Characterisation of the Anti-Inflammatory Effects of Marine Extracts

A thesis submitted for the degree of Ph.D.

By

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Declaration

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…It is impossible to start….

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ABBREVIATIONS

AA	Arachidonic acid
AIA	Antigen-induced arthritis
AICD	Activation-induced cell death
APC	Antigen presenting cell
BSA	Bovine serum albumin
BTK	Bruton's Tyrosine kinase
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Crohn's disease
CD14	Cluster of differentiation
CIA	Collagen induced arthritis
CLA	Conjugated linoleic acid
CNS	Central nervous system
COX	Cyclo-oxygenase
CpG	Cytosine phosphate guanine
CREB	Ca^{2+}/cAMP response element binding protein
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DC	Dendritic cell
DHA	Docosahexanoic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal-related kinase
FCS	Foetal calf serum
Foxp3	Fork-head box P3
GATA-3	GATA-binding protein 3
GMCSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
HEK	Human Embryonic Kidney
HRP	Horseradish peroxidase
HSV-1	Herpes Simplex Virus-1
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IFN	Inteferon

IKKα/β	IkB kinase α/β
IL	Interleukin
IL-1Ra	IL-1 receptor anatgonist
IP-10	Interferon-inducible protein 10
IRF	Interferon regulatory family
IRAK	Interleukin 1 receptor associated kinases
ISRE	IFN-stimulated response element
JAK	Janus kinases
JNK	c-Jun N-terminal kinase
kDA	Kilodalton
KO	Knockout
LB broth	Luria-Bertani broth
LBP	LPS binding protein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTA	Lipoteichoic acid
LTB	Leukotriene
MAPK	Mitogen-activated protein kinase
MCP	Macrophage chemoattractant protein
MDP	Muramyldipeptide
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLN	Mesenteric lymph node
MS	Multiple sclerosis
MUFA	Monounsaturated fatty acid
NEMO	NFκB essential modulator
NFAT	Nuclear factor of activated T cells
NFkBD	NFκB decoy
NFκB	Nuclear factor-κB
NK	Natural killer
NOD	Nucleotide oligomerization domain
OVA	Ovalbumin
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PG	Prostaglandin
PGN	Peptidoglycan
PMA	Phorbol 12-Myristate 13-Acetate
PP	Peyer's patches
PPAR	Peroxisome proliferator-activated receptor

PPRE	PPAR response element
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid arthritis
RBC	Red blood cell
RHD	REL-homology domain
RSG	Rosiglitazone
RT	Room temperature
RXR	Retinoic X receptors
SDS	Sodium dodecylsulphate
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TMB	3,3'5,5'-tetramethyl-benzidine
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
Treg	T regulatory
TXB	Thromboxane
UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule

PRESENTATIONS AT CONFERENCES/MEETINGS

- MESTECH workshop, NCSR, Dublin City University, March 2011- **Poster presentation**
- 14th International Congress of Immunology, Kobe, Japan, August 2010- **Poster presentation**
- 1st IRCSET Symposium, Dublin, November 2009- **Poster presentation**
- 25th Irish society of immunology meeting (ISI), Trinity College Dublin, October 2010- **Oral presentation**
- Biochemical Society IAS meeting, University College Dublin, November 2009- **Oral presentation**
- 1st Biotechnology Research Day, Dublin City University, January 2009- **Oral presentation**
- 1st Marine Biodiscovery Rearchers Workshop, Galway, December 2008- **Oral presentation**

PUBLICATIONS

Published

Synthesis and Biological Evaluation of Pyridine-Containing Lipoxin A4 Analogues
*Duffy CD, Maderna P, **McCarthy C**, Loscher CE, Godson C, Guiry PJ.*

A role for TLR4 in *Clostridium difficile* infection and the recognition of surface layer proteins.
*Ryan A, Lynch M, Smith S.M, Amu S, Hendrik J.N, McCoy CE, Dowling JK, Draper E, O'Reilly V,**McCarthy C**, Smith SM, O'Brien J, Ní Eidhin D, O'Connell M.J, Keogh B, Morton CO, Rogers T.R , Fallon PG, O'Neill LA, Kelleher D and Loscher CE.*
PLoS Pathogens

In preparation

A purified marine compound isolated from the marine sponge *Membranipora membranacea* suppresses macrophage function following exposure to bacterial but not viral ligands.
***McCarthy C**, Murphy C, Canavan M, Dowling JK, Guiry PJ, Loscher CE*

A purified marine compound isolated from the marine sponge *Membranipora membranacea* suppresses dendritic cell activation by targeting the adaptor Mal
***McCarthy C**, Dowling JK, Murphy C, Guiry PJ, O'Neill LA, Loscher CE*

Commercialisation of research

Invention disclosure – A purified compound isolated from the marine sponge *Membranipora membranacea* exerts its anti-inflammatory abilities by targeting the Mal adaptor protein (MyD88 adaptor like protein).

ABSTRACT

The ocean is a major source of structurally unique natural products that are mainly found in marine organisms. Numerous compounds already isolated from these marine organisms have been shown to possess anti-inflammatory activities. The objective of this work was to examine a range of marine organisms in order to identify compounds that had anti-inflammatory potential and also to elucidate the mechanism of their actions. Inflammatory diseases account for significant ill health and morbidity worldwide and are typically associated with dysregulated T cell responses, which are activated by dendritic cells (DC). Therefore, novel strategies that modulate the activation of DCs may have therapeutic potential. We screened a number of marine species by examining their effects on DC function and from this focussed on candidate marine extracts for further assessment. These were then fractionated and further purified until we had a single compound sample (INV013). This purified compound was then used to determine the intracellular target of INV013.

DCs were exposed to marine extract INV013 and then activated with a panel of TLR ligands (TLR2/1, TLR2/6, TLR3, TLR4, TLR5, TLR7 and TLR9). INV013 specifically suppressed the IL-12 family of cytokines (IL-12p40, IL-12p70 and IL-23), IL-1β, IL-6 but not TNF-α, and enhanced IL-10 production following activation only with TLR4 or TLR2 ligands. The marine compounds also suppressed DC migration in response to TLR4 and inhibited production of chemokines. INV013 had no effect on DCs stimulated with ligands to the other TLRs. While there are some common pathways downstream of TLR activation TLR2 and TLR4 use an adaptor protein called MyD88 adaptor-like molecule (Mal), which is not used by the other TLRs.

Examination of the TLR downstream signalling pathways revealed that INV013 selectively inhibited NF-κB activation but had no effect on IRF3 activation. Furthermore, INV013 was also able to block Mal-driven activation of NFκB. These findings demonstrate that marine extract INV013 has specific effects on DCs and indicates that it exerts these effects by targeting Mal. This suggests that INV013 has the potential to be developed as a therapeutic for treatment of inflammatory disease.

CHAPTER 1

GENERAL INTRODUCTION

1.0 THE IMMUNE SYSTEM

The immune system is an extremely complex system of lymphoid organs, cells, humoral factors and cytokines. An effective immune response is provided by means of innate 'early' and adaptive 'delayed' immunity. Although considered independent, there is a high level of interaction and collaboration between the two.

1.1 OVERVIEW OF INNATE IMMUNITY

The innate immune system comprises the first line of defence to the body in a non-specific manner. Most organisms exist with only innate mechanisms however vertebrates have alternative systems for pathogen recognition and elimination, collectively known as adaptive immunity (Kimbrell and Beutler 2001) The principal components of innate immunity include physical, chemical and anti-microbial barriers to infection (Beutler 2004). Innate immunity is mostly dependant on phagocytosis, a process which engulfs and destroys invading pathogens. Phagocytic cells (macrophages, neutrophils) also produce a wide range of inflammatory mediators, including cytokines and chemokines, which simultaneously direct other host responses. Macrophages are found throughout the body (e.g. heart, lung, brain and liver), and upon breach of the epithelial barrier are the first cells to encounter invading microbes (Beutler 2004). The majority of neutrophils are found circulating in the blood and have a short life span. They are attracted to the site of infection by activated macrophages and upon activation they release cytokines and chemokines which in turn amplify inflammatory reactions by several other cell types (Janeway et al. 2008)

1.2 MACROPHAGE FUNCTION AND ACTIVATION

Macrophages (MØ) are key effector innate immune cells that play a fundamental role in tissue homeostasis, pathogen clearance, wound healing and resolution of inflammation. MØ have been a continuous source of investigation for immunologists since their discovery by Elie Metchnikoff in 1905 (HIRSCH 1959). MØ are prodigious phagocytic immune cells that perform several different functions throughout the innate and adaptive immune response. They are present in lymphoid and non-lymphoid tissues and their primary role is to recognise, engulf and kill invading pathogens. MØ express numerous pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) which enable them to recognise pattern associated molecular patterns (PAMPs) resulting in phagocytosis and the initiation of intracellular signalling pathways, leading to the production of inflammatory mediators such as cytokines and chemokines (Gordon 2007). In addition MØ are capable of initiating an adaptive immune response by presenting antigen to $CD4^+$ T cells via major histocompatibility class (MHC) II molecules. Phagocytosis is a fundamental process that is involved in the clearance of cellular debris from tissue remodelling and apoptosis and without this process the host would not survive (Janeway et al. 2008). The receptors that are involved in this homeostatic clearance include scavenger receptors (SRs), phosphatidyl serine receptors, integrins and complement receptors (Erwig and Henson 2007). MØ activation is dependant on the type of pathogen encountered or the environment in which the macrophage resides. Currently MØ can be classified into three different subsets classically activated (M1 or host defence), alternatively activated (M2 or wound healing) and regulatory MØ (Fairweather and Cihakova 2009, Gordon and Martinez 2010) [see figure 1.1]. However they are not permanently differentiated but have plasticity and are capable of moving from

classically activated to alternatively activated and back again depending on the local environment (Stout and Suttles 2004)

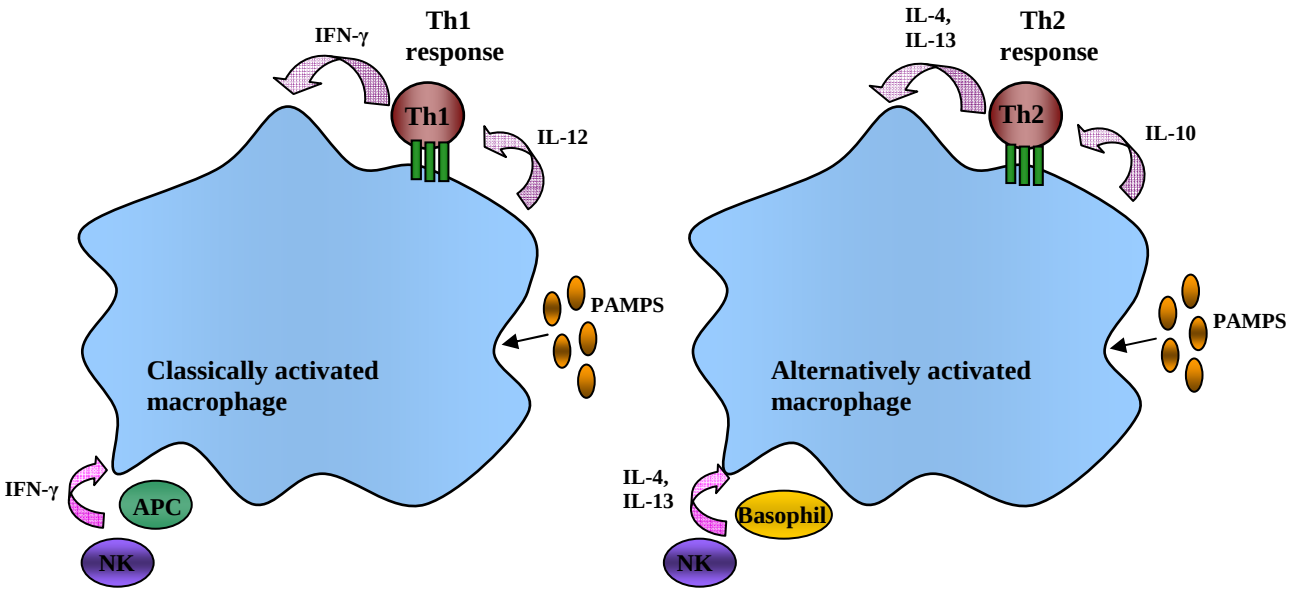

Figure 1.1: *Diagrammatic representation of cytokines produced by immune cells and their effect on macrophage physiology.*

1.2.1 CLASSICAL AND ALTERNATIVE ACTIVATION

MØ that are produced during cell-mediated immune responses are termed 'classically activated.' Classically activated MØ are generated following stimulation with pro-inflammatory cytokines IFN-γ and TNF-α or LPS. Cells of both the innate and adaptive immune system are involved in the production of IFN-γ whereas TNF-α is produced by the ligation of a TLR to its respective ligand, activating the MyD88-dependant signalling pathway (O'Shea and Murray 2008, Ma, et al. 2003). IFN-γ primes MØ to secrete pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12, and the chemokines MIP-1α/CCL3, IP-10/CXCL10 and MCP-1 (Fairweather and Cihakova 2009). In addition, classical activation is associated with increased

expression of the antigen presentation receptor, MHCII and also the co-stimulatory marker, CD86 (Martinez, Helming and Gordon 2009). Although these MØ are vital components of host defence there activation must be firmly controlled as over-production of pro-inflammatory cytokines can lead to host-tissue damage resulting in autoimmune diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (Szekanecz and Koch 2007, Zhang and Mosser 2008).

Contrastingly, alternative activation of macrophages is induced by IL-4 and IL-13, cytokines that are secreted in a Th2 response particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens (Martinez, Helming and Gordon 2009). Similar to classically activated macrophages they can develop in response to innate and adaptive signals. Innate immune cells including basophils, mast cells and granulocytes are fundamental early sources of IL-4 secretion following tissue injury (Louis, et al. 1999, Brombacher, et al. 2009). Similar to classically activated macrophages, these MØ can be detrimental to the host when their matrix-enhancing activity is dysregulated leading to autoimmune diseases such as RA. In addition alternatively activated macrophages are more susceptible to intracellular infections for example *M.tuberculosis* (Gordon and Martinez 2010).

1.3 OVERVIEW OF ADAPTIVE IMMUNITY

In addition to innate immunity, vertebrates have evolved an adaptive immune system, utilizing antigen-specific receptors on T and B cells to direct effector responses. T and B lymphocytes are the recognized cellular pillars of adaptive immunity. T lymphocytes are chiefly responsible for cellular immunity and B lymphocytes for humoral immunity but they work together with other cell types to mediate an effective adaptive immune response (Pancer and Cooper 2006). T and B lymphocytes originate in the bone marrow from progenitor cells. T cells migrate and mature in the thymus while B cells remain in the bone marrow undertaking further development. The antigen recognition of T cells is attributed to the T cell receptor (TCR) while B cell specificity is due to membrane bound antibody (Janeway et al. 2008). Specificity of both T and B cells for TCR and antibody is a result of random reorganization of genes encoding them, thus resulting in the expression of a vast repertoire of receptors (Nemazee 2006). Naïve lymphocytes migrate to peripheral lymphoid organs including lymph nodes, spleen and the mucosa associated lymphoid tissues such as Peyer's patches (PP) in the gut. It is in these particular locations where the majority of the immune responses occur. Both lymphocytes display surface receptors for a single specific antigen but due to a high level of specificity, only a reasonably small number of lymphocytes are able to recognize any given antigen. Lymphocytes are constantly circulating between the blood and the peripheral lymphoid tissues in order to increase their chances of encountering their specific antigen. When the lymphocytes recognize their specific antigen which is coupled to the MHC receptor on the surface of an antigen presenting cell, it ceases to migrate. For several days it proliferates, undergoing clonal expansion and differentiation eventually giving rise to effector T cells of identical antigen specificity. The effector T cells then re-enter the bloodstream

and migrate to the sites of infection (Janeway et al. 2008). Upon re-exposure to a particular antigen, memory T and B lymphocytes initiate an important and effective immune response a process often referred to as 'immunological memory'(Gourley, et al. 2004).

1.3.1 LINKING INNATE AND ADAPTIVE IMMUNITY

The co-ordination of innate and adaptive responses is assisted by signalling among different PRRs (Reise Sousa 2004).While innate immunity is essential as the first line of defence against infection, adaptive immunity provides specific targeted response to the invading pathogen. Furthermore adaptive immunity provides long-lasting protection against pathogens (Janeway et al. 2008). Dendritic cells which are the main antigen presenting cells of the immune system play a key role in linking innate and adaptive immunity (Gottenberg and Chiocchia 2007). Figure 1.1a illustrates various cells of the immune system and the differentiation from stem cells.

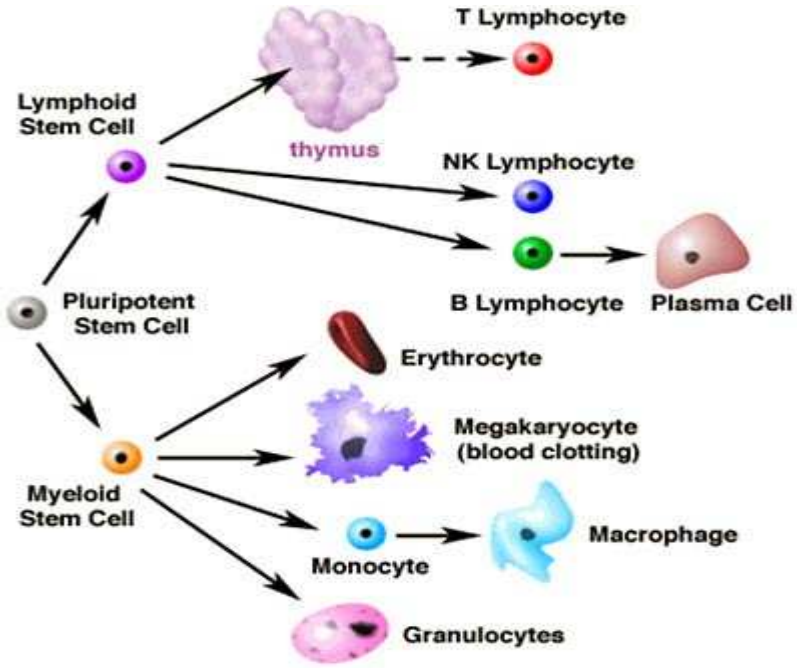

Figure 1.1a: *Diagrammatic representation of the differentiation of immune cells from a myeloid stem cells* (Geissmann, et al. 2010)

1.4 DENDRITIC CELLS (DC)

Dendritic cells (DC) are a heterogeneous family of cells that are present in low quantities in those tissues which are in contact with the environment e.g. the skin (where they are often called Langerhans cells). They are considered the most influential antigen presenting cells (APC) in the body because they are capable of internalizing, processing endogenous or exogenous antigen and their unique ability to present antigen-MHC complexes to the peptide specific T cells thereby initiating adaptive immunity (Banchereau, et al. 2000). DC have a plethora of other functions which include, induction of tolerance, control of T cell polarization and recruitment of T cells to the site of infection. They start out as immature DCs where they are constantly patrolling for pathogens such as viruses and bacteria (Knight, Burke and Bedford 2002). Once a DC detects a pathogen, the initial interaction is between a specific PAMP found on the pathogen and its relevant receptor such a toll-like receptor (TLR) on the surface of the DC (Beutler 2009). TLRs are essential transmembrane proteins that detect pathogens and alert the immune system to the presence of invading microbes (Goldstein 2004). TLR ligation leads to the activation of DCs and the instantaneous production of pro-inflammatory cytokines such as IL-12, TNF-α and IL-1β that facilitate the enhancement of innate and adaptive immune responses. Throughout DC maturation there is an increase in the levels of MHC molecules bearing pathogen-derived antigen on the surface of DCs which engages with naïve pathogen-specific T cells (signal 1), in addition to an up-regulation in co-stimulatory molecules (CD40, CD80, CD86), greatly enhancing their ability to activate naïve T cells (signal 2). Finally, DC maturation also results in inflammatory mediators (i.e. cytokines, co-stimulatory molecules) that can act on naïve T cells to promote their differentiation into effector T cells such as Th1, Th2, Th17 or

regulatory T cells (signal 3), summarised in figure 1.2 (Joffre, et al. 2009). DCs play a central role in the controlling immunity and are thus targets for clinical research examining T –cell mediated events e.g. transplantation, allergy and autoimmune diseases (Banchereau and Steinman 1998b).

Immature DCs capture antigens by phagocytosis, macropinocytosis or via interaction with a variety of cells surface receptors and endocytosis. The most prevalent antigen receptors expressed by DCs include members of the C-type lectin family, such as mannose receptor, DEC-205 and Fcγ receptors (Banchereau, et al. 2000). These receptors allow efficient capture of IgG immune complexes and opsonised particles (Cella, Sallusto and Lanzavecchia 1997). Internalization of foreign antigens by these receptors eventually leads to DC activation. Once DCs become activated the maturation process commences, DCs gain the ability to migrate to the lymph nodes where they stimulate naïve T cells.

1.4.1 DENDRITIC CELL MATURATION

The process of DC maturation coordinates a series of significant changes resulting in the loss of endocytic/phagocytic activity, up-regulation of adhesion and co-stimulatory molecules (CD40, CD80, CD86), redistribution of MHC II molecules and a change in morphology (Banchereau and Steinman 1998a). The interaction of co-stimulatory molecules will be discussed later in chapter 3**.** The maturation process also results in changes in the expression of chemokine receptors on the surface of DCs. Immature DCs express high levels of chemokine receptors including CCR1, CCR2, and CCR5 which are essential in assisting the migration of DC to T cell areas.

Once DCs reach the lymph node area they lose their migratory capacity and produce T and B cell stimulatory cytokines such as Interferons (IFN), IL-12 and IL-6. When DCs have acquired the ability to activate T cells they have completed their core function and thus commit suicide by apoptosis (Matsue, et al. 1999).

1.4.2 ANTIGEN PROCESSING AND PRESENTATION

Antigen processing and presentation is an intriguing combination of cellular functions and specialized processes. DCs are capable of processing and presenting peptides in the context of either MHC I or II. Two generally distinct pathways are used by MHC I and II molecules in order for the presentation of antigens to T cells. If the antigen is found endogenously i.e. within the cell, it is complexed to MHC I through an intracellular processing pathway. On the other hand exogenous antigens taken up by APCs (macrophages, DC, B cells) through endocytosis are processed onto MHC II and then expressed on the surface of the cell. DC-LAMP, a lysosomal glycoprotein, is specifically expressed in the lysosomal MHC II compartment and aids in the processing of exogenous antigen or in assisting transport of antigen-loaded MHC II complexes to the cell surface (Banchereau, et al. 2000). Immature DCs synthesize high levels of MHC II but instead of being expressed on the surface of APC they are expressed in the endosomal compartments where they have a short life. When immature DCs are activated by inflammatory stimuli, such as lipopolysacharide (LPS), the antigen is degraded within the MHC II endosomal compartment, coupled to MHC II molecules and then transported to the surface of the APC where it remains stably complexed to peptide for days and available for $CD4^+$ T cells (Steinman, et al. 1999).

1.4.3 ACTIVATION OF T CELLS

T cells express a specific antigen receptor termed TCR which interacts with DCs. This interaction is fundamental for initiating cell mediated adaptive immune responses. However, full activation of T cells critically depends on three signals. The TCR is composed of a α- and β-chain that form the TCR heterodimer which is responsible for ligand recognition. These α- and β-chains are non-covalently associated with a complex of low molecular weight transmembrane proteins, referred to as the CD3 complex (Schmitz and Krappmann 2006). The first signal is initiated upon binding of the TCR with antigenic peptides complexed to MHC molecules. The TCR-CD3 complex interaction plays an essential role in mediating cell recognition and inducing signal transduction into the nucleus, activating genes involved in T cell proliferation. Signal 2 is provided by the co-stimulatory molecules CD80 and CD86 which engage with the accessory molecule, CD28 on the T cell. Co-stimulatory molecules are only present on activated DCs ensuring that T-cell immunity is not activated unnecessarily (Smith-Garvin, Koretzky and Jordan 2009). These co-receptors are essential to enhance a productive immune response, which leads to the secretion of cytokines and increased survival and clonal expansion of T cells. However, in the absence of these costimulatory signals, the T cells will either become anergic or die by apoptosis, thereby promoting T cell tolerance. Anergy can occur in two distinct ways, one being if the TCR is engaged by a non-professional APC lacking costimulatory molecules on its surface (Macian, et al. 2004, Schwartz 2003); the second is if TCR ligation is followed by the binding of cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) with CD28 on the T cell (Inobe and Schwartz 2004).

Signal 3, relies on the ligation of a polarizing cytokine to its corresponding receptor on the T cell allowing naïve T cells to finally become effector T cells (Corthay 2006). Naïve T cells require stimulation for at least 20 hrs in order to be committed to proliferate whereas memory/effector cells are able to respond within 0.5-2hrs. Following engagement with antigen, the TCR is internalised and degraded and the differentiated cells lose their lymph-node homing receptors and acquire receptors that control their migration to inflamed tissues where they execute their effector functions (Sallusto and Lanzavecchia 2002). In contrast, T cells that receive a short stimulation proliferate, but fail to differentiate to effector cells and maintain the lymph node homing characteristics of naïve T cells. Division and clonal expansion of each T cell results in up to 1000 progeny, most of which are armed effector cells (Parkin and Cohen 2001a).

1.5 T CELLS

T cells are a subset of lymphocytes that arise from the bone marrow and undergo a cascade of events in the thymus including, differentiation and proliferation before returning to the circulation as mature T cells. They are found throughout the body, they often reside in the secondary lymphoid organs (the lymph nodes and spleen) as sites where activation occurs, but they are also found in other tissues of the body, most conspicuously the liver, lung, blood, and intestinal and reproductive tracts (Bousso 2008). T cells can be divided into two major subsets that are functionally and phenotypically different; T-helper cells (Th) and cytotoxic T-cells (CTLs) characterised by the expression of CD4 and CD8 respectively on their cell surface [see table 1.1]. T cells are able to recognise antigen bound to MHC molecules. $CD4^+$ T cells recognise antigen complexed to MHCII molecules whereas $CD8^+$ T cells recognise antigen complexed to MHCI molecules (Parkin and Cohen 2001a). As mentioned in section 1.4 these T cells are only activated if accessory molecules on the T cell are ligated to costimulatory molecules on the APC.

$CD8^+$ T cells exhibit cytotoxic activity, which is fundamental in directly killing certain tumour cells, viral-infected cells, any cells displaying foreign antigen coupled to MHCI and occasionally parasites. The $CD8^+$ T cells are also essential in down-regulation of immune responses (Foulds, et al. 2002). $CD4^+$ T cells are important in directing the immune response. $CD4^+$ T lymphocytes differentiate into distinct effector cell subsets characterised by their function and cytokine production profile. Defects in the appropriate regulation of $CD4^+$ T cell function have been implicated in the exacerbation of numerous autoimmune diseases such as RA and IBD (Egwuagu 2009, Palmer and Weaver 2010). Until recently the general agreement was that there

were only 2 types of T helper lineages; Th1 and Th2, however new lineages of T cells are emerging including Th17, Th9 and Th22. Figure 1.2 below depicts the processes involved in the polarisation of Th1 and Th2 cells.

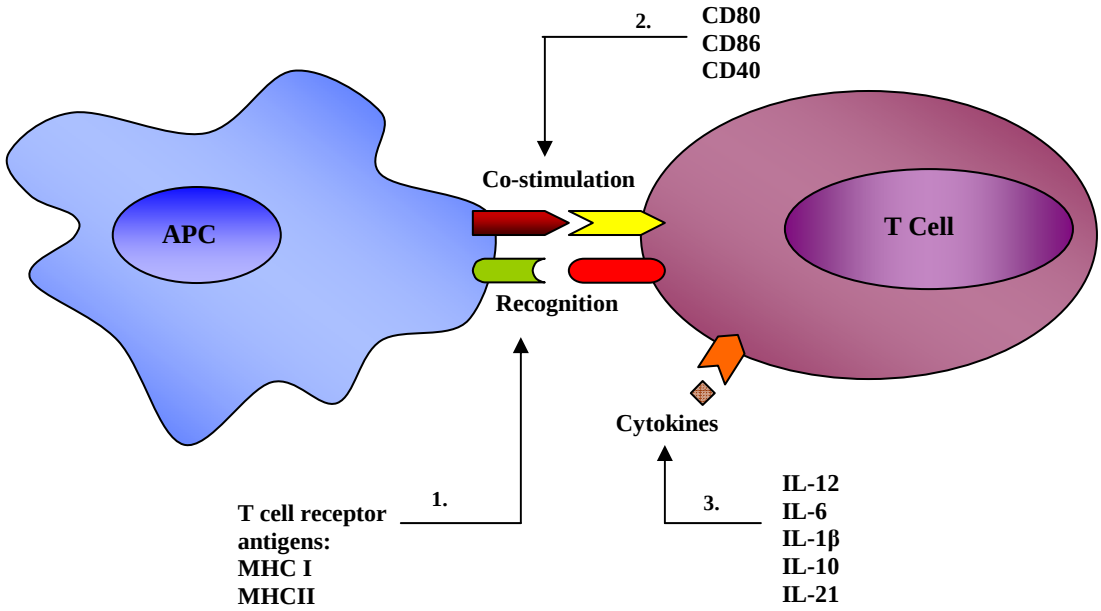

Figure 1.2 *T-cell activation and TH1/TH2-cell differentiation require three dendritic cell-derived signals; Signal 1 is the recognition and ligation of the TCR receptor to MHC peptide complexes. Signal 2 is the co-stimulatory signal, mainly mediated by ligation of CD28 by CD80 and CD86 on APC and, signal 3 secretion of cytokines by APC that bind to cytokine receptors on the surface.*

1.5.1 TH1 AND TH2 CELL DEVELOPMENT

Numerous factors are involved in the polarisation of naïve T cells into a Th1 or a Th2 phenotype. The cytokines IL-12, IL-27, IFN-γ and the transcription factors, signal transducer and activator of transcription- (STAT)-1, STAT4, the adhesion molecule, ICAM-1 (intracellular adhesion molecule-1) and T-bet promote the development of Th1 cells, where as the differentiation of Th2 is largely dependant on the cytokine IL-4 in combination with the transcription factors STAT6 and GATA3 (Murphy and Reiner 2002, Callard 2007).

The conventional definition of Th1 and Th2 cells depends firmly on the cytokines that they produce. Th1 cells produce IL-2, IL-15, TNF-β, and IFN-γ which activate macrophages, NK cells, and CTLs. The primary role of Th1 cells is regulating protective immune responses against infection with intracellular pathogens (Murphy and Reiner 2002). In addition, they are principal effectors of cell-mediated immunity and delayed type hypersensitivity (DTH) (Agnello, et al. 2003). Conversely, Th2 cells secrete IL-4, IL-5 and IL-13, which promote humoral immunity, allergic responses and constrain cell mediated immunity and inflammation. During Th2 responses, IL-4 and IL-13 stimulate B cells to produce IgE, in contrast IL-5 is the main eosinophil-activating cytokine (Trinchieri 2003b). Extracellular pathogens, such as helminthic worms, tend to induce differentiation along the Th2 pathway (see table 1.1). IFN-γ and IL-4 act in regulatory feedback loops promoting further differentiation of Th1 and Th2 cells, respectively, in an anticrine manner (Murphy and Reiner 2002). One vital characteristic of the Th1/Th2 paradigm is that they can antagonise each other, Thl-type cytokines, especially IFN-γ, inhibit the differentiation of Th2 cells, while Th2-type cytokines, especially IL-4, inhibit the differentiation of Thl cells (Ho and Glimcher 2002).

Several diseases have been associated with the dysregulated function of Th1 and Th2 cells. For example, Th1 cells have been implicated in the development and pathogenesis of autoimmune diseases such as multiple sclerosis (MS) while Th2 cells contribute atopic diseases such as allergy and asthma (Deenick and Tangye 2007).

1.5.2 TH17 CELL DEVELOPMENT

Recently a subset of Th cells have been identified, termed Th17 cells (Bettelli, Korn and Kuchroo 2007, McGeachy and Cua 2008). In the same way as Th1 and Th2 cells, Th17 cells require specific cytokines and transcription factors for their differentiation. Th17 cells are characterised by the secretion of a distinct cytokine profile, including IL-17A, IL-17F, IL-21 and IL-22 (see table 1.1). IL-17 has emerged as a key pro-inflammatory cytokine and is known to induce the production of IL-1, TNF-α, and GMCSF. Differentiation of this subset is controlled by transforming growth factor-(TGF)-β and IL-6 (Smith-Garvin, Koretzky and Jordan 2009). These cytokines act in a STAT3-dependant manner to induce the expression of the orphan nuclear receptor-RORγt which subsequently upregulates the production of IL-17 and the IL-23R (Deenick and Tangye 2007). In addition, the production of IL-21 by Th17 cells together with TGF-β and in the absence of IL-6 are able to initiate Th17 differentiation, therefore IL-21 may be a positive feedback loop to amplify the precursor frequency of Th17 cells (McGeachy and Cua 2008). The differentiation of this subset was shown to be inhibited by IL-4 and IFN-γ as naïve T cells cultured with TGF-β and IL-6, in the absence of IFN-γ and IL-4 were found to differentiate into Th17 cells (Kikly, et al. 2006). Development of Th17 cells can also be suppressed by the presence of IL-27, a member of the IL-12 family of cytokine, as mice lacking the IL-27 receptor are highly susceptible to EAE because of increased production of IL-17 (Batten, et al. 2006).

Initially IL-23, a member of the IL-12 family, was thought to be involved in the development of Th17, however later studies showed that IL-23 acts on previously differentiated Th17 cells to induce expansion and survival of the Th17 phenotype

(Kikly, et al. 2007). Th17 cells are fundamental cells in host defence against certain pathogens such as *Candida albicans* and specific extracellular bacteria and fungi and have been implicated as being responsible for many inflammatory disorders previously attributed to Th1 cells, including RA and EAE (Himer, et al. 2010, Lubberts 2010, Segal 2010, Furuzawa-Carballeda, Vargas-Rojas and Cabral 2007).

1.5.3 TH9 CELL DEVELOPMENT

The complexity of Th cells increased further with the identification of a new subset of Th cells, termed Th9 cells (Veldhoen, et al. 2008). The combination of TGF-β and IL-4 induced the differentiation of naïve $CD4^+$ T cell into a Th9 subset which are characterised by the secretion of a distinct cytokine profile including IL-9 and IL-10. The generation of these cells requires the expression of the transcription factor, PU.1 and secretion of IL-9 is upregulated by IL-25 (Lloyd and Hessel 2010). Both human and murine Th9 cells do not co-express the characteristics of other Th subsets such as IFN-γ produced by Th1 cells. IFN-γ and/or IL-27 impeded the generation of Th9 cells. Th9 cells have been demonstrated to be involved in promoting inflammation and allergic reaction, mostly by recruiting macrophages, mast cells and eosinophils. Conversely, IL-9 modulates virus initiated inflammation and enhances the immunosuppressive activity of natural T regulatory cells. However, Vijay Kuchroo and workers have demonstrated that Th9 may elicit inflammation, particularly following re-stimulation (Jager, et al. 2009).

1.5.4 T REGULATORY CELL DEVELOPMENT

T regulatory cells or Tregs play a fundamental role in suppressing T cell responses to foreign and self antigens, preventing autoimmune diseases and limiting chronic inflammatory diseases (Mills 2004).

In general, Tregs can directly suppress the activation of effector T cells, compete with pathogenic T cells for access to APCs, or directly target APCs (Rutella and Lemoli 2004). Treg cells can be divided into two subsets based on the expression of cell surface markers, production of cytokines and mechanism of action; $CD4^+CD25^+$ Tregs that are developmentally programmed and suppress the activation of other self reactive T cells, and those that secrete abundant amounts of IL-10 and TGF-β which have shown to be essential for the prevention of colitis in mouse models of IBD (Izcue and Powrie 2008, Iwasaki and Medzhitov 2004).

T-Helper cell subsets	Differentiated by	Cytokines produced	Inhibited by	Protective activity
Th1	IL-12 ICAM-1 IL-27	IFN-γ IL-2 TNF-α/β	IL-4 I L-17	Cell-mediated immunity against intracellular bacteria & some viruses
Th2	IL-10 OX40L IL-4	IL-4 IL-5 IL-6 IL-13	IFN-γ IL-12	Humoral responses against helminth infections & multicellular organisms
Th17	IL-23 IL-1β, IL-6 TNF-α, TGF-β	IL-17A IL-17F IL-22 IL-6	IFN-γ IL-4	Extracellular bacteria & some fungi (Klebsiella pneumoniae; Candida albicans)
Treg	IL-10 TGF-β	IL-10 TGF-β	IL-6	Homeostasis – self-tolerance & control of excessive responses against non-self

TABLE 1.1 *Summary of the $CD4^+$ T-helper subsets, the cytokines they secrete, the cytokines that influence their differentiation, and their main immunological functions.*

1.6 CYTOKINES

Cytokines are a group of low molecular weight polypeptides or glycoproteins that are produced primarily by immune cells and encompass a wide range of functions. These molecules facilitate communication between cells, stimulate proliferation of antigen specific effector cells and mediate local and systemic inflammation in an autocrine, paracrine and endocrine manner (Sanchez-Munoz, Dominguez-Lopez and Yamamoto-Furusho 2008). Cytokines that act as molecular messengers between leukocytes are termed interleukins, while those that act against viruses and regulate cellular activation are characterized as interferons (IFN). Cytokines that cause differentiation and proliferation of stem cells are called colony stimulating factors. The cytokine system is a very potent force in homeostasis, when cytokine production is sustained they contribute to the pathology of inflammation. Cytokines can be classified according to their biological effects; pro-inflammatory and anti-inflammatory cytokines (Parkin and Cohen 2001a). Pro-inflammatory cytokines are involved in the up-regulation of inflammatory reactions (i.e. Th1 and Th17 responses) which comprise IL-12, IFN-γ, IL-17 and IL-1β whereas anti-inflammatory cytokines are involved in the down-regulation of inflammatory reactions (i.e. Th2 and T regulatory responses) which include IL-4, IL-10 and TGF-β (Zidek, Anzenbacher and Kmonickova 2009a) [see figure 1.3]. Below I will describe in detail some of the cytokines and chemokines relevant to this study table 1.2

Cytokine	Source	Modes 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})

TABLE 1.2 *Representative cytokines produced by different cells and their mode of action. Main reference sources (Janeway, Charles 2008; Borish,L.C. 2003; Nicod,L.P. 1993)*

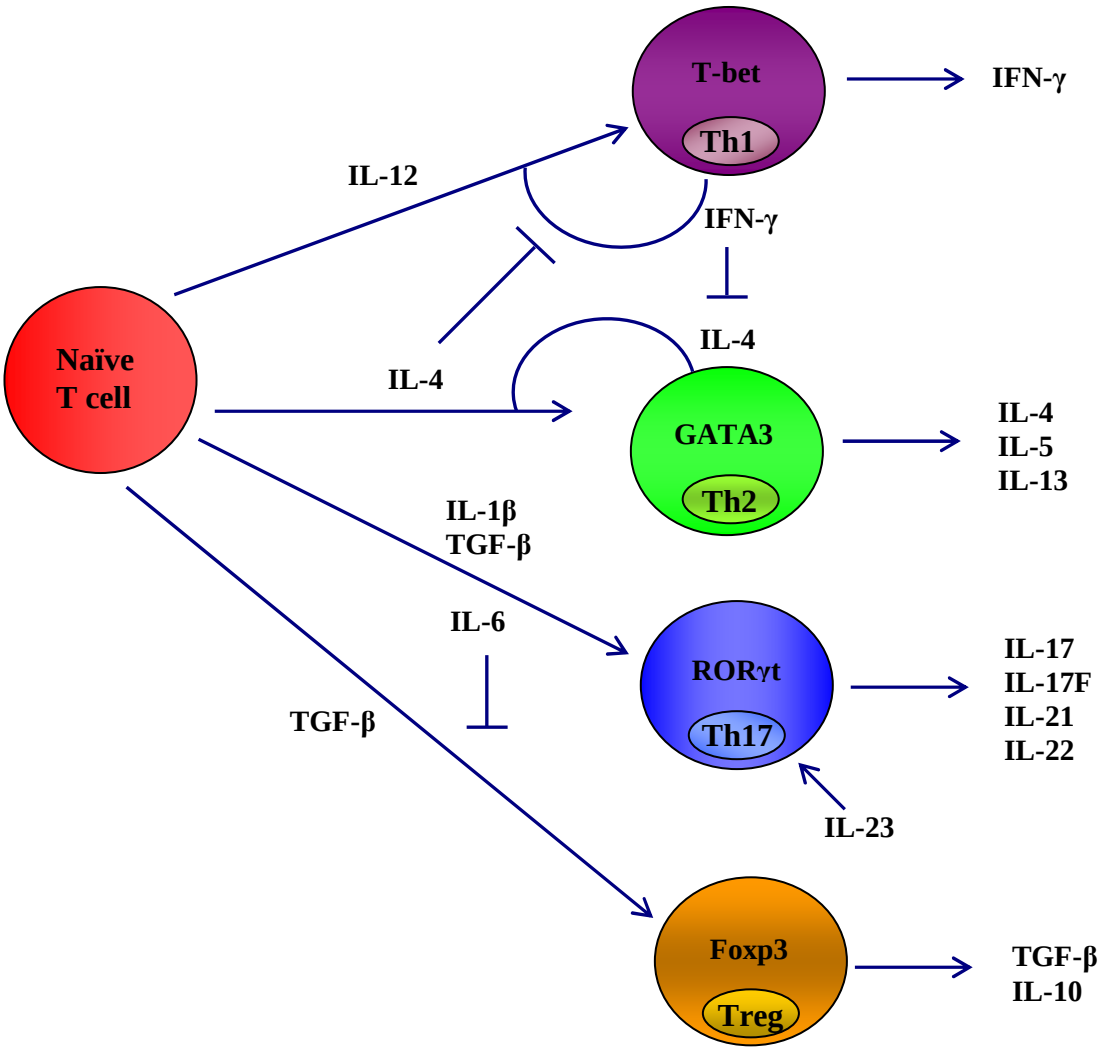

Figure 1.3 *Diagrammatic representations of the cytokines involved in promoting and inhibiting the differentiation of the distinct T helper cell subsets*

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1.6.1 INTERLEUKIN-12 (IL-12)

IL-12 was independently discovered by Trinchieri and colleagues in 1989 as a natural killer–stimulatory factor (Kobayashi, et al. 1989). The production of IL-12 by phagocytes and antigen presenting cells is essential for host defence against a wide range of pathogens. IL-12 functions in both innate and adaptive mechanisms, enhancing the phagocytic and bactericidal activities of phagocytes and the generation of other pro-inflammatory cytokines. IL-12 is a heterodimeric cytokine formed by a 35kDa light chain termed p35 and a 40kDa heavy chain termed p40. The p35 subunit

has homology to single- chain cytokines while the p40 subunit is homologous to the extracellular domain of cytokine receptors (Watford, et al. 2003). The genes encoding these subunits are located on different chromosomes and protein expression is therefore independently regulated. However, when both genes are co-expressed in the same cells, these subunits form the biologically active p70 heterodimer (Agnello, et al. 2003). In addition to forming IL-12p70, p40 can associate with a p19 subunit to form IL-23, which has similar biological effects to IL-12, but yet are distinc t(Kastelein, Hunter and Cua 2007). Induction of IL-12 occurs following stimulation with a variety of purified microbial products (i.e. LPS), parasites, viruses and fungi. T-cell activation also drives IL-12 production through CD40-CD40L interactions and stimulation through CD28 augments the expression of IL-12R (Trinchieri 2003a) . Furthermore, IL-12 secretion stimulates the production of a variety of cytokines, most significantly IFN-γ but also TNF-α, IL-18 and GM-CSF from T cells, NK cells (Bettelli and Kuchroo 2005) [see figure 1.4].

A critical function of IL-12 is its regulation of the adaptive immune response, as IL-12 is the classic cytokine that directs naïve T helper cells to a Th1 phenotype. In T cells IL-12 is synergistic with many activating stimuli, including, IL-2, TCR-CD3 complex and CD28 ligation for inducing rapid and efficient secretion of IFN-γ. In addition IL-12 augments the cytotoxic activity of CTLs and NK cells (Trinchieri 2003a, Trinchieri 1998). [See Figure 1.4 for a diagrammatic illustration of the main roles of IL-12]

IL-12 has been shown to be directly and predominantly involved in autoimmune diseases including MS, IBD, and RA (Gee, et al. 2009), consequently it represents a potential therapeutic target for the treatment of these inflammatory disorders.

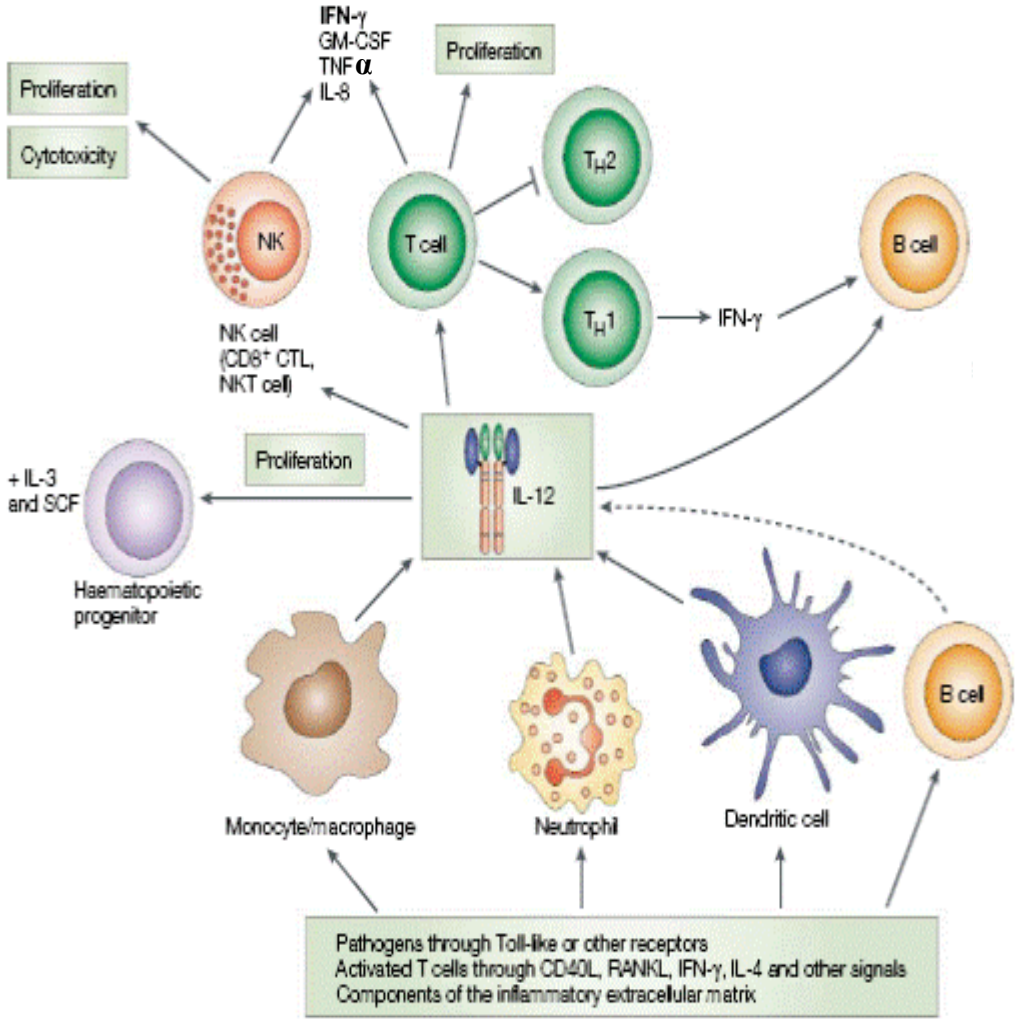

Figure 1.4: *Diagrammatic representation of the major biological effects of the cytokine IL-12. [Adapted from* (Trinchieri 2003a)*]*

1.6.2 IL-23

IL-23 is a heterodimeric protein that is a member of the IL-12 family of cytokines. It is composed of a unique p19 subunit along with a p40 subunit, which is also a

component of IL-12. Just like IL-12, the formation of biologically active IL-23 requires the co-expression of both subunits (p19 and P40) within the same cell (Collison and Vignali 2008). The p40-p19 complex is produced by activated DCs and macrophages. It is now accepted that IL-23 is not the differentiation factor for IL-17 producing T helper cells but it does act on previously differentiated Th17 cells to induce expansion and survival of the Th17 phenotype. Naïve T cells cultured in the presence of TGF-β and IL-6 and in the absence of Th1 producing cytokines IFN-γ or IL-4 are polarized to a Th17 phenotype, in addition the IL-23R is up-regulated (Langrish, et al. 2004)

Emerging evidence indicates the definitive role of IL-23 and not IL-12 in autoimmune pathogenesis, this has been demonstrated using knock out mice. Mice deficient in the subunit p19 but not p40 showed resistance to the induction of EAE (Wong, et al. 2008). Furthermore, elevated levels of IL-17, IL-6, IL-23p19 and TNF-α have been found in the biopsies from patients with RA thus implicating IL-23 as a major agent in the progression of inflammatory disorders (Li, et al. 2010).

1.6.3 IL-10

IL-10 is the crucial immunoregulator during infection with bacteria, viruses, fungi and helminths ameliorating the excessive Th1 and CD8+ T cell responses. IL-10 modulates expression of cytokines, soluble mediators and cell surface markers by a variety of cells including T and B lymphocytes, macrophages, dendritic cells and mast cells (Borish and Steinke 2003).

IL-10 limits the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, GMCSF, and TNF-α, it also acts directly on Th1 cells, inhibiting the proliferation and production of IL-2 and IFN-γ. IL-10 also reduces the production of chemokines including, MCP-1α, MIP-1, MIP-2, RANTES and IP-10, this prevents DCs migrating to lymph nodes and inducing Th1 differentiation of naïve T cells. In addition IL-10 inhibits expression of MHC II antigens and co-stimulatory molecules CD80, CD86 and ICAM-1, all of which impaired T cell activation (Pretolani 1999). Mice deficient in IL-10 signalling leads to the excessive production of pro-inflammatory cytokines directing the development of chronic inflammatory diseases such as Crohn's disease (CD), psoriasis and RA (Asadullah, et al. 1998).

1.6.4 TNF-α

TNF-α is a 17kDa pleiotropic cytokine produced by numerous cells such as macrophages, dendritic cells, monocytes and activated lymphocytes (Sanchez-Munoz, Dominguez-Lopez and Yamamoto-Furusho 2008). The biological responses to TNF-α are mediated through two distinct receptors, TNFR1 and TNFR2 (Palladino, et al. 2003). TNF-α activates a variety of cells including macrophages and NK cells and induces the production of pro-inflammatory cytokines (IL-1β and IL-6) and chemokines (MCP-1α). TNF-α also interacts with endothelial cells to induce adhesion molecules such as ICAM-1, stimulates proliferation of fibroblasts as well as the initiation of cytotoxic, apoptotic, acute phase responses and inhibition of apoptosis. TNF-α also influences the function of APC by augmenting antigen-presenting capability and up-regulating the expression of co-stimulatory molecules but conversely inhibits the properties of mature DCs by inducing their apoptosis and impairing antigen presentation (O'Shea, Ma and Lipsky 2002) .

Over-production of TNF-α has been extensively documented in RA, CD, MS and in many other autoimmune diseases and consequently its inhibition or blockade has proved efficacious in treating many of these disorders (Palladino, et al. 2003).

1.6.5 INTERFERON (IFN)-γ

IFN-γ is a pleiotropic cytokine and its production by APCs not only enhances innate immunity but also adaptive immunity. One of the primary roles of IFN-γ is to activate macrophage and dendritic cells leading to increased expression of MHC class I and II molecules, and the elevated production of IL-12, which induces the production of IFN-γ by NK cells and directs naïve T cells to differentiate into Th1 cells (Borish and Steinke 2003). In addition, IFN-γ aids macrophage and neutrophil intracellular killing by increasing NO and superoxide production and by phagocytosis. It also induces the production of TNF-α and IL-1 from APC, and upregulates the secretion of pro-inflammatory chemokines to augment the inflammatory process (Parkin and Cohen 2001b).

IFN-γ is primarily produced by $CD4^+$ T cells (mainly by Th1 cells), $CD8^+$ T cells and NK cells, however other cells including macrophages and DCs have been shown to secrete this cytokine but to lesser degree, following CD40-CD40 ligand costimulation (Choy and Panayi 2001). The production of IFN-γ by NK cells might play an important role in the initial stages of infection, prior to specific recognition of T cells. The IL-12 family of cytokines including the newly identified member, IL-35, share the common feature of inducing the production of IFN-γ by T cells and NK cells. For instance, IL-35 effectively attenuated established collagen-induced arthritis in mice, with concomitant suppression of IL-17 production but enhanced IFN-γ. IFN-γ in turn promotes IL-12 production from APCs creating a positive feedback loop between the

Th1 cytokines and enhancing the Th1 response. Since the production of IFN-γ by Th1 cells is involved in the pathogenesis of inflammatory disease such as, IBD, antibodies that target this specific cytokine could be of therapeutic value. For instance, fontolizumab is an anti- IFN-γ drug that has proved efficacious in patients with moderate to severe active IBD (Hommes, et al. 2006). Extensive research has also indicated IFN-γ as a mediator for disease as the genetic deletion of IFN-γ and/or the IFN-γR actually increased disease severity (for example in EAE and collagen-induced arthritis (CIA) (Guedez, et al. 2001) however conflicting studies by Komiyama and colleagues demonstrated that IFN-γ may be involved in the initiation of EAE (Komiyama, et al. 2006)

1.6.6 IL-17

IL-17 has numerous activities including the proliferation, maturation and chemotaxis of neutrophils, co-stimulates T cells, and enhances the maturation of dendritic cells, the production of pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-8 and GMCSF and chemokine monocyte chemoattractant protein (MCP-1) and MIP-2 (Korn, et al. 2009). IL-17 is mainly secreted by $CD4^+$ Th17 cells. In addition, $CD8^+$ T cells lacking T-bet can differentiate into an IL-17 secreting lineage. NK cells have been shown to be able to produce high levels of IL-17 rapidly upon activation and rapid secretion of IL-17 has also been detected upon stimulation of lymphoid tissue inducer cells by zymosan (Pernis 2009). IL-17 shares transcriptional pathways with IL-1β and TNF-α. IL-17 binding to the IL-17R can activate NFκB and MAPK (p38, JNK, ERK) resulting in T cell proliferation and the production of pro-inflammatory cytokines and chemokines (Paradowska, et al. 2007). IL-17 has been implicated in the pathology of autoimmune diseases such as RA, IBD and MS (Segal 2010, Li, et al. 2010, Kolls 2010). For instance, numerous studies have detected elevated levels of IL-17 in the

sera and synovial fluid in patients with RA also studies by Langrish and colleagues demonstrated that mice deficient in IL-17 are resistant to the development of EAE (Langrish, et al. 2005)

1.7 CHEMOKINES

Chemokines are a superfamily of small proteins (8-12kDa) able to induce chemotaxis in a variety of cells including dendritic cells, macrophages, monocytes and lymphocytes (Borish and Steinke 2003). These molecules regulate activity through interactions with members of the 7- transmembrane, G-coupled receptor (GPCR) superfamily (Lodowski and Palczewski 2009). Chemokines are produced in response to Th1 cytokines IFN-γ and IL-2 and other pro-inflammatory cytokines such as IL-1 and TNF-α. In contrast, the Th2 cytokine IL-4 and Treg cytokines, IL-10 and TGF-β are known to down-regulate secretion of chemokines. Chemokines execute numerous functions aside from chemotaxis (Sozzani, et al. 1999). They have direct effects on T cell differentiation by altering APC migration or cytokine secretion. Chemokines such as MIP-1α and RANTES can promote the development of Th1 lymphocytes while in contrast chemokines including MCP can inhibit IL-12 production and enhance secretion of IL-4 directing a Th2 response (Lodowski and Palczewski 2009, Foti, et al. 1999). Representative Chemokines, their modes of action and the cells that produce them are listed in table 1.3

Chemokines	Source	Modes 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 1.3 *Representative chemokines produced by different cells and their mode of action. Main reference sources {{91 Lodowski,D.T. 2009; 326 Viola,A. 2008; 345 Wang,Y. 2006}}*

1.8 INNATE IMMUNE RECEPTORS

The primary role of the immune system is to detect and eliminate microbial components following recognition of conserved molecular patterns referred to as ‘pathogen associated molecular patterns’ (PAMPs). Since PAMPs are highly conserved components of microbes, they are not readily altered by mutation or selection and provide ideal targets for innate recognition. PAMPs are recognised by a limited number of germ line encoded receptors known as ‘pattern recognition receptors’ (PRRs). There are numerous types of PRRs which include TLRs, NOD-like receptors (NLRs), Scavenger receptors (SRs) and RIG-like receptors (RLRs) (Gordon 2002). These PRRs are expressed by many cells of the immune system and they all play a fundamental role in innate and adaptive immune responses. PRRs can recognise and respond to components derived from bacterial, fungal and viral pathogens. In addition PRRs respond to danger associated molecules (DAMPs) including heat shock proteins, DNA and uric acid (Bianchi 2007). Signalling through PRRs activates specific intracellular signalling pathways leading to a cascade of events including the production of inflammatory cytokines and chemokines, upregulation of surface markers and also the maturation of immune cells.

1.9 THE IL-1 (IL-1R)/TOLL-LIKE RECEPTOR (TLR) SUPERFAMILY

Toll-like receptors are a family of PRRs that recognize PAMPs. The ligation of these receptors involves the maturation of immune cells and activation of intracellular signalling pathways leading to the production of cytokines and other inflammatory mediators (Sandor and Buc 2005). TLRs were discovered with the identification of toll, a receptor expressed by *Drosophila meganogaster* (Hashimoto, Hudson and Anderson 1988**).** It was observed that Toll displayed remarkable sequence similarity in its intracellular domain to that of a mammalian receptor for the IL-1R1, toll was shown to have a role in host defence and to be fundamental for antifungal immunity in *Drosphila* (Akira and Takeda 2004a). TLRs play a key role in the initiation of innate immunity which is essential for the induction of an adaptive immune response. TLRs are expressed on sentinel cells of the immune system, most notably, DCs, macrophages and neutrophils. They are also expressed on a variety of other cells including vascular endothelial cells, adipocytes and intestinal epithelial cells (O'Neill 2006).

TLRs are type 1 transmembrane proteins that are evolutionarily conserved between insects and humans. They encompass a subfamily within the larger superfamily of interleukins (IL-1) receptors based on the similarity within their cytoplasmic regions, which is now referred to as the Toll/IL-1 receptor (TIR) domain (O'Neill 2008). In contrast, the extracellular (ectodomains) portions of both types of receptors are quite distinct. The IL-1 receptors possess three immunoglobulin (Ig) like domains, whereas TLRs ectodomains are characterized by the presence of leucine rich regions referred

to as leucine rich repeats (LRRs) [see figure 1.5] (Beutler 2009, Akira and Takeda 2004a)(Beutler 2009, Akira and Takeda 2004b)

Ten human TLRs have been found to date and they individually have a distinct function in innate immune recognition. Based on the chromosomal location, genomic structures and amino acid sequence, the human TLRs can be divided into 5 subfamilies; TLR2, TLR3, TLR4, TLR5, TLR9 (Sandor and Buc 2005). Each TLR has the ability to recognise a specific ligand or discrete set of ligands as outline in section 1.9.1. The assembly of LRR side chains presents a sole combinational code to each TLR facilitating its binding to a particular ligand (Goldstein 2004, Kawai and Akira 2006). Imperatively, Charles Janeway and Ruslan Medzhitov identified human toll, htoll, now called TLR4, as the fist human toll to be discovered.

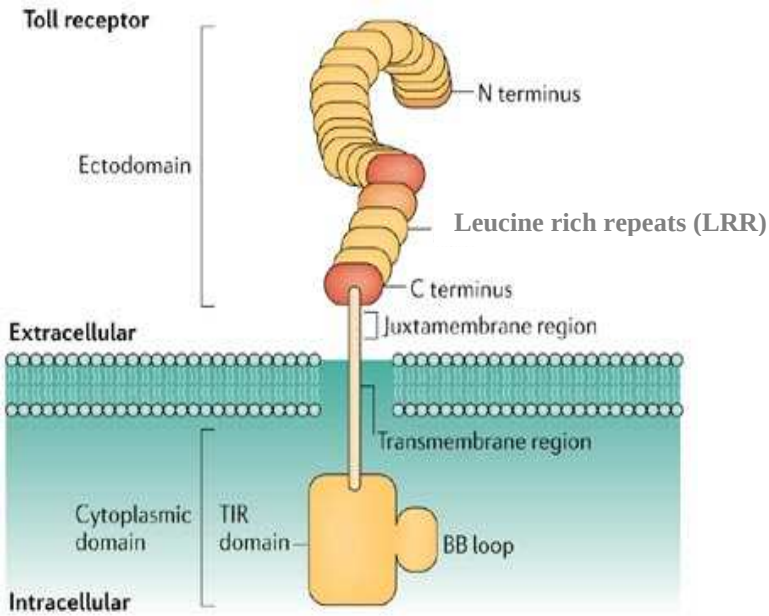

Figure 1.5: *Schematic diagram of toll and toll-like receptors adapted from* (Gay, Gangloff and Weber 2006)

1.9.1 TOLL LIKE RECEPTORS (TLRs)

So far, 10 and 12 functional TLRs have been identified in humans and mice, respectively (Georgel, Macquin and Bahram 2009). The most extensively studied is TLR4, which recognises the gram negative bacteria lipopolysaccharide (LPS) (Chow, et al. 1999). TLR2 is also involved in the recognition of bacterial components which, in combination with TLR1, recognises triacylated lipopeptides, or in combination with TLR6, recognises diacylated lipopeptides. TLR5 recognises flagellin, a protein component of bacterial flagellum (Gomez-Gomez and Boller 2002). Several TLRs are involved in the recognition of viral components including TLR3 which recognises double stranded RNA (dsRNA), TLR7 and TLR8 which recognise single stranded RNA (ssRNA), and TLR9 which recognises bacterial and viral DNA containing unmethylated CpG motifs (Yamamoto and Takeda 2010). TLRs, relative adaptor molecules, signalling pathways and inducible genes are listed in table 1.2.

1.9.1.1 TLR1, TLR2, TLR5 AND TLR6

TLR2 recognises numerous different microbial components including lipoproteins derived from *M.tuberculosis*, *Treponema pallidum* and *Mycoplasma fermantans*. In addition, TLR2 mediates a cellular response from whole gram positive and gram negative bacterium, the yeast cell wall component zymosan and peptidoglycan (PGN), the *Trypanosoma cruzi* glycophosphatidylinositol anchor (Aliprantis, et al. 1999, Takeuchi, et al. 2000, Underhill, et al. 1999). TLR2 also recognises viral components such as herpes simplex virus 1 (HSV-1) and measles virus (MV) wild-type H protein (Kurt-Jones, et al. 2000, Bieback, et al. 2002). Amazingly, TLR2 is also able to recognise various kinds of LPS that are not recognised by TLR4 for example LPS

derived from *Porphyromonas gingivalis* (Ogawa, et al. 2002). The ability of TLR2 to recognise a variety of components and to initiate signalling is based on TLR2's unique ability to form heterodimers with other TLR family members. The TLR2/TLR1 heterodimer recognises triacylated lipoproteins such as $Pam_3C_sK_4$ whereas TLR2/TLR6 recognises diacylated lipoproteins such as zymosan (West, Koblansky and Ghosh 2006). Generally activation of TLR2 leads to the production of inflammatory cytokines and not type I interferon by macrophages and dendritic cells (DCs), however it can mediate the production of type I interferon by inflammatory monocytes following infection with vaccinia virus, which suggests a cell type specific role for TLR2 in antiviral responses (Barbalat, et al. 2009). Flagellin is a 55kda protein from bacterial flagella which is found on the outer membrane of gram positive and gram negative bacteria and is recognised by TLR5. A wide range of acute infections are caused by flagellated bacteria such as respiratory tract infection (de C Ventura, et al. 2008). The vital function of flagella in bacterial motility creates a situation in which the capacity for the molecule to mutate is constrained, and this renders it an ideal candidate for innate immune recognition (Hayashi, et al. 2001).

1.9.1.2 TLR3, TLR7, TLR8 AND TLR9

TLR3, TLR7, TLR8 and TLR9 are antiviral receptors that execute their functions in the intracellular compartment of the cell. TLR3 recognises a synthetic analog termed polyinosinic acid (Poly:IC), which is widely used to mimic viral infection. In addition TLR3 has been shown to recognise (Koyama, et al. 2008). West Nile Virus (WNV). WNV is a positive ssRNA virus that produces dsRNA in its life cycle. WNV causes a breakdown in the blood brain barrier leading to an enhanced systemic infection in the brain resulting in encephalitis and death. Mice deficient in TLR3 are more resistant to lethality after WNV infection (Wang, et al. 2004). TLR7 and TLR8 are structurally

and phylogenetically related. Synthetic compounds of the imidazoquinoline family, including imiquimod (R-837) and resquimod (R-848) demonstrate potent anti-viral and anti-tumour activities in animal models (Smits, et al. 2008). Hemmi and colleagues have shown that TLR7 is involved in the recognition of both imiquimod (R-837) and resquimod (R-848) (Hemmi, et al. 2002). However human TLR8 only recognises R-837 whereas mouse TLR8 does not recognise either, indicating that TLR8 is non-functional in mice (Hemmi, et al. 2002). Both TLR7 and TLR8 have recently been discovered as natural receptors for ssRNA including vesicular stomatisis virus and influenza virus. Furthermore, Heil and colleagues have demonstrated that the guanosine analog, loxoribine (7-allyl-7,8-dihydro-8-oxo-guanosine), activates NFκB via TLR7 but not TLR8 in a Myd88-dependant manner (Heil, et al. 2004). Unmethylated CpG DNA motifs are present in bacterial and viral but not vertebrate genomic DNA. Synthetic CpG oligodeoxynucleotides (ODN) are recognised by TLR9 which activates host defence mechanisms leading to innate and adaptive immune responses (Hemmi, et al. 2000). TLR9 is expressed by numerous cells of the immune system including pDCs and B cells. The TLR profile in pDC is limited to TLR7 and TLR9 and it is for this reason that these cells are frequently used to examine the immunostimulatory effects of CpG DNA and the associated role of TLR9 (Rothenfusser, et al. 2002). In addition to recognising DNA, TLR9 directly recognises a non-DNA ligand termed hemozoin generated following digestion of host haemoglobin by malaria parasites (Coban, et al. 2010)

1.9.1.3 TLR 4

The founding member of the TLR family, TLR4 recognises and is activated by LPS, a component found on the outer membrane of gram negative bacteria such as

Escherichia coli and *Salmonella Minnesota*. LPS is a complex glycolipid composed of a hydrophilic polysaccharide and a hydrophobic domain termed lipid A which is primarily responsible for the biological activity of LPS and which is responsible for instigating the life threatening condition called endotoxic shock (Erridge, et al. 2008b). In order for TLR4 to initiate signalling LPS needs to bind to LPS-binding protein (LBP), an acute phase protein that is found in the bloodstream and produced by the liver. The LPS-LBP complex then interacts with cluster of differentiation 14 (CD14), a leucine rich repeat containing protein, which enables LPS to be transferred to the LPS receptor complex TLR4-MD-2 (Akashi-Takamura and Miyake 2008, Palsson-McDermott and O'Neill 2004) . In addition TLR4 is involved in the recognition of a wide range of molecules including heat shock proteins, fibrinogen, taxol and also viruses such as respiratory syncytial virus fusion proteins, mouse mammary tumour virus envelope proteins, *Streptococcus pneumoniae* pneumolysin and the plant-derived cytostatic drug paclitaxel, however, LPS remains its most potent ligand (Kurt-Jones, et al. 2000, Kawai and Akira 2010).

TLR4 was identified through the examination of a mouse strain C3H/HeJ that was hyporesponsive to LPS. This C3H/HeJ mouse strain carries a missense point mutation within the *tlr4* gene region encoding the cytoplasmic tail. This mutation alters a highly conserved proline to a histidine therefore allowing for the generation of mice with non-functional TLR4. These TLR4 $^{-/-}$ mice were hyporesponsive to LPS, confirming that TLR4 is essential for LPS signalling (Poltorak, et al. 1998).

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, TNF-α, 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 1.2: *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.*

1.10 TLR ACTIVATION AND SIGNALLING

TLR detection of PAMPs initiates the intracellular signalling pathways that induce a cascade of events including the up-regulation of pro-inflammatory cytokines and chemokines, enhancement in the expression of co-stimulatory molecules, activation of complement, recruitment of phagocytic cells and mobilization of APCs. There is clear evidence that if TLRs are over activated infectious and inflammatory diseases can develop therefore they must be tightly controlled (O'Neill 2006, O'Neill 2005). TLRs occur as dimers and to date several crystal structures of TLR dimers have been elucidated, TLR2 heterodimerize with either TLR1 or TLR6 while TLR4, TLR5 and TLR9 homodimerize. The preliminary step in signal transduction of TLRs involves the binding of the ligand resulting in dimerization of two receptor chains. This induces a conformational change in the dimer, which brings the TIR domains of the two TLRs into close proximity [see figure 1.6]. Eventually, association of the TIR domains provides a platform that is essential for the recruitment of specific adaptor molecules which also contain a TIR domain. Once TIR-adaptors are engaged to TLR-TIR domain, signalling is initiated which will ultimately lead to the activation of specific transcription factors (O'Neill 2008, Miggin and O'Neill 2006).

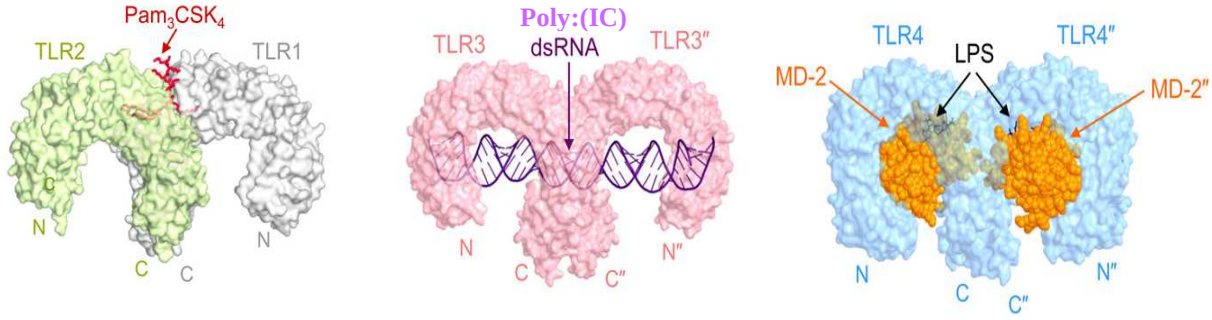

Figure 1.6: *The crystal structures of 'm' shaped TLR dimers induced by ligand binding (A) TLR1-TLR2-Pam3CSK4, (B) TLR3-dsRNA and (C) a model of TLR4-MD-2-Eritorian complex. Double apostrophes mark the second TLR or associated molecule (MD-2) in the receptor complex adapted from* (Jin and Lee 2008)

Signalling through TLRs via their TIR-domain involves five adaptor molecules and include; myeloid differentiating protein 88 (MyD88) (Verstak, et al. 2009), 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 and Takeda 2010), Trif-related adaptor molecule/TIR-containing adaptor molecule-2 (TRAM/TICAM-2) (Oshiumi, et al. 2003), and sterile alpha (SAM), HEAT/Armidillo motif and TIR-containing adaptor protein (SARM) (Jenkins and Mansell 2010). Some TLRs do not utilize the same set of adaptors and the adaptors chosen determine the transcriptional response induced following microbial recognition as TLRs lead to the activation of two major signalling cascades, namely the MyD88- dependant pathway and the MyD88-independent pathway (Bagchi, et al. 2007).

1.10.1 TLR SIGNALLING VIA THE MyD88-DEPENDANT PATHWAY

MyD88 was the first TLR adaptor protein to be described (Burns, et al. 1998). Due to the degree of conservation within the TIR domain between family members, it is not surprising to discover that signalling via the TLRs paralleled the downstream events following ligand binding of the IL-1 or IL-18 receptor complexes, both of which have TIR domains. The MyD88- dependant pathway eventually leads to the activation of the transcription factor, NFκB, initiating the production of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α. MyD88 is involved in NFκB activation of every TLR identified so far with the exception of TLR3, TLR3 induced activation by poly(I:C) was normal in the absence of MyD88 (Matsumoto and Seya 2008). Signalling via the MyD88 dependant pathway is initiated by the recruitment of MyD88 adaptor. Upon stimulation IL-1R associated kinase (IRAK-4) interacts with

the death domain of MyD88. IRAK-4 is required for the recruitment of IRAK-1 to the receptor complex which causes the activation and phosphorylation of IRAK-1. However the molecular mechanisms of IRAK-4 signalling and the role of its kinase activity remain unknown. Phosphorylated IRAK-1 leads to the association of the TNF-receptor associated factor 6 (TRAF6) (Akira and Takeda 2004a). TRAF6 was established to have a role in the IL-1R signalling pathway when a dominant negative form of the protein inhibited activation of NFκB by IL-1. TRAF6 and IRAK1 then dissociate from the receptor complex and interact with a membrane bound pre-associated of TGF-β-activated kinase (TAK-1) and two TAK-1 binding proteins, referred to as TAB-1 and TAB-2. A series of ubiquitinylation reactions occur on TRAF-6 and TAK-1. This then activates inhibitor of NFκB protein (IκB) kinase (IKK), IKKα and IKKβ (O'Neill 2008). Once activated the IKK complex induces phosphorylation and subsequent degradation of IκB which leads to the release of NFκB enabling it to translocate into the nucleus culminating in NFκB activation and the expression of pro-inflammatory cytokines. Activation of the Myd88- dependant pathway also allows for the activation of mitogen activated protein kinases (MAPK) including JNK, P38 and ERK (Takeda and Akira 2007, Watters, Kenny and O'Neill 2007). TLR7, TLR8 and TLR9 act through MyD88 to induce pro-inflammatory cytokine secretion and the IFNs.

1.10.2 TLR SIGNALLING VIA THE MyD88-INDEPENDANT PATHWAY

Experiments in MyD88 deficient mice show no production of inflammatory cytokines and no activation of NFκB in response to TLR2, TLR7 and TLR9 ligands and LPS induced activation of NFκB is observed with delayed kinetics when compared with wild type mice [see figure 1.7]. These findings led to the identification of an alternative

pathway utilised by TLR4, independent of MyD88 which leads to the activation of the transcription factor (IFN) regulatory factor-3, (IRF3) (Takeda and Akira 2005). Ultimately IRF3 activation leads to the production of type-1 interferons, IFN-α and IFN-β, and other IFN inducible genes. The MyD88 independent pathway requires the recruitment of the adaptor TRIF. TRIF is the sole adaptor used by TLR-3 in the activation of IRF3 and activation of NFκB (Yamamoto, et al. 2003a)

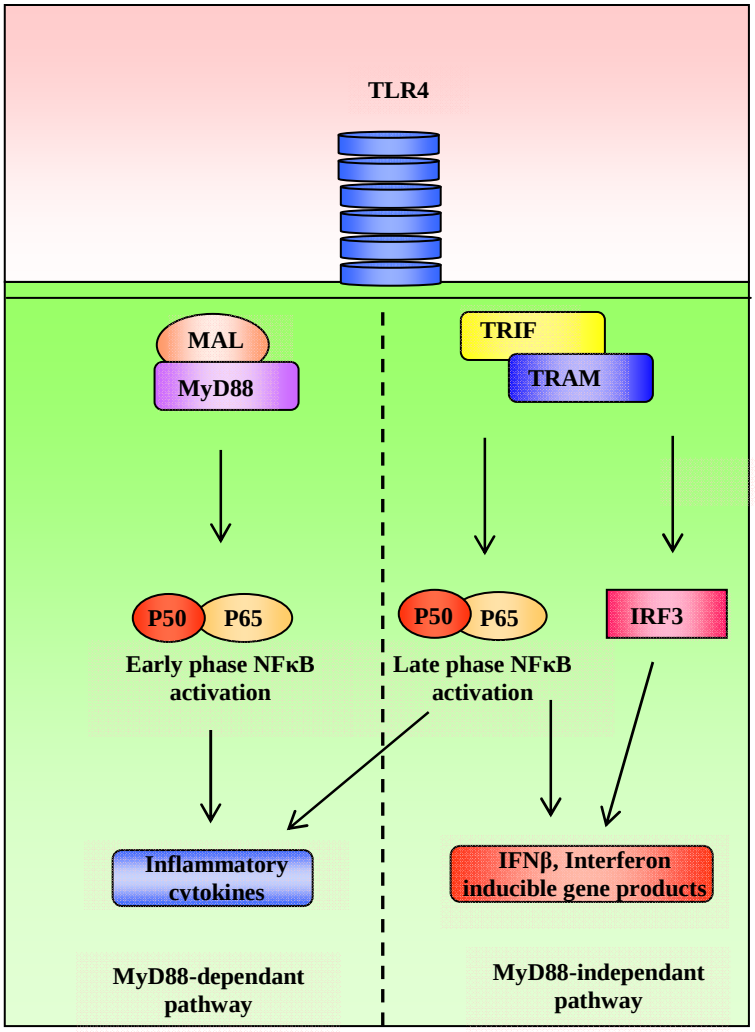

Figure 1.7: *Diagrammatic representation of TLR4 signalling through the MYD88 dependant and Independent pathways. MYD88-independent pathway activates IRF-3 and the late phase activation of NFκB*

In contrast to TLR3 signalling, TLR4 signalling independent of MyD88 utilises both TRIF and TRAM to activate IRF3. TLR4 is unique in that it is the only know TLR

that engages all four TIR containing adaptors (Palsson-McDermott, et al. 2009). TRIF uses some shared and unique signalling molecules compared with MyD88. TRIF allows for the activation of IRF3, NFκB and is also involved in the induction of apoptosis. TRIF binds to TLR3 and recruits TRAF6 by binding to its N-terminal domain in an IRAK1 and IRAK4 independent manner. This leads to TAK1 activation and subsequent NFκB activation. However, to mediate IRF3 activation the N-terminal region of TRIF engages with tank-binding-kinase-1 (TBK-1) and IKKi (Yamamoto, et al. 2003a). These kinases then phosphorylate IRF3 resulting in its dimerization and translocation to the nucleus initiating in the transcription of IFN-inducible genes via the binding to an interferon stimulated response element (Beutler 2009, Akira and Takeda 2004a, Brikos and O'Neill 2008). Schematic representation of TLR activation of MyD88 dependant and independent pathways is outlined in figure 1.8.

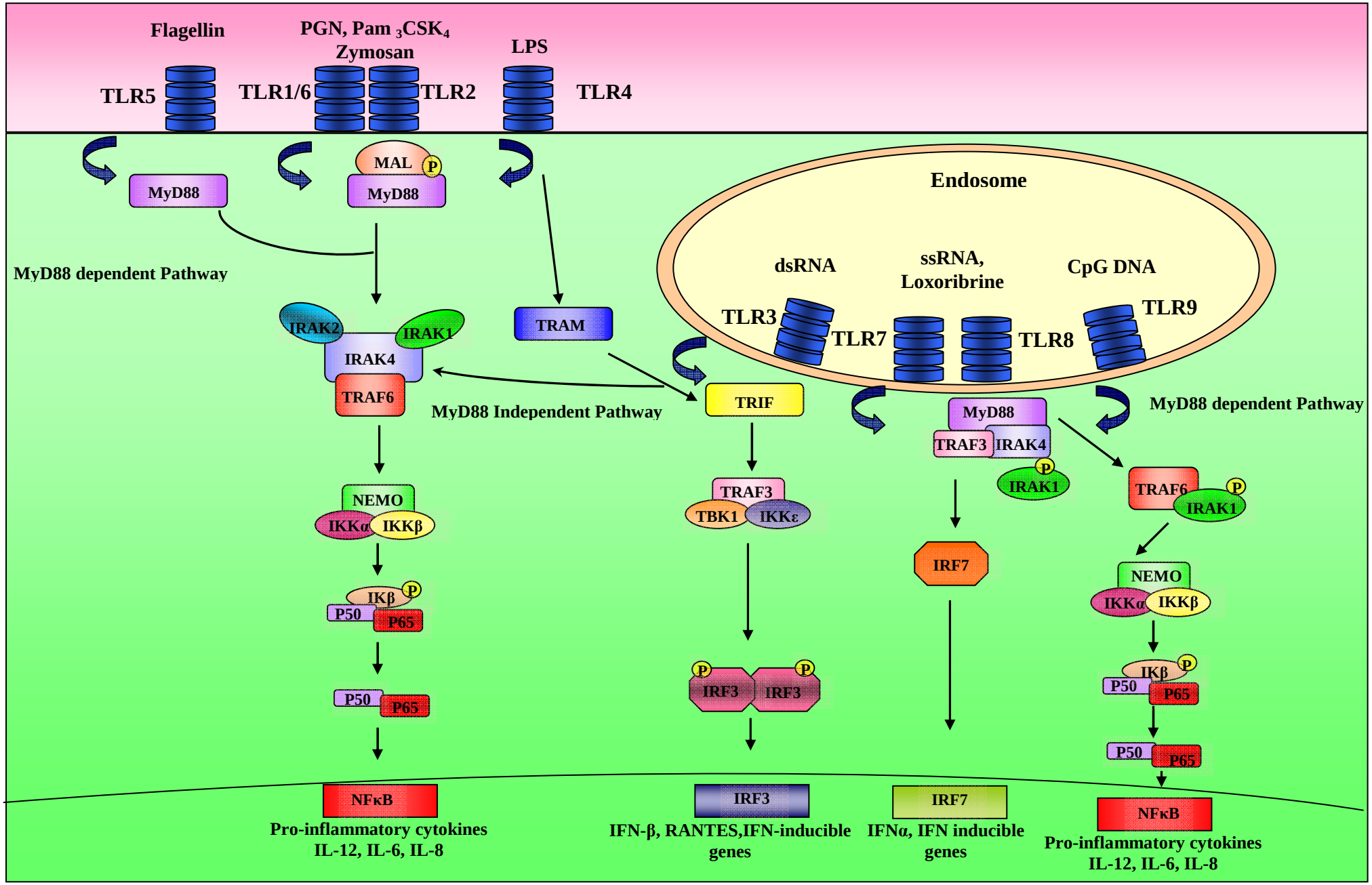

Figure 1.8: *Diagrammatic representation of TLR signalling with specific TLR ligands through the MyD88 dependant and independant pathways*

1.11 MAL

Mal was the second TIR domain containing adaptor to be identified which is capable of driving NFκB activation. It is a 25kDa protein, 235 amino acids in length and contains a TIR domain at the C-terminus (Fitzgerald, et al. 2001). It was initially assumed that Mal was involved in the TLR4 MyD88-independent pathway. However, mice deficient in Mal demonstrated that Mal is not involved in the MyD88-independent pathway but is critically involved in the MyD88-dependant pathway. In Mal deficient mice, IL-1, TLR5, TLR7 and TLR9 signalling were normal whereas deficiency in the activation of NFκB and cytokine production was observed following stimulation with LPS, though with delayed kinetics (Horng, et al. 2002a). Furthermore, overexpression of Mal in human embryonic kidney cells (HEK293) leads to the activation of NFκB, MAPK, JNK and p38, indicating its involvement with TLR4, but not with other receptors of the TLR family (Fitzgerald, et al. 2001, Horng, Barton and Medzhitov 2001b). Interestingly, Mal deficient mice showed impairment in response to TLR2 activation, indicating that Mal is an essential adaptor for TLR2-mediated innate host defence (Yamamoto, et al. 2002). This led to the conclusion that Mal is fundamental for signalling only through TLR4 and TLR2 (figure 1.9).

It has been clearly demonstrated that Mal serves as a bridging adaptor for MyD88, aiding the recruitment of MyD88 to the plasma membrane, where it can activate downstream signalling components. Mal contains a phosphatidylinositol-4,5-bisphosphate (PIP2)-binding domain in its N-terminus which mediates the recruitment of Mal to PIP2-rich regions in the plasma membrane (Kagan and Medzhitov 2006).

Obstructing this localisation of Mal to PIP2-rich regions inhibits TLR4-MyD88-dependant signalling (McLaughlin, et al. 2002). TLR4 does not bind directly with MyD88 but alternatively interacts with Mal in association with TLR4. This is consistent with the electrostatic surfaces of the TIR domains of Mal and MyD88. MyD88, TLR2 and TLR4 are greatly electropositive on their surfaces therefore MyD88 is unable to bind these TLRs. Whereas Mal is mainly electronegative on its surface enabling it to interact with TLR2 and TLR4 and bring MyD88 into the signalling complex (Sheedy and O'Neill 2007). LPS signalling in Mal deficient mice was restored when PIP2-binding domain was grafted onto the N-terminus of MyD88, which indicates that the sole function of Mal in relation to the NFκB pathway is to recruit MyD88 (McLaughlin, et al. 2002)

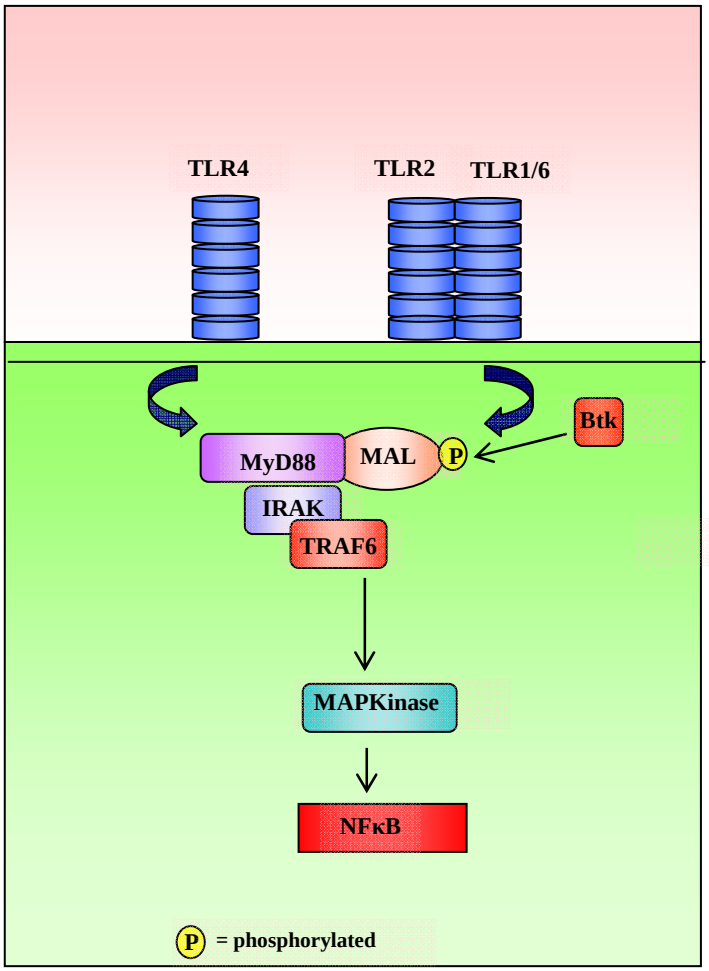

Figure 1.9: *Diagrammatic representation of TLR2 and TLR4 Mal mediated-signalling*

Mal contains a caspase-1 cleavage site in its C terminus, which is an essential inflammatory caspase responsible for the cleavage of pro-IL-1β into their bioactive forms (Kenny and O'Neill 2008). The caspase-1 inhibitor YVAD-Cmk prevented NFκB activation, in response to LPS and phosphorylation of p38 in response to Pam_3Cys. Moreover, TLR7 or IL-1 NFκB activation was not impaired, indicating that cleavage of Mal by caspase-1 is fundamental for Mal to signal (Miggin, et al. 2007) [see figure 1.10].

Mal contains a TRAF6-binding domain within its TIR domain. Mal can interact with TRAF6 directly and mutating this TRAF6 binding inhibits Mal-induced MAPK activation and NFκB activation and was able to impede TLR2- and TLR4-mediated activation of NFκB (Verstak, et al. 2009). A study by Gray and colleagues demonstrated that Mal undergoes tyrosine-phosphorylation at residues 86 and 106 by Bruton's tyrosine kinase (Btk) during TLR2 and TLR4 signalling (Gray, et al. 2006). This phosphorylation of Mal is fundamental for the activation of a pathway culminating in transactivation of the NFκB subunit p65 by phosphorylation on serine 536 and mutations in Mal at these residues results in decreased ability to activate phosphorylation of p38, IκBα degradation and NFκB (Doyle, Jefferies and O'Neill 2005). In addition, Tyrosine-phosphorylated Mal is subsequently degraded following stimulation of TLR2 and TLR4. This degradation is mediated by suppressor of cytokine signalling 1 (SOCS1) and requires tyrosine phosphorylation of Mal by Btk. Cells deficient in SOCS1 demonstrate enhanced NFκB activation and excessive cytokine production (Baetz, et al. 2004, Nakagawa, et al. 2002) [see figure 1.10]

A variant of Mal was discovered in humans in which the serine at position 180 is mutated to a leucine (Watters, Kenny and O'Neill 2007). It was discovered by Khor and colleagues that heterozygous carriage of this variant confers resistance against numerous infections diseases including malaria, tuberculosis (TB), bacteraemia and pneumococcal pneumonia (Khor, et al. 2007). In addition it was observed that S180L mutation inhibited TLR2 signalling. Therefore, due to the decreased level of TLR2 and TLR4 signal heterozygotes are protected from diseases which are sufficient for host defence but not for inflammatory diseases such as RA (Sheedy and O'Neill 2007).

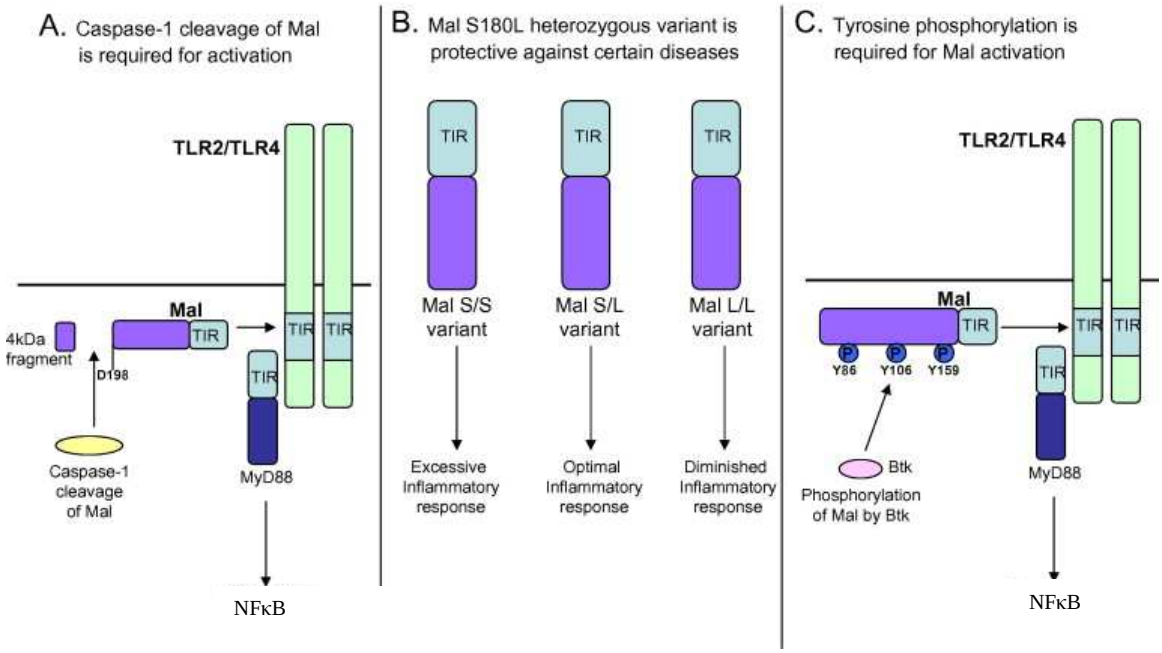

Figure 1.10: *Diagrammatic representation of the three main findings in Mal signalling: (A) Caspase-1 cleavage of Mal is required for signalling, (B) A mutation in Mal at the serine residue to a leucine results in protection against certain disease, (C) Mal is phosphorylated at tyrosine residues in order for the activation of the MyD88-dependant pathway adapted from* (Kenny and O'Neill 2008)

1.12 INTRACELLULAR SIGNALLING

Intracellular signalling is the fundamental mechanism by which cellular activation occurs. Innate immune stimuli, including pathogens, cytokines and stress signals function through distinct intracellular signalling pathways to activate protein kinases, instigating phosphorylation and ubquitination events that lead to the activation of specific transcription factors controlling gene expression. There are numerous signalling pathways and transcription factors that are known to be involved in the development of inflammatory disease such as IBD and RA (Brown, Claudio and Siebenlist 2008). The two transcription factors relevant to this study will be discussed in details below.

1.12.1 NUCLEAR FACTOR (Fib)

NFκB is a vital transcription factor in inflammation, regulating genes that encode pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-12 and IL-6, chemokines, adhesion molecules, immune receptors, matrix metalloproteinases (MMPs) and cyclooxygenase (COX) (Tak and Firestein 2001, Plevy, et al. 1997). NFκB was originally identified by its ability to bind to the promoter of the kappa light chain in B cells (Sen and Baltimore 2006) but it has since been shown to be present and functional in many cells types including DC, macrophages, neutrophils and mast cells (Wan and Lenardo 2010). Five related transcription factors of the NFκB family have been identified to date; NFκB1 (p50/p105), NFκB2 (p52/p100), RelA (p65), RelB, and c-Rel. The p50 and p52 subunits are transcriptionally inactive but can induce gene expression when they form heterodimers with p65, c-Rel, or RelB. Homodimers of p50/p50 or p52/p52 subunits can repress transcription, however the most frequently observed active form of NFκB is the p65/p50 heterodimer (Yan and Polk 2010). The IL-12 p40 promoter

has been reported to bind NFκB (p50/p65 and p50/c-Rel) in macrophages activated by a number of IL-12 activating pathogens, including LPS and *S. aureus* (Murphy, et al. 1995). All NFκB family members contain a highly conserved REL-homology domain (RHD) which is responsible for DNA binding, dimerization and interaction with inhibitors of NFκB (IκB) (Ghosh and Karin 2002).

Homodimers or heterodimers of NFκB are bound to NFκB (IκB) inhibitory proteins. This binding causes the dimers to be sequestered in the cytoplasm and are unable to initiate transcription of target genes. Like NFκB, IκBs are also members of a multigene family containing eight members IκBα, IκBβ, IκBγ, IκBε, IκBζ, Bcl-3 and the precursor Rel-proteins p105 and p100. Stimulation by numerous innate stimuli, such as LPS and cytokines such as TNF-α and IL-1 results in the activation of NFκB (Li and Verma 2002). Ultimately, these stimuli lead to the activation of IκB kinase (IKK), which is a complex composed of two catalytic subunits, IKKα and IKKβ, and a scaffold protein called NEMO (NFκB essential modulator - also known as IKKγ). IKKβ activation phosphorylates IκB which is then ubquitinated, which targets it for degradation by the 26S proteasome, releasing NFκB dimers from the cytoplasm and allowing to translocate to the nucleus (Yan and Polk 2010). Due to the phosphorylation of the p65 subunit of NFκB, NFκB allows the basal transcription initiation complex to interact with cofactors and coactivators and subsequently allows for full transactivation of its target genes (Chen 2005). IκBα is then degraded and is rapidly resynthesised to allow inhibition of the pathway. IKK also phosphorylates IκBβ leading to prolonged NFκB activation whereas phosphorylation of IκBα regulates transient NFκB activation (Krappmann and Scheidereit 1997). The role of NFκB as a key regulator in innate and adaptive immunity is demonstrated by studies

using transgenic mice whose T cells lack the NFκB signalling pathway. This shows that NFκB plays a vital role in Th1 responses. Furthermore DC maturation appears to be NFκB-dependant; following *in vitro* stimulation, NFκB is activated to facilitate DC maturation which is characterised by an up-regulation of co-stimulatory molecules and MHC II expression. Interestingly, NFκB activation can have anti-inflammatory roles by directly inhibiting expression of pro-inflammatory genes and by directing the expression of anti-inflammatory cytokines such as IL-10 thus inhibiting NFκB during the resolution stage of inflammation (Tak and Firestein 2001). Excessive activation of NFκB is extensively implicated in inflammatory diseases such as RA, IBD and MS and much attention has focused on the development of anti-inflammatory drugs targeting NFκB (Yan and Greer 2008, Wei and Feng 2010, Boyce, Yao and Xing 2010).

1.12.2 INTERFERON REGULATORY FACTOR 3 (IRF3)

The interferon regulatory factors (IRF) are a family of unique transcription factors and they are present in high levels in the cytoplasm of all cell types. IRF play a critical role in anti-viral responses and are responsible for the production of type I interferons, which include IFN-α and IFN-β (Taniguchi, et al. 2001). In addition to the anti-viral responses, IFN have further pleiotropic effects, including essential roles in apoptosis and growth inhibition via increased expression of MHC class I. IRF-3 and IRF-7 are closely related and are the only members of the IRF family that can initiate production of both IFN-α and IFN-β (Yoneyama, Suhara and Fujita 2002). Human type I interferon promotes differentiation of DCs and induces Th1 polarization. IRF-3 is activated in many ways including through TLR3 by recognition of viral dsRNA, by intracellular receptors such as retinoic acid-inducible gene I (RIG-I) and recognition of bacterial components (McCoy, et al. 2008). Upon stimulation IRF-3 is

phosphorylated at multiple sites at the C terminus of the protein by two non-canonical IκB kinases; TBK-1 and IKKε, resulting in IRF-3 dimerization, allowing for association with co-factor CAMP-response element binding protein (CREB), and following translocation to the nucleus (McCoy, et al. 2008, Servant, Grandvaux and Hiscott 2002). Entry of IRF3 into the nucleus facilitates binding to a consensus DNA sequence known as the interferon-response-element (ISRE) and the induction of IFN-inducible genes including; IFN-α, IFN-β, CXCL10 and RANTES (Servant, et al. 2001, Servant, Tenoever and Lin 2002).

1.13 THE MARINE INDUSTRY

Three quarters of the Earth's surface is covered by oceans and life on Earth has its origin in the sea. It contains an extraordinary reservoir of biological and chemical diversity and it is estimated that the biological diversity is higher than in tropical rainforests. The ocean represents a rich source of novel compounds with huge potential as pharmaceuticals, nutritional supplements, cosmetics, molecular probes, agrichemicals and enzymes with each marine bioproduct having huge market potential (Faulkner 2002). Already compounds isolated from marine species have been shown to have enormous potential in the clinical setting for treatment of many diseases and disorders (Blunt, et al. 2011).

Many marine species are soft bodied and produce novel secondary metabolites and compounds. This is due to the harsh conditions in which they live which require them to synthesize these unique compounds to survive. They are also linked to the defensive mechanisms by which the species protects itself from predators (Haefner 2003). The marine environment is the source of a large group of unique natural products that are mainly found in sessile marine invertebrates such as sponges, tunicates and bryozoans. A number of these molecules have been shown to possess a variety of functions including anti-tumour, anti-bacterial, anti-viral and anti-inflammatory activities and are therefore candidates for the development of therapeutics (Haefner 2003, Bhadury and Wright 2004). Attention has been focused on sponges as a source of biomedically important metabolites due to the discovery of spongouridine, a potent tumour inhibitor isolated from the Caribbean sponge *Cryptotethia crypta* (Kaul and Daftari 1986). Since then marine sponges have become a rich source of biologically active compounds with almost 100 anti-inflammatory

compounds isolated to date. Two of these, bolinaquinone and petrosaspongiolide, have recently been shown to have anti-inflammatory activity in animal models of RA and IBD (Busserolles, et al. 2005, Garcia-Pastor, et al. 1999, Lucas, et al. 2003).

1.13.1 COMPOUNDS IN PRECLINICAL AND CLINICIAL EVALUATION

In recent years numerous marine species have been shown to be candidates for anti-cancer and anti-inflammatory therapies [see table 1.4]

Chemical compound	Source organism	Application
Bryostatin-1	*Bugula neritina sp*	Anti-cancer
Ara-C	*Cryptotethya crypta sp*	Anti-cancer
Salinosporamide A	*Salinospora sp*	Anti-cancer
Manoalide	*Luffariella variabilis sp*	Anti-inflammatory
Avarol	*Dysidea avara sp*	Anti-inflammatory
Contignasterol	*Petrosia contignata sp*	Anti-inflammatory
Capnellene	*Capnella imbricate sp*	Anti-inflammatory

Table 1.4: *Anti-cancer/anti-inflammatory compounds isolated from marine organisms in clinical trials*

1.13.2 BRYOSTATIN-1

The bryostatins are macrocyclic lactones isolated from the bryozoans, *Bugula neritina*. They have been extensively studied for their therapeutic potential since 1986 (Hale, et al. 2002). Bryostatin-1 is the most studied compound of this species. It is a potent anti-cancer compound in combination with other drugs showing remarkable

selectivity against human leukaemia, renal cancer and melanoma. It has also been shown to bind to and modulate the signal transduction enzyme protein kinase-C (PKC) (Barr, et al. 2009). Owing to their therapeutic potential, scientists at Stanford University have synthesised numerous analogues which have been shown to be more potent against tumour cell lines (Haefner 2003). Additionally Neristatin-1, an analogue of bryostatin-1, reduced the production of IL-8 and prostaglandin E_2 in human cells, showing that it has anti-inflammatory activity (Breton and Chabot-Fletcher 1997).

1.13.2 NFκB INHIBITORS

NFκB is a crucial mediator in the inflammatory process and this transcription factor is an attractive target in suppressing an overactive inflammatory response (Atreya, Atreya and Neurath 2008). Many products isolated from marine species have been found to possess potent anti-inflammatory effects through their inhibition of NFκB [see figure 1.11]. One of the interesting explanations for the discovery of NFκB inhibitors from marine species comes from an evolutionary point of view (Folmer, et al. 2008). Marine invertebrates in many cases possess NFκB or closely related analogues and are therefore more likely to have developed metabolites that can counterbalance the effect of NFκB. Sea urchins have been shown to use spNFκB, an analogue of NFκB, to protect themselves against predators and also to respond to bacterial infection (Pancer, Rast and Davidson 1999).

Marine compounds that act as NFκB inhibitors can be divided into four categories: (1) compounds targeting IκB degradation, (2) compounds that impede the proteolytic activity of the 26S proteasome, (3) compounds that interfere with the translocation of

NFκB to the nucleus, and (4) compounds with unknown molecular targets (Folmer, et al. 2008). Scytonemin, Cacospongioloide B and Petrospongiolide M have all been shown to interfere with the degradation of IκB (Stevenson, et al. 2002, Posadas, et al. 2003). Marine products that have been shown to interfere with the proteosome include Salinosporamide A, Mycalolide A and acetate agosterol C (Folmer, et al. 2008, Macherla, et al. 2005, Tsukamoto, et al. 2003). However the mechanisms of action of most of the NFκB inhibitors remain unknown including the isolates Curacin A, Ilimaquinone, Verracurin A and Cycloprodigiosin Hydrochloride (Folmer, et al. 2008). Helenaquinone has been shown to inhibit PI3K, and the sesterterpene scalaradial has been shown to impede the phosphorylation of Akt. Akt is phosphorylated downstream of PI3K and both have been shown to be potent activators of NKκB (Xie, et al. 2005).

The anti-inflammatory potential of *bolinaquinone* and *petrosaspongiolide M* were assessed by (Busserolles, et al. 2005). Bolinaquinone was acquired from the Dysidea species of marine sponge while Petrosaspongiolide was acquired from the Petrosaspongia nigra marine sponge. These experiments were performed on mice treated with 2,4,6-trinitrobenzenesulphonic acid (TNBS) which induced Crohn's disease like symptoms. After administration with TNBS the size of the colon increased due to inflammation. Administration of bolinaquinone and petrosaspongiolide decreased the size of the colon.. The level of NFκB expression in the nucleus was found to be low after treatment with petrosaspongiolide which shows that it must inhibit its translocation to the nucleus by blocking the degradation of IκB(Busserolles, et al. 2005). These marine products have so far shown no

complications or side-effects which make them a potential candidate in the future treatment of inflammatory conditions (Busserolles, et al. 2005).

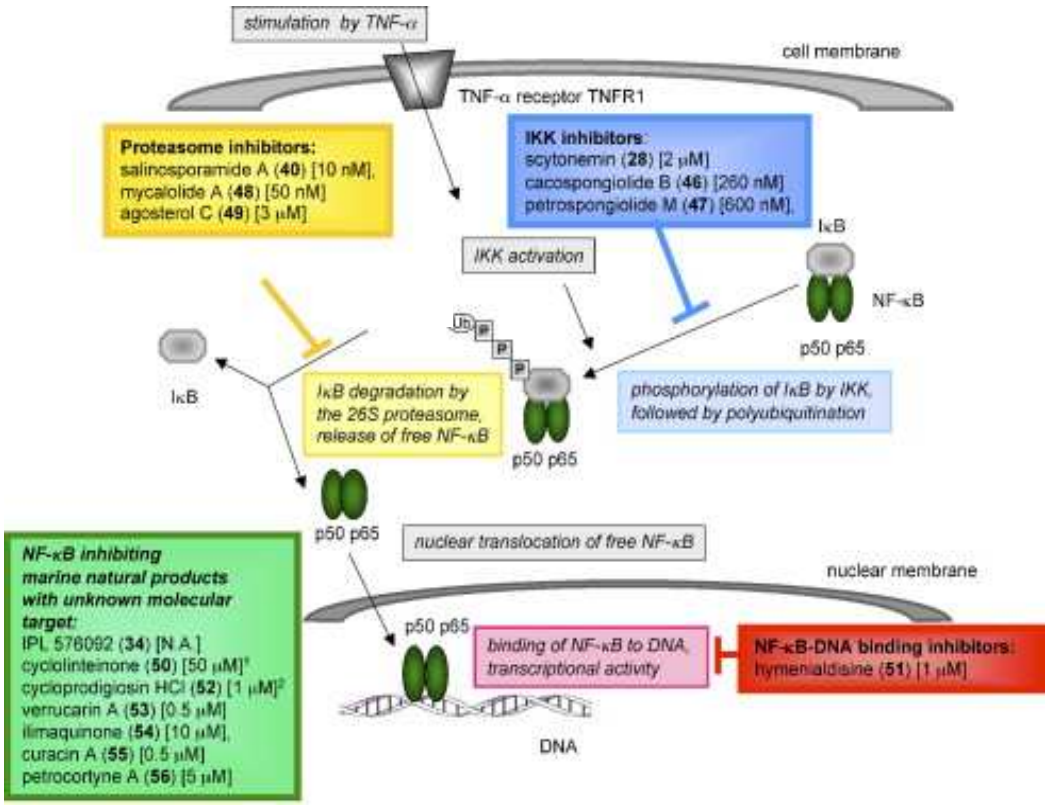

Figure 1.11: *Diagrammatic representation of the molecular targets of marine compounds known to inhibit NFκB activation adapted from* (Folmer, et al. 2008)

AIMS AND OBJECTIVES

Although numerous marine natural products have been reported to have pharmaceutical activity, relatively few marine compounds have been shown to have therapeutic effects in inflammatory disorders. This work aims to identify a novel marine compound with anti-inflammatory effects and also to elucidate its molecular target.

- To screen crude and fractionated marine extracts to find an individual compound showing anti-inflammatory potential, by examining the effects of marine extracts on macrophage, DC maturation, migration, phagocytosis, cytokine and chemokine production

- To ascertain the possible mechanisms utilized by the marine compound to elicit its anti-inflammatory effects in DC, by examining alterations in NFκB and IRF-3 downstream of TLR4

- To determine if the marine extract elicits its anti-inflammatory effects by interacting with the adaptor molecule, Mal.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

TISSUE CULTURE MATERIALS/REAGENTS

Materials	Source
Tissue culture flasks T-75 cm^2/T-175cm^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®
OVA Peptide 323-339	Genescript Corp
GMCSF	J558 GMCSF producing cell line
CCL19	R&D Systems®
GM-CSF	R&D Systems®
Recombinant IL-2	BD Pharmigen
Trypan blue (0.4% v/v)	Sigma®
Anti-mouse CD3e monoclonal antibody	BD Pharmingen
Anti-mouse CD28 monoclonal antibody	BD Pharmingen
CellTiter 96® Aqueous One Solution	Pierce
RPMI-1640	Invitrogen™
Foetal Calf Serum (FCS)	Invitrogen™
Penicillin Streptomycin	Invitrogen™
LPS (*E.Coli* serotype R515)	Alexis Biochemicals
PGN	Invitrogen™
$PamC_3S_4$	Invitrogen™
Poly:IC	Invitrogen™
Zymosan	Invitrogen™
Flagellin	Invitrogen™
CPG	Invitrogen™
DMEM	Invitrogen™
LPS (*E.Coli* serotype R515)	Alexis Biochemicals
Hygrogold	Invitrogen™
Blasticidin	Invitrogen™

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

PROTEIN PURIFICATION REAGENTS

Materials	Source
BCA Protein Assay	Pierce
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®
Ammonium persulphate (APS)	Sigma®
Phenylmethanesulfonyl fluoride (PMSF)	Sigma®
Propan-2-ol (isopropanol)	VWR International Ltd.
30 % Acrylamide/Bis solution	Bio-Rad
Sodium Orthovanidate	Sigma®
Leupeptin	Sigma®
Aprotinin	Sigma®
Immobilon Western HRP Substrate	Millipore
Re-Blot Plus Solution (10 X)	Millipore
Ponceau S Solution	Sigma®
Nitrocellulose membranes	Biosciences
Fuji SuperRX film	FujiFilm Ireland Ltd.
Precision Plus Protein™ Dual Color Standard	Bio-Rad
Nitrocellulose membrane	Biosciences

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

WESTERN BLOTTING ANTIBODIES

Antibody	Source
Anti-Phospho NFκB-p65	Cell Signalling
Anti-Phospho-p38	Cell Signalling
Anti-IκB	Cell Signalling
Anti-Phospho-IRF3	Cell Signalling
Anti-NFκBp65 (C-21)	Santa-Cruz Biotechnology Inc.
Anti- p38	Cell Signalling
Anti-βatin	Sigma®
Anti-Phosphotyrosine, clone 4G10	Millipore
HA-MAL	Sigma®
Anti-mouse IgG peroxidase	Sigma®
Anti-rabbit IgG peroxidase	Sigma®

TABLE 2.3: *All antibodies used for western blotting analysis.*

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®
1X PBS	Biosciences

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

FLOW CYTOMETRY

Antibody	Fluorochrome	Source	Isotype Control	Concentration/ 10^6 cells
TLR4-MD-2	PE	BD	RatIgG2a	0.5 µg
CCR5	PE	BD	Rat IgG2a	0.5 µg
CD11c	APC	Caltag	Rat IgG2b	0.5 µg
CD40	FITC/PE	BD	Ham IgM	0.5 µg
CD80	PE	BD	Ham IgG	0.5 µg
CD86	FITC	BD	Rat IgG2a	0.5 µg
MHCII	FITC/PE	BD	Rat IgG2a	0.5 µg

TABLE 2.5: *Antibodies used for FACs analysis of cell surface markers; suppliers and concentrations used.*

FACS MACHINE/PREPARATION FACS MACHINE/PREPARATION

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

TABLE 2.6: *Materials/reagents used for flow cytometry and FACS preparation.*

DNA MANIPULATION AND LUCIFERASE ASSAYS

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

TABLE 2.7: *Materials used for manipulation of DNA plasmids in transient transfections.*

2.2 METHODS

PREPARATION OF MAIN BUFFERS/ ELECTROPHORESIS GELS

Buffer	Composition
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
Electrode Running Buffer (10X)	25mM Tris Base, 200mM Glycine, 17mM SDS
Low Stringency Lysis Buffer	50mM HEPES, 100mM NaCl, 10% glycerol, 0.4% NP40, 1mM EDTA
Sample Buffer (5X)	0.5mM Tris-HCL pH 6.8, 10% Glycerol, 10% SDS, 5% β-mercaptoethanol, 0.05% w/v Bromophenol blue
Separating Gel (12%)	33% v/v Bisacrylamide (30% stock), 1.5M Tris-HCL pH 8.8, 1% w/v SDS, 1% w/vAmmonium persulphate, dH_2O, 0.1% TEMED
Stacking Gel	6.5% v/v Bisacrylamide (30% stock), 0.5M Tris-HCL pH 6.8 1% w/v SDS, 1% w/vAmmonium persulphate, dH_2O, 0.1% TEMED

TABLE 2.8: *Composition of most commonly used buffers.*

2.3 CELL CULTURE

All tissue culture was carried out using aseptic techniques in a class II laminar airflow unit (Holten 2010- ThermoElectron Corporation, OH, USA). Cells were maintained in a 37°C incubator with 5% CO_2 and 95% humidified air (Model381- ThermoElectron Corporation, OH, USA).Cells were grown in complete RPMI-1640 medium as indicated in Appendix A. FCS was heat inactivated (56°C for 30mins) to order to inactivate complement and then aliquoted for storage at -20°C. Supplemented medium was stored at 4°C.

2.3.1 CELL ENUMERATION AND VIABILITY ASSESSMENT

Cell viability was assessed using the trypan blue dye exclusion test. This test is based on the principle that live cells possess intact cell membranes that exclude certain dyes were as dead cells are unable to exclude the dye and appear blue when viewed under a microscope. 100µl of cell suspension was mixed with 150µl PBS and 250µl trypan blue solution (0.4% (v/v)). After ~2mins cells were applied to a brightline haemocytometer (Sigