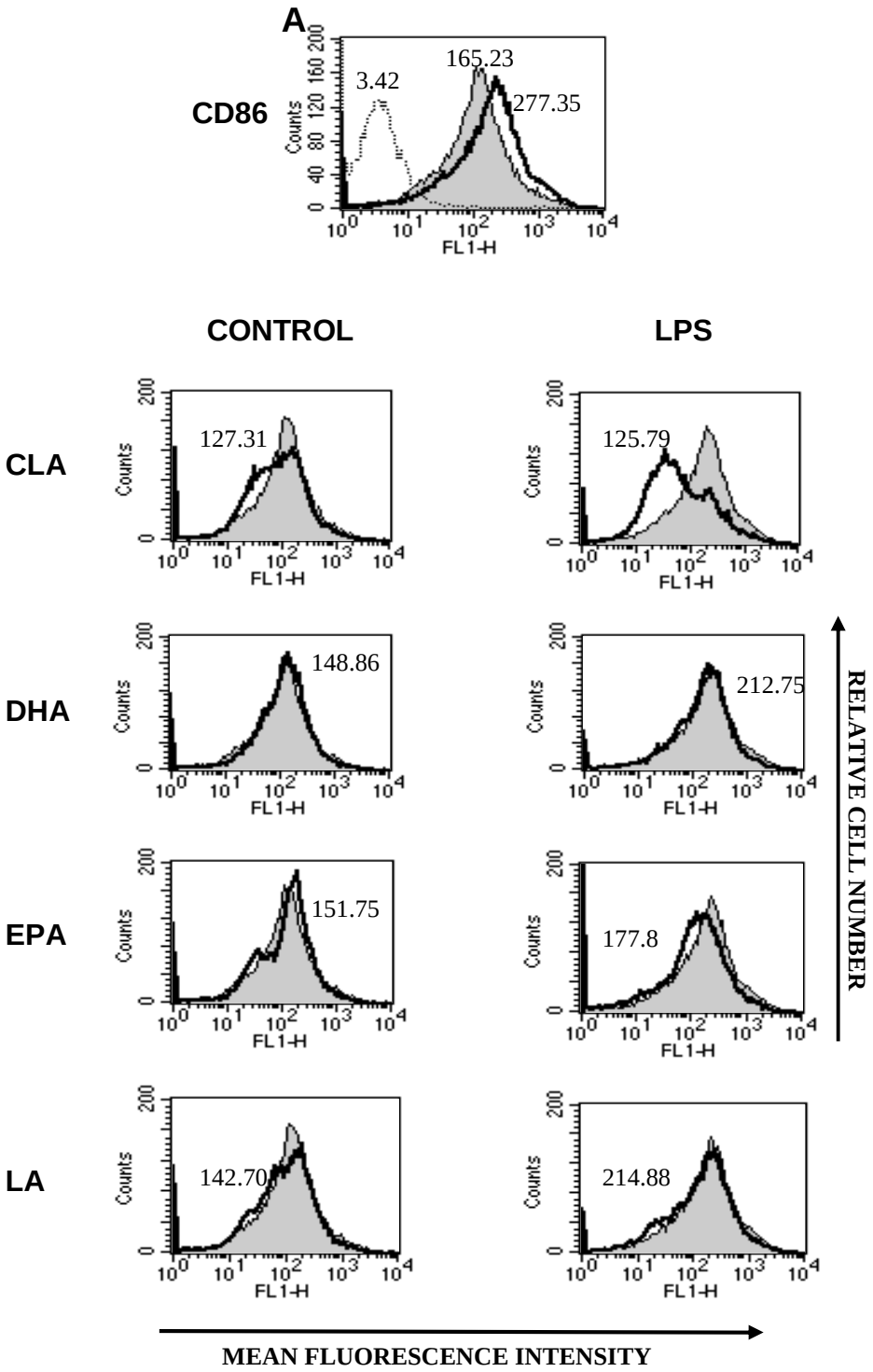


FIGURE 3.6: PUFA modulate the expression of CD86 on the surface of mature J774 macrophage. MØ were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml). Subsequently, cells were washed and stained with antibody specific for CD86 or with an isotype matched control. Results of flow cytometric analysis are shown for unstimulated DMSO-treated MØ (filled histogram), stimulated MØ (thin black line) and isotype control (dotted line) **[A]**. Results are also shown for DMSO-treated MØ (shaded histogram) vs. PUFA-treated MØ (black line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. Mean Flourescent Intensity (MFI) values are also presented for each histogram.
Profiles are shown for a single experiment and are representative of 3 experiments.

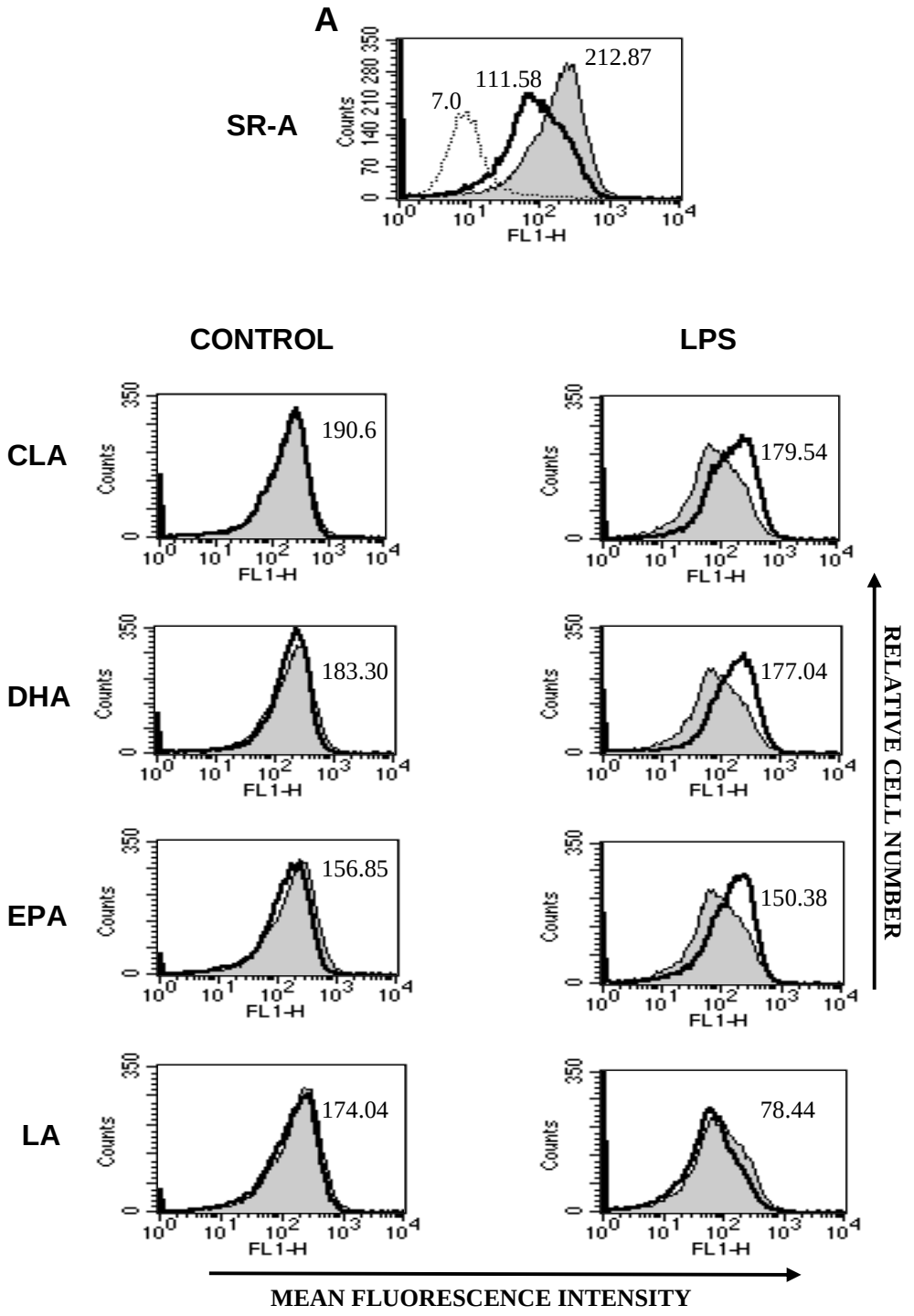


FIGURE 3.7: PUFA modulate the expression of CD204 (SR-A) on the surface of mature J774 macrophage. MØ were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml). Subsequently, cells were washed and stained with antibody specific for SR-A or with an isotype matched control. Results of flow cytometric analysis are shown for unstimulated DMSO-treated MØ (filled histogram), stimulated MØ (thin black line) and isotype control (dotted line) **[A]**. Results are also shown for DMSO-treated MØ (shaded histogram) vs. PUFA-treated MØ (black line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. Mean Flourescent Intensity (MFI) values are also presented for each histogram.
Profiles are shown for a single experiment and are representative of 3 experiments.

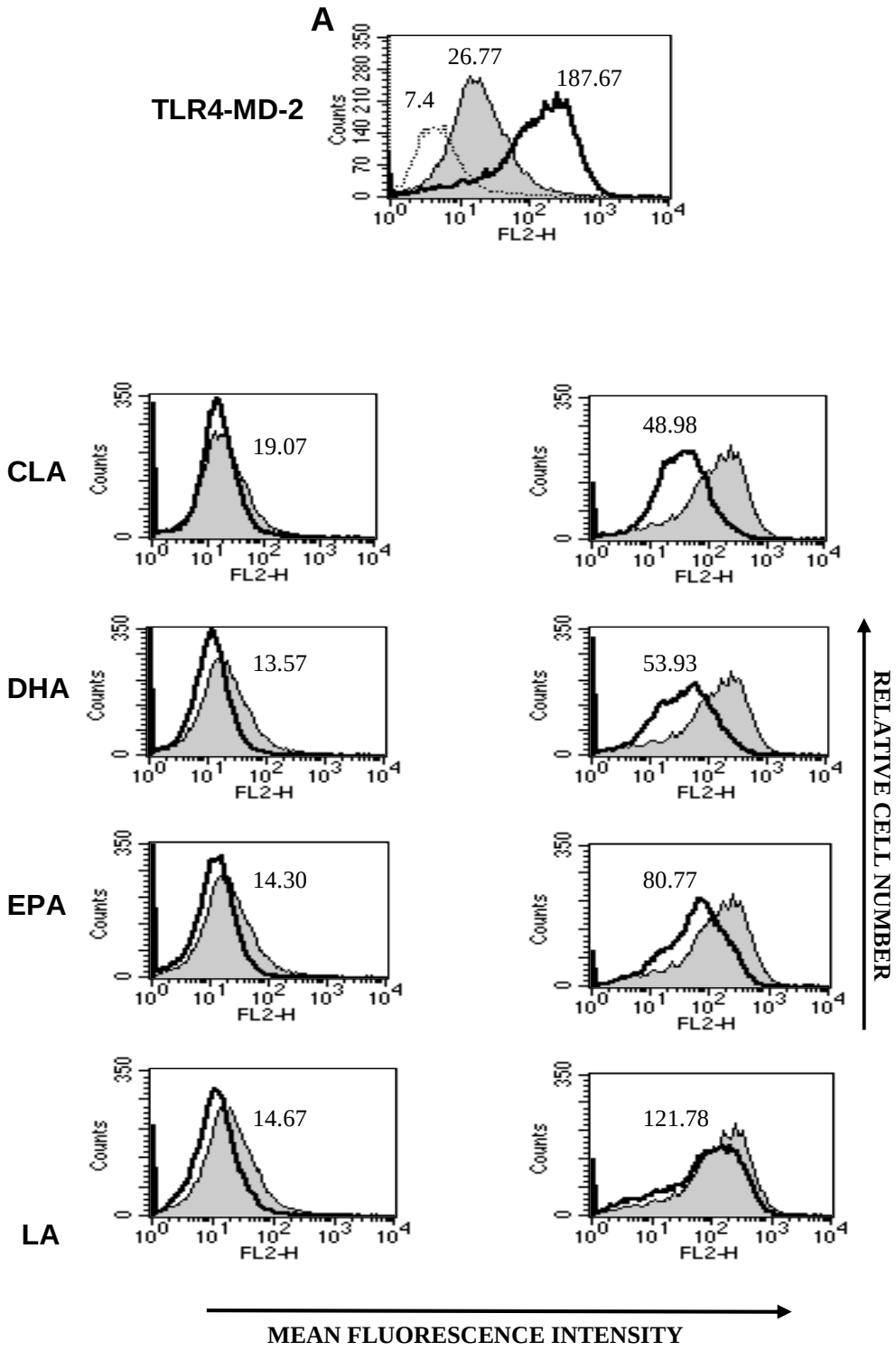


FIGURE 3.8: PUFA modulate the expression of cell surface marker, TLR4-MD-2 on the surface of mature J774 macrophage. MØ were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml). Subsequently, cells were washed and stained with antibody specific for TLR4-MD-2 or with an isotype matched control. Results of flow cytometric analysis are shown for unstimulated DMSO-treated MØ (filled histogram), stimulated MØ (thin black line) and isotype control (dotted line) **[A]**. Results are also shown for DMSO-treated MØ (shaded histogram) vs. PUFA-treated MØ (black line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. Mean Flourescent Intensity (MFI) values are also presented for each histogram.
Profiles are shown for a single experiment and are representative of 3 experiments.

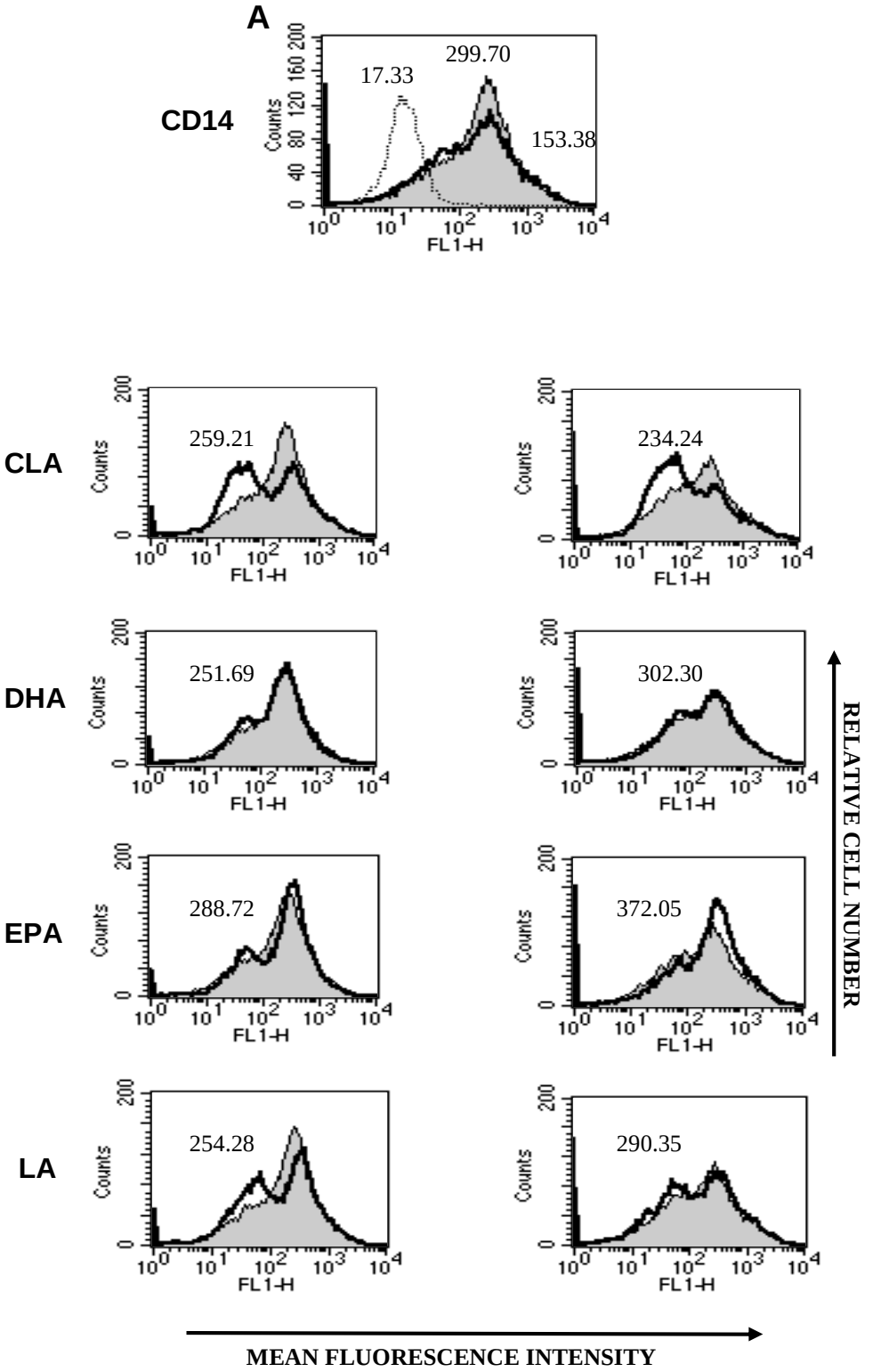


FIGURE 3.9: PUFA modulate the expression of CD14 on the surface of stimulated and unstimulated J774 macrophage. MØ were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml). Subsequently, cells were washed and stained with antibody specific for CD14 or with an isotype matched control. Results of flow cytometric analysis are shown for unstimulated DMSO-treated MØ (filled histogram), stimulated MØ (thin black line) and isotype control (dotted line) **[A]**. Results are also shown for DMSO-treated MØ (shaded histogram) vs. PUFA-treated MØ (black line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. Mean Flourescent Intensity (MFI) values are also presented for each histogram.
Profiles are shown for a single experiment and are representative of 3 experiments.

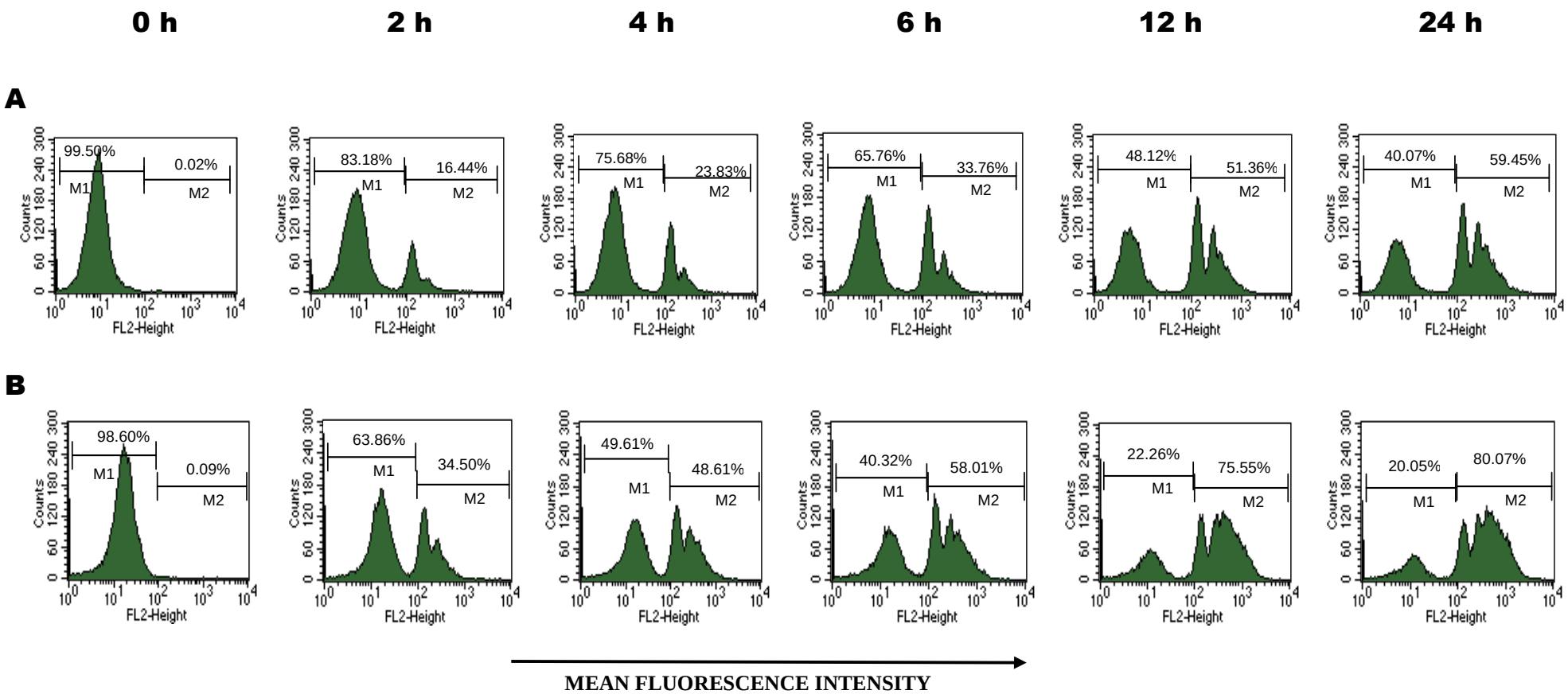


FIGURE 3.10: The rate of phagocytosis in unstimulated **[A]** and stimulated, LPS (100 ng/ml) **[B]** J774 macrophage is enhanced over time. Macrophage were plated at 5 x 10^5 cell/well in a 6-well plate and left to rest overnight. On the next day, 2.5 x 10^6 fluorescently labelled latex beads (1 x 10^6 beads/µl) (Sigma®) were added to each well at the indicated times. After the completed time course all wells were washed to remove excess beads and fixed in 200 µl 4 % (v/v) paraformaldehyde/PBS. The rate of phagocytosis over time in macrophage was assessed using a BD FACSCalibur™. Note: In **[B]**, macrophage were stimulated with LPS (100 ng/ml) for 24 h prior to the addition of latex beads.

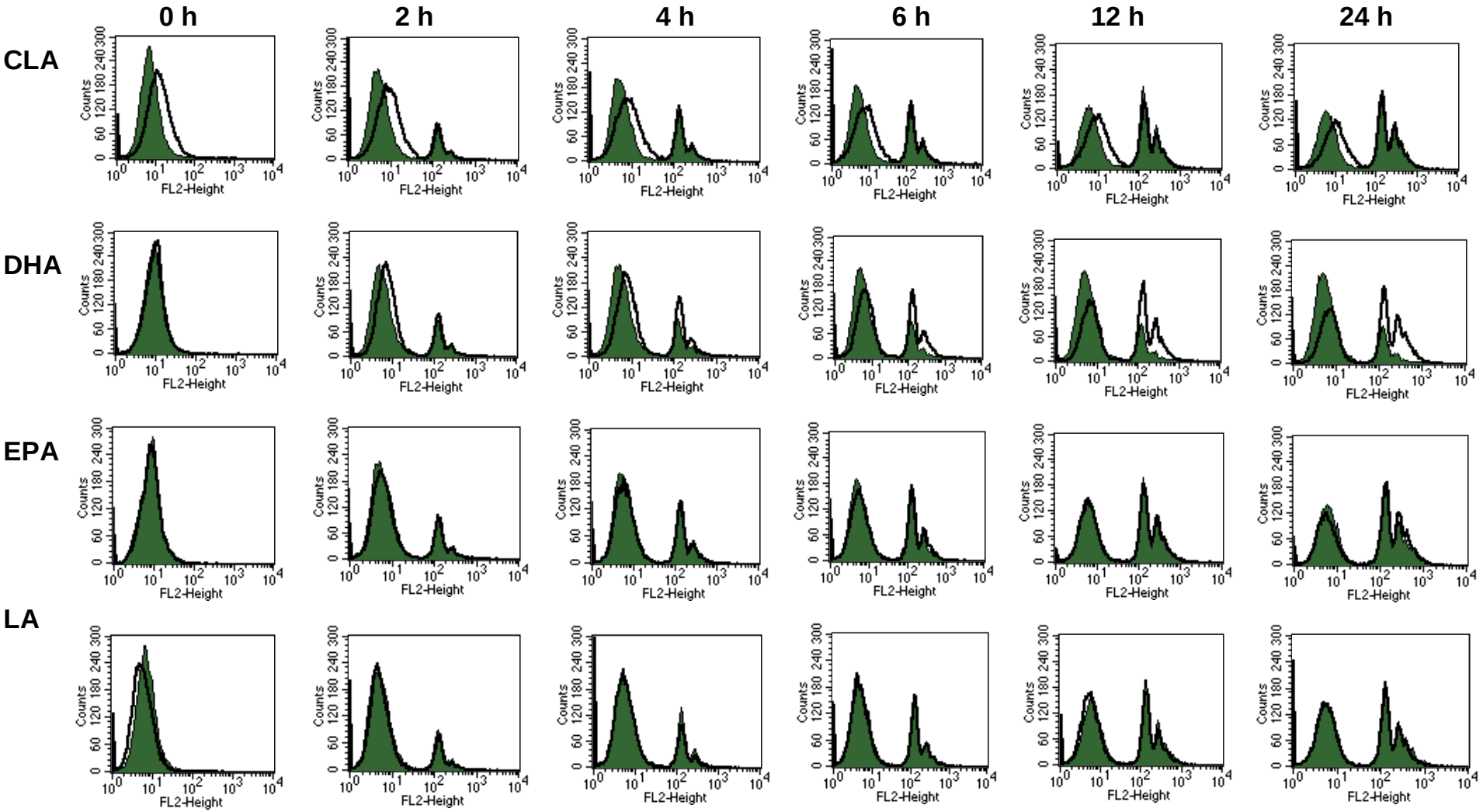


FIGURE 3.11: PUFA modulate the rate of phagocytosis in unstimulated J774 macrophage. Macrophage were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days. Cells were then plated at 5 x 10^5 cell/well in a 6-well plate and left to rest overnight. On the next day 2.5 x 10^6 fluorescently labelled latex beads (Sigma®) were added to each well at the indicated times. At 0 h all wells were washed to remove excess beads and fixed in 200 μl 4 % (v/v) paraformaldehyde/PBS. The rate of phagocytosis over time in macrophage was assessed using a BD FACSCalibur™.

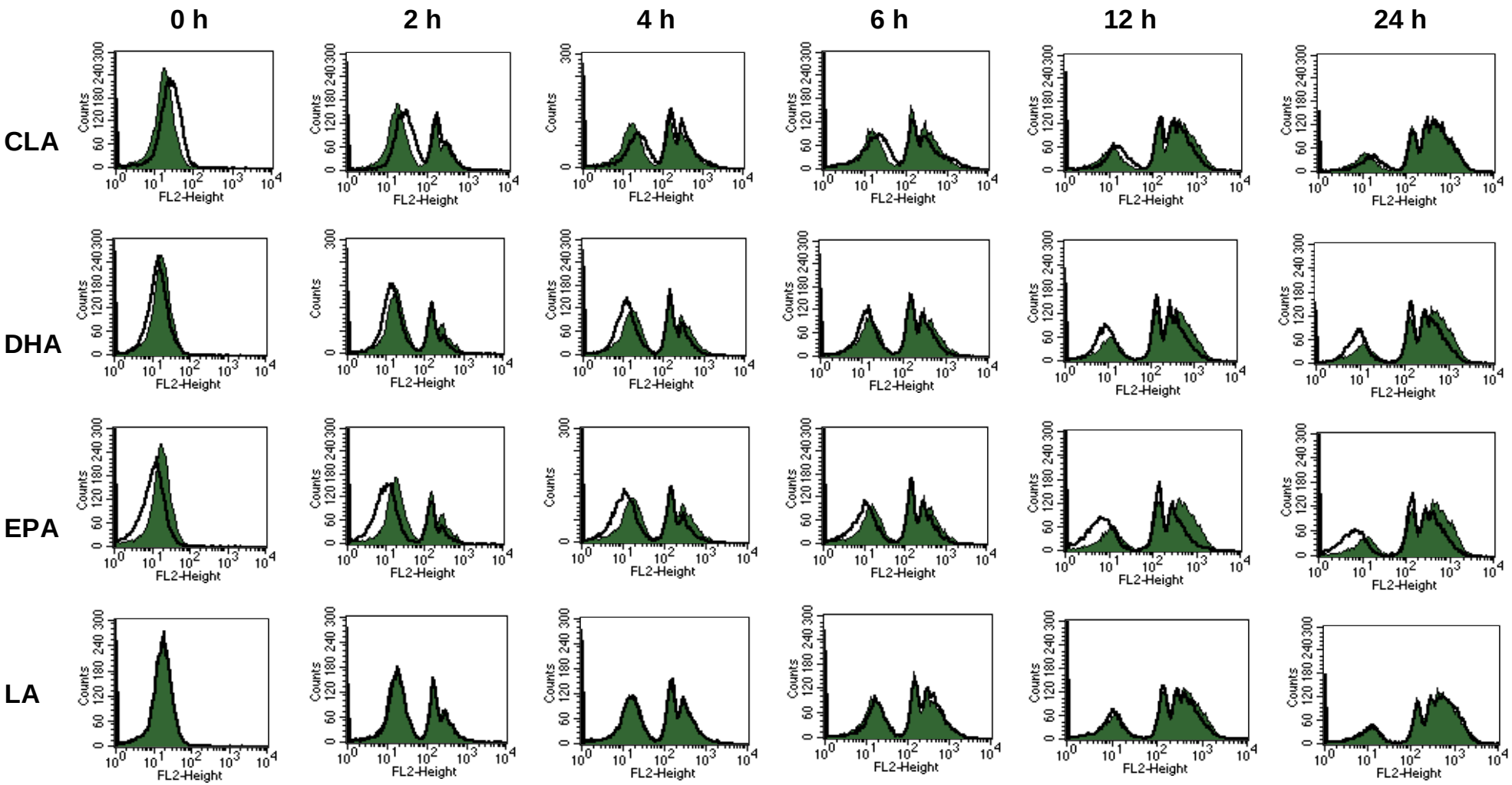


FIGURE 3.12: PUFA modulate the rate of phagocytosis in J774 macrophage stimulated with LPS. Macrophage were cultured in DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days. Cells were then plated at 5 x 10^5 cell/well in a 6-well plate and left to rest overnight. On the next day, cells were stimulated for 24 h with LPS, 100 ng/ml. On the third day 2.5 x 10^6 fluorescently labelled latex beads (Sigma®) were added to each well at the indicated times. After the completed time course all wells were washed to remove excess beads and fixed in 200 μl 4 % (v/v) paraformaldehyde/PBS. The rate of phagocytosis over time in macrophage was assessed using a BD FACSCalibur™.

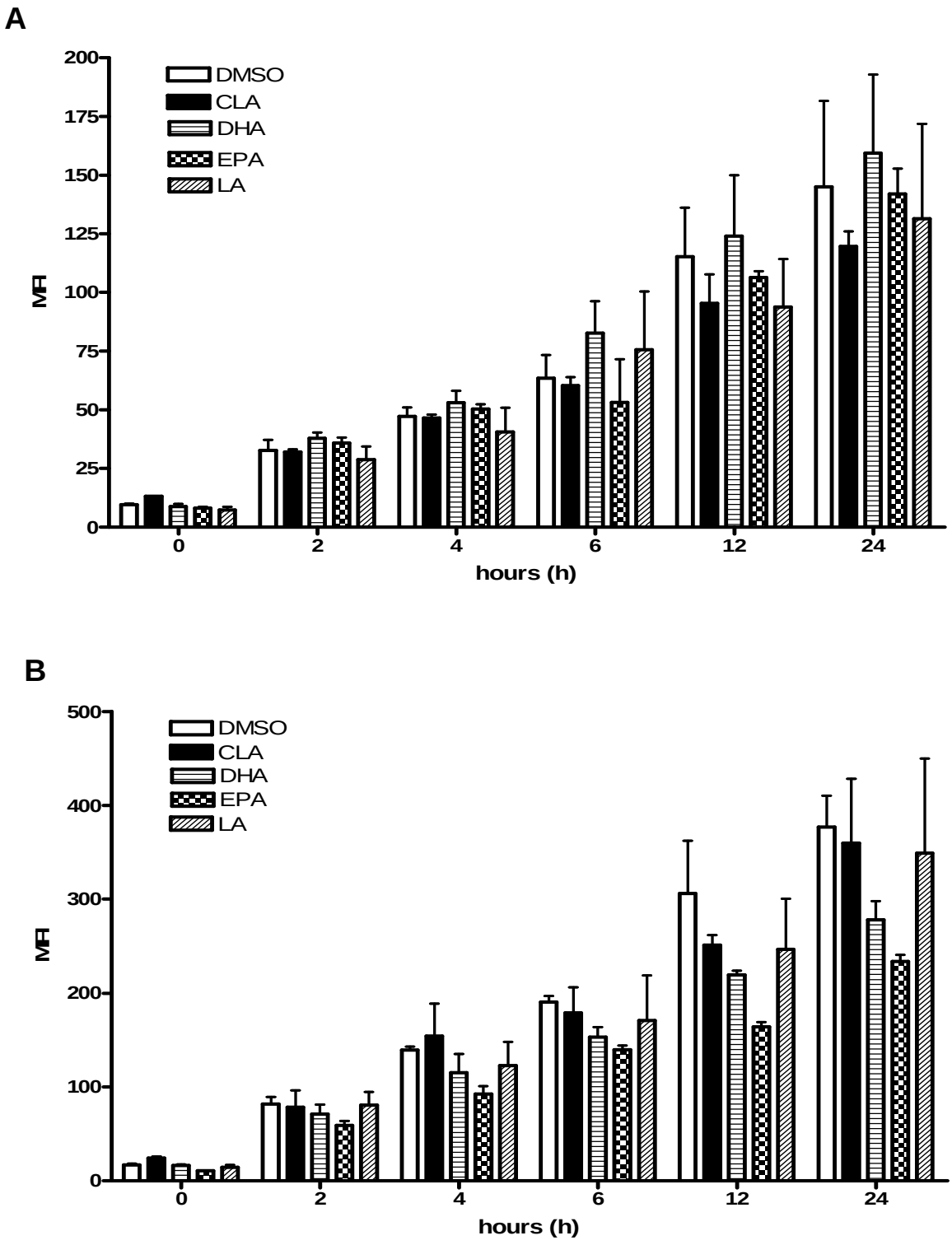


Figure 3.13: Graphical representation of mean fluorescent intensity values (MFI) for unstimulated **[A]** and stimulated **[B]** macrophage phagocytosis assays. MFI values of [$_{M2}$] have been plotted, indicative of the rate of phagocytosis as shown in figure 3.15.

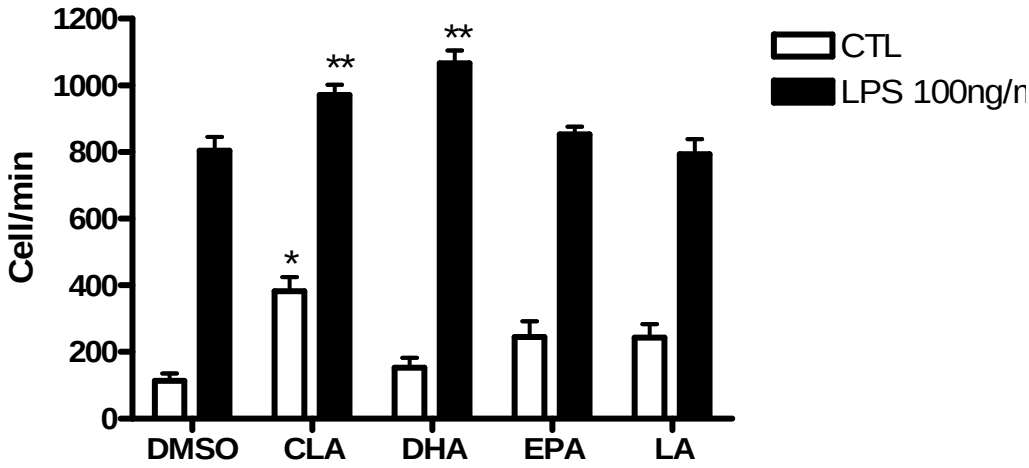


FIGURE 3.14: CLA and DHA enhance the chemotaxis of MØ iN response to LPS. MØ were cultured in the presence of fatty acids and stimulated with LPS (100 ng/ml). 3 x 10^5 cells were placed in the upper chamber of a Transwell® plate (8.0 μm). Media containing recombinant GMCSF (10 ng/ml) and IL-2 (10 BRMP/ml; were 1 BRMP = 40 pg/ml) was added to the lower chamber and plates were incubated for 5 hours at 37 ºC. To determine the number of migrated cells, media from the bottom well was collected and events (cells) counted for 1 min on a BD FACsCalibur™.
***P<0.001, **P<0.01, *P<0.05 vs. DMSO vehicle control determined by one-way ANOVA test.

3.3 DISCUSSION

The findings of this study demonstrate that polyunsaturated fatty acids (PUFA), CLA, EPA and DHA have the ability to modulate LPS-induced responses in macrophage. Exacerbated macrophage activation results in extensive tissue damage associated with autoimmune diseases such as rheumatoid arthritis (RA) (Szekanecz and Koch 2007) and with infection, such as schistosmiasis (Hesse et al. 2001). Furthermore, several studies strongly suggest that ongoing activation of macrophage in inflamed lamina propria mucosa is central to the immunopathology of inflammatory bowel disease (IBD) (Papadakis and Targan 2000, Papadakis and Targan 1999, Podolsky 2002). Therefore, modulating macrophage responses holds great therapeutic potential. Critically, the parameters assessed here have broad implications and importance in both macrophage activation in inflammatory disease and their involvement in the immune response to infection. Furthermore, this comparative study has revealed that exposure to n-3 versus n-6-derived PUFA results in distinct functional effects on macrophage.

Results here provide evidence that the n-3 PUFA, EPA robustly inhibits the biologically active subunit of potent pro-inflammatory cytokine IL-12, IL-12p40 in macrophage. IL-12 is a vital link between innate and adaptive immunity, favouring the differentiation of a T_H1 phenotype (Trinchieri 2003a). As such, IL-12 is largely implicated in autoimmune and T_H1 mediated diseases including; multiple sclerosis (MS), IBD and RA (La Cava and Sarvetnick 1999, Papadakis and Targan 2000). It is also one of many pro-atherogenic cytokines found at sites of atherosclerotic lesions (Kleemann, Zadelaar and Kooistra 2008). Targeting this cytokine may hold therapeutic potential. EPA-mediated inhibition of IL-12 has been reported in murine dendritic cells (DC) (Wang et al. 2007) but not macrophage. Administration

of EPA in combination with aspirin has been shown to afford protection against sulfonic acid-induced colitis in balb/c mice mediated by a reduction in nitric oxide synthase and IL-12 (Arita et al. 2005). DHA, CLA and saturated fatty acid, LA showed no effect on LPS induced IL-12 production. However, CLA has previously been shown to suppress IL-12 production in murine DC and a caco-2 cell model of human intestinal epithelium (Loscher et al. 2005a, Reynolds et al. 2008). IL-12 is a potent inducer of IFNγ (Trinchieri 2003a) which directs the induction of classically activated macrophage by inactivating feedback inhibitory mechanisms, such as those mediated by IL-10 (Mosser and Edwards 2008, Hu, Chakravarty and Ivashkiv 2008). The unchanged response of IL-12 production in CLA and DHA treated cells suggests that IL-12 is not a mechanism utilised by these PUFA to direct an anti-inflammatory phenotype in macrophage.

IL-23 is a cytokine consisting of the IL-12p40 sub-unit and a unique p19 sub-unit. It binds to the IL-23R on $CD4^+$ T cells promoting their development into T_H17 cells, characterised by the production of IL-17 and IL-6 (Langrish et al. 2005, Kikly et al. 2006). IL-23 has emerged as a key player in a number of chronic inflammatory diseases including IBD (Neurath 2007) and collagen-induced arthritis (Yago et al. 2007). Marked production of IL-12 and IL-23 by DC and macrophage are believed to play a critical pathogenic role in psoriasis, a chronic immune mediated skin disease (Yawalkar et al. 2009). The n-3 PUFA, EPA and n-6 derivative, CLA significantly suppressed LPS induced IL-23 production in macrophage. This may suggest a possible downstream role for PUFA-treated macrophage on T_H17 cell development or maintenance. Furthermore, it implies that PUFA may also help ameliorate the symptoms and/or onset of diseases such as psoriasis. Importantly, the saturated fatty acid, LA significantly enhanced IL-23

production post stimulation. This is indicative of the pro-inflammatory effects of saturated fats, which have been reported (Lee et al. 2001, Kennedy et al. 2009, Milanski et al. 2009).

EPA and DHA slightly suppressed TNFα and IL-1 in macrophage. In addition, statistical significance was observed in suppression of LPS-induced IL-6. A previous study has demonstrated that EPA can suppress TNFα production by murine macrophage (Babcock et al. 2002)(Babcock et al. 2002). Specifically, this suppression was reported to be a direct result of EPA altered NFκB activity (Lo et al. 1999). In addition, studies employing human macrophage models have reported that EPA and DHA inhibit TNFα, IL-6 and IL-1β production (Goua et al. 2008, Chu et al. 1999). Exacerbation of LPS recognition results in elevated levels of these cytokine in endotoxemia. In line with other studies, our data suggests that daily supplementation of n-3 PUFA may help ameliorate chronic inflammation caused during sepsis.

In contrast, CLA significantly enhanced LPS-induced IL-1β production, which was surprising given that many of its effects are anti-inflammatory and IL-1β is a pro-inflammatory cytokine. Other studies in our laboratory have also shown CLA to enhance IL-1β production by DC, however the implications of this are still not clear. Furthermore, although not statistically significant, CLA slightly enhanced IL-6 production pre- and post stimulation. A linear correlation between elevated IL-1 and IL-6 levels is associated with increased fibroblast proliferation and collagen synthesis associated with wound healing (McDaniel et al. 2008). The high levels of IL-1 reported here in CLA-treated cells, may suggest a pathway through which CLA promotes a wound healing phenotype in macrophage. However, IL-1 is

a potent pro-inflammatory cytokine and the elevated levels reported here raise concerns for the use of CLA in treating inflammatory disease.

It is widely accepted that inhibition of pro-inflammatory cytokines by PUFA may partly explain their anti-inflammatory effects. Some studies have already assessed the efficacy of n-3 PUFA and specifically the n-6 derivative, CLA (*cis-9,trans-11*) on cytokine production in murine macrohages (Babcock et al. 2002, Lo et al. 1999, Novak et al. 2003) and indeed human macrophage (Weldon et al. 2007, Chu et al. 1999, Zhao and Chen 2005). However, what is evident in the present study, in conjunction with most recent literature, is a clear dependency on cell type for PUFA exerted effects and a distinct difference between the effects of n-3 versus n-6 PUFA in murine macrophage.

In contrast to the above mentioned cytokines, IL-10 is considered an anti-inflammatory or regulatory cytokine. IL-10 critically inhibits macrophage and NK activity, T cell proliferation and chemotaxis (Conti et al. 2003, Mocellin et al. 2004). It suppresses the production of inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α from antigen presenting cells (APC) and also reduces the secretion of IL-2 and IFNγ from T_H1 cells (Papadakis and Targan 2000). Indeed, IL-10 deficiency in mice leads to overproduction of pro-inflammatory cytokines and the development of chronic inflammatory diseases (Conti et al. 2003). CLA significantly enhanced IL-10 production in macrophage pre- and post stimulation with LPS. EPA also enhanced LPS induced IL-10 production but to a lesser extent than CLA. This presents a possible mechanism used by CLA and EPA to elicit their anti-inflammatory effects. Principally, the increase in IL-10 suggests subsequent regulatory effects on T helper (T_H) cell differentiation as IL-10 inhibits

T_H1 development (Conti et al. 2003). The effect of PUFA on IL-10 in macrophages is previously unreported. Interestingly, it has been shown that CLA enhances LPS induced IL-10 production in murine DC (Loscher et al. 2005a). With DC being the most potent APC this emphasises the potential of PUFA in directing subsequent adaptive immune responses.

Further to the effects of PUFA on cytokine production, cytokines themselves have regulatory roles. IL-10 can alter surface marker expression. It has been shown to inhibit the full maturation of DC by down-regulating CD80, CD86 and ICAM-1 expression (Esther, Hermelijn and Martien 2005). Interaction of surface markers CD40, CD80 and CD86 with T cells plays a major role in the activation and expansion of all effector and regulatory T_H cell subsets (Janeway et al. 2008). Furthermore, the interaction of CD40 and CD40 ligand activates APC and enhances T cell activation (van Kooten and Banchereau 2000). Overexpression of these surface markers has been implicated in the pathology of tissues from patients with IBD, RA and MS (Maerten, Liu and Ceuppens 2003, Liu et al. 2001). As such, these markers represent important targets for treating inflammatory disorders.

Particularly, blocking T cell co-stimulatory signals is an attractive approach to treat autoimmune disease. CD86 enhances severity of arthritis by enhancing IL-17 production and increasing the accumulation of effector T cells in joints without affecting Th1/Th2 development (Odobasic et al. 2008). Monoclonal antibodies to CD80 and CD86 have been shown to differentially modulate the activation of T_H1 and T_H2 cells (Kuchroo et al. 1995). However, antibodies to CD80 and CD86 are known to exacerbate autoimmune disease, in part through enhanced production of TNF in macrophages (Khan et al. 2007). Indeed, this study demonstrates both CLA

and the n-3 PUFAs, EPA and DHA suppress CD40 and CD86 post stimulation. CLA had the most dramatic effect and suppressed CD86 significantly prior to stimulation It is possible this robust suppression of CD86 observed in CLA treated macrophage in both resting and LPS stimulated cells may be attributed to the significantly enhanced IL-10 production in CLA treated cells. Furthermore, CLA suppression of CD86 offers an alternative mode of suppressing T cell activation which may be beneficial in the treatment of autoimmune disease.

There is overwhelming evidence for the role of dysregulated and overexpressed chemokines in inflammatory disease. This study has assessed the modulatory effects of PUFA on potent chemokines MIP-1α, MIP-2 and MCP. MIP-1α has strong chemotactic effects on a vast number of leukocytes and high levels of this chemokine are common in inflammatory disorders and infection including; meningococcal disease (Moller et al. 2005) and rheumatoid arthritis (Kunkel et al. 1996). Similarly, MCP is implicated in rheumatoid arthritis and other inflammatory disorders and is known to heavily recruit 'lipid-laden' macrophage in atheroma (Kunkel et al. 1996, Ross 1993). MIP-2 plays a major role in the infiltration of neutrophils in response to viral infections. Particularly, MIP-2 enhances the infiltration of neutrophils in HSV-1 infected cornea were they indirectly cause tissue injury (Yan et al. 1998). However, questions remain as to the benefits of targeting chemokines in the resolution of disease. It is unclear as to whether 'anti' chemokine strategies have the ability to reverse established inflammation. Furthermore, recruitment and activation of leukocytes by chemokines enhance inherent protective responses to invading pathogens. Leukocyte infiltration is of paramount importance during innate host defence. The interplay between phagocytes and bacteria leads to the clearance of infection. Depleted levels of MIP-

2 are associated with impaired bacterial clearance of *Klebsiella pneumonia* (Strieter et al. 1996). Similarly, low levels of MCP-1 markedly reduce clearance of *Cryptococcus neoforms*. This highlights the benefits of these chemokines in antibacterial and antifungal host defence. Notably, impaired bacterial clearance and increased mortality in mouse models of bacterial pneumonia have been reported (Strieter et al. 1996).

Effects on chemokines were not dramatic however, CLA significantly enhanced expression of MIP-2 pre- and post stimulation, while DHA-enhanced MIP-2 expression was observed in activated cells. An unexpected role for MIP-2 in the control of mucosal lymphocyte migration has also been reported (Ohtsuka et al. 2001). Additionally, groups report MIP-2 expression is enhanced in the intestinal (Ohtsuka et al. 2001) and corneal (Yan et al. 1998) epithelium by IL-1. High levels of IL-1 in CLA-treated macrophage reported here may account for the increase in MIP-2 observed in CLA-treated macrophage. Similarly, MCP is regulated by IL-1 and TNF (Adams and Lloyd 1997). As the majority of chemokines are only expressed upon cellular stimulation, CLA enhanced IL-1 may partly account for the significantly elevated MCP expression pre-stimulation. In contrast to these findings, EPA and DHA have previously been reported to suppress MIP-2 and MCP in mycotoxin deoxynivalenol (DON) infected murine lymphoid tissues (Kinser et al. 2005). MCP suppression was also observed by EPA and DHA in LDL receptor null mice and a diabetic mouse model (Wang et al. 2009, Garman et al. 2009) and was beneficial to the resolution of both disease states. However, as chemokines assist rapid inflammatory response to pathogens and bacterial clearance, the administration of PUFA may have to be tailored to the particular

inflammatory state/disease. As such PUFA exerted effects on macrophage function warrants further investigation.

Phagocytosis is a fundamental role of all macrophage populations, important in the ingestion and clearing of pathogens. Membrane fluidity strongly influences this process. As such several studies have investigated the role of PUFA membrane incorporation on phagocytosis activity. Here we report a suppressed ability of LPS-stimulated macrophage to phagocytose as a result of EPA and DHA treatment. However, enhanced phagocytic activity among CLA- treated cells was observed at several time points following LPS stimulation. Importantly, LA suppressed phagocytosis in unstimulated cells and no change was observed in response to LPS comparative to control. To the best of our knowledge this is the first report of modulated phagocytic activity in CLA-treated cells. Conflicting evidence on the effects of n-3 PUFA on phagocytosis have been observed. Feeding studies incorporating n-3 PUFA into mouse diets report elevated PUFA content in lipid membrane but no significant effect on monocyte or neutrophil phagocytic activity (Kew et al. 2003). Similarly, EPA and DHA were described as having no effect on the ability of alveolar macrophage to phagocytose (D'Ambola et al. 1991). Alternatively, very early studies in the area demonstrated that altering the fatty acid composition of murine macrophages *in vitro* with PUFA made macrophage more phagocytically active (Lokesh and Wrann 1984, Calder et al. 1990, Mahoney et al. 1977). The discrepencies described here may be due to the varied doses used among these studies. Phagocytosis remains an important event in bacterial clearance and maintaining homeostasis. Results reported here suggest that the n-6 PUFA derivative, CLA enhanced phagocytosis and therefore may be beneficial in innate protection from infection.

Further to this we report enhanced chemotaxis of CLA treated macrophage both pre- and post stimulation with LPS, demonstrating that the ability to move to the site of infection aswell as phagocytosis, is enhanced. IL-6 strongly enhances the ability of cells to migrate (Clahsen and Schaper 2008). The small enhancement in IL-6 in CLA-treated cells reported here may be a mechanism utilised by CLA to increase chemotaxis and support the recruitment of cells to site of inflammation. Little augmentative effect was displayed in EPA- and LA-treated cells. However, DHA significantly enhanced chemotaxis in response to LPS.

Several critical parameters of macrophage activation and functional status have been assessed in this study. However, it was also important to define any effects on the pathogen recognition ability of these cells. As LPS, which activates TLR4, was used throughout this study the effect of PUFA on this receptor as well as associated molecule CD14 and SR-A were investigated. Both CLA and n-3 PUFA, EPA and DHA robustly suppressed TLR4-MD-2 surface expression following stimulation with LPS, while the saturated fatty acid, LA exhibited no effect. To the best of our knowledge this is an unreported finding for both CLA and n-3 PUFA. Studies to date have focused on the assessment of PUFA-modulation on TLR4 downstream signalling events, particularly NFκB and COX-2 gene expression (Lee et al. 2001, Lee et al. 2003, Weatherill et al. 2005). Of further significant interest was the observed upregulation of SR-A by all PUFA, with no observed change by LA-treated cells. Particularly as *in vitro* studies have demonstrated the ability of SR-A to bind LPS suggesting a possible role for SR-A in LPS clearance (Hampton et al. 1991). Further to our findings regarding TLR4, its co-receptor CD14 was enhanced by EPA following stimulation with LPS. In contrast, CLA significantly suppressed

CD14 both in resting and LPS stimulated cells. This finding in relation to altered CD14 expression was pivotal in the study. It confirmed distinct immunomodulatory effects of n-6 derived CLA isomer (*cis-9, trans-11*) and n-3 PUFA upstream of the parameters previously assessed. Therefore, the regulation of TLR4, CD14 and SR-A by PUFA presented important mechanisms through which fatty acids may exert their anti-flammatory effects. This is investigated thoroughly in chapters 4 and 5.

The focus of this study was to assess the immunomodulatory properties of PUFA regarding macrophage activation. Our findings highlight for the first time distinct effects of n-3 PUFA, EPA and DHA and n-6 derivative CLA in this regard. A lack of experimental and clinical studies in the area complicates any implication of their beneficial use in prevention and treatment of disease. As such, it remains for further studies to be carried out which may make feasible the administration of PUFA and the tailoring of such for the treatment of inflammatory disease and infection.

CHAPTER 4

THE EFFECTS OF PUFA ON CELL SURFACE MARKERS AND BINDING

4.1 INTRODUCTION

Intracellular signalling is the ultimate mechanism for the induction of cellular activation. However, this is preceded by the activation of receptors at the cell membrane following their ligation by various stimuli. By far, the most widely recognised and characterised stimuli of the innate immune response is lipopolysaccharide (LPS) (Janeway et al. 2008). LPS has been used extensively throughout our study and is a potent activator of the pathogen recognition receptor (PRR), Toll-like receptor 4 (TLR4) (Poltorak et al. 1998). Thus far we report significant downstream effects of PUFA on the activation and inflammatory status of macrophage, MØ. In the field of PUFA research to date, reports have focused on the downstream signalling effects of PUFA on TLR signalling, specifically in relation to NFκB and COX-2 expression (Lee et al. 2001, Lee et al. 2001, Lee and Hwang 2006). Significantly, due to the very nature of PUFA and their ability to become incorporated within the plasma membrane (Li et al. 2006, Li et al. 2005), we postulate that markers at the cell membrane present important targets for PUFA-mediated immune effects upstream of signalling. Here, we examine over time the effect of PUFA on surface marker expression to further delineate the different effects of EPA, DHA and CLA and determine whether these are early events.

This is important given that prolonged activation of TLR4 is broadly accepted to contribute to the deleterious effects associated with chronic infection, sepsis and inflammatory disease (Karima et al. 1999, Salomao et al. 2008, Leon et al. 2008). Furthermore, CD14, an associated molecule of the TLR4 receptor complex plays an important role in the maximal responsiveness of TLR4 to LPS (Kirkland et al. 1993, Landmann, Müller and Zimmerli 2000, Miyake 2006b). As such, PUFA-

mediated effects on CD14 were investigated further by examining the levels of this protein in membrane and cytosolic fractions from PUFA-treated macrophage.

The activation of macrophage and other leukocytes by LPS occurs after LPS molecules bind to CD14 followed by loading of LPS to TLR4-MD-2. This interaction of CD14 and LPS leads to optimal LPS induced signalling (Kim et al. 2005). Indeed, CD14 mutants display impaired responses to LPS in HEK 293 cells (Juan et al. 1995, Stelter et al. 1999, Ohnishi, Muroi and Tanamoto 2001, Muroi, Ohnishi and Tanamoto 2002). Considering the modulatory effects of PUFA on CD14 and TLR4-MD-2 expression reported in the previous chapter, we deemed it important to examine the effects of PUFA on the ability of LPS to bind to TLR4. This chapter describes the optimisation of a protocol to assess the binding of LPS to the TLR4 receptor complex by flow cytometry.

By the very nature of their structure PUFA become incorporated into the plasma membrane. As such the membrane provides an important point from which they may exert their modulatory effects. As such the possibility of a PUFA modulated interaction between LPS and the TLR4 receptor complex at the membrane is investigated by examining surface marker expression over time, LPS-binding and

4.2 RESULTS

4.2.1 PUFA MODULATE EXPRESSION OF KEY CELL SURFACE MARKERS AT EARLY TIMEPOINTS

J774 macrophage were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), or LA (50 μM). The resultant fatty acid-treated macrophage were harvested and plated at a concentration of 1 x 10^6 cell/ml before stimulating with 100 ng/ml LPS (*E.Coli* serotype R515) for 0, 2, 4, 6 and 12 h. Control and LPS-stimulated cells were subsequently stained with fluorochrome-labelled monoclonal antibodies for numerous cell surface markers (i.e., CD40, CD80, CD86, CD204 (SR-A), TLR4-MD-2 and CD14) [See **Table 2.7**].

PUFA modulated the levels of key cell surface markers in unstimulated and stimulated J774 macrophage over time. **[Figure 4.1]** and **[Figure 4.2]** shows that CD40 and CD80 expression was upregulated in cells over time from 0 h to 12 h. There was no suppression of CD40 in any of the PUFA-treated groups or the saturated fatty acid control, LA, at any of the early time points examined **[Figure 4.1]**. However, there was a marked enhancement of CD40 in DHA treated cells at 4 h. This indicates that the observed suppression of CD40 in PUFA-treated cells reported in Chapter 3 is a late event. In addition, neither the PUFA or LA exerted any effect on CD80 expression between 0 and 12 h stimulation with LPS **[Figure 4.2]**.

Conversely, expression of CD86 was significantly inhibited in CLA-treated cells prior to stimulation with LPS and indeed over the complete time course **[Figure**

4.3]. From the previous chapter, we can see that this effect is maintained at 24 h. CD86 expression was unaltered in both DHA- and EPA-treated cells with the exception of a slight suppression at 2 h in the EPA group. Our previous study indicated a suppression of CD86 in EPA treated cells stimulated with LPS for 24 h. Furthermore, LA exerted no effect on CD86 expression over the course of LPS stimulation.

The effects of PUFA on SR-A expression were not observed over this early time course following LPS stimulation **[Figure 4.4]**, indicating that the increase in SR-A in PUFA-treated cells reported in Chapter 3 is a late event. The previous chapter also demonstrated a clear decrease in expression of TLR4 in PUFA-treated macrophage. In this study we show that these changes are not obvious at the early time points examined, however by 12 h, DHA- and EPA-treated cells begin to show a small decrease in the expression of this receptor **[Figure 4.5]**. This indicates that the effects of PUFA on TLR4 expression is a late event.

Given our previous data that CLA suppressed CD14 expression in both resting and LPS-activated macrophage, we were particularly interested to see if this change was indeed an early event. Our data revealed that CLA suppressed CD14 at early time points (0 h, 2 h, 12 h) **[Figure 4.6]**, confirming that these changes are an early event. In contrast, enhancement of CD14 was observed in EPA and DHA treated macrophage from 2 h onwards comparative to control, while treatment with saturated fatty acid, LA enhanced CD14 expression from 4 h onwards.

4.2.2 PUFA DIFFERENTIALLY MODULATE EXPRESSION OF CD14 AT THE CELL MEMBRANE

J774 macrophage were cultured in the absence of fatty acids, as described [section 2.3.1] and plated at a concentration of 1 x 10^6 cell/ml or 2 x 10^5 cell/ml in a 6-well plate (3 ml/well) and left to rest overnight. Cells were stimulated with LPS (100 ng/ml) for 0, 2, 4 and 6 h prior to scraping and fractionating membranes from cytosolic fractions as described [section 2.6.1]. Levels of CD14 were assessed by western blot. Upregulation of CD14 could not be observed following stimulation with LPS in the membrane fraction of cells plated at 1 x 10^6 cell/ml **[See APPENDIX A]**. However, change in the levels of both membrane and cytosolic CD14 was clearly evident in cells plated at 2 x 10^5 cell/ml **[See APPENDIX A]**. As such it was decided that 2 x 10^5 cell/ml be used in further experiments investigating the effect of PUFA-treatment on cytosolic and membrane bound levels of CD14.

Further to this J774 macrophage were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), or LA (50 μM). The resultant fatty acid-treated macrophage were harvested and plated at a concentration of 2 x 10^5 cell/ml (3 ml/well) and stimulated with LPS (100 ng/ml) for 0, 2, 4 and 6 h. Membrane and cytosolic fractions were generated as described [section 2.6.1] and levels of CD14 assessed by western blot and densitometric analysis.

LPS stimulation resulted in enhanced expression of CD14 in the membrane fractions of DMSO treated cells between 0 and 6 h **[Figure 4.8]**, **[Figure 4.9]**, **[Figure 4.10]** and **[Figure 4.11]**. This was accompanied by a drop in cytosolic

levels of CD14 at 0, 2 and 4 h and subsequent enhancement at 6 h LPS stimulation. PUFA-treatments differentially modulated the expression of membrane-bound and cytosolic CD14. Membrane CD14 was significantly decreased at 0 h in CLA-treated cells **[Figure 4.8]** which agrees with our flow cytometry data. Furthermore, levels of CD14 in these cells are reduced after LPS activation compared to the DMSO controls. The opposite was observed in the cytosol of these cells with CD14 being increased after LPS stimulation compared to the DMSO control. Similar to the DMSO group, EPA-, DHA- and LA-treated cells displayed enhanced expression of membrane bound CD14 over the course of LPS stimulation with no significant differences between the groups. However, in contrast to CLA, EPA and DHA treated cells displayed significantly higher levels of CD14 at the membrane at early time points compared to DMSO (control), **[Figure 4.9]** and **[Figure 4.10]**, respectively. The cytosolic fractions of DHA-treated cells had decreased levels of CD14 particularly at 4 h and 6 h following LPS stimulation **[Figure 4.9]** with EPA-treated cells showing no clear differences **[Figure 4.10]**. The levels of CD14 in the cytosol of the LA-treated cells were increased at some of the time points examined with little change in the membrane CD14 **[Figure 4.11]**.

4.2.3 OPTIMISATION OF LPS-BINDING IN HEK 293 CELLS

HEK 293 and HEK MTC cells were incubated with 5, 10 or 50 μg/ml of various biotinylated conjugates as described [section 2.4.4]. Binding of conjugates to cells was assessed by flow cytometry on a FACSCalibur™. As expected, binding to HEK 293 cells was negligible for all conjugates and free biotin **[Figure 4.12B]**. On the other hand binding to HEK-MTC cells was negligible for all conjugates and free biotin except Biotin-LPS (Invivogen™) **[Figure 4.13B]**. Binding was observed at 5, 10 and 50 μg/ml Biotin-LPS. The range of conjugates used in the experiment

significantly ruled out non specific binding (NSB). Furthermore, NSB in both cell lines was further ruled out by incubating cells solely with the streptavidin-FITC conjugate, **[Figure 4.12A]** and **[Figure 4.13A]**. Substantial binding at low doses of Biotin-LPS (5 μg/ml) lead to the use of this concentration of the conjugate being used in experiments to assess the effect of PUFA on binding of LPS in this cell line.

4.2.4 PUFA DO NOT MODULATE THE BINDING OF LPS IN HEK 293 CELLS

HEK-MTC cells were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), or LA (50 μM). Following this HEK 293 and HEK MTC cells were incubated with 5 μg/ml of Biotin-LPS (Invivogen™) and assessed for binding by flow cytometry on a FACSCalibur™, as described [section 2.4.5]. Internal controls similar to those performed in the optimisation experiments were included. As expected, Biotin-LPS (Invivogen™) was the only conjugate to bind to the HEK-MTC cells **[Figure 4.14A]**. Furthermore, no significant change in binding was observed in PUFA treated cells compared to DMSO (control) **[Figure 4.14B]**.

CD40

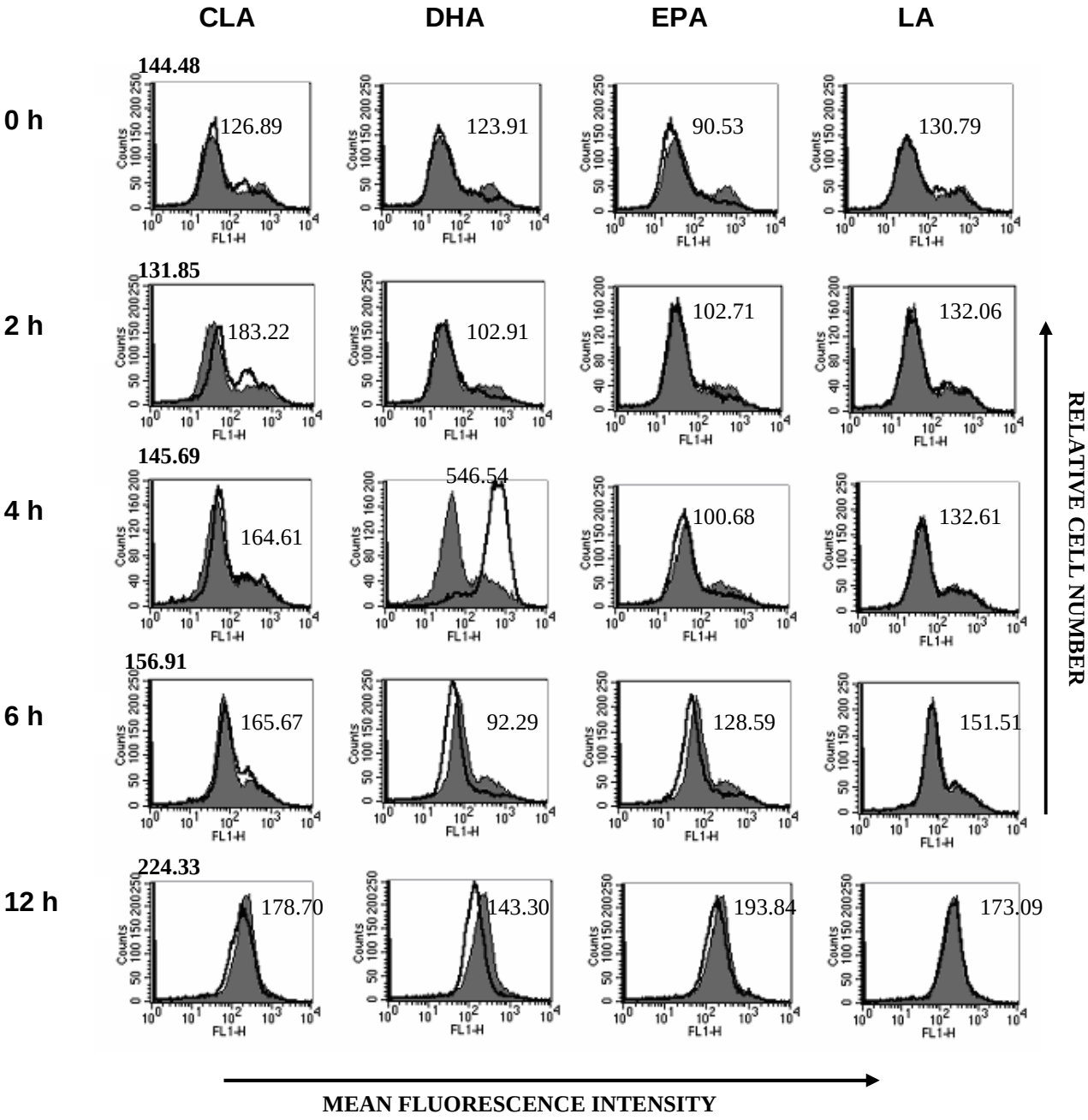


FIGURE 4.1: Effects of PUFA on CD40 expression on J774 macrophage (MØ). Macrophage were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml) for 0, 2, 4, 6 and 12 h. Subsequently, cells were washed and stained with antibody specific for CD40. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line). Mean Flourescent Intensity (MFI) values are also presented for DMSO (bold black) and PUFA groups (on histogram) over the time course.
Profiles are shown for a single experiment and are representative of 3 experiments.

CD80

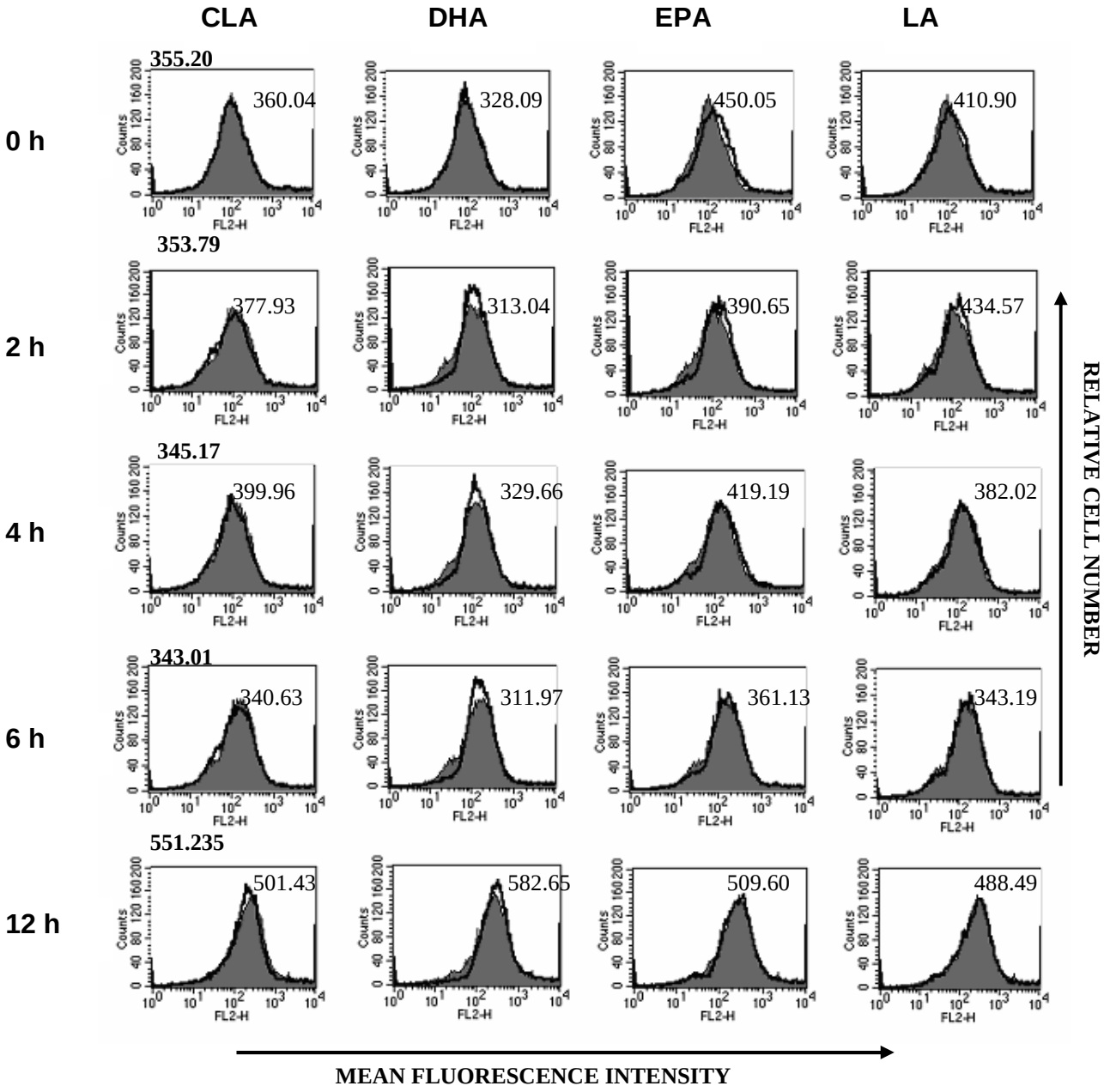


FIGURE 4.2: Effects of PUFA on CD80 expression on J774 macrophage. Macrophage were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml) for 0, 2, 4, 6 and 12 h. Subsequently, cells were washed and stained with antibody specific for CD80. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line). Mean Flourescent Intensity (MFI) values are also presented for DMSO (bold black) and PUFA groups (on histogram) over the time course.
Profiles are shown for a single experiment and are representative of 3 experiments.

CD86

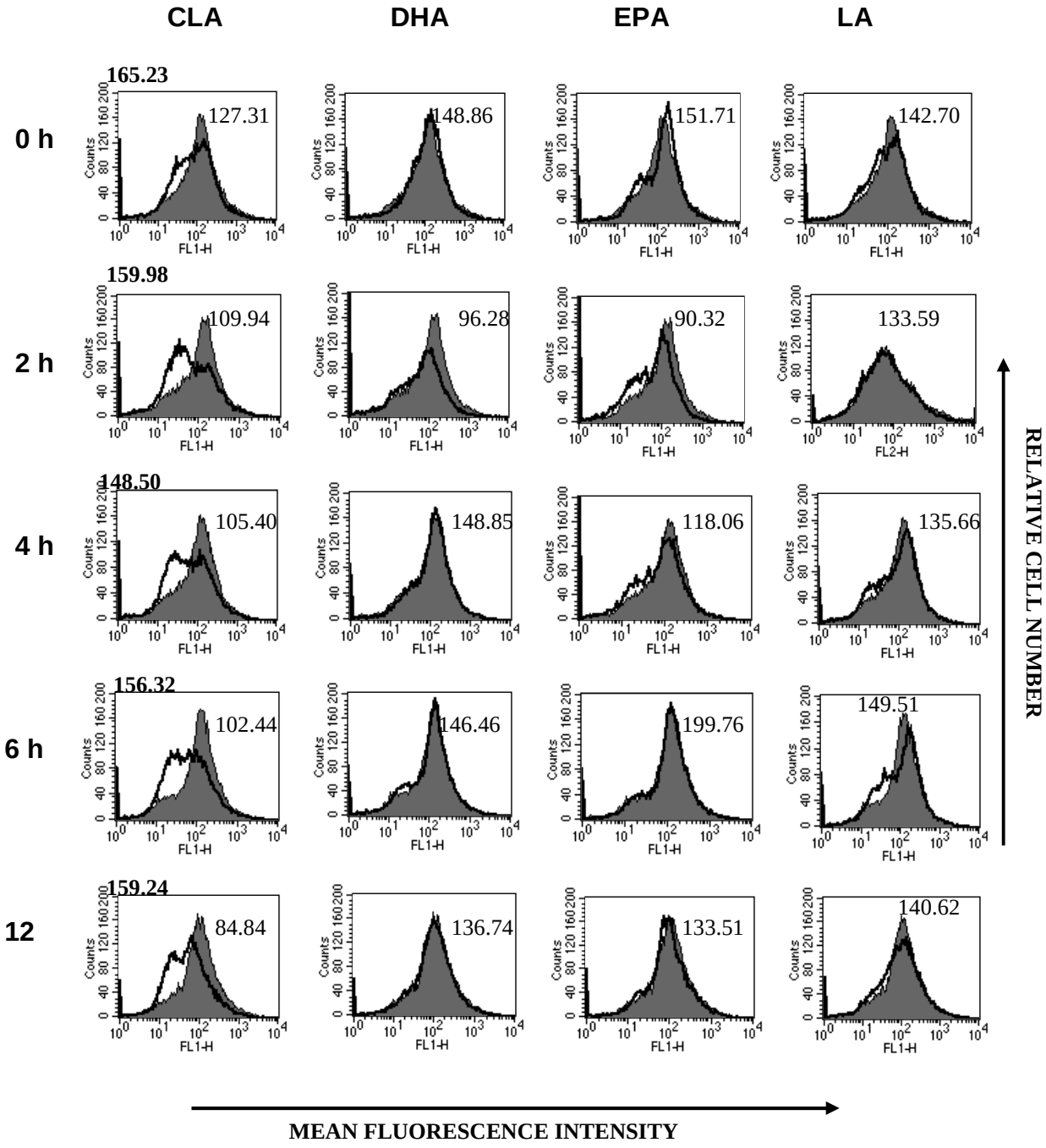


FIGURE 4.3: Effects of PUFA on CD86 expression on J774 macrophage. Macrophage were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml) for 0, 2, 4, 6 and 12 h. Subsequently, cells were washed and stained with antibody specific for CD86. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line). Mean Flourescent Intensity (MFI) values are also presented for DMSO (bold black) and PUFA groups (on histogram) over the time course.
Profiles are shown for a single experiment and are representative of 3 experiments.

SR-A

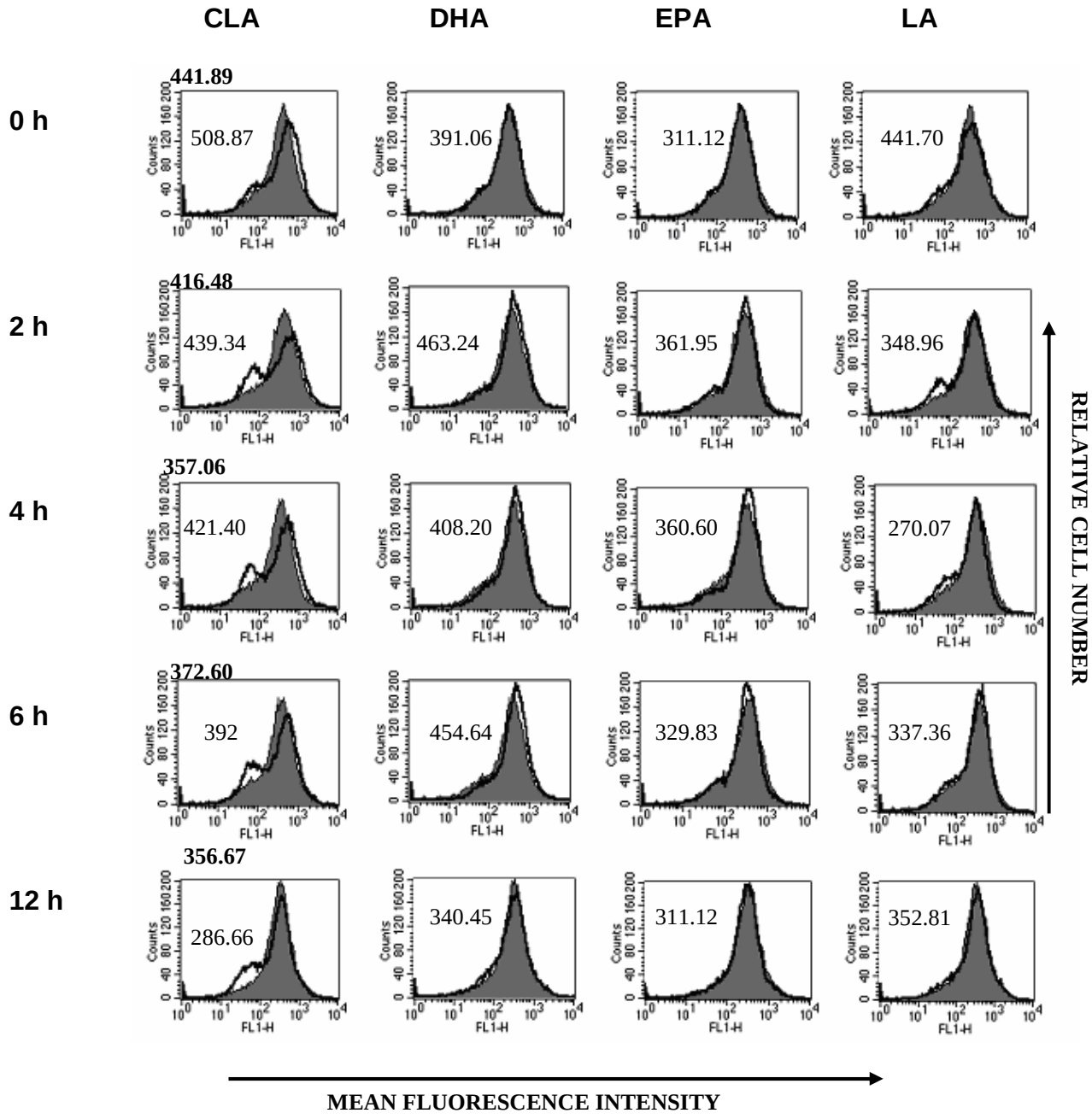


FIGURE 4.4: Effects of PUFA on SR-A (CD204) expression on J774 macrophage. Macrophage were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml) for 0, 2, 4, 6 and 12 h. Subsequently, cells were washed and stained with antibody specific for SR-A. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line). Mean Flourescent Intensity (MFI) values are also presented for DMSO (bold black) and PUFA groups (on histogram) over the time course.
Profiles are shown for a single experiment and are representative of 3 experiments.

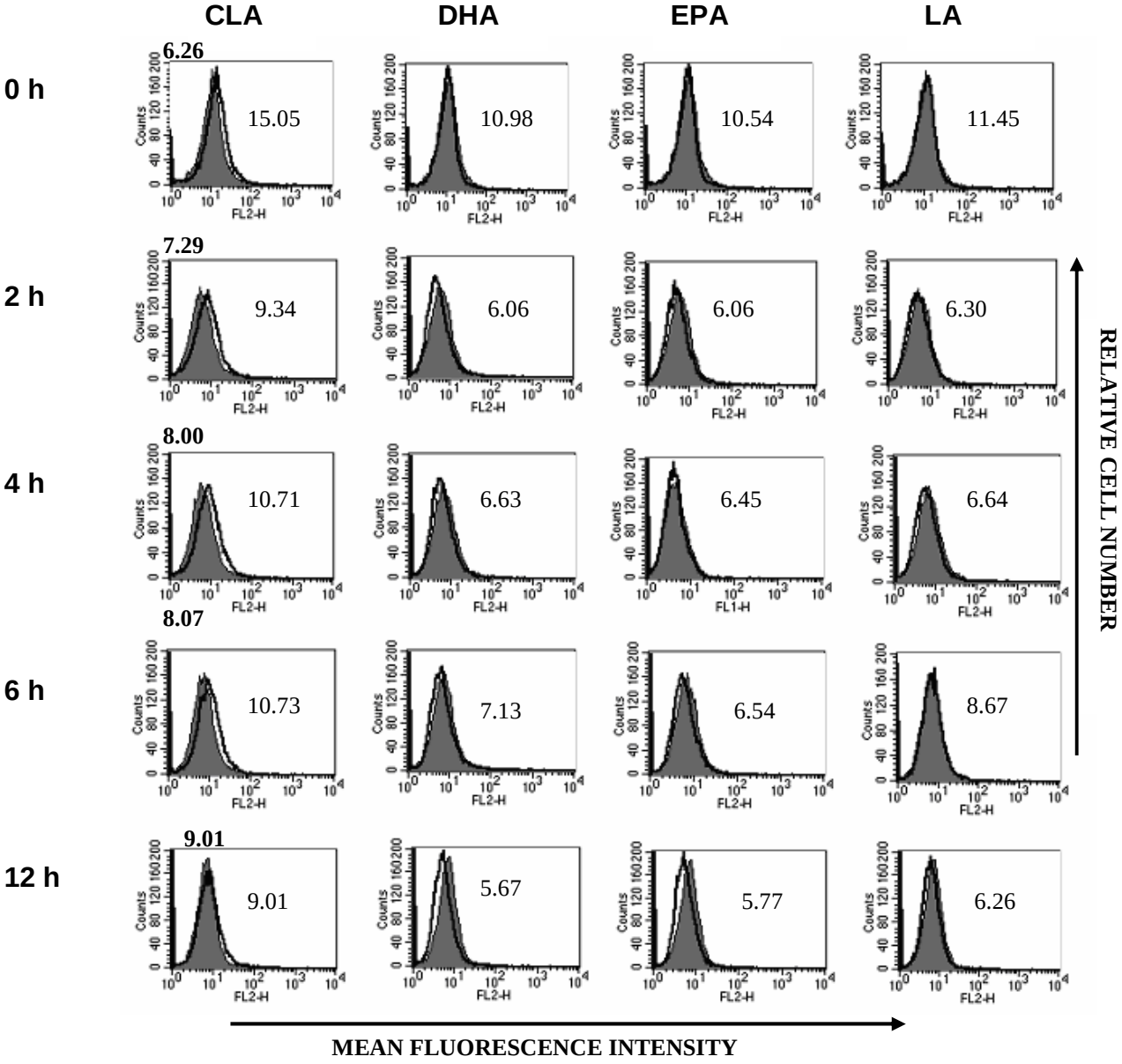


FIGURE 4.5: Effects of PUFA on TLR4-MD-2 expression on J774 macrophage (MØ). Macrophage were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml) for 0, 2, 4, 6 and 12 h. Subsequently, cells were washed and stained with antibody specific for TLR4-MD-2. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line). Mean Flourescent Intensity (MFI) values are also presented for DMSO (bold black) and PUFA groups (on histogram) over the time course.
Profiles are shown for a single experiment and are representative of 3 experiments.

CD14

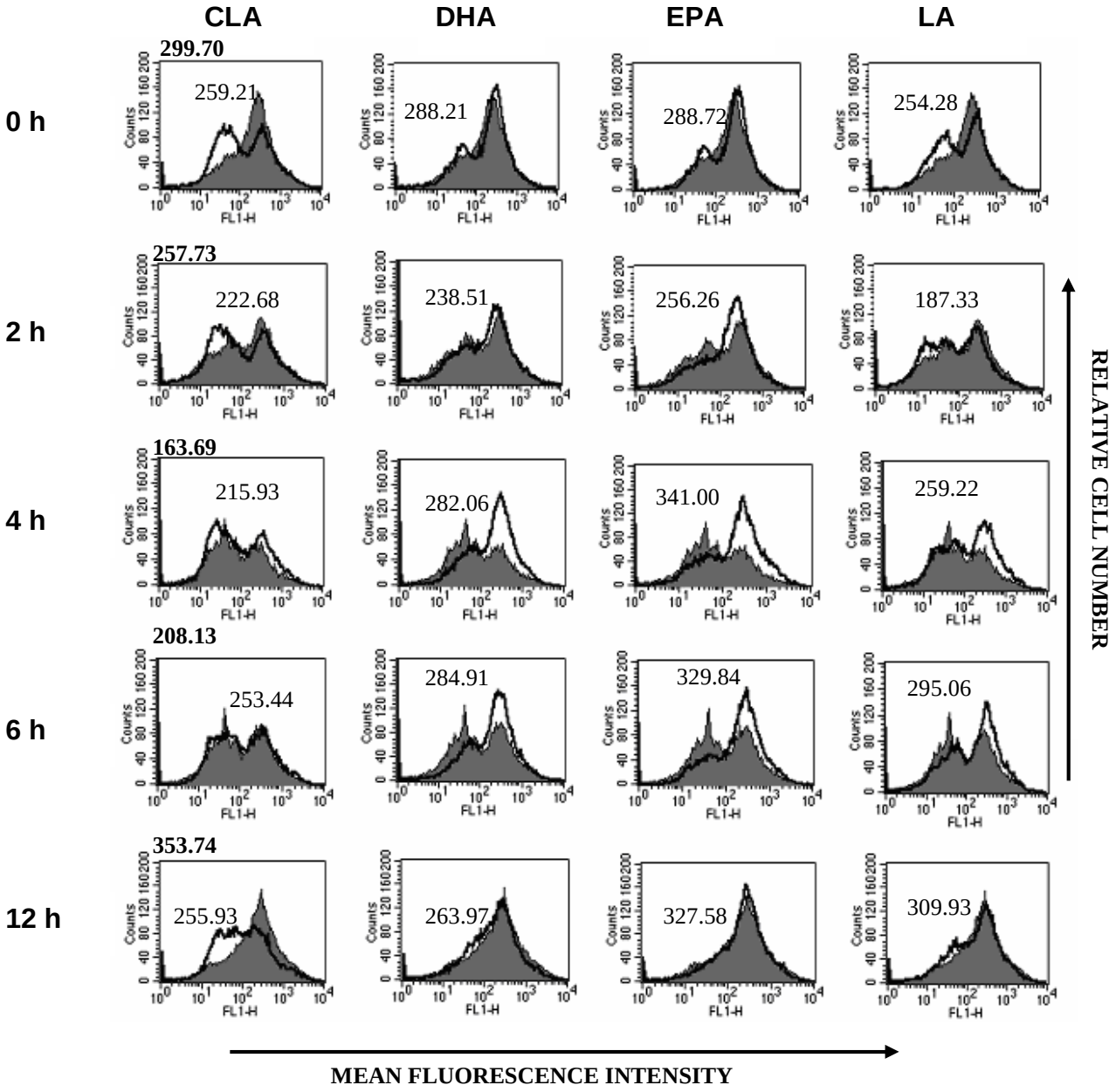


FIGURE 4.6: Effects of PUFA CD14 expression on J774 macrophage (MØ). Macrophage were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days, before activation with LPS (100 ng/ml) for 0, 2, 4, 6 and 12 h. Subsequently, cells were washed and stained with antibody specific for CD14. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line). Mean Flourescent Intensity (MFI) values are also presented for DMSO (bold black) and PUFA groups (on histogram) over the time course.
Profiles are shown for a single experiment and are representative of 3 experiments.

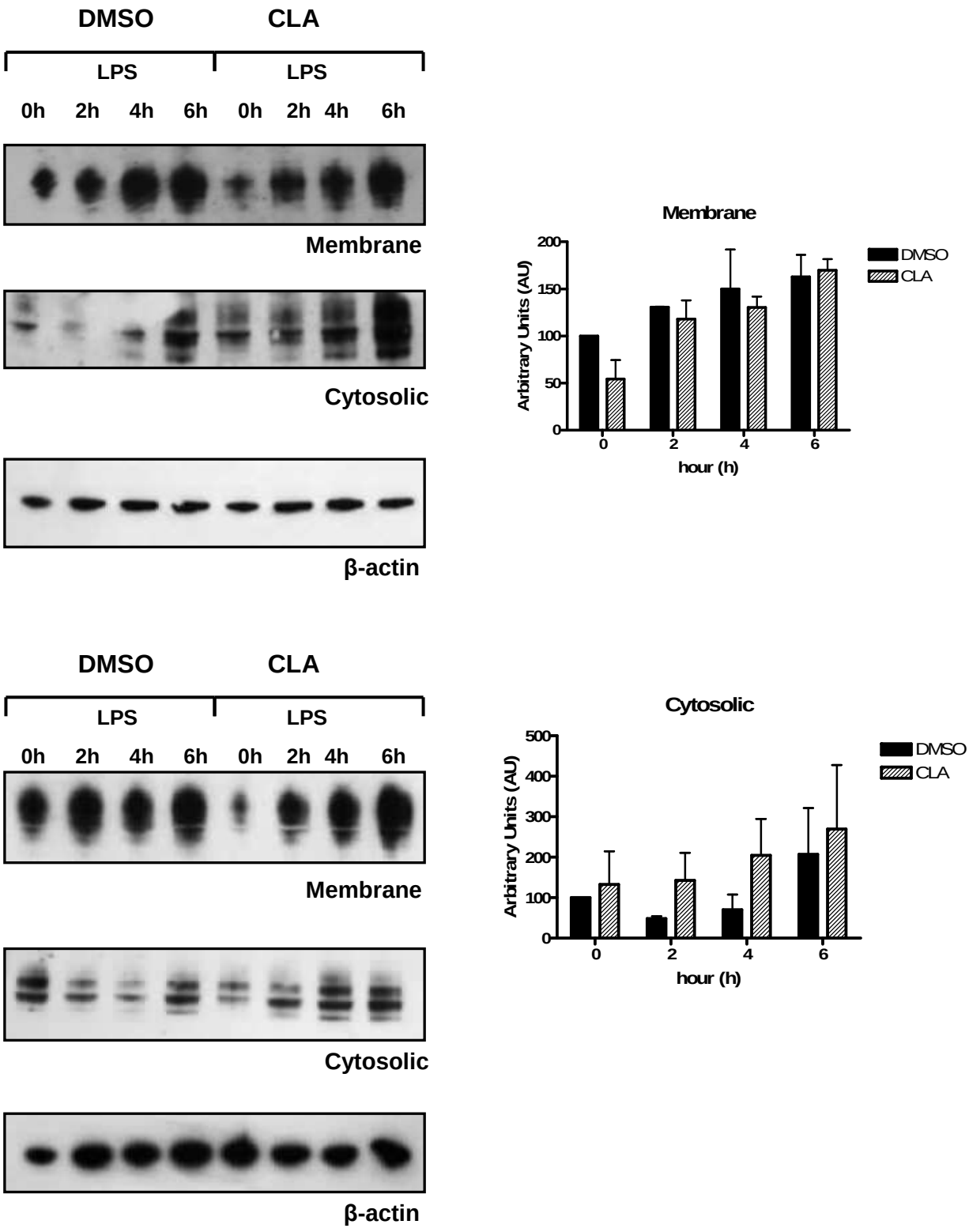


FIGURE 4.8: CLA modulated expression of membrane and cytosolic CD14 in macrophage. Macrophage were cultured with DMSO (vehicle control) or CLA (50 μM) for 7 days. Subsequently, cells were plated at 2 x 10^5 cell/ml, (3 ml/well) and left to rest overnight. Cells were then stimulated with LPS (100 ng/ml) over 6 h, as indicated, after which cell lysates were harvested and membrane fractionation performed. Total cellular levels of β-actin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of CD14 expression in arbitrary units (AU) is given for both membrane and cytosolic fractions. Results are shown for two experiments and are representative of 3 independent experiments.

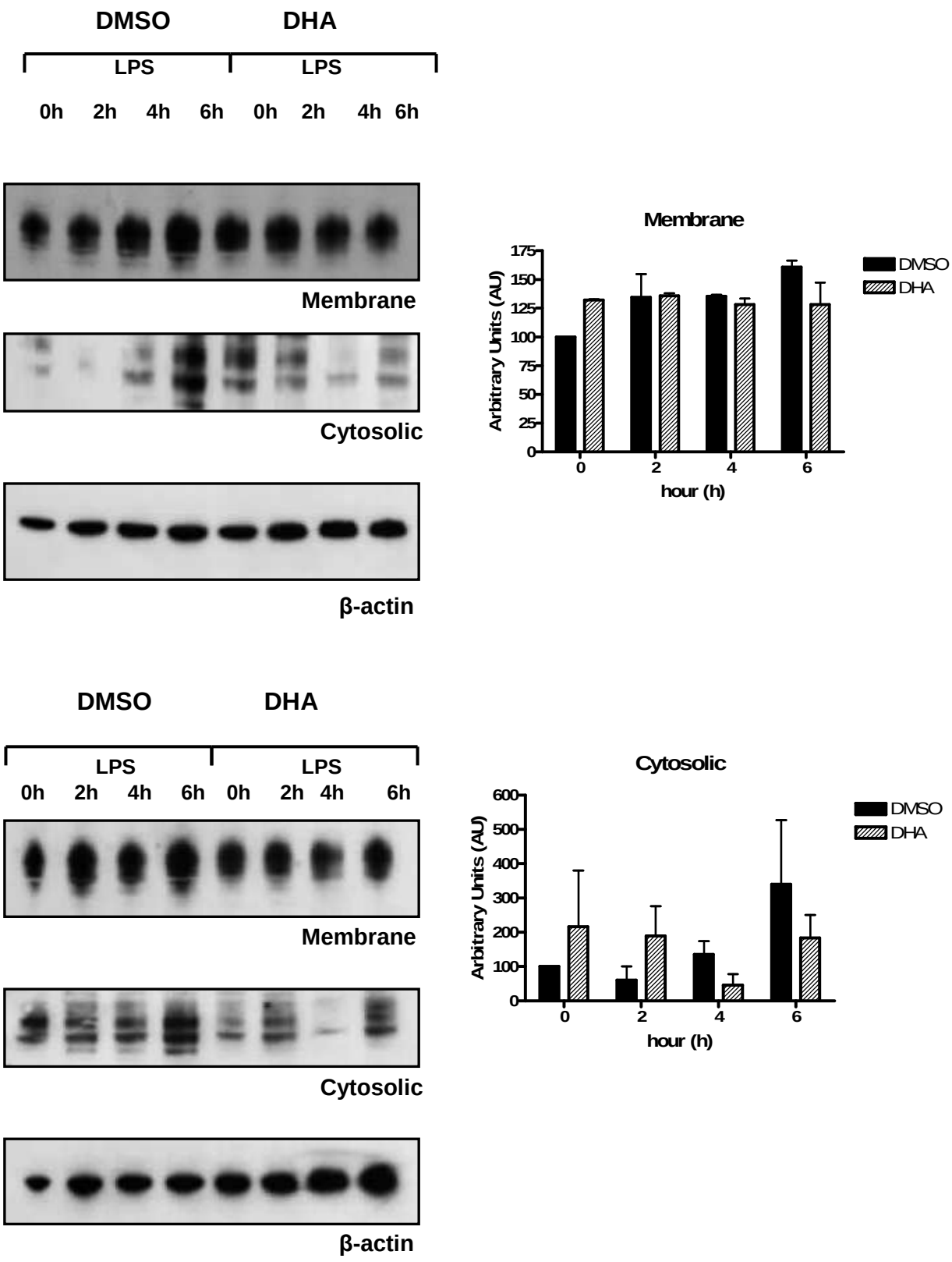


FIGURE 4.9: DHA modulated expression of membrane and cytosolic CD14 in macrophage. Macrophage were cultured with DMSO (vehicle control) or DHA (25 μM) for 7 days. Subsequently, cells were plated at 2 x 10^5 cell/ml, (3 ml/well) and left to rest overnight. Cells were then stimulated with LPS (100 ng/ml) over 6 h, as indicated, after which cell lysates were harvested and membrane fractionation performed. Total cellular levels of β-actin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of CD14 expression in arbitrary units (AU) is given for both membrane and cytosolic fractions. Results are shown for two experiments and are representative of 3 independent experiments.

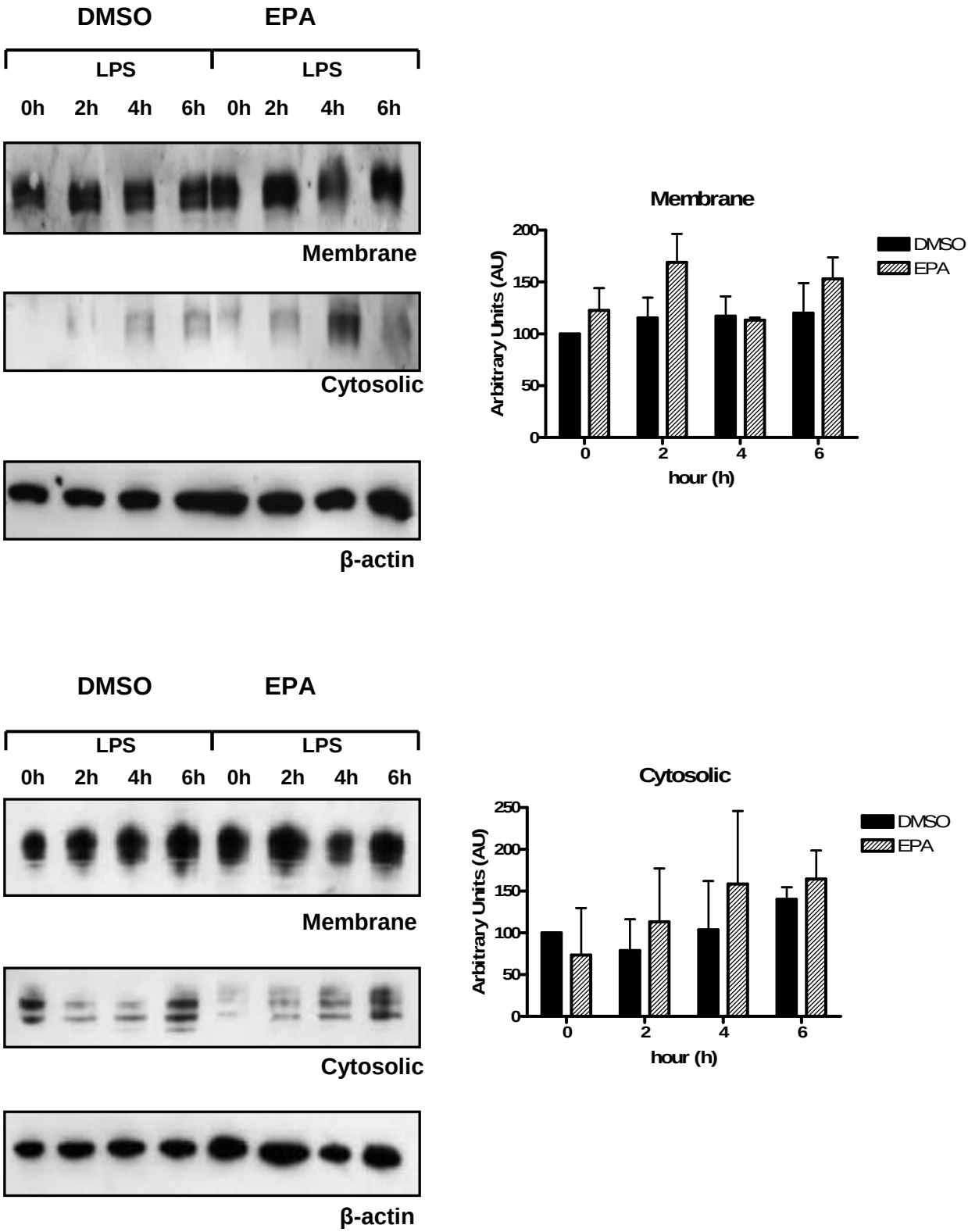


FIGURE 4.10: EPA modulated expression of membrane and cytosolic CD14 in macrophage. Macrophage were cultured with DMSO (vehicle control) or EPA (25 μM) for 7 days. Subsequently, cells were plated at 2 x 10^5 cell/ml, (3 ml/well) and left to rest overnight. Cells were then stimulated with LPS (100 ng/ml) over 6 h, as indicated, after which cell lysates were harvested and membrane fractionation performed. Total cellular levels of β-actin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of CD14 expression in arbitrary units (AU) is given for both membrane and cytosolic fractions. Results are shown for two experiments and are representative of 3 independent experiments.

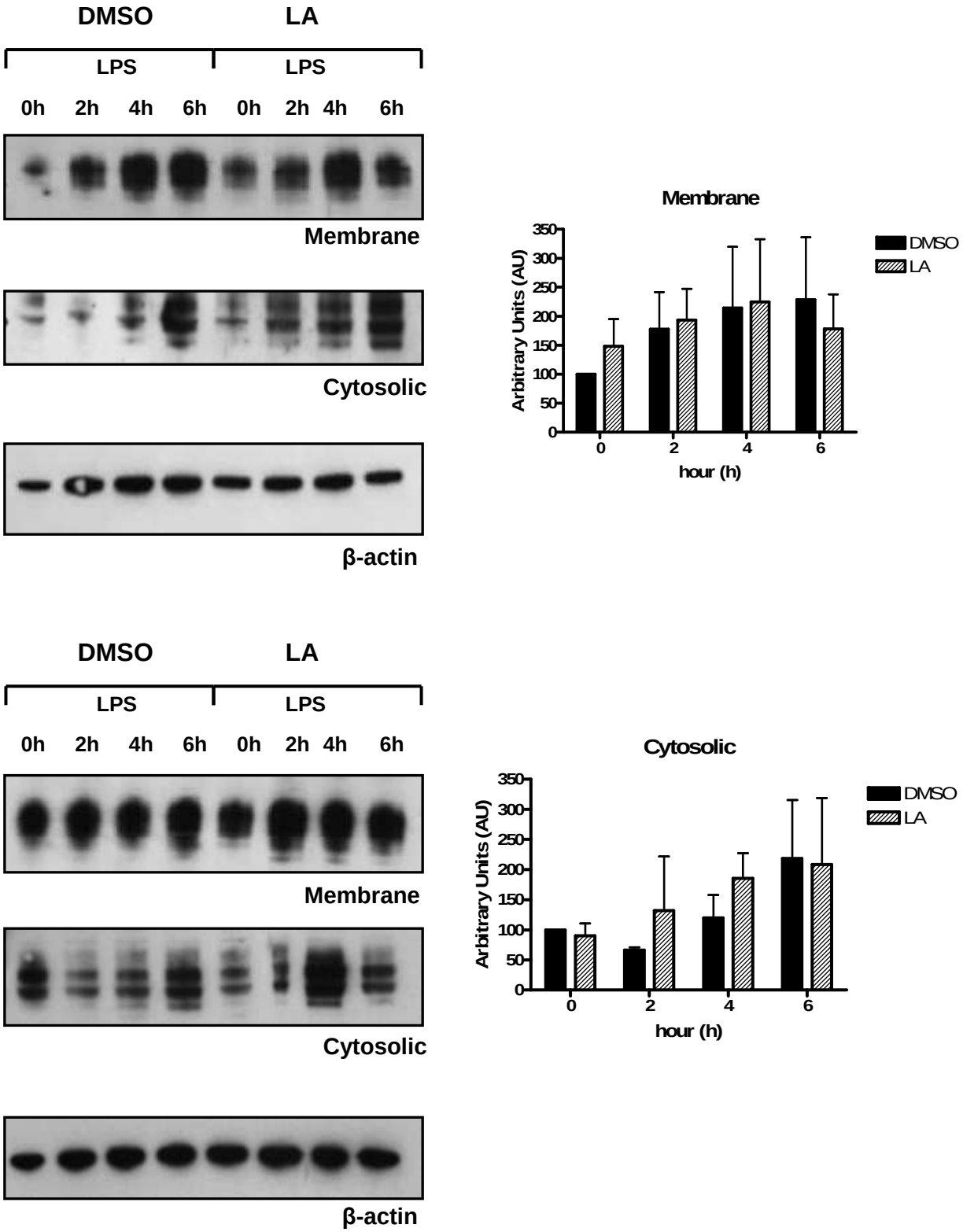


FIGURE 4.11: Effects of LA on expression of membrane and cytosolic CD14 in macrophage. Macrophage were cultured with DMSO (vehicle control) or LA (50 μM) for 7 days. Subsequently, cells were plated at 2 x 10^5 cell/ml, (3 ml/well) and left to rest overnight. Cells were then stimulated with LPS (100 ng/ml) over 6 h, as indicated, after which cell lysates were harvested and membrane fractionation performed. Total cellular levels of β-actin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of CD14 expression in arbitrary units (AU) is given for both membrane and cytosolic fractions. Results are shown for two experiments and are representative of 3 independent experiments.

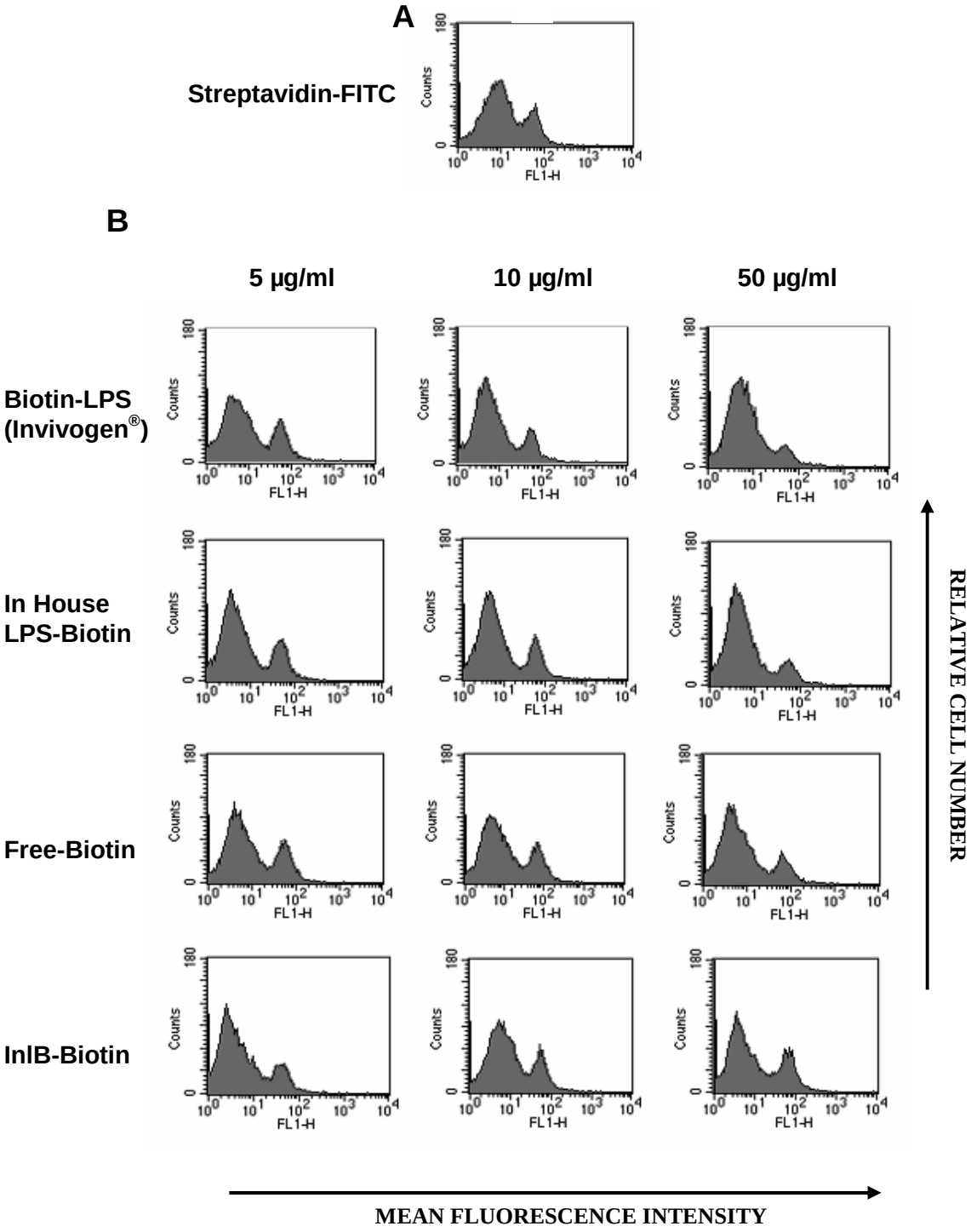


FIGURE 4.12: Optimisation of LPS-binding in HEK 293 cells by flow cytometry. 1 x 10^6 cells were incubated with 5, 10 or 50 µg/ml of various biotinylated conjugates. Following this a streptavidin-FITC probe was used to detect binding of the conjugates by flow cytometry **[B]**. HEK 293 cells were also incubated with strptavidin-FITC alone to rule out any non-specific binding **[A]**. Results of flow cytometric analysis are shown. Profiles are shown for a single experiment and are representative of 3 experiments.

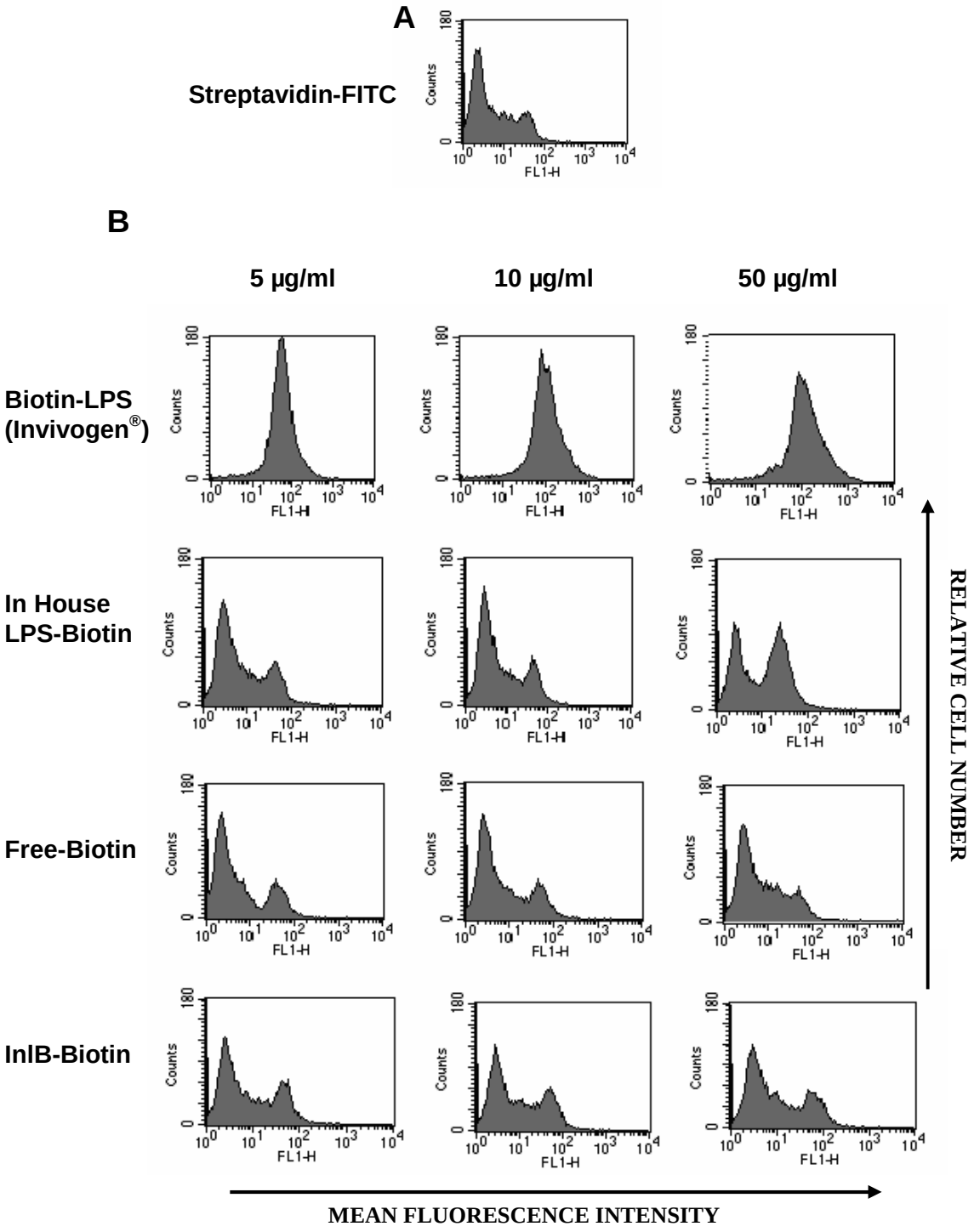


FIGURE 4.13: Optimisation of LPS-binding in HEK-MTC cells by flow cytometry. 1 x 10^6 cells were incubated with 5, 10 or 50 µg/ml of various biotinylated conjugates. Following this a streptavidin-FITC probe was used to detect binding of the conjugates by flow cytometry **[B]**. HEK MTC cells were also incubated with streptavidin-FITC alone to rule out any non-specific binding **[A]**. Results of flow cytometric analysis are shown. Profiles are shown for a single experiment and are representative of 3 experiments.

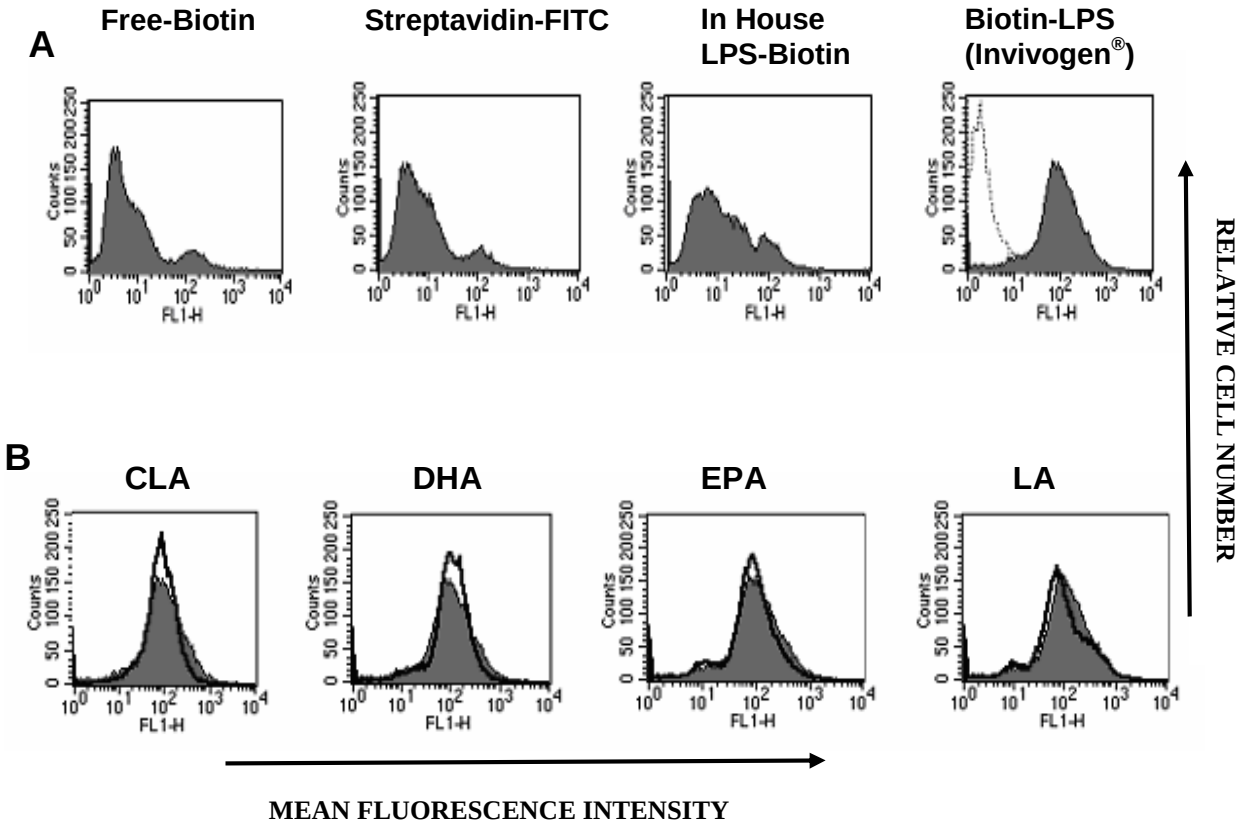


FIGURE 4.14: PUFA do not affect LPS binding to TLR4. Internal controls for binding of LPS in HEK-MTC cells are shown (filled histograms) and unstained cells (dotted line) **[A]**. HEK-MTC cells were cultured with DMSO (vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days. Subsequently, cells were incubated with Biotin-LPS conjugate and binding assessed using a streptavidin-FITC probe. Results of flow cytometric analysis are shown for DMSO-treated MØ (filled histogram) and PUFA-treated MØ (thin black line) **[B]**. Profiles are shown for a single experiment and are representative of 3 experiments.

4.3 DISCUSSION

The focus of this study was to investigate the possible mechanisms underlying the PUFA-induced effects on the activation status of macrophage reported in chapter 3. To this end, PUFA-modulated expression of cell surface markers, particularly TLR4 and CD14, represented important targets upstream of signalling events through which PUFA may exert their effects. Furthermore, binding of LPS to CD14 and subsequent transfer and loading to TLR4-MD-2 initiates cellular activation (Kirkland et al. 1993, Miyake 2006a). Therefore, we also set out to assess whether the previously observed downstream effects of PUFA treatment, such as altered cytokine and chemokine production were linked to any changes in this putative binding event.

While it is clear that PUFA affect signalling events which are downstream of TLR4, their exact mechanism of action is still unclear and some studies suggest that they exert their effects upstream of the signalling events, at the TLR4 complex itself (Lee et al. 2003). Studies have shown that a number of molecules affect LPS binding. Specifically, the antibacterial peptides, cathelicidins, secreted by mammalian cells have been shown to exert protective actions against endotoxin shock by blocking the binding of LPS (Nagaoka et al. 2001). Moreover, when preincubated with RAW264.7 cells they bound to the cell surface and inhibited binding of FITC-LPS to the cells. In addition, aside from its commonly known function as an anticoagulant, heparin has been shown to inhibit LPS binding and subsequent cytokine production (Anastase-Ravion et al. 2003a). Therefore, we firstly examined the effects of PUFA on LPS binding.

To this end, the protocol for these experiments was rigorously optimised. Specifically, a HEK 293 cell line stably expressing TLR4, CD14 and MD-2 (HEK-MTC) was employed to rule out any alternate binding to additional LPS receptors (e.g. SR-A). Our data presented here demonstrates that PUFA do not modulate the binding of LPS to the TLR4 receptor complex. In addition, no significant effect on LPS binding was observed in saturated fatty acid, LA treated cells. Given that our data from the previous chapter showed that PUFA decreased expression of TLR4, we were initially surprised to find that the amount of LPS binding to TLR4 was not reduced. However, further experiments in this chapter reveal that the effects of PUFA on TLR4 are not early events and changes in the expression of this receptor were not obvious until 12 h post LPS-stimulation. SR-A is another receptor present on macrophages, which has been shown to bind LPS without any subsequent downstream signalling (Haworth et al. 1997). Our previous chapter showed that PUFA increased SR-A and indicated the possibility that upregulation of SR-A by PUFA may be a mechanism whereby LPS can bind to SR-A instead of TLR4, thus decreasing the cells response to LPS stimulation. It was therefore important to determine whether these changes occurred early. This study demonstrates that changes in SR-A expression are not an early event and therefore cannot be responsible for the decreased response of macrophage to LPS following treatment with PUFA.

This study also revealed that similar to our observation for SR-A and TLR4-MD-2, the effects of PUFA on CD40 and CD80 expression are also not early events and are likely to be as a result of the overall diminished response of the macrophage to LPS stimulation. However, the expression of CD40 was markedly enhanced in DHA treated cells after 4 h stimulation. This is previously unreported, nonetheless,

studies involving DHA have looked at the effect of this fatty acid on CD40 after 24 h stimulation and correlate with our observation of suppressed CD40 at this time (Chapter 3) (Wang et al. 2007).

This study so far has demonstrates that many of the changes in cell surface receptor expression induced by PUFA are not early events and are therefore likely to be as a result of the decreased response of the cells to LPS. In direct contrast to the above, CLA suppressed CD14 and CD86 prior to and throughout the course of stimulation. Conversely, n-3 PUFA, EPA and DHA did not alter CD86 however; both n-3 PUFA were seen to enhance CD14 from as early as 4 h stimulation. As discussed before, blocking T cell co-stimulatory signals by targeting CD86 is an attractive approach to treat autoimmune disease (Odobasic et al. 2008). On the other hand, lack of co-stimulatory signals results in the failure of cytotoxic T cell activation against tumor specific antigens (Luque, Reyburn and Strominger 2000, Suzuki et al. 2003). Furthermore, high levels of CD86 are found in the bone marrow and peripheral blood of patients with chronic graft-versus-host disease (Arpinati et al. 2008). Our finding in relation to CD86 further emphasises not only the differential effects of fatty acids but the importance of tailoring their administration in the treatment of individual inflammatory diseases. The consequences of suppressed CD86 in macrophages warrants further investigation.

Our finding regarding the alternate effects of CLA and n-3 PUFA on CD14 expression and the fact that it is an early event is the most significant piece of data generated here and was further assessed in the analysis of membrane versus cytosolic expression of the protein. There are continuously emerging roles for CD14 in the exacerbation of infection and inflammatory disease. Indeed, high

levels of CD14 are indicative of intestinal macrophage populations from patients suffering with IBD (Kamada et al. 2008). Signalling through CD14 has been shown to play an obligate role in cardiac inflammation occurring after major burn injuries (Barber et al. 2008). (Panaro et al. 2008) demonstrate the overexpression of CD14 in specific areas of the central nervous system (CNS) within an endotoxin induced mouse model of Parkinson's-like disease. Furthermore, Wegener's granulomatosis is partially characterised by the upregulation of CD14 and CD18 on monocytes by antineutrophil cytoplasmic auto antibodies (ANCA) (Yard et al. 2002). The findings presented here demonstrate by flow cytometry and western blot that CLA suppresses surface expression of CD14 prior to and during stimulation. Taken together with our earlier findings regarding the effects of CLA on activation status (Chapter 3) this implies a cellular phenotype less responsive to LPS and therefore less likely to produce exacerbated pro-inflammatory signals in the presence of immune stimuli. Interestingly, other studies in our laboratory have recently shown that CLA reduces the symptoms of endotoxic shock and that this is associated with a decreased expression of CD14 on dendritic cells (unpublished observation). In this regard, administration of CLA may offer an alternative to anti-CD14 therapies suggested by many of the groups cited above.

It cannot be concluded from the results presented here whether PUFA-modulated expression of CD14 occurs at the transcriptional level. Indeed, suppressed levels of CD14 at the membrane in CLA treated cells is accompanied by significantly enhanced levels in the cytosol. This is perhaps due to an inability of transcribed CD14 to become incorporated into the membrane and is therefore retained in the cytosol. This is a novel finding in relation to CLA. Furthermore, after attachment of the GPI-anchor, GPI-anchored proteins are transported to the cell surface via the

'canonical' secretory pathway (Lippincott-Schwartz, Roberts and Hirschberg 2000). Thus far, fatty acids have not been reported to interfere with this translocation event.

In contrast to CLA, n-3 PUFA, EPA- and DHA-treated cells showed enhancement of CD14 at early time points at the membrane along with decreased levels in the cytosol. In contrast to our findings (De Smedt-Peyrusse et al. 2008) report suppressed levels of membrane CD14 and enhanced cytosolic levels following DHA treatment in microglial cells. However, while this was indeed prior to stimulation, cells were treated exogenously with DHA (30 μmol/L for 24 h). Findings presented here significantly emphasize the different modulatory effects of CLA and n-3 PUFA, EPA and DHA on LPS receptor CD14 and requires further clarification. Studies assessing the effect of PUFA on CD14 mRNA levels may help elucidate the mechanisms involved.

Most significantly, in conjunction with our previous findings, PUFA modulated effects on CD14 may be a key point upstream of signalling through which PUFA exert their anti-inflammatory effects. Moreover, it is vitally important to assess this further. Certainly, (Ji et al. 2006) report suppression of CD14 by components of Qinggan Huoxue Recipe (QGHXR), however, the effects of these compounds on CD14 was not associated with subsequent suppression of NFκB.

While CD14 is required for the maximum responsiveness of the TLR4 receptor complex to LPS, is it absolutely required for TLR4 endocytosis and subsequent activation of interferon-β via adaptors Trif and TRAM (Jiang et al. 2005, Kagan et al. 2008, Godowski 2005). Lipid rafts represent important microdomains within the

plasma membrane of cells that constitutively express GPI-anchored proteins. In addition, translocation of key signalling receptors including TLR4 and indeed the expression of GPI-anchored proteins is enhanced upon stimulation in these domains. As a result, in the following chapter, expression of CD14 in lipid raft microdomains of PUFA treated macrophage will be examined. Furthermore, the effect of PUFA on TLR4 endocytosis and the induction of key inflammatory transcription factors NFκB and IRF will also be assessed.

CHAPTER 5

THE EFFECTS OF PUFA ON THE TLR4 RECEPTOR COMPLEX AND DOWNSTREAM SIGNALLING

5.1 INTRODUCTION

Throughout this work we have endeavoured to elucidate the pivotal mechanisms through which fatty acids exert their anti-inflammatory effects. Thus far our finding regarding PUFA modulated expression of CD14 has been of great significance. Not only do CLA and n-3 PUFA differentially modulate the expression of this protein, in particular suppression can be observed at very early stages in CLA-treated cells. This finding and the fundamental role of CD14 in TLR4 signalling warranted further investigation. As such the rationale for the current study has been to concentrate on PUFA-mediated effects in relation to TLR4 endocytosis, downstream signalling and indeed targeting of CD14 itself to microdomains of the plasma membrane.

With regard to signalling, TLR4 is unique in that it activates both MyD88 dependant and independent pathways leading to the induction of the transcription factors NFκB and IRF3, respectively (Brikos and O'Neill 2008, Akira, Yamamoto and Takeda 2003). This facilitates the production of pro-inflammatory cytokines via NFκB (O'Neill 2006) and type I interferons via IRF3 (McCoy and O'Neill 2008). Activation of the MyD88 dependant pathway is mainly an event initiated at the plasma membrane. Furthermore, induction of IRF via the MyD88-independent pathway is dependant on the endocyotosis of TLR4 an event requiring the presence of CD14 (Jiang et al. 2005, Kagan et al. 2008).

In the previous chapter we describe the suppression of membrane-bound CD14 in CLA-treated cells both prior to and at very early stages following stimulation with LPS. In this chapter we investigate the effect of PUFA-treatment on the activation of IRF3, a completely novel area in the field of PUFA research. In conjunction

with this we also assess the modulatory effects of PUFA used in our study on NFκB, given that it is broadly accepted that PUFA mediate part of their anti-inflammatory effects through the suppression of NFκB (Sampath and Ntambi 2005). Furthermore, we postulate that the effect of CLA on CD14 may indeed modulate TLR4 signalling by limiting the ability of TLR4 to become endocytosed leading to a suppression of IRF. Importantly, in support of this (Lee et al. 2003) demonstrate that the target for PUFA mediated effects may in fact lie upstream at the receptor complex itself.

Interestingly, the activation of several signalling complexes has been linked with their specific recruitment to microdomains within the plasma membrane known as lipid rafts. In particular, this is widely documented regarding immune cell signalling (Dykstra et al. 2003, Goebel et al. 2002). Indeed, lipid rafts have been widely implicated in T-cell activation (Horejsi et al. 1999). Specifically, the recruitment of TLR2 (Soong et al. 2004)(Soong et al. 2004) and TLR4 and associated molecule CD14 (Triantafilou et al. 2002) to lipid rafts following stimulation with their respective ligands has been demonstrated. As a result, we aim here to assess the effect of PUFA on the recruitment of CD14 to lipid raft micro-domains following stimulation with LPS.

5.2 RESULTS

5.2.1 OPTIMISATION OF LIPID RAFT ISOLATION FROM J774 MACROPHAGE

J744 macrophage were cultured and left unstimulated or stimulated with LPS for 30 min. Following this lipid raft fractions were isolated as described [see section 2.6.3]. As a means of optimising the isolation procedure various concentrations of Triton X-100 were employed and lipid raft fractions assessed by western blot. Results demonstrate that 0.2 % (v/v) Triton X-100 was an insufficient amount of detergent to successfully solubilize cellular material and leave lipid rafts free to float to the interface of the sucrose gradient (Fractions 4 -6) **[Figure 5.1]**. Indeed this is indicated by the lack of alkaline phosphatase activity, indicative of lipid rafts and lipid raft marker, flotillin-1, FL-1 localisation within these fractions. Furthermore, CD14 and the non-raft marker β-actin were also detected throughout fractions 2- 11.

Alternatively, resuspending cellular membranes in 0.5 % (v/v) Triton X-100 provided sufficient solubilization for partitioning of lipid rafts to the sucrose interface **[Figure 5.2]**. Indeed, high levels of alkaline phsophatase are indicated in fractions (4-6) in both unstimulated and stimulated cell samples. Furthermore, specific localisation of lipid raft marker, FL-1 and GPI-anchored protein, CD14 can also be observed in these fractions. In addition, the non-raft marker, β-actin is only localised within the bottom fraction or ‘pellet’. In contrast, employing 1 % (v/v) Triton X-100 completely solubilized non-raft and raft material **[Figure 5.3]**. In a case such as this, both raft and non-raft domains coalescence with detergent and result in disordered flotation of proteins throughout the gradient (Shogomori and

Brown 2003). Certainly, in support of this, CD14, FL-1 and β-actin were all detected throughout the gradient. Furthermore, alkaline phosphatase activity is completely skewed in both resting and stimulated cells. Therefore, for the investigation of lipid rafts from PUFA-treated macrophage 0.5 % (v/v) Triton X-100 was employed.

5.2.3 ANALYSIS OF LIPID RAFTS FROM PUFA TREATED J774 MACROPHAGE

J774 macrophage were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), CLA (50 μM), or LA (50 μM) after which approx. 100 x 10^6 cells were left unstimulated or stimulated with LPS for 30 min. Following this, lipid raft fractions were isolated as described [see section 2.6.3] and assessed by western blot. The lipid raft marker, FL-1 was successfully detected at the interface of sucrose gradients for DMSO (vehicle control) and PUFA-treated cells **[Figure 5.4 – 5.7]**. Importantly, the non-raft marker, β-actin was also detected distinctly apart from FL-1 at the bottom of gradients, mainly in cell pellets **[Figure 5.4 – 5.7]**.

Furthermore, significant alkaline phosphatase activity was present for lipid raft fractions (Fraction 4-6) in DMSO treated cells (vehicle control) both before and after stimulation **[Figure 5.4]**. In contrast PUFA-treatment significantly altered alkaline phosphatase activity pellets **[Figure 5.5– 5.6]**. This was particularly prominent in CLA-treated cells **[Figure 5.5]**. Moreover, LA-treated cells also displayed randomised effects on alkaline phosphatase activity throughout the sucrose gradient **[Figure 5.7]**. Importantly, stimulation with LPS resulted in an increase in CD14 in the lipid rafts in all of the groups examined **[Figure 5.4 – 5.7]**.

The method employed here afforded qualitative assessment of raft associated proteins. Nonetheless, even with the restriction of qualitative assessment, CD14 'appeared' to be suppressed in lipid raft fractions of CLA treated cells following stimulation **[Figure 5.5]** unlike raft fractions from EPA treated cells **[Figure 5.6]**. In conjunction with observed effects of CLA on alkaline phosphatase activity this warranted further investigation.

5.2.4 QUANTITATIVE ASSESSMENT OF CD14 LEVELS IN LIPID RAFT FRACTIONS OF PUFA TREATED J774 MACROPHAGE

The direct effects of PUFA on the recruitment of CD14 to lipid rafts following stimulation with LPS was quantitatively assessed by western blot. Protein levels were normalised following quantitative analysis via a NanoDrop3300 [see section 2.3.6.4]. Levels of CD14 within the lipid raft fractions of LPS-stimulated CLA-treated cells were significantly lower compared the same fractions from DMSO-treated cells (vehicle control) **[Figure 5.8B and C]**. Conversely, EPA-treated cells displayed elevated levels of CD14 in lipid raft fractions compared to the DMSO group following LPS stimulation **[Figure 5.8B and C]**.

5.2.5 PUFA SELECTIVELY INHIBIT NFKB AND IRF3 EXPRESSION

HEK 293 cells and HEK 293 cells stably expressing TLR4-CD14-MD-2 (HEK-MTC) were cultured for 7 days with either DMSO (50 μM, vehicle control), EPA (25 μM), DHA (25 μM), CLA (50 μM), or LA (50 μM). The resultant fatty acid-treated cells were plated and transiently transfected with either an NFκB or ISRE luciferase reporter plasmid as described [see section 2.8.3]. The IFN-stimulated response element (ISRE) provides a specific readout for the induction of IRF3.

As expected, no induction of NFκB or ISRE was detected in HEK 293 cells stimulated with LPS **[Figure 5.9A]** and **[Figure 5.9B]**, respectively. Analysis by means of unpaired t-test demonstrated that CLA significantly ($p<0.001$) suppressed NFκB in comparison to DMSO (vehicle control) in HEK-MTC cells **[Figure 5.9C]**. In addition, the n-3 PUFA, DHA ($p<0.05$) and EPA ($p<0.01$) also suppressed NFκB in HEK-MTC cells but to a lesser extant than CLA **[Figure 5.9C]**. The saturated fatty acid, LA, exerted no effect on NFκB or ISRE in HEK-MTC cells.

CLA ($p<0.01$) also significantly suppressed the activation of IRF3 compared to the DMSO (vehicle control) in stimulated cells, by unpaired t-test **[Figure 5.9D]**. Furthermore, DHA and EPA treatments exerted no effect on IRF3 induction in response to LPS **[Figure 5.9D]**. Similarly, CLA was observed to suppress levels of IRF3 in U373 cells, while n-3 PUFA exerted no effect **[Figure 5.10A]**. Significantly, in a U373 cell line stably overexpressing CD14, the inhibitory effect of CLA on IRF3 was completely reversed **[Figure 5.10B]**.

5.2.6 EFFECTS OF PUFA ON LPS INDUCED ENDOCYTOSIS OF TLR4

HEK-MTC cells were cultured for 7 days with either DMSO (50 μM, vehicle control), CLA (50 μM), EPA (25 μM) or LA (50 μM). Subsequently, for the investigation of LPS-induced endocytosis of TLR4, HEK-MTC cells were transiently transfected with TLR4-YFP and EEA1-CFP (early endosomal antigen) constructs as described [see sections 2.8.3 – 2.9]. Cells were either stimulated for 7.5 min, 15 min or left unstimulated and analysed using an Olympus FluoView™ FV1000.

Results demonstrate that in resting cells treated with DMSO (vehicle control), TLR4 is localised on the plasma membrane with few early endosomes present **[Figure 5.11]**. In addition, there is little co-localisation of TLR4 and EEA1. As expected, following 7.5 min stimulation with LPS, TLR4 was rapidly endocytosed and is almost completely inside the cell. At this point, early endosome formation and co-localisation of EEA1 with TLR4 is more prominent. After 15 min of stimulation TLR4 has not returned to the plasma membrane however, there is significantly less co-localisation of the two molecules and less endosomes present.

In direct comparison to DMSO, PUFA treated cells displayed distinct patterns of TLR4 localisation and endocytosis. Indeed, while TLR4 was clearly localised on the plasma membrane in resting cells treated with CLA there was significantly more early endosome formation and co-localisation of EEA1 with TLR4 compared to DMSO **[Figure 5.12]**. Following stimulation with LPS at 7.5 min early endosome formation and EEA1 and TLR4 co-localisation was comparative to DMSO treated cells at this time point. However, significantly more TLR4 was retained at the plasma membrane. Similarly, at 15 min stimulation early endosome formation and EEA1 co-localisation with TLR4 was comparative to that of DMSO however; again TLR4 was seen to remain significantly at the membrane.

Conversely, TLR4 was extremely difficult to detect on the plasma membrane of resting cells treated with EPA **[Figure 5.13]**. Co-localisation of EEA1 and TLR4 was similar to that of resting cells treated with DMSO. Similar to CLA, treatment with this PUFA enhanced the number of endosomes in resting cells. Following 7.5 min stimulation practically all TRL4 was observed to be inside cells and co-

localised with EEA1. In addition, at 15 min stimulation there is a definite shift of TLR4 away from endosomes, however, more co-localisation is observed at this time point in EPA treated cells compared to DMSO. Interestingly, virtually no TLR4 is evident on the membrane. Cells treated with the saturated fatty acid control, LA, displayed patterns of TLR4 endocytosis and early endosome formation similar to those of DMSO-treated cells **[Figure 5.14]**.

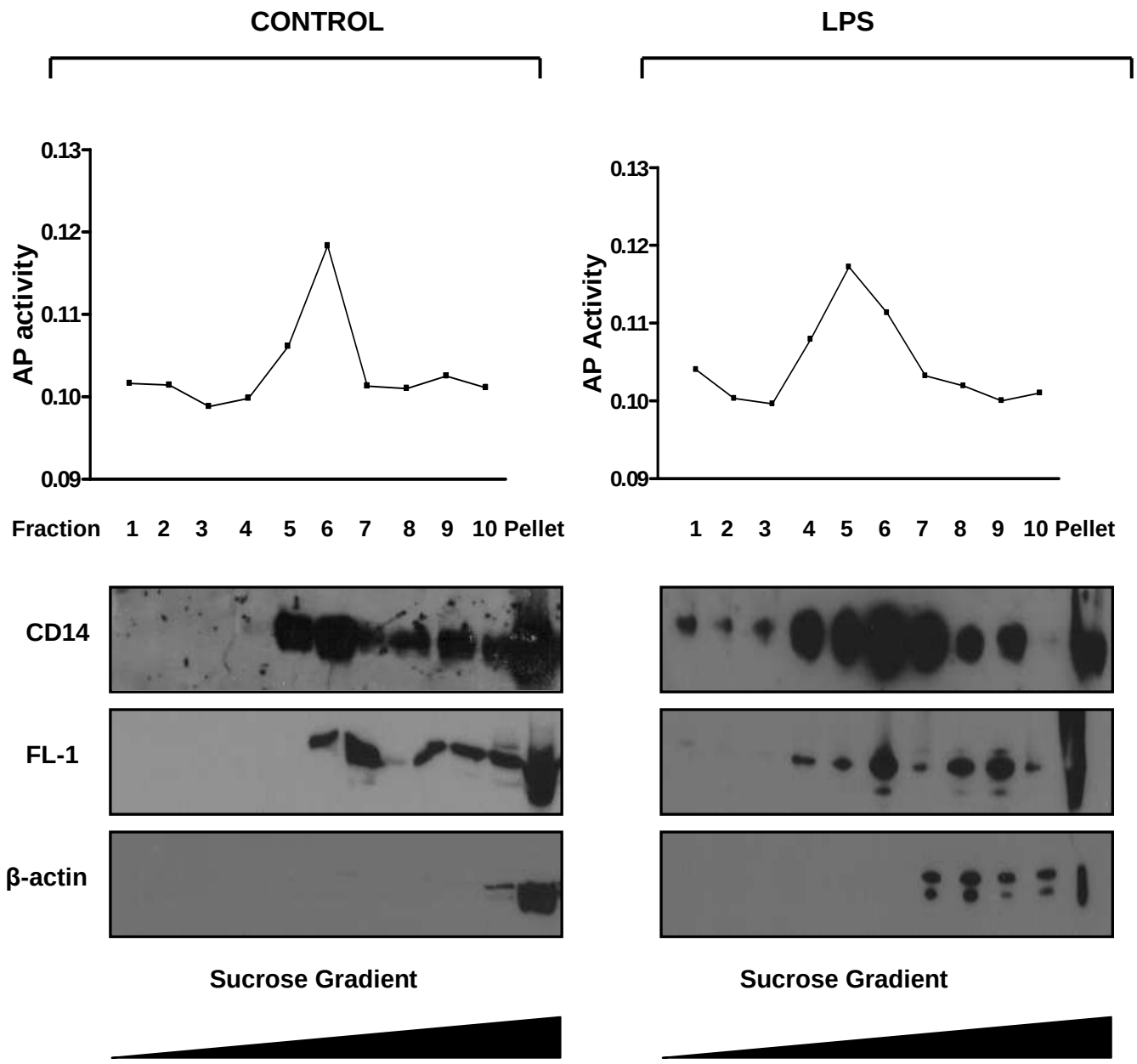


FIGURE 5.1: Isolation of lipid raft fractions by sucrose density gradient. Membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** J774 macrophage were solubilised in 0.2 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top (Fraction 1) to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

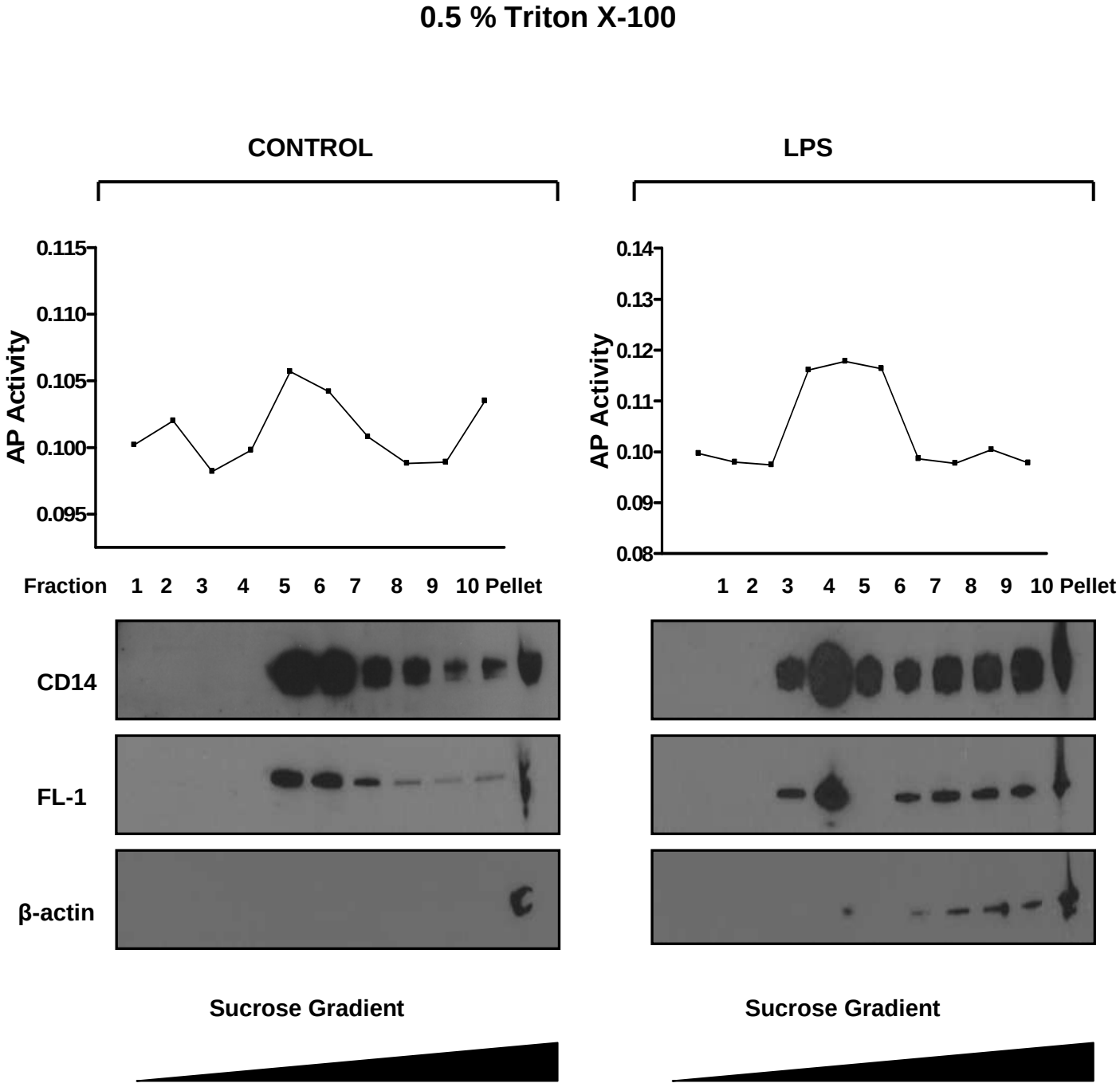


FIGURE 5.2: Isolation of lipid raft fractions by sucrose density gradient. Membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** J774 macrophage were solubilised in 0.5 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top (Fraction 1) to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

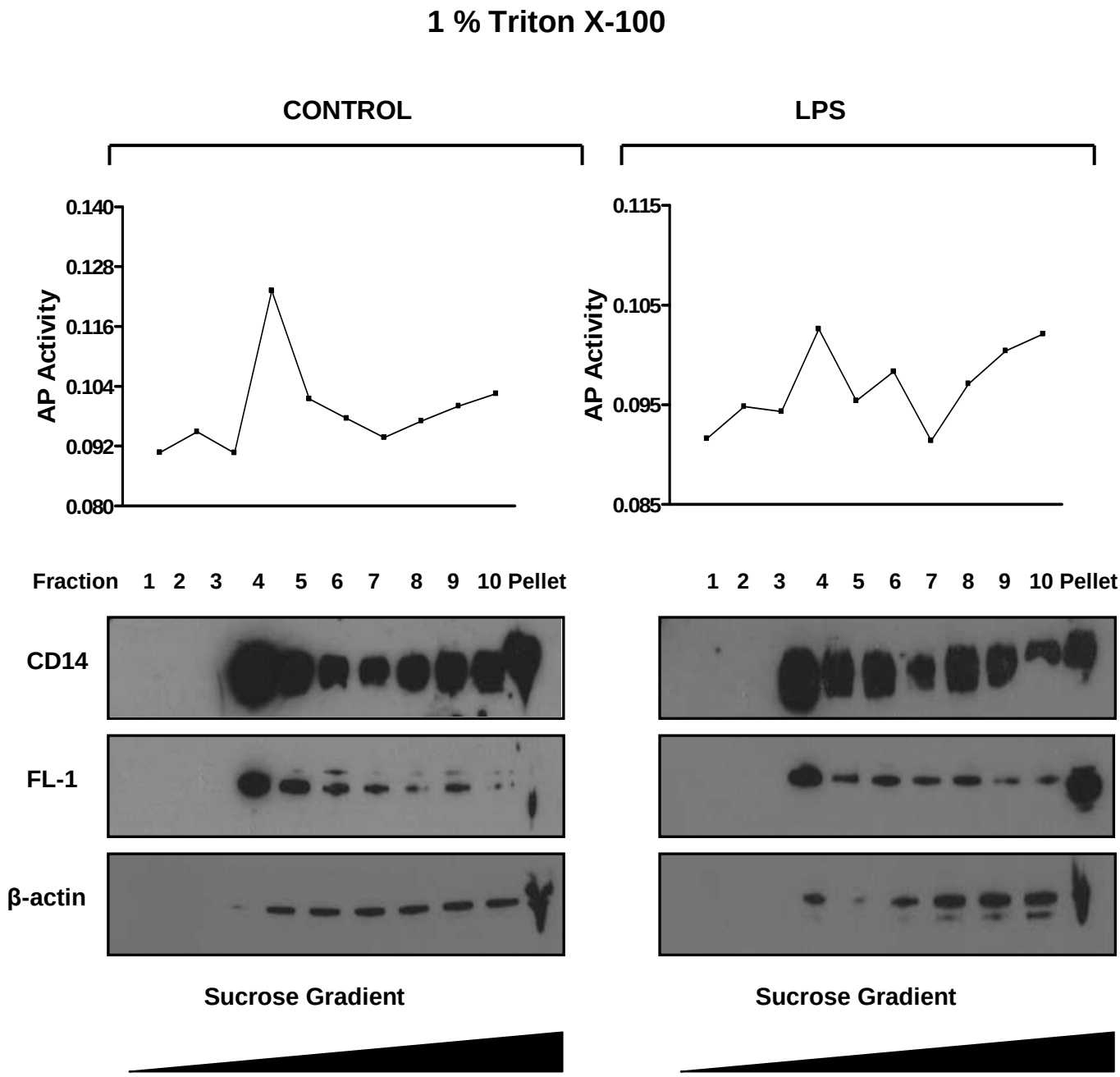


FIGURE 5.3 Isolation of lipid raft fractions by sucrose density gradient. Membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** J774 macrophage were solubilised in 1 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top (Fraction 1) to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

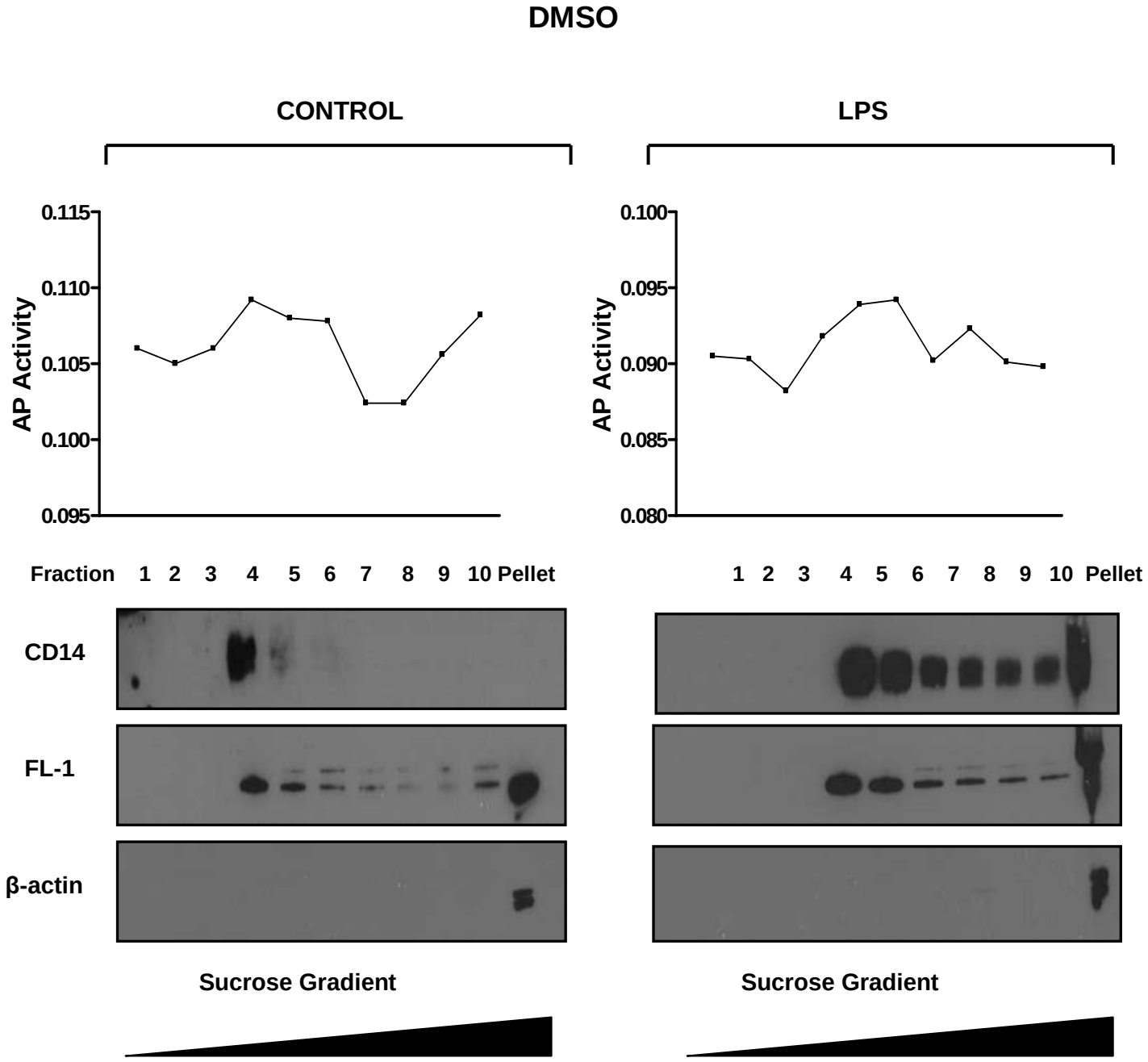


FIGURE 5.4: Isolation of lipid raft fractions by sucrose density gradient. J774 macrophage were treated with DMSO (50 μM) for 7 days. Subsequently, membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** cells were solubilised in 0.5 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

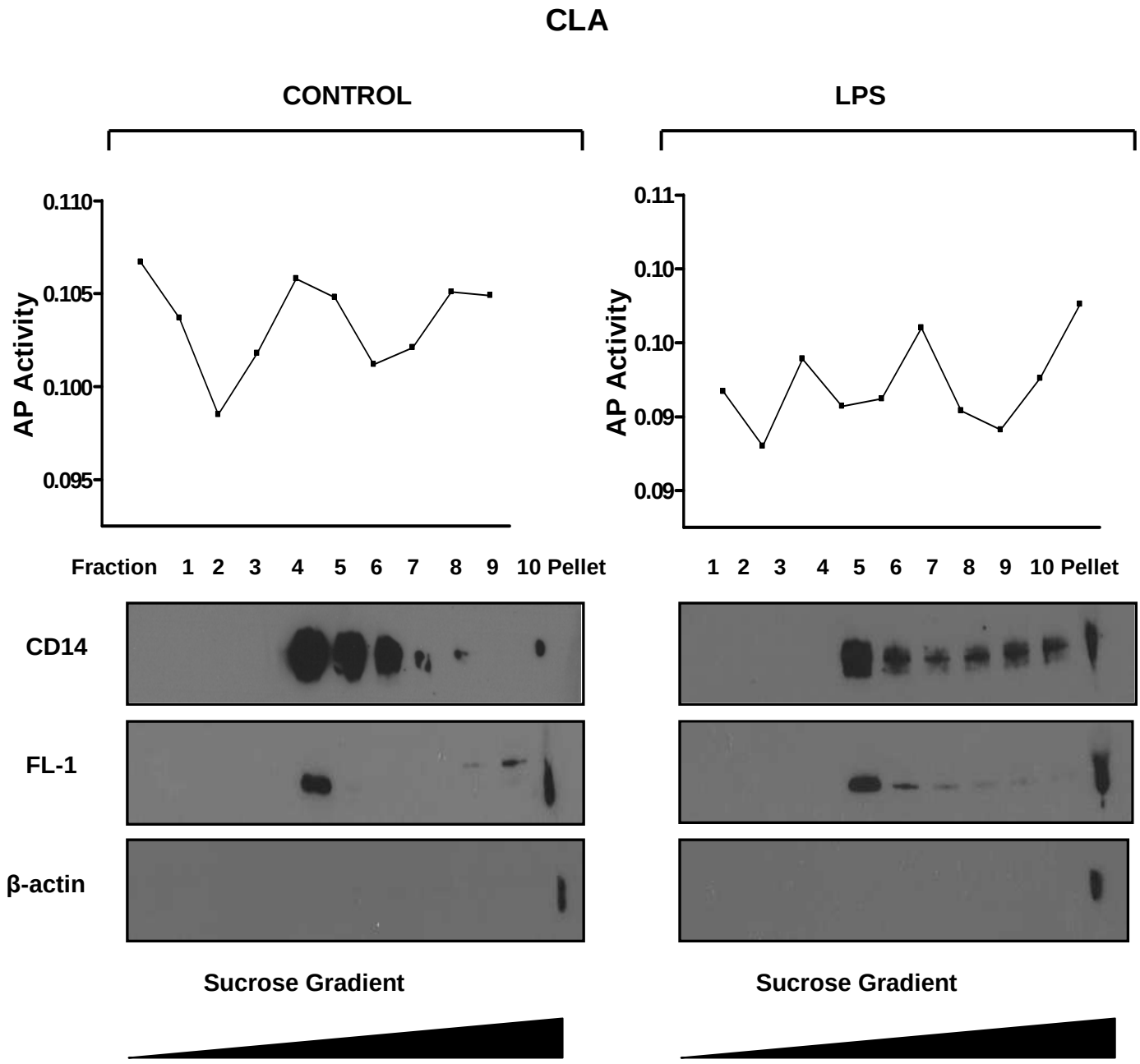


FIGURE 5.5: Isolation of lipid raft fractions by sucrose density gradient. J774 macrophage were treated with CLA (50 μM) for 7 days. Subsequently, membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** cells were solubilised in 0.5 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top (Fraction 1) to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

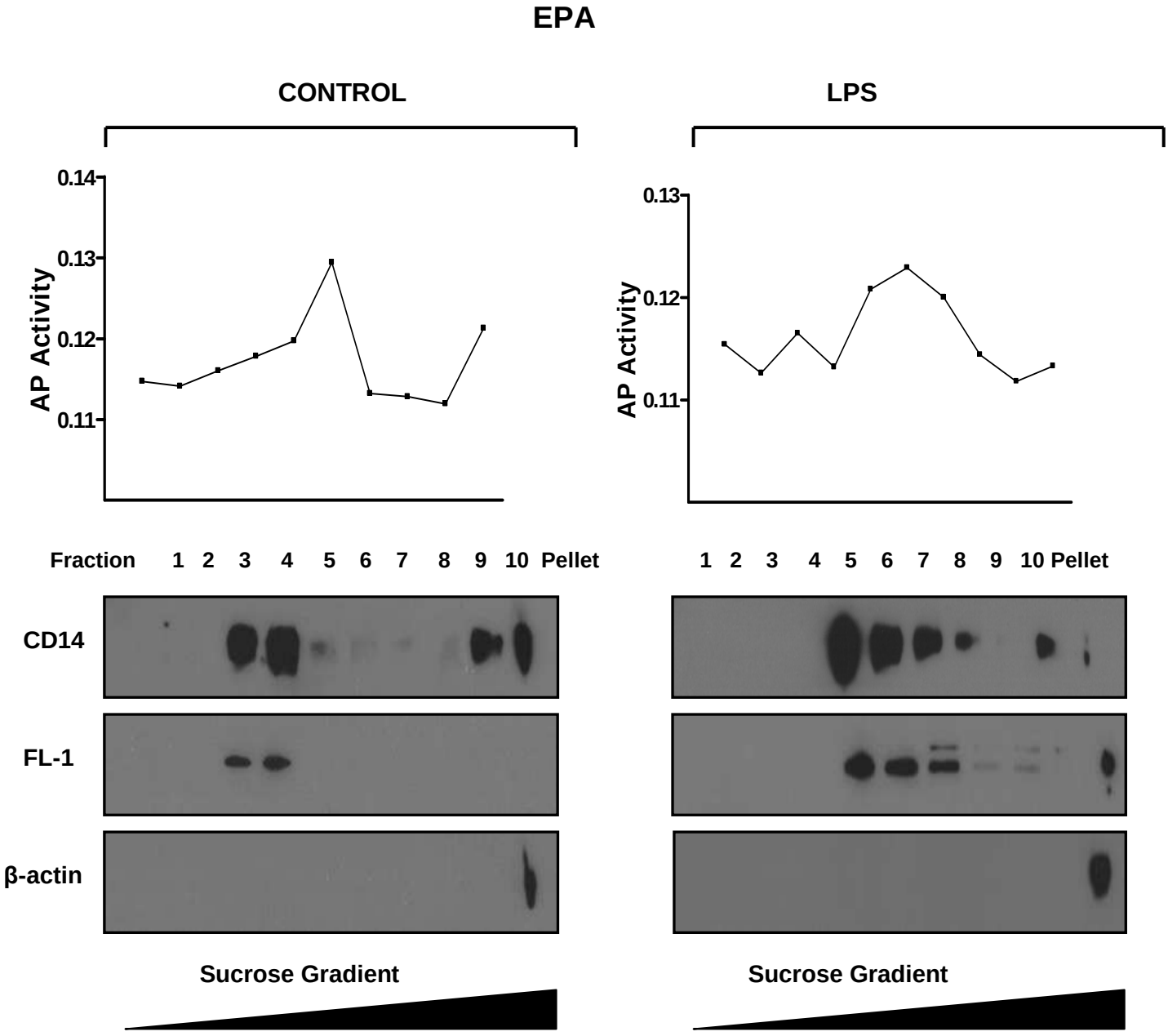


FIGURE 5.6: Isolation of lipid raft fractions by sucrose density gradient. J774 macrophage were treated with EPA (25 μM) for 7 days. Subsequently, membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** cells were solubilised in 0.5 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top (Fraction 1) to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

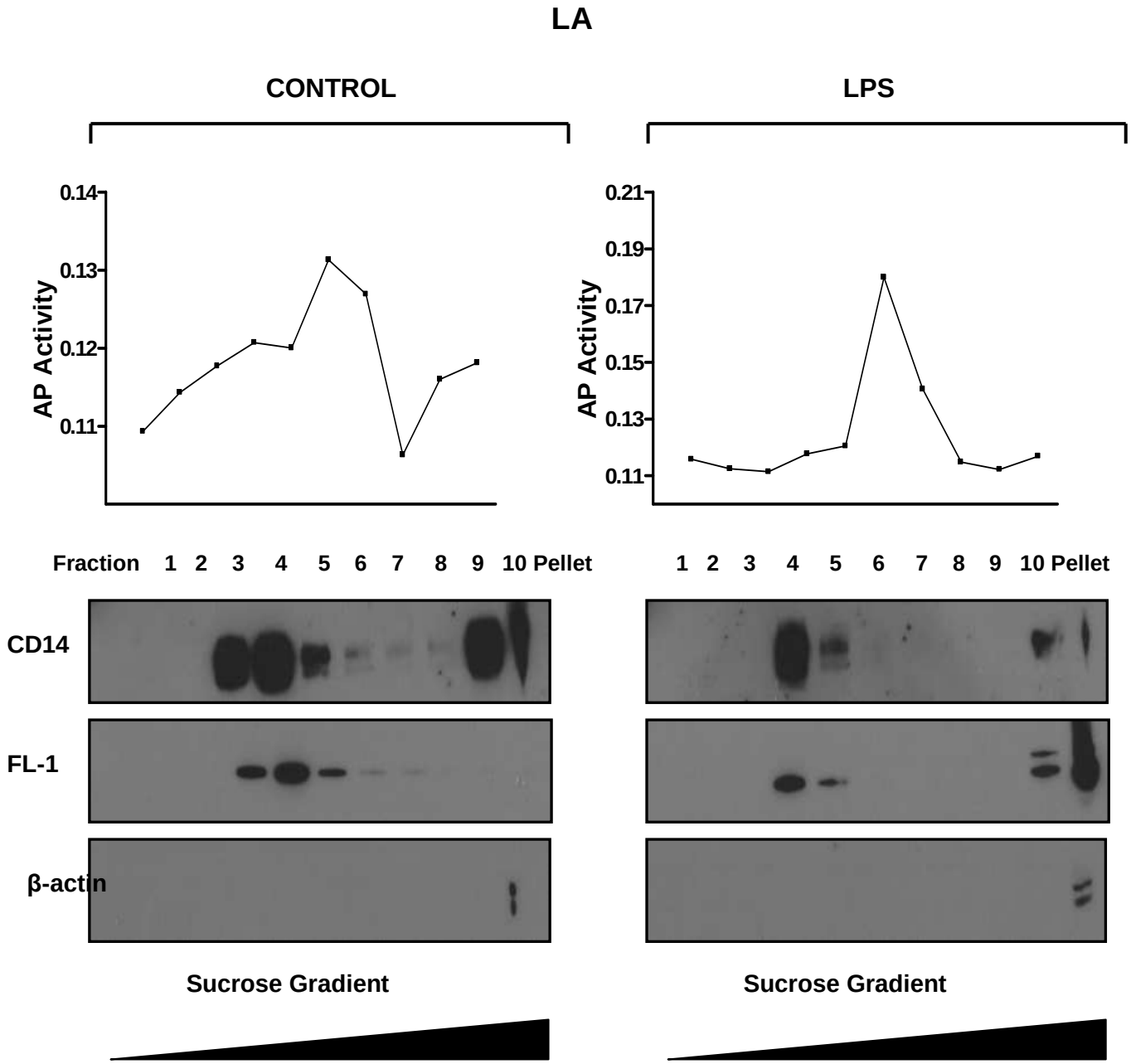


FIGURE 5.7: Isolation of lipid raft fractions by sucrose density gradient. J774 macrophage were treated with LA (50 μM) for 7 days. Subsequently, membrane preparations from unstimulated **[CONTROL]** and stimulated **[LPS]** cells were solubilised in 0.5 % (v/v) Triton X-100 before application on a discontinuous sucrose gradient. Following 16 h of centrifugation, 1 ml fractions were harvested from the top (Fraction 1) to the bottom (pellet) of the gradient. Fractions were analysed for alkaline phosphatase activity (graph), raft marker flotillin-1 (FL-1), non-raft marker β-actin and GPI-anchored protein, CD14 (blots).

PUFA LIPID RAFTS FRACTIONS AND CD14

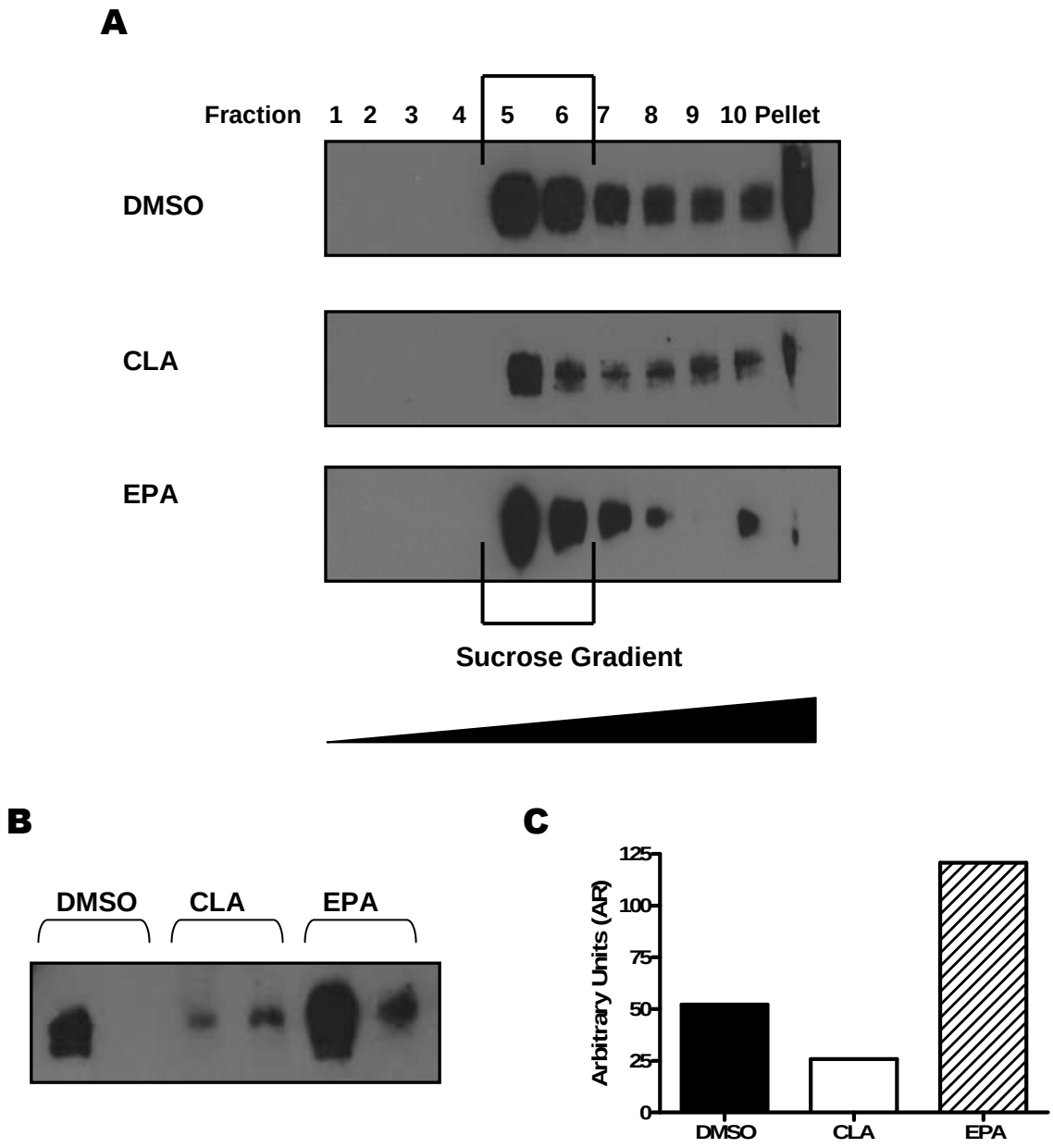


FIGURE 5.8: Quantitative analysis of CD14 levels in lipid rafts from PUFA-treated cells stimulated with LPS. J774 macrophage were treated with DMSO (50 μM), CLA (50 μM) or EPA (25 μM) for 7 days and stimulated with LPS (100ng/ml) for 30 min. Qualitative detection of CD14 in lipid rafts from PUFA treated cells is shown **[A]**. Additionally, protein levels were equalised and quantitative analysis of CD14 levels in lipid rafts (Fractions 5-6) was performed by western blot **[B]**. Densitometric analysis was conducted on the immunoblot and graphical representation of CD14 expression in arbitrary units (AU) is given **[C]**.

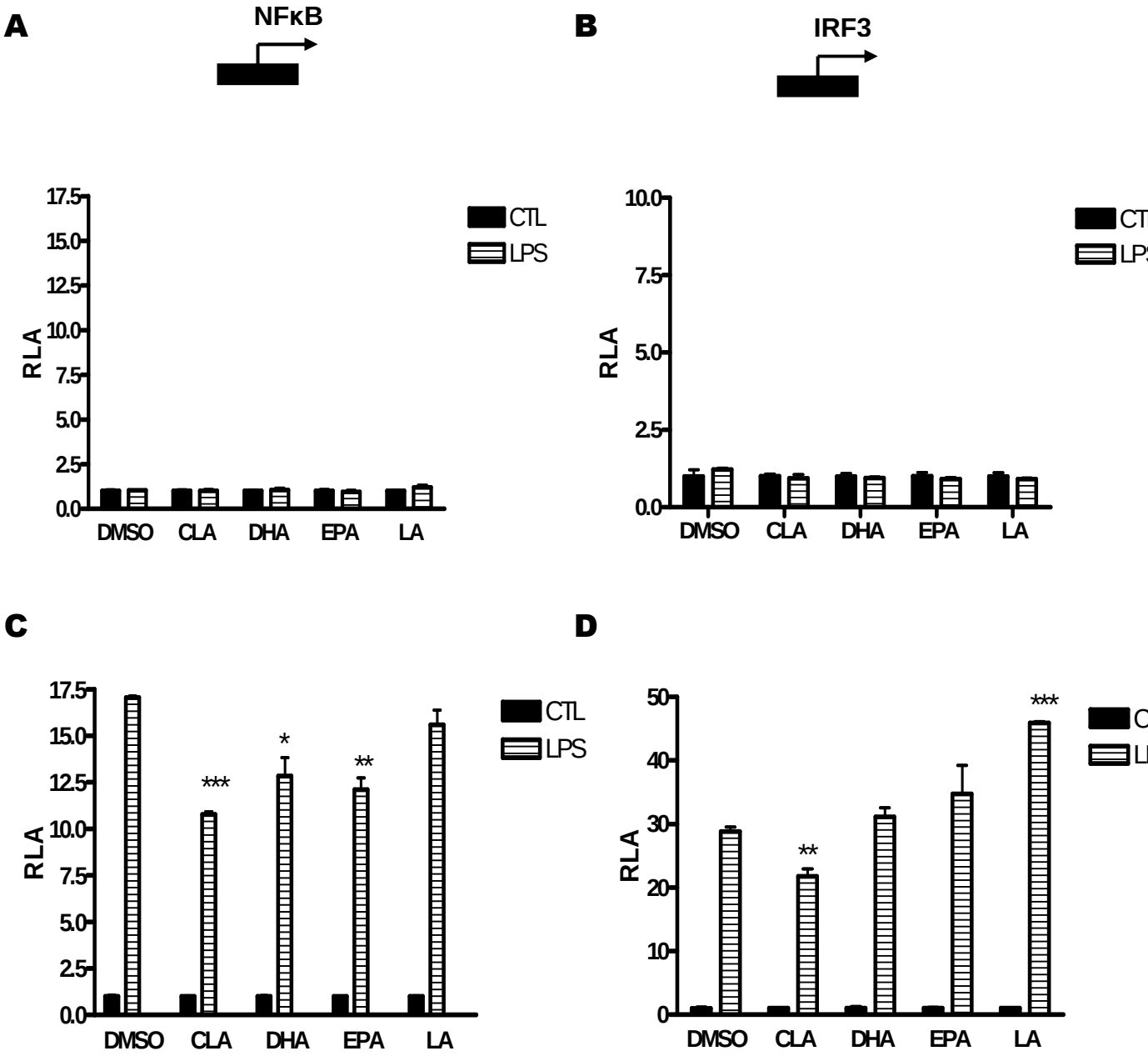


FIGURE 5.9: The effect of PUFA on the induction of NFκB and ISRE (IRF3 response element) was assessed using luciferase reporter assays. HEK 293 **[A&B]** and HEK-MTC cells **[C&D]** were cultured for 7 days with either DMSO (50 µM, vehicle control), EPA (25 µM), DHA (25 µM), CLA (50 µM) or LA (50 µM). Subsequently, cells were transiently transfected with either an NFκB or ISRE luciferase reporter plasmid. Induction of both transcription factors was assessed following 6 h stimulation with LPS (100 ng/ml).

Statistical analysis was carried out between DMSO and PUFA-treated groups using an unpaired t-test.

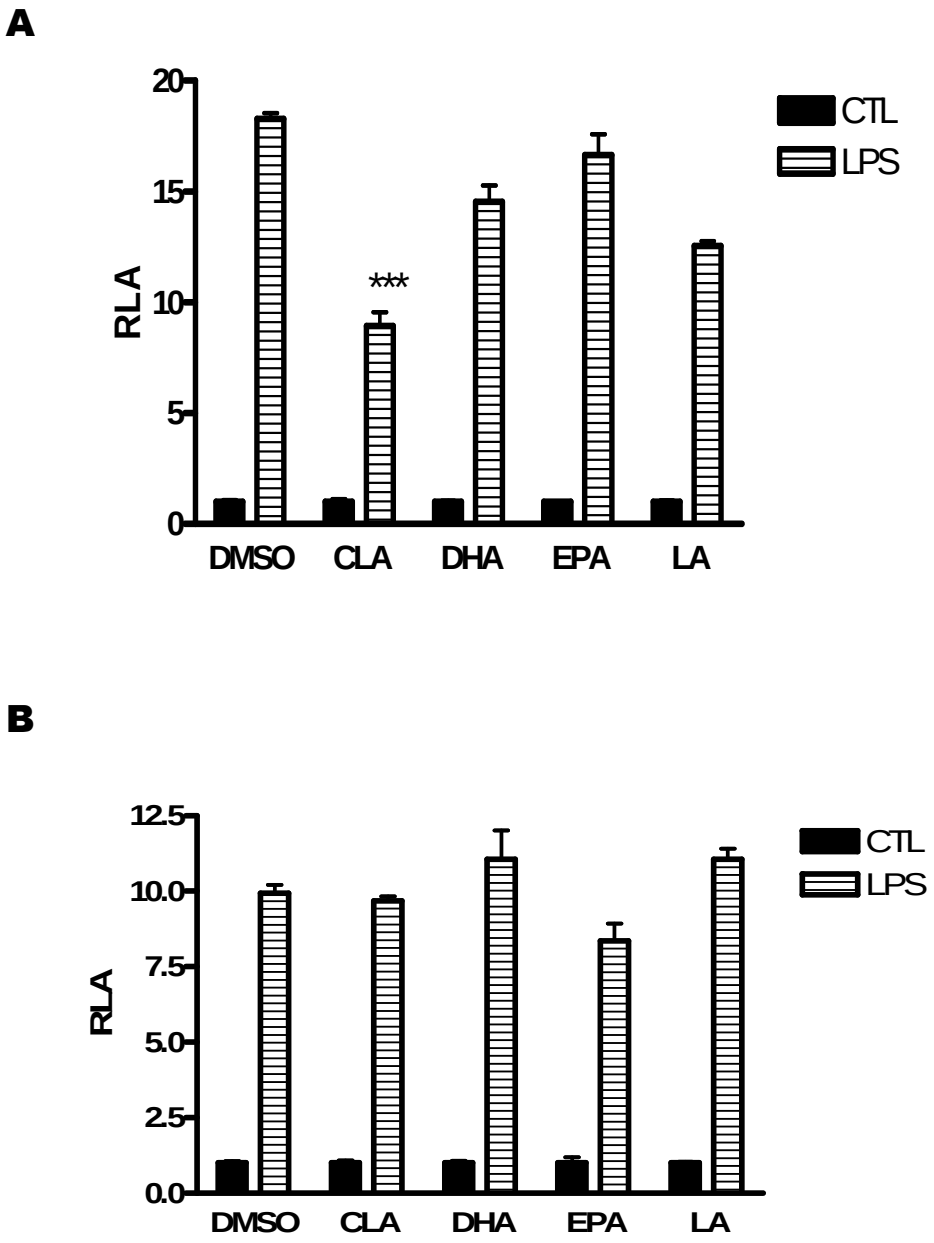


FIGURE 5.10: Over-expression of CD14 reverses the inhibitory effect of CLA on IRF3 actiavtion. U373 cells **[A]** and U373-CD14 cells **[B]** were cultured for 7 days with either DMSO (50 µM, vehicle control), EPA (25 µM), DHA (25 µM), CLA (50 µM) or LA (50 µM). Subsequently, cells were transiently transfected with an ISRE luciferase reporter plasmid. Induction of IRF3 was assessed following 6 h stimulation with LPS (100 ng/ml). Statistical analysis was carried out between DMSO and PUFA-treated groups using an unpaired t-test.

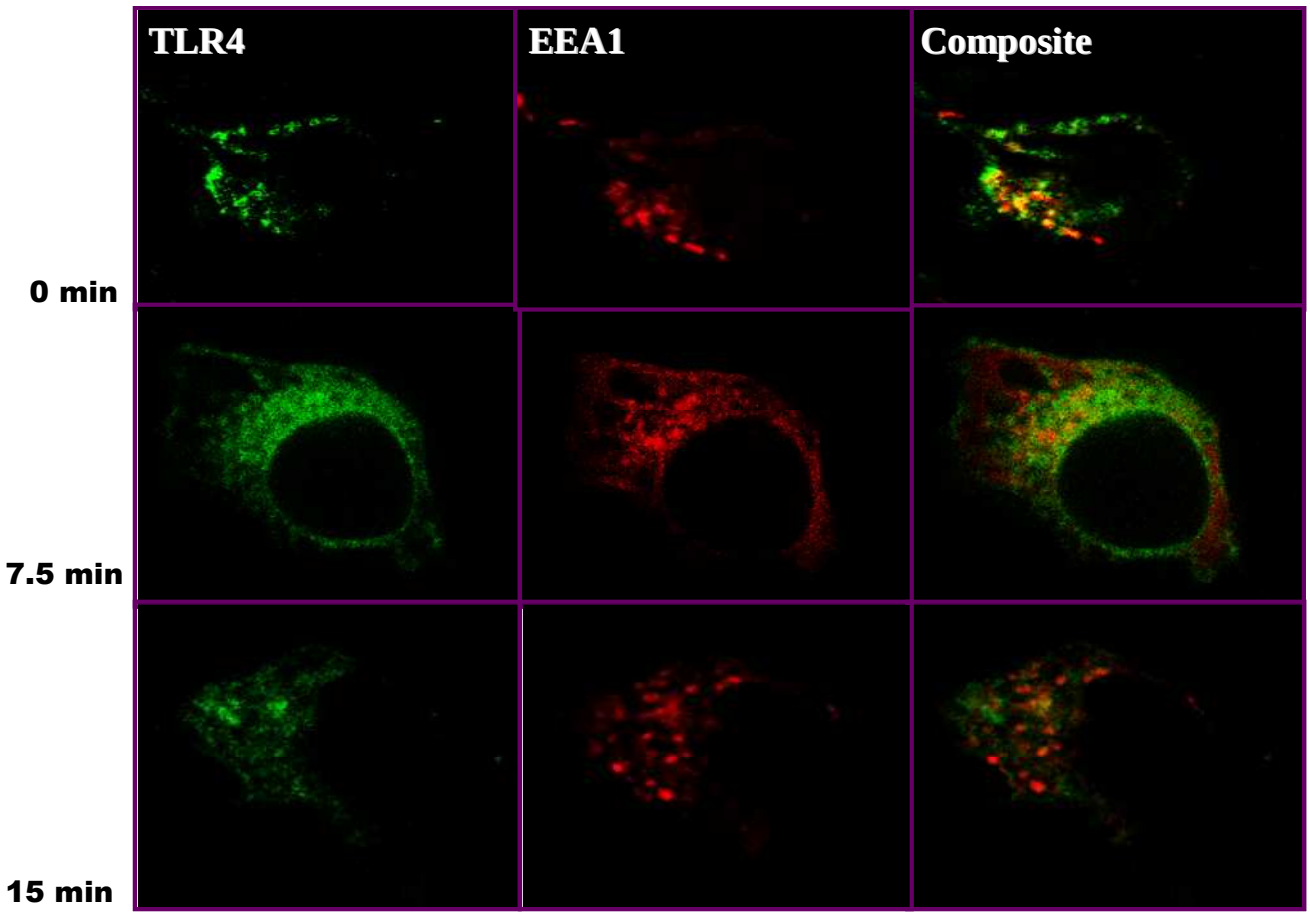


FIGURE 5.11: LPS-induced endocytosis of TLR4 in DMSO treated cells. HEK-MTC cells were cultured in DMSO (50 μM) for 7 days. Subsequently cells were transiently transfected with TLR4-YFP (green) and EEA1-CFP (red) plasmids. Cells were left unstimulated or stimulated with LPS (250 ng/ml) for 7.5 and 15 min. Endocytosis of TLR4 and its localisation relevant to EEA1 was assessed by confocal microscopy. Co-localisation of TLR4 and EEA1 is shown in the composite image (yellow).

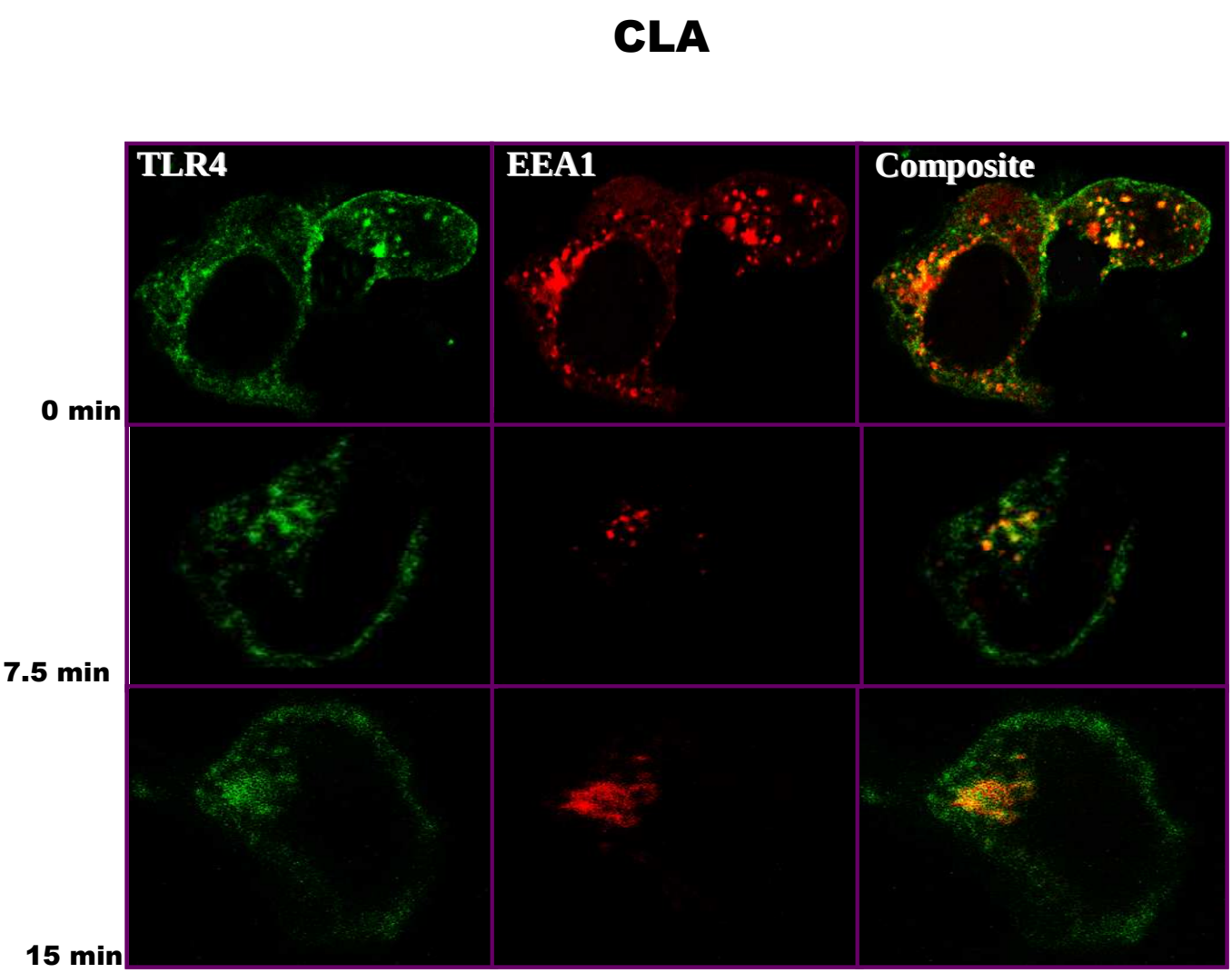


FIGURE 5.12: LPS-induced endocytosis of TLR4 in CLA-treated cells. HEK-MTC cells were cultured in CLA (50 μM) for 7 days. Subsequently cells were transiently transfected with TLR4-YFP (green) and EEA1-CFP (red) plasmids. Cells were left unstimulated or stimulated with LPS (250 ng/ml) for 7.5 and 15 min. Endocytosis of TLR4 and its localisation relevant to EEA1 was assessed by confocal microscopy. Co-localisation of TLR4 and EEA1 is shown in the composite image (yellow).

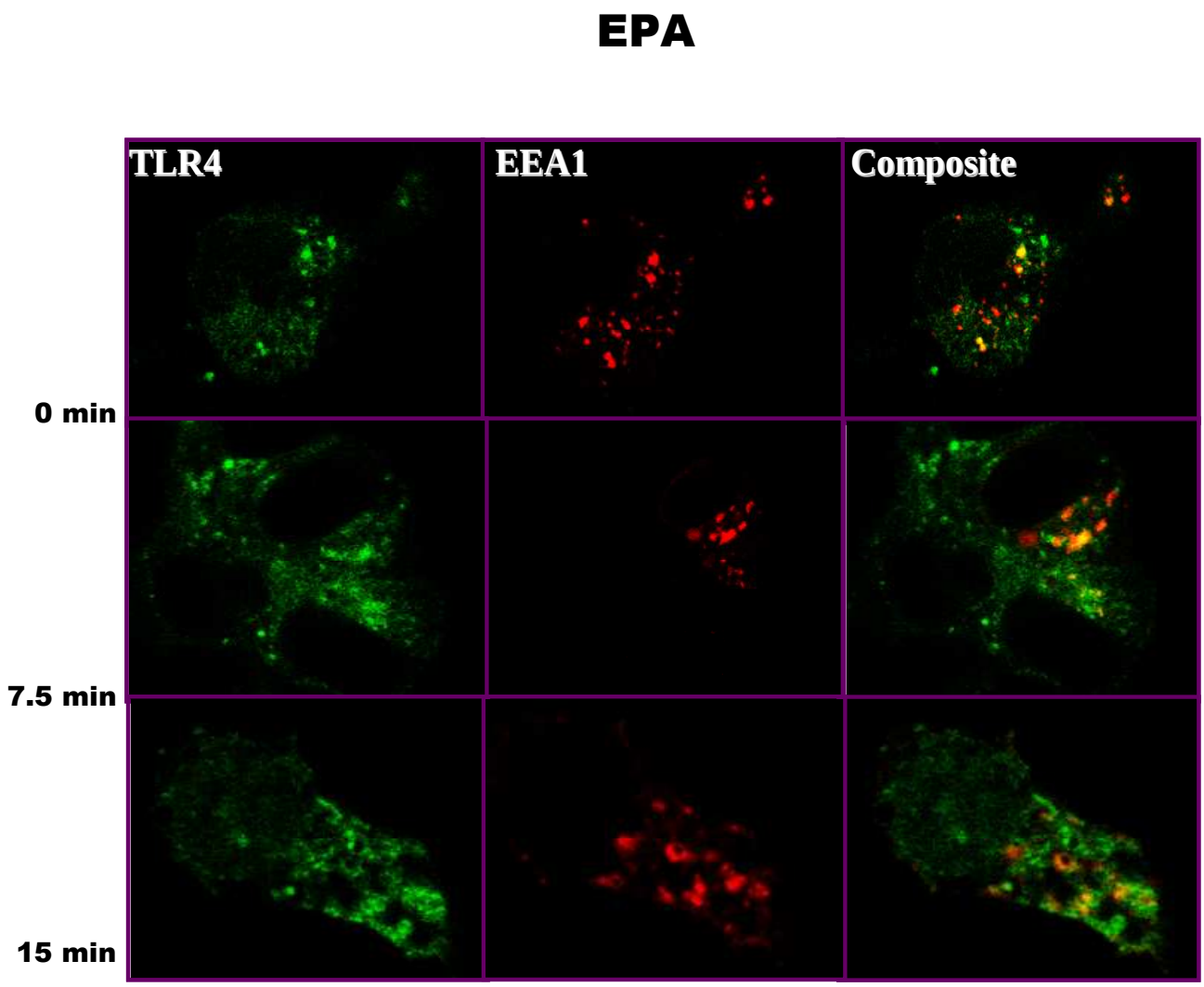


FIGURE 5.13: LPS-induced endocytosis of TLR4 in EPA-treated cells. HEK-MTC cells were cultured in EPA (25 μM) for 7 days. Subsequently cells were transiently transfected with TLR4-YFP (green) and EEA1-CFP (red) plasmids. Cells were left unstimulated or stimulated with LPS (250 ng/ml) for 7.5 and 15 min. Endocytosis of TLR4 and its localisation relevant to EEA1 was assessed by confocal microscopy. Co-localisation of TLR4 and EEA1 is shown in the composite image (yellow).

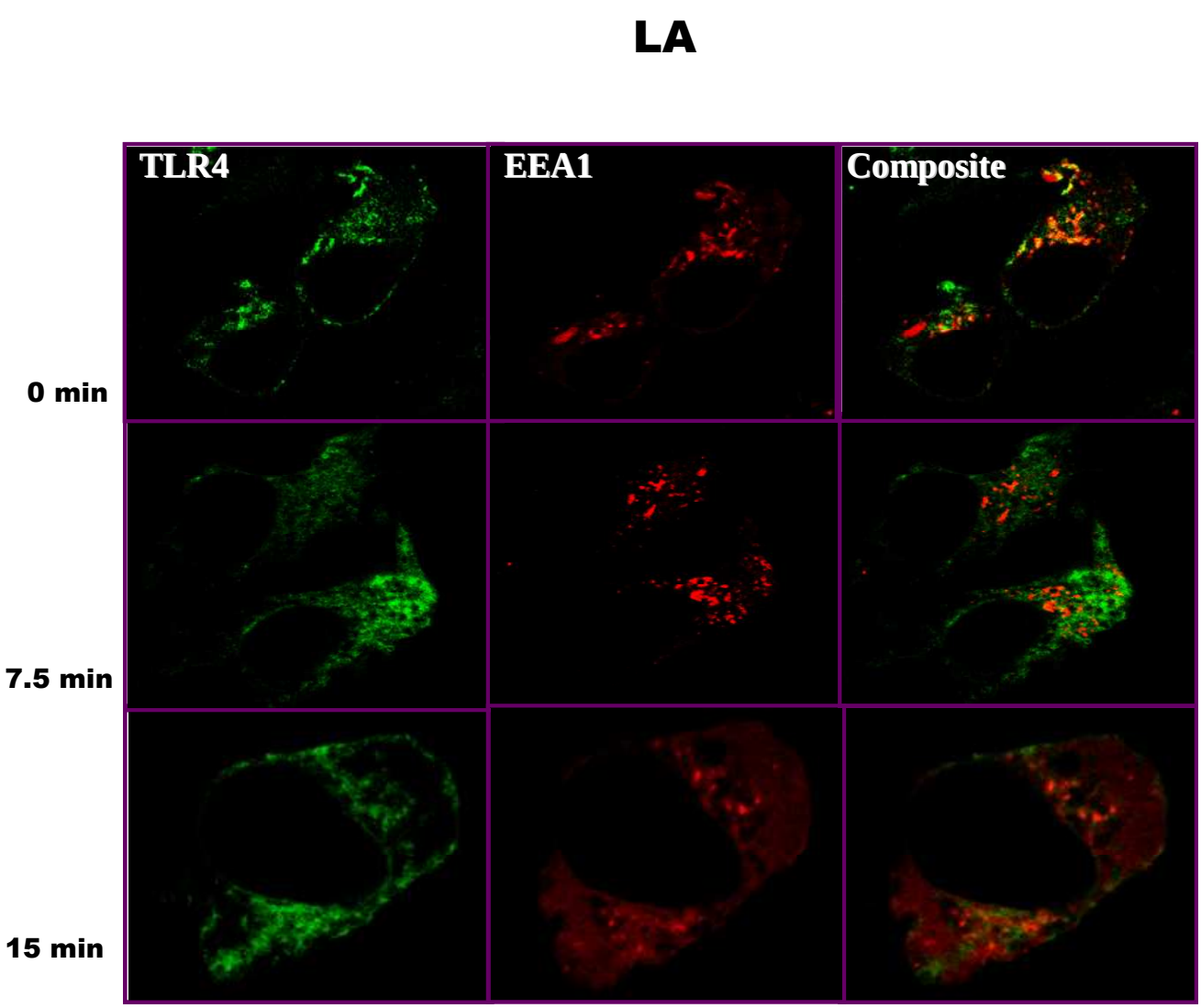


FIGURE 5.14: LPS-induced endocytosis of TLR4 in LA-treated cells. HEK-MTC cells were cultured in LA (50 μM) for 7 days. Subsequently cells were transiently transfected with TLR4-YFP (green) and EEA1-CFP (red) plasmids. Cells were left unstimulated or stimulated with LPS (250 ng/ml) for 7.5 and 15 min. Endocytosis of TLR4 and its localisation relevant to EEA1 was assessed by confocal microscopy. Co-localisation of TLR4 and EEA1 is shown in the composite image (yellow).

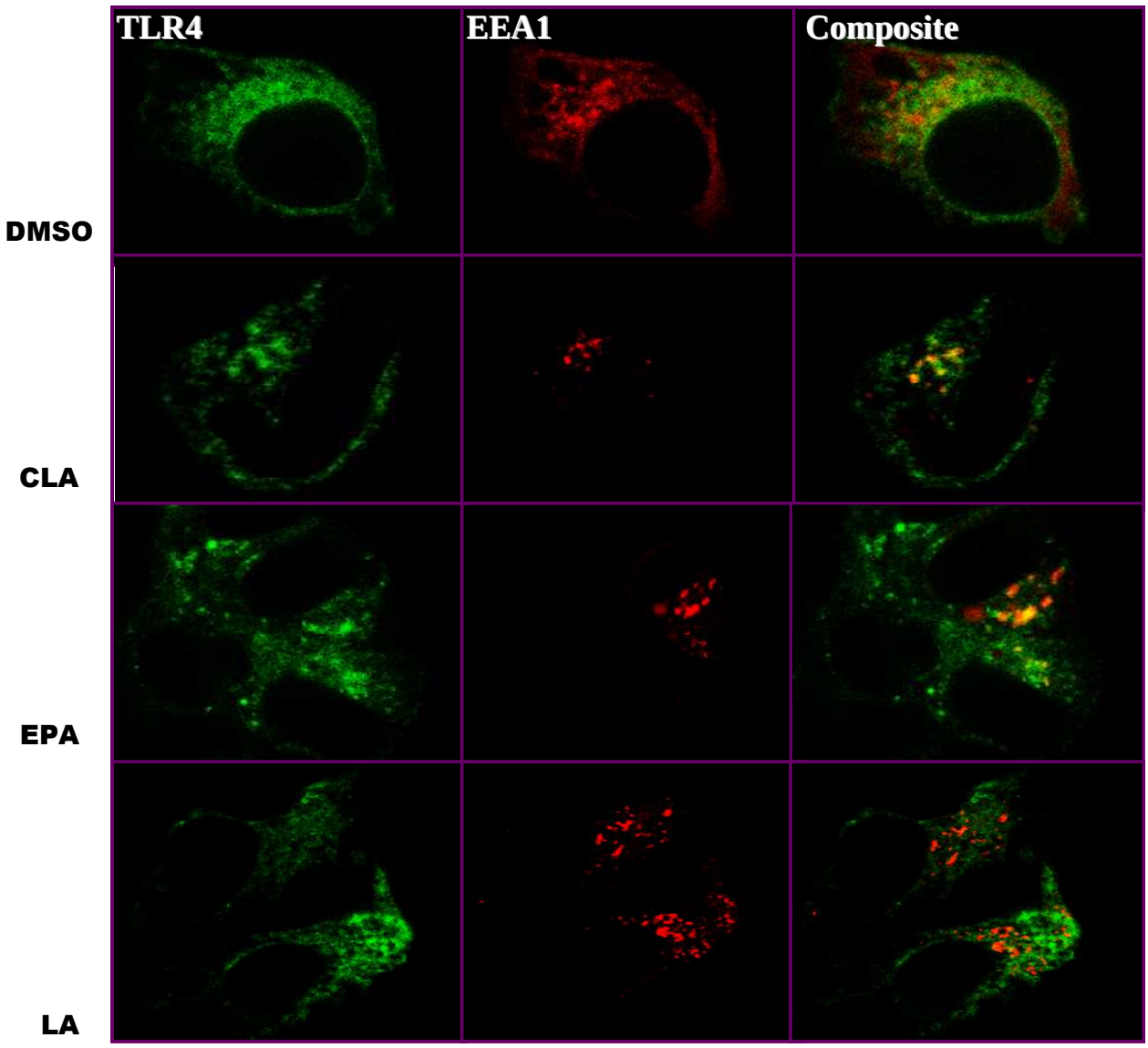


FIGURE 5.15: Representative LPS-induced endocytosis of TLR4 in PUFAtreated cells after 15 min of stimulation. HEK-MTC cells were cultured in DMSO (vehicle control), EPA (25 μM), CLA (50 μM), – or a saturated fatty acid control, LA (50 μM), for 7 days. Subsequently cells were transiently transfected with TLR4-YFP (green) and EEA1-CFP (red) plasmids. Cells were stimulated with LPS (250 ng/ml) 15 min. Endocytosis of TLR4 and its localisation relevant to EEA1 was assessed by confocal microscopy. Co-localisation of TLR4 and EEA1 is shown in the composite image (yellow).

5.3 DISCUSSION

Mounting evidence points towards the beneficial effects of PUFA in the prevention and treatment of inflammatory disease. This has prompted widespread research in relation to PUFA-mediated anti-inflammatory effects with the hope of elucidating the exact mechanisms which can in turn be harnessed for therapeutic use. Currently, the field has focused its attention on the effects of PUFA on the events downstream of receptor activation, specifically those mediated by modified eicosanoid production and gene expression, (Calder 2006, Sampath and Ntambi 2005, Schmitz and Ecker 2008).

However, thus far our results highlight the direct effects of PUFA on key surface markers, particularly CD14. As a result, we hypothesized, that the plasma membrane may be a principal target through which the modulatory effects of PUFA are mediated. Indeed, it is well established that by their very nature PUFA become incorporated into the plasma membrane. Furthermore, a keynote paper published by (Lee et al. 2003) and colleagues supports this hypothesis. Fundamentally, the group demonstrated that in RAW 264.7 cells, DHA inhibited LPS-induced NFκB activation in the presence of key signaling dominant negative mutants with the introduction of constitutively activate TLR4 (TLR4-CA). Furthermore, DHA failed to inhibit NFκB activation in the presence of dominant negatives and introduction of MyD88-CA or AKT-CA constructs. This indicated that PUFA may exert their effects at the membrane, at the receptor complex itself.

Given these findings and the overwhelming observations of our study regarding PUFA modulated membrane-bound CD14, the targeting of CD14 to key signaling domains known as ‘lipid rafts’ became the first focus of this study. Importantly, in

order to critically assess the effects of PUFA on CD14 lipid raft recruitment; a lipid raft isolation procedure was stringently optimized. Lipid rafts are isolated based on the insolubility of raft proteins and lipids in the presence of non-ionic detergents. It is crucially important to obtain a detergent concentration that remains low enough not to solubilise tightly packed lipid raft domains and yet solubilise the remainder of the membrane or 'disordered domain'. Detergent insolubility is a relatively, cheap and widely used method for the isolation of these domains. However, the type and concentration of detergent used will vary from cell type and must be appropriately tested to insure separation of raft and non-raft proteins (Shogomori and Brown 2003, Chamberlain 2004). Indeed, results presented here demonstrate that concentrations lower that 0.5 % Triton X-100 were insufficient to solubilise non-raft material. Furthermore, concentrations any higher resulted in complete disruption of lipid raft ordered domains.

As such, 0.5 % Triton X-100 was successfully used for the isolation of lipid raft domains from J774 macrophage. This concentration of detergent afforded complete separation of raft marker flotillin-1, FL-1 from non-raft marker, β-actin. In addition, alkaline phosphatase activity, indicative of lipid rafts, was notably high in fractions in which FL-1 was detected. As expected, being a GPI-anchored protein, CD14 could be successfully detected in raft fractions. Notably, alkaline phosphatase activity was used in this study as a marker of lipid raft partitioning at the interphase of sucrose gradients (Gargalovic and Dory 2003). While activity corresponded to FL-1 localisation in untreated cells, PUFA treated cells displayed skewed alkaline phosphatase activity specifically when treated with CLA. As such, we suggest that PUFA in some way compromise alkaline phosphatase in lipid rafts and that its activity should be used solely during optimisation procedures.

In keeping with our previous findings, PUFA distinctly modulated recruitment of CD14 to lipid raft domains in LPS-stimulated cells. CLA was seen to significantly suppress recruitment of CD14 to lipid raft domains following stimulation. Furthermore, EPA acutely enhanced levels of CD14 in lipid raft fractions of stimulated cells compared to control. These are completely novel findings in relation to PUFA and lipid rafts, specifically regarding CLA.

Certainly to date, studies have documented the effect of PUFA, particularly, n-3 PUFA on membrane composition and report alterations in size and distribution of rafts (Li et al. 2005, Chapkin et al. 2008b, Ma et al. 2004). However, studies have also generally focused on the effect of PUFA on T cell function via alteration of the lipid raft structure and indeed translocation of signalling molecules. Given the potent inflammatory potential of T cells this research is no doubt warranted. Nevertheless, mechanisms utilised by PUFA regarding T cells and their lipid raft status may indeed be extended to other cell types. The effects of PUFA on T cell lipid rafts has been extensively reviewed (Yaqoob 2009). For example, it has been demonstrated that changes in the fatty acid composition of lipid rafts was associated with a decrease in the translocation of protein kinase C, PKC to lipid rafts, a key molecule regulating $CD4^{+}$ T cell activation(Chapkin et al. 2008a)(Fan et al. 2004).

To the best of our knowledge no other anti-inflammatory molecules have been reported to alter lipid raft composition or function to the extent of PUFA. With regard to our finding in relation to CD14, two studies are of particular interest. (De Smedt-Peyrusse et al. 2008) report that DHA affects the proteins presentation but

not its localisation to lipid rafts. In contrast to this, (Chapkin et al. 2008b) report that DHA specifically increases clustering of signalling proteins to lipid raft domains. Furthermore, only one study has cited alterations in the partitioning of CD14 to lipid rafts by an anti-inflammatory molecule. (Dai, Zhang and Pruett 2005) demonstrate that ethanol, EtOH suppresses LPS-induced TNFα and CD14 recruitment to lipid rafts in RAW 264.7 cells. Dai and colleagues suggest that EtOH may cause subtle changes in the lipid portion of rafts, causing changes in clustering or sequestration of proteins within them. Recently it has been shown that GPI-anchored proteins contain two saturated fatty acid chains in their phosphatidylinositol, PI moiety which allow them to be incorporated to lipid rafts (Maeda et al. 2007). The remodelling of the GPI anchor takes place between the ER and Golgi with the unsaturated sn-2 chain being removed and a saturated one added. This process is mediated by post GPI-attachment proteins (PGAP2 and PGAP3). Furthermore, S-acylation of proteins with heterogenous unsaturated fatty acids has been proposed as a mechanism by which cells regulate signalt transduction by altering the association of proteins with rafts (Webb, Hermida-Matsumoto and Resh 2000, Liang et al. 2001). Our findings that CLA reduced the incorporation of CD14 into the lipid raft, may suggest that the fatty acid (Webb, Hermida-Matsumoto and Resh 2000, Liang et al. 2001)can interfere with these processes.

We suggest the membrane action of CLA regarding CD14 recruitment to lipid rafts may be a principal mechanism by which this fatty acid suppresses TLR4 signalling. Indeed this finding compounded our interest regarding the effects of CLA on TLR4 downstream signalling. Thus far the field has focused completely on the suppressive effects of PUFA on NFκB, however TLR4 signalling is unique in its

ability to activate both NFκB and IRF3 via MyD88 dependant and independent pathways, respectively (Brikos and O'Neill 2008, Akira and Takeda 2004). We have previously demonstrated that CLA can suppress NFκB in dendritic cells (Loscher et al. 2005b) and in adipose tissue from mice fed on diets of CLA (Moloney et al. 2007). Furthermore, we have also reported the suppressive effects of EPA and DHA on NFκB in a human macrophage cell line (Weldon et al. 2007, Mullen, Loscher and Roche 2009). The data generated here agree with these previous studies and demonstrate that CLA, EPA and DHA all suppress NFκB activation. However, interestingly our data also demonstrates a completely novel and selective inhibition of IRF3 in PUFA-treated cells. While both n-3 PUFA and CLA suppressed levels of NFκB, CLA was the only fatty acid to significantly suppress IRF3. Furthermore, we postulated that this was indeed related to the suppression of CD14 at the membrane in CLA-treated cells. Particularly as the activation of IRF3 downstream of TLR4 requires endocytosis of the TLR4 complex and its subsequent association with TRIF and TRAM (Kagan et al. 2008), a process that is dependent on CD14 (Shuto et al. 2005). Certainly, in cells over-expressing CD14 the inhibitory effect of CLA on IRF3 was completely reversed.

In order to assess whether the suppression of CD14 was associated with reduced endocytosis of TLR4, which would explain the decrease in IRF3 activation, we used confocal microscopy to examine TLR4 expression at the membrane and in early endosomes in cells treated with CLA and EPA. Given that the involvement of CD14 in TLR4 endocytosis has only been reported in the last few years (Shuto et al. 2005), there is a lack of literature examining the effects of anti-inflammatory compounds on these parameters as a potential mechanism. Indeed the only paper we found reported a positive correlation between increased CD14 with increased

consequences of TLR4 activation. Specifically, (Hua et al. 2007) report that an extract of Ganoderma lucidum polysaccharides enhances CD14 expression which results in a subsequent increase in pro-inflammatory cytokine production downstream of TLR4. In our study we clearly demonstrate retention of TLR4 on the plasma membrane following stimulation with LPS in CLA-treated cells indicating a decrease in endocytosis of TLR4 compared to the DMSO control. This was not the case in EPA-treated cells. Our data has revealed a novel mechanism for how CLA exerts its anti-inflammatory effects in macrophage. Furthermore, to our knowledge this is the first report of an anti-inflammatory compound exerting its effects via a CD14-dependent suppression of TLR4 endocytosis. A schematic illustrating our proposed model for PUFA-mediated effects on TLR4 signalling is presented in figure 5.15.

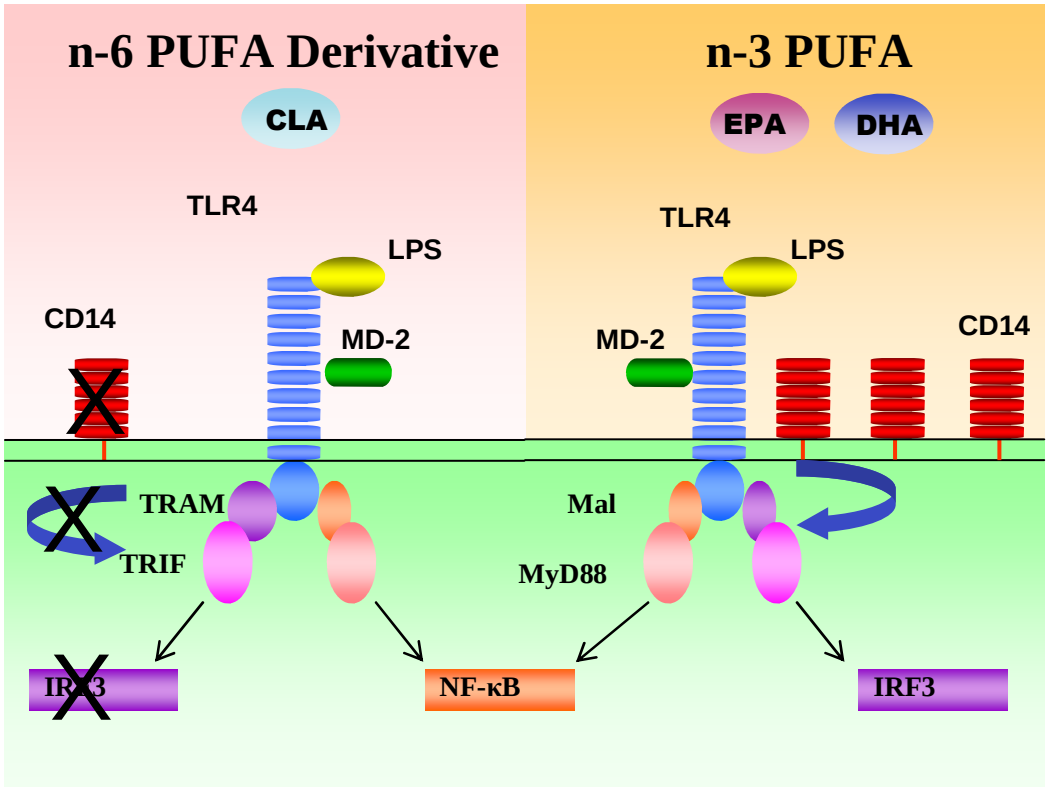


FIGURE 5.15: Illustration of our proposed model of PUFA-mediated effects on TLR4 signalling. Both CLA and n-3 PUFA, EPA and DHA suppress NFκB. CLA selectively inhibits signalling through adaptors TRAM and TRIF suppressing IRF3 by means of inhibiting recruitment of membrane bound CD14 to the TLR4 complex in lipid rafts.

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

Inflammation is the body's natural response to infection or injury in most circumstances. However, when it becomes dysregulated this can lead to extensive tissue damage, uncontrolled infection and chronic disease. Ironically, inflammation is also vital in order to promote resolution (Serhan et al. 2007) and considerable efforts are placed on elucidating mechanisms by which anti-inflammatory pathways may be enhanced to promote this process. Furthermore, contemporary treatments of inflammatory disease can have severe deleterious effects and research now focuses on the development of more natural therapeutic agents. Interestingly, evidence continues to point towards the benefits of PUFA in the prevention and treatment of inflammatory disease (MacLean et al. 2004, Schachter et al. 2004). This study has uncovered a number of PUFA-mediated effects on macrophage *in vitro* that suggest possible pathways used by these molecules to elicit their immunomodulatory actions. Furthermore, a novel mechanism implicating CLA in the modulation of CD14 within lipid rafts was elucidated and may represent the ultimate mechanism through which this fatty acid exerts its anti-inflammatory effects.

Macrophage, MØ are highly sophisticated phagocytes and one of the many leukocytes recruited to sites of inflammation in order to neutralise and eliminate potentially harmful stimuli (Beutler 2004, Mosser 2003). It is not surprising that exacerbated MØ responses play a role in various inflammatory disorders including; inflammatory bowel disease (Zhang and Mosser 2008), schistosmiasis (Hesse et al. 2001) and atherosclerosis (Wilson, Barker and Erwig 2009). In this study we have found a modulatory role for PUFA regarding the functional status of macrophage

with potential implications for inflammatory disease and immune responses to infection.

Throughout this study we have found distinct effects of CLA and n-3 PUFA on macrophage. This has led us to equally distinct conclusions with regard to their possible roles in modulating an immune response. We found that macrophage exposed to CLA *in vitro* produced significantly more IL-10 in response to LPS. This anti-inflammatory cytokine has a potent regulatory and immunosuppressive role. Administration of IL-10 to patients with Crohn's disease has been reported to reduce bowel inflammation (Mocellin et al. 2004). Furthermore, differentiation of T cells into various subsets is partly determined by IL-10 and it has been shown to inhibit T_H1 responses. Indeed, work in our lab has demonstrated that enhanced IL-10 production in CLA-treated DC results in diminished T helper cell cytokine production (unpublished observation). Our finding in relation to enhanced IL-10 in CLA-treated MØ was the first of our results to suggest the potential use of CLA in the treatment of inflammatory disease. In support of this, our study also demonstrates that CLA-treated MØ display suppressed levels of IL-23 following stimulation. The involvement of this cytokine in promoting T_H17 development is well established. Furthermore, IL-23 is implicated as a major causative agent of inflammatory pathology in IBD (Kikly et al. 2006), EAE (Langrish et al. 2005) and collagen-induced arthritis (Yago et al. 2007). In addition, CLA treatment resulted in significant inhibition of surface CD86. This co-stimulatory molecule enhances severity of arthritis by enhancing IL-17 production and increasing the accumulation of effector T cells in joints (Odobasic et al. 2008). Conventional CD86 antibody therapies have been shown to exacerbate autoimmune disease. We suggest CLA

may provide an alternative mode of suppressing T cell activation which may be beneficial in the treatment of these diseases.

One of the results we obtained with regard to cytokine production was very surprising. The fact that CLA enhanced the production of IL-1β by macrophage was unexpected and raises concerns regarding the administration of CLA during inflammatory diseases in which IL-1β is known to play a key role. This increase in IL-1β by CLA is a recurrent observation in our laboratory in both macrophage and dendritic cell studies and something we cannot explain at this time. We suggest that while CLA may in fact be extremely beneficial in the prevention/treatment of inflammatory disease its administration may have to be tailored for the treatment of specific disease states.

With regard to the more extensively studied n-3 PUFA, DHA and EPA, our study demonstrates multiple anti-inflammatory effects of these fatty acids in macrophage. Specifically, EPA markedly suppressed levels of the potent pro-inflammatory cytokine IL-12 in response to LPS. IL-12 favours the induction of a T_H1 phenotype (Trinchieri 2003a) and as such IL-12 is largely implicated in autoimmune and T_H1 mediated diseases including; multiple sclerosis (MS), IBD and RA (La Cava and Sarvetnick 1999, Papadakis and Targan 2000). EPA-mediated inhibition of IL-12 has been reported in murine dendritic cells (DC) (Wang et al. 2007) but not macrophage and this finding may indicate its therapeutic potential. In addition EPA-treated macrophage displayed significantly suppressed levels of IL-23. The inflammatory actions of this cytokine are implicated in the pathogenesis of inflammatory diseases such as IBD, EAE and arthritis (as discussed). Furthermore, blocking T cell co-stimulatory signals is an attractive approach for the treatment of

autoimmune disease. Indeed, we found that EPA and DHA suppress CD40 expression in macrophage following stimulation. Collectively, our study suggests that the n-3 PUFA modify cytokine production and surface marker expression in macrophage. This may lead to altered(Neurath et al. 1998) antigen presentation and/or co-stimulation with the possibility of modulating the expansion and cytokine production profile of T cells. We postulate that these critical events are mechanisms through which n-3 PUFA prove beneficial in inflammatory disease.

Certainly, many studies involving n-3 PUFA indicate their consumption can benefit persons with various inflammatory diseases including; cardiovascular disease and rheumatoid arthritis (MacLean et al. 2004, Mantzioris et al. 2000). Unfortunately, few have examined their role in macrophage and those that have are commonly found to be contradictory. Most recent literature also indicates a clear discrepancy between PUFA-induced effects in different cells types. To this end in order to be used beneficially in inflammatory disease, it remains for more powerful and controlled studies to be carried out. Importantly, (Neurath et al. 1998) suggest that targeting NFκB may be a novel molecular approach for the treatment of IBD, particularly because over-expression of NFκB by macrophages is common among IBD patients. We found that CLA and indeed the n-3 PUFA, DHA and EPA suppressed activation of NFκB. As such this represents another potential beneficial effect of PUFA in an inflammatory disease situation.

Further to this, we have found the immunomodulatory actions of CLA and n-3 PUFA in macrophage may afford protective effects during infection. In this regard, the enhanced levels of IL-10 and IL-1 production in CLA treated cell may be very useful during and after injury and infection. IL-10 is notably involved in the

resolution stages of infection (Serhan et al. 2007)(Serhan et al. 2007, Serhan 2007) while IL-1 commonly promotes wound healing actions in macrophage populations (Mosser and Edwards 2008). CLA treatment also enhanced the ability of macrophage to phagocytose, an important event in bacterial clearance and homeostasis. Furthermore, we found CLA also significantly enhance chemotaxis and MIP-2 production. The infiltration of leukocytes to the site of infection is crucial in mounting an immune response. Furthermore, depleted levels of MIP-2 have been broadly implicated in impaired bacterial clearance (Strieter et al. 1996). Therefore, not only do our findings implicate a therapeutic role for CLA in inflammatory disease, it also suggests that CLA may be beneficial in innate protection from infection.

On the other hand, we found alternative effects mediated by n-3 PUFA that may prove beneficial to or hamper innate responses during infection. DHA and EPA significantly suppressed levels of IL-6 in response to LPS stimulation. Certainly, studies employing human macrophage models have reported that EPA and DHA inhibit TNFα, IL-6 and IL-1β production (Goua et al. 2008, Chu et al. 1999). Elevated levels of these cytokines are characteristic of LPS-induced endotoxemia. In line with other studies, our data suggests that daily supplementation of n-3 PUFA may help ameliorate chronic inflammation caused during sepsis. Furthermore, DHA markedly enhanced expression of MIP-2 an important chemokine in the process of bacterial clearance (as discussed). However, we also found that EPA and DHA suppressed the rate of phagocytosis in macrophage. This suggests that n-3 PUFA may delay bacterial clearance by impeding the ability of macrophage to phagocytose. This conflicts with previous studies employing n-3 PUFA treatments in macrophage (Calder et al. 1990, Kew et al. 2003, D'Ambola et

al. 1991). Certainly, such discrepancies are common in the literature. It is believed to be due variations in dose and cell lines used and presents an arguable challenge in PUFA research.

In conclusion, there are conflicting reports as to whether PUFA collectively or individually have the ability to enhance or diminish immune functions. Furthermore, it is notable that PUFA have differential effects on immune function, as reviewed extensively by (Fritsche 2006, Calder 2006, Yaqoob 2009). Importantly however, it its broadly accepted that the ratio between n-3 and n-6 fatty acids is significant in determining cell function, whole body physiology and human health (Simopoulos 2002, Burdge and Calder 2006, Simopoulos 2008). Certainly a lower ratio of n-6/n 3 fatty acids is more desirable in reducing the risk of many of the chronic diseases of high prevalence in Western societies. There is need not only to examine the effect of dose on immune and inflammatory responses, but also to separate the effects of individual PUFA and their combination with others in different ratios. These investigations will promote an understanding of the mechanisms employed by PUFA to exert their effects. Our data certainly suggest that CLA and the n-3 PUFA, DHA and EPA may have immunomodulatory effects and could be useful in inflammatory situations. The compelling evidence in the literature regarding the many immunomodulatory effects of PUFA is certainly difficult to explain and warrants further investigation.

A lack of experimental and clinical studies in the area complicates any implication of their beneficial use in prevention and treatment of disease. Indeed, many clinical studies cannot account for the background diets of their subjects. In the majority if not all animal studies, the diet is completely controlled. Furthermore, many

diseases are the result of genetic predispositions. Quite possibly the dose of PUFA administered may depend on the degree of severity of disease on a case by case basis. In realistic terms it is foreseeable that PUFA may not be administered for the complete prevention or treatment of a disease or infection. But the evident anti-inflammatory benefits of PUFA suggest that they may help ameliorate symptoms and certainly assist in their resolution.

The secondary focus of this work was to define a mechanism through which PUFA may exert anti-inflammatory effects with particular interest to CLA. Previous investigations in the field have paid particular attention to downstream signalling components, and suggest these as pinnacle targets through which PUFA exert their effects. Indeed DHA and EPA have been shown to increase phosphorylation of p38 and epidermal growth factor receptor, EGFR, a beneficial effect considering sustained activation of the EGFR and p38 MAPK has been associated with apoptosis in human breast cancer cells (Schley, Brindley and Field 2007). Furthermore, several groups have demonstrated the anti-inflammatory effects of PUFA via the involvement of PI3K/Akt (Wan et al. 2007, Weaver et al. 2009). In addition, numerous studies report PUFA mediated suppression of transcription factors particularly NFκB (Sampath and Ntambi 2005) and activation of peroxisome proliferator activated receptors, PPARs (Jump 2002b).

However, an important observation was made by (Lee et al. 2003) and colleagues. Based on their work, with various downstream dominant negative mutants and constitutively active molecules, they suggest that PUFA may exert their effects upstream at the membrane, or even at the TLR4 receptor complex itself. Notably, PUFA-modulated effects are mediated via modifying eicoanoid production.

However, PUFA perform this modulation primarily by competing with arachidonic acid at the ‘membrane’ leading to the decreased production of the inflammatory eicosanoid products (Albers et al. 2002, Mantzioris et al. 2000). Certainly in early chapters we found the modulation of CD14 in resting cells treated with CLA to be a pivotal finding. This GPI-anchored protein resides in the membrane and acts as an associated molecule of TLR4 (Wright et al. 1990), as such it plays a fundamental role in TLR4 signalling (Shuto et al. 2005, Juan et al. 1995).

We also found that CLA suppressed surface expression of CD14 at very early stages of stimulation with LPS. This suppression of CD14 at the membrane in CLA-treated cells was accompanied by enhanced levels within the cytosol. Further to this our study focused on clarifying if modulation of CD14 by CLA involved altered targeting of the protein to lipid raft microdomains in the plasma membrane. Indeed we found this to be the case. CD14 was markedly suppressed in lipid raft fractions isolated from stimulated macrophage treated with CLA. With the exception of disrupted raft association of CD14 in EtOH treated cells (Dai, Zhang and Pruett 2005), this is the only example of an anti-inflammatory molecule modulating CD14 in this way. In addition, we also assessed the consequences of this effect on signalling pathways downstream of TLR4 and its possible effects on the ability of TLR4 to become endocytosed.

We found direct downstream implications for the activation of NFκB and IRF3, with CLA treatment suppressing the induction of both transcription factors. However, an exact mechanism was elucidated regarding the suppression of IRF3. Indeed, CD14 is a requirement for LPS induced endocytosis of TLR4 and subsequent activation of IRF3 (Kagan et al. 2008, Shuto et al. 2005). We found the inhibitory effect of CLA on IRF3 was reversed by the over-expression of CD14 in

U373 cells. Furthermore, we found that the ability of TLR4 to become endocytosed upon activation was diminished significantly in CLA-treated cells. As such, our study revealed a novel mechanism for CLA-induced anti-inflammatory effects. Furthermore, to our knowledge this is the first report of an anti-inflammatory compound exerting its effects via a CD14-dependent suppression of TLR4 endocytosis. While it was clear that EPA and DHA did not suppress CD14, this study did not extend to determining their exact effects at the cell membrane. However we did report that these fatty acids suppressed expression of TLR4, therefore rendering the macrophage less responsive to LPS. Furthermore, our observation that all of the PUFA we examined enhanced the expression of SR-A is an interestingly observation, given that this receptor is known to bind LPS, and warrants further investigation. These changes in receptor expression at the membrane may explain some of the anti-inflammatory effects of these fatty acids.

We postulate that with emerging roles for CD14 in the exacerbation of infection and inflammatory disease our finding regarding CLA will have direct implications for the possible use of this fatty acid as a treatment. High levels of CD14 are indicative of intestinal macrophage populations from patients suffering with IBD (Kamada et al. 2008). Signalling through CD14 has been shown to play an obligate role in cardiac inflammation occurring after major burn injuries (Barber et al. 2008). The over-expression of CD14 in specific areas of the central nervous system (CNS) within an endotoxin induced mouse model of Parkinson's-like disease has also been demonstrated (Panaro et al. 2008). Furthermore, diseases such as Wegener’s granulomatosis are partially characterised by the up-regulation of CD14 on monocytes (Yard et al. 2002). In this regard, we suggest the possibility that administration of CLA may offer an alternative to conventional CD14 antibody

therapies for inflammatory disease. Importantly, production of IFN-β (downstream of IRF3 activation) is essential for induction of endotoxic shock and the activation of a full immune response (Pechine et al. 2007). Since CLA can suppress IRF3, our data suggests that CLA may be useful in endotoxic shock. Interestingly a recent study in our laboratory has revealed that feeding mice with CLA protects them from endotoxic shock (Loscher et al., unpublished).

This work has uncovered a completely novel mechanism regarding the anti-inflammatory effects exerted by CLA. Furthermore, this is highly relevant to research within our field of nutritional immunology. It was over thirty years ago that investigations first examined and tried to elucidate the possible anti-inflammatory potential of fatty acids (Palmblad and Gyllenhammar 1988). At that time it was a well held belief that fatty acid-induced changes in the cell membrane were responsible for subsequent changes observed in membrane-dependent functions including; phagocytosis and cell signalling events. All were thought to be a direct result of alterations in membrane composition and fluidity (Peck 1994). However, this theory rapidly lost favour as; 'it failed to explain why n-6 and n-3 PUFAs have contradictory actions on the immune system' (Fritsche 2006, Palmblad and Gyllenhammar 1988). Currently, this theory has re-emerged with the recent discovery of discrete lipid domains or 'lipid rafts' in the plasma membrane. We believe that the work in our study reinforces this renewed interest. Furthermore, we postulate this will be a critical focus within the field of nutritionally based immunological research and prove fundamental in efforts to elucidate the multiple mechanisms used by PUFA to elicit their anti-inflammatory effects.

CHAPTER 7

FUTURE PERSPECTIVES

7.1 FUTURE PERSPECTIVES

Within chapter 4 a study to assess the binding of LPS to the TLR4 receptor complex by flow cytometry is described. Various other groups have conducted studies with the premise of elucidating the exact nature of LPS binding *in vitro*, particularly emplying neutrophils and monocytes (Anastase-Ravion et al. 2003b, Kitchens et al. 2000). While several knockout mice models have revealed unexpected features of LPS action *in vivo* (Fenton and Golenbock 1998), to the best of our knowledge there are no reports citing the examination of LPS binding events in an artificial environment outside the living organism (i.e. *ex vivo*). Consequently, we describe herein as a future perspective to the work conducted on LPS binding in chapter 4, the development of an *ex vivo* model to assess LPS binding to brush border membrane from the small intestine of BALB/C mice. In order to do this, the Biacore3000™ surface plasmon resonance (SPR) system was employed, providing a highly sensitive platform for looking at real time binding events. The results of this work are presented in appendix C and discussed below.

Brush border membrane vesicles (BBMVs) are gaining recognition as an alternative tool to investigate active transport interactions as they contain phospholipids, hydrolytic enzymes, carrier proteins (Baroni et al. 2006, Balon, Riebesehl and Muller 1999, Kramer et al. 1994) and lipid rafts (Danielsen and Hansen 2003) that are responsible for many biologic interactions and binding events. BBMV surfaces have been useful for the estimation of binding events, particularly of orally administered drugs as they possess structural and functional similarity to real intestinal membranes (Kim et al. 2004, Cho et al. 2004). They are of particular interest as over activated and/or dysregulated function of toll-like

receptors including TLR3, TLR4 and TLR5 are largely implicated in many diseases of the gut (Lodes et al. 2004, Cario and Podolsky 2000). However, the exact expression of TLRs and indeed interaction with their respective ligands in BBMVs has not been investigated.

The possibility of using the Biacore 3000™ SPR-based biosensor to monitor *ex vivo* real-time binding of LPS to BBMVs was investigated. Using the L1 chip it was possible to successfully capture significant levels of BBMVs. A pilot experiment suggested significant LPS binding to the immobilised BBMVs. However, follow-up experiments indicated that there was substantial NSB of LPS to the L1 chip surface itself. A series of scouting experiments was then undertaken to determine the most appropriate strategy to counteract the NSB. A high concentration solution (12 mg/ml) CM-dextran and BSA blocking buffer was passed across the surface. In addition, the LPS was diluted in this same blocking buffer. BSA is a commonly employed blocking protein and the CM-dextran mimicked the actual surface matrix of the L1 chip and thus, it was postulated that any LPS with non-specific affinity to the CM-detxtran surface would be effectively blocked by the high concentration of CM-dextran in the blocking buffer. However, this strategy alone did not fully eliminate the NSB contribution. (Anderluh et al. 2005) previously reported that blocking with lipids was an effective approach to reduce NSB. We found that a combination of CM-dextran/BSA blocking buffer and pre-blocking the L1 chip surface with a concentrated liposome solution was effective in eliminating NSB.

Unfortunately, when the blocking steps were incorporated no binding of LPS was discernable. One possible reason for this is that the CM-dextran/BSA and liposome blocking agents also effectively coated and blocked the BBMV surface. This could

be overcome by employing an alternative blocking strategy. It is also possible that the integrity of the BBMVs is somehow compromised when immobilised. This could be due to aggregation and degradation either on chip or during prolonged storage in buffer. It is also important to note that the expression of TLRs on BBMVs has not yet been characterised, therefore other ligands, apart from the TLR4 ligand used here, will be used during further optimisation. Although at present it will not supplant cellular-based assays, it is confidently predicted that with further optimisation and amendment this model platform will provide a novel strategy for investigating the interactions of other toll ligands in a simple and rapid real-time *ex vivo* assay format.

CHAPTER 8

APPENDICES

APPENDICES

APPENDIX A

CELL CULTURE MEDIA

COMPLETE RPMI 1640	500 ml
5% Heat inactivated Foetal Calf Serum (FCS)	25ml
Penicillin/streptomycin/L-glutamine Culture Cocktail (Gives a final concentration of 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin)	10 ml

COMPLETE DMEM	500 ml
5% Heat inactivated Foetal Calf Serum (FCS)	25ml
Penicillin/streptomycin/L-glutamine Culture Cocktail (Gives a final concentration of 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin)	10 ml

10X PHOSPHATE BUFFERED SALINE (PBS)

$Na_2HPO_4.2H_2O$ (8 mM)	23.2 g
KH_2PO_4 (1.5 mM)	4 g
NaCl (137 mM)	160 g
KCl (2.7 mM)	4 g

Make up to 2 L pH to 7.4

10 X TRIS BUFFERED SALINE (TBS) pH 7.6

NaCl	48.4 g
Trizma Base	160 g

Dissolve in 2 L dH_2O pH to 7.6

2N H_2SO_4

H_2SO_4 (36 N)	11.1 ml
dH_2O	88.9 ml

FACS BUFFER

2%FCS

0.05% NaN_3

PBS

5X SAMPLE BUFFER

125 mM Tris	6.25 ml 1M Tris HCl pH 6.8
10 % Glycerol	5 ml
2 % Sodium dodecyl sulphate (SDS)	10 ml (10 % (w/v) SDS)
0.05 % (w/v) Bromophenol Blue	0.01 g
dH_2O	28.75 ml

0.25 M Dithiothreitol (DTT)*	250 µl 1 M DTT S

* Added to 1 ml 5X Sample Buffer just before use

SEPARATING GEL (10 % (v/v))

33% w/v Bisacrylamide (30% stock)

1.5M Tris-HCl pH8.8

1% w/v SDS

0.5% w/v Ammonium persulpate

dH_2O

0.1% v/v TEMED

STACKING GEL

6.5% v/v Acrylamide/Bisacrylamide (30% stock)

0.5M Tris-HCl pH6.8

1% w/v SDS

0.5% w/v Ammonium persulphate

dH_2O

0.1% v/v TEMED

ELECTRODE RUNNING BUFFER

25mM Tris base

200mM Glycine

17mM SDS

LIPOSOMES

Liposomes: 30:30:10 % molar ratio DSPC:Cholesterol:DSPE-PEG

DSPC: Sigma®; DSPE-PEG: Avanti Polar Lipids Inc.,; Cholesterol: Sigma®.

APPENDIX B

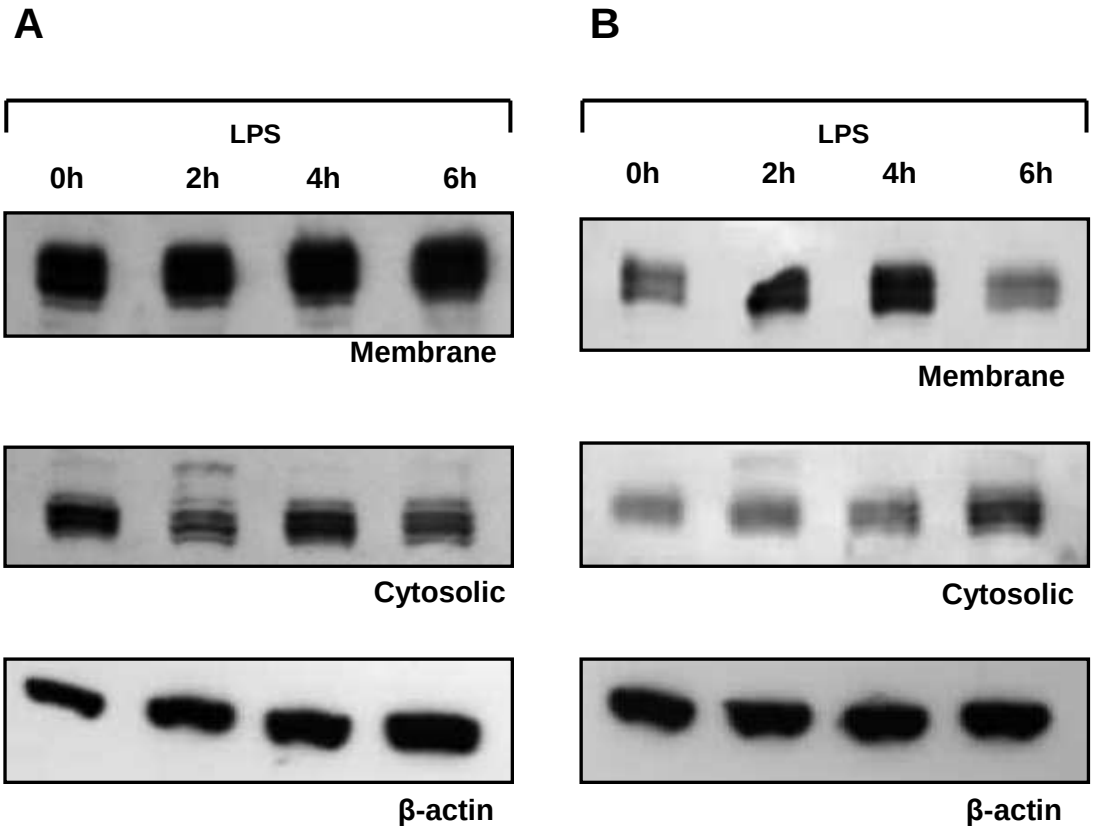


FIGURE 8.1: Membrane and cytosolic expression of CD14 in J774 macrophage. Cells were plated at 1 x 10^6 cell/ml, (3 ml/well) **[A]** or 2 x 10^5 cell/ml, (3 ml/well) **[B]** and left to rest overnight. On the following day cells were stimulated with LPS (100 ng/ml) over 6 h, as indicated. After the completed time course cell lysates were harvested and membrane fractionation performed. Total cellular levels of β-actin were used as loading control.

APPENDIX C

BRUSH BORDER MEMBRANE CHARACTERISATION

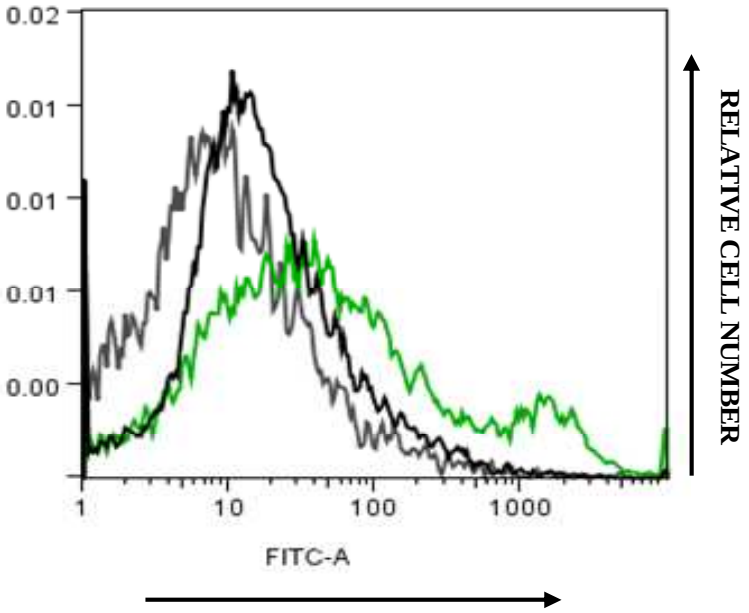
Brush border membrane vesicles (BBMV) were isolated as described [section 2.10.1] and characterised with a monoclonal antibody against CD66a which is constitutively expressed on BBMV. Following incubation with CD66a antibody BBMV were stained with an anti-mouse FITC conjugate and analysed by flow cytometry on a BD FACSAria™. CD66a bound significantly to BBMV preparations **[Figure 8.2]**. In addition binding of solely the anti-mouse FITC conjugate to BBMV was negligible.

ESTABLISHING AN *EX VIVO* MODEL TO INVESTIGATE LPS BINDING

In an attempt to establish an *ex vivo* model for the investigation of binding of LPS, BBMV were immobilised on the surface of an L1 chip using a Biarcore3000™, as described [section 2.10.2]. BBMV were successfully immobilised on the surface with a final level of 2166 response units (RU) of covalently attached BBMV achieved **[Figure 8.2A]**. Subsequently, LPS (10 μg/ml) was passed over the surface resulting in a binding event to the significance of 216 RU **[Figure 8.2A and B]**. Following this, any non-specific binding (NSB) of LPS to the surface of the L1 chip was assessed [section 2.10.4]. LPS bound significantly to the surface of a blank flowcell **[Figure 8.3A]**. In addition, blocking the flowcell with CM-dextran BSA and preincubation of LPS with the same resulted in binding of LPS to the surface of the flowcell by approx. 100 RU **[Figure 8.3B]**. Furthermore, full analysis of a BBMV-functionalised, liposome and CM-dextran BSA blocked chip resulted in complete elimination of LPS to the surface **[Figure 8.4]**. This suggests

that the previously observed LPS binding event to immobilised BBMVs was non-specific **[Figure 8.2A]**.

A



B

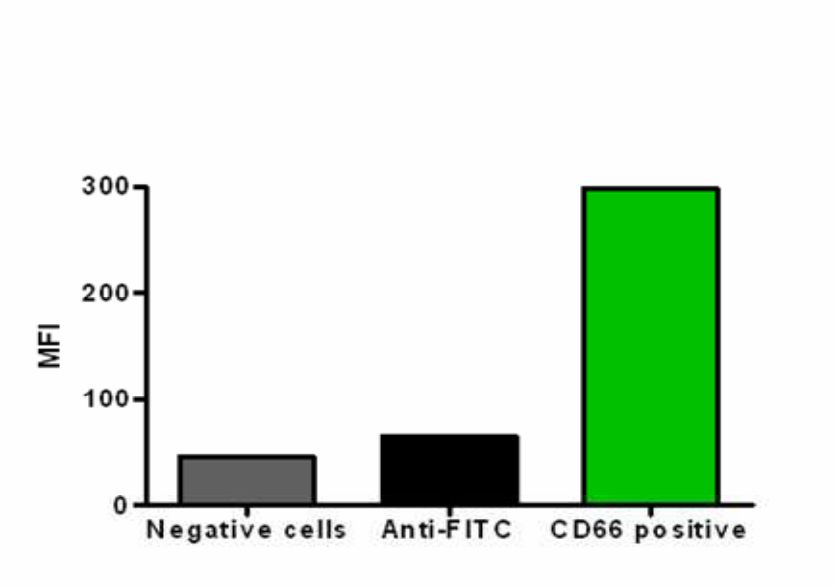


FIGURE 8.2: Flow cytometric analysis of BBMV isolated from the small intestine of BALB/C mice **[A]**. Unstained BBMV are represented by the grey histogram. Non-specific binding of the anti-FITC conjugate used in characterisation was assessed (black histogram). Specific binding of CD66a to BBMV is displayed (green histogram). Mean fluorescence intensity values are also represented above **[B]**.

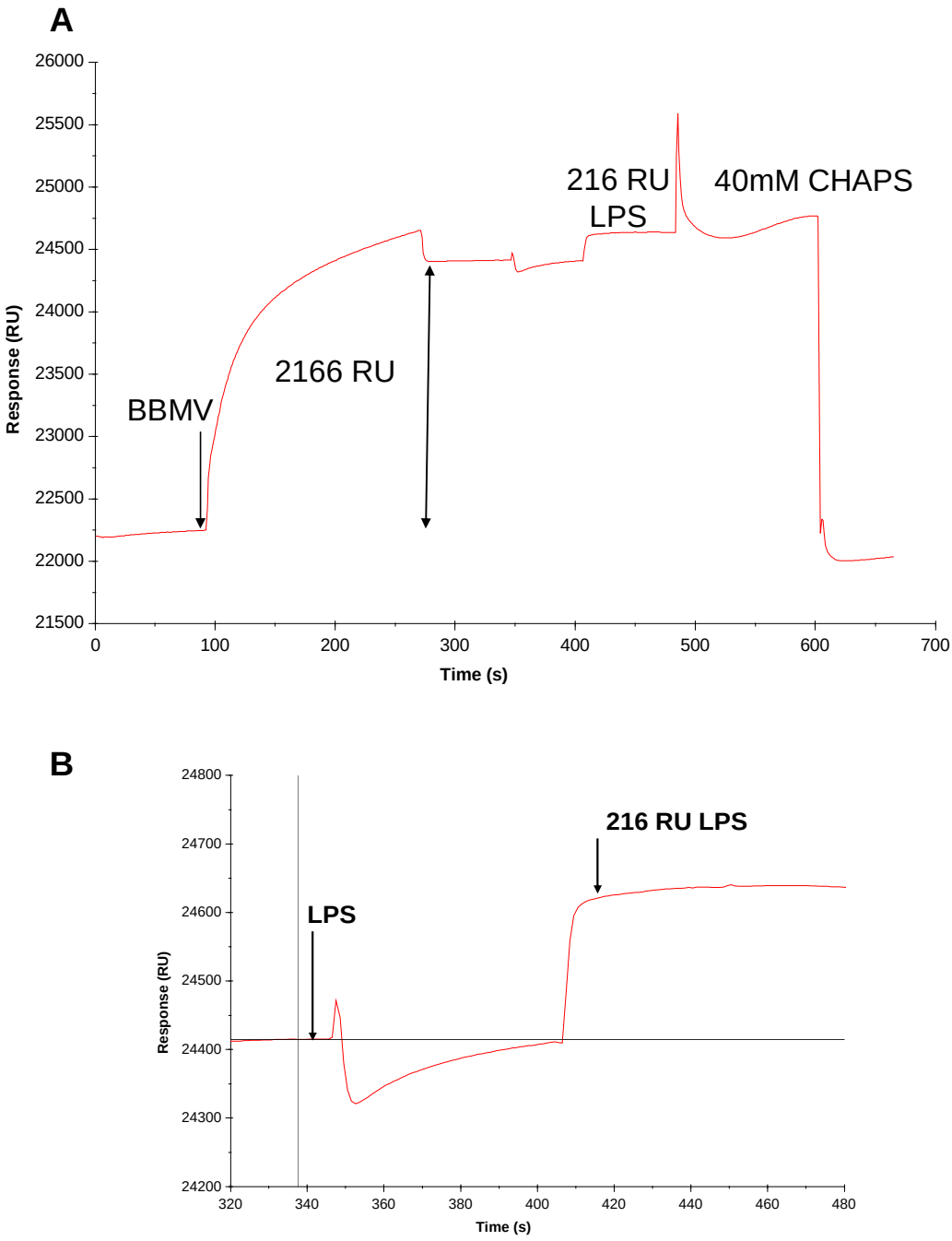


FIGURE 8.3: Binding sensorgram demonstrating capture of BBMV and subsequent binding of LPS in HBS-DF buffer **[A]**. After passing BBMVs over the surface of the L1 chip a final level of 2166 RUs of covalently attached BBMVs was achieved. Subsequently, LPS (10 µg/ml) was passed over the surface leading to a binding event to the significance of 216 RU change. Binding of LPS is highlighted in **[B]**. The surface of the L1 chip was regenerated by injecting 40 mM CHAPS.

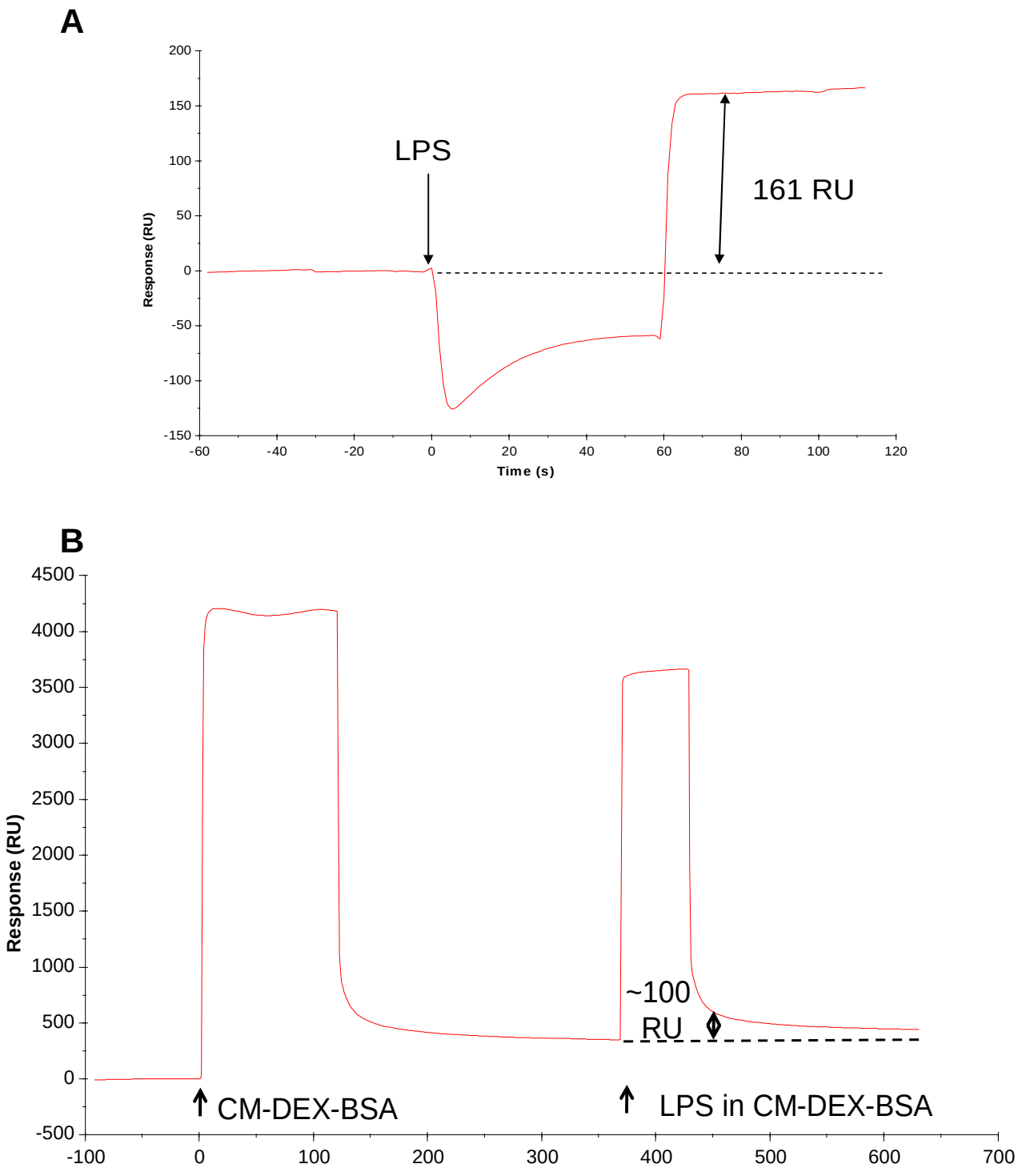


FIGURE 8.4: Testing for binding of LPS on a blank L1 chip surface **[A]**. Injection of LPS (10 µg/ml) over the blank flowcell resulted in binding response of 161 RU. Non-specific binding (NSB) was recognised as a significant problem and in an attempt to overcome this, blocking agent CM-dextran BSA was passed over the surface prior to injecting LPS diluted in blocking buffer **[B]**. However, LPS still bound significantly to the surface.

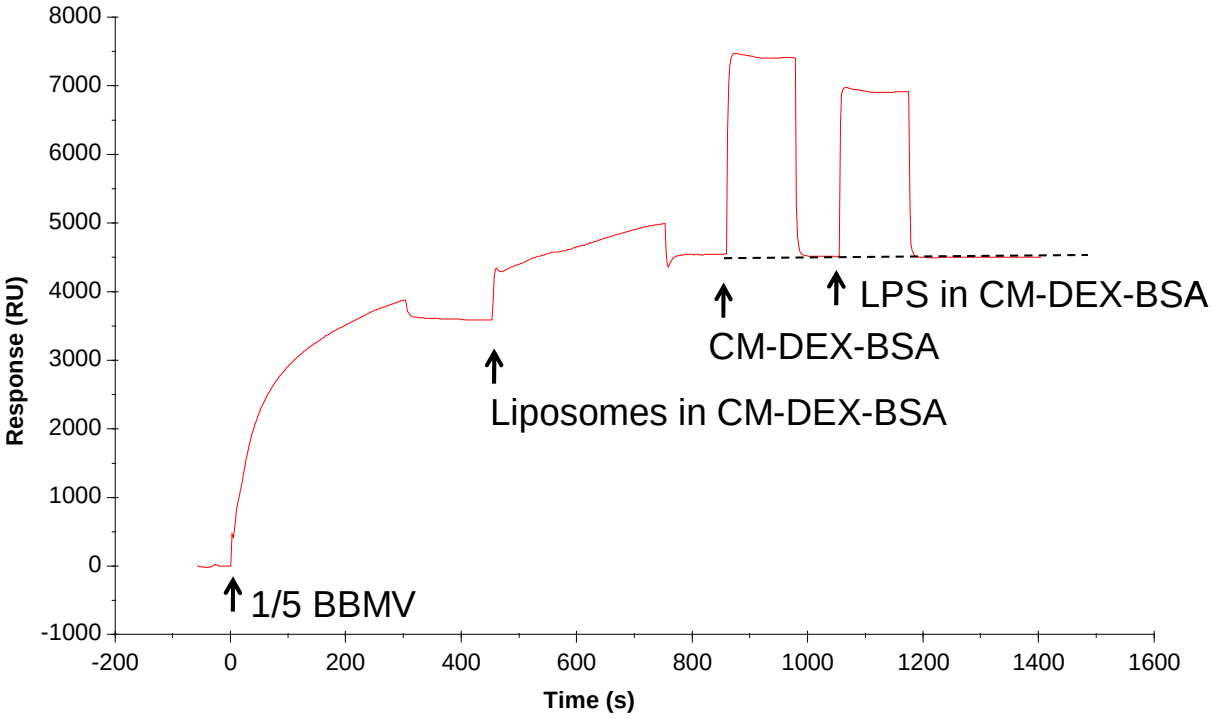


FIGURE 8.5: Full analysis of BBMV-functionalised and liposome-blocked chip. Following immobilisation of BBMV to the L1 chip surface, non-specific sites were blocked using a liposome solution followed by CM-dextran BSA. Blocking procedures efficiently abrogated NSB, however there was also a concomitant abrogation of LPS binding to captured BBMVs.

CHAPTER 9

BIBLIOGRAPHY

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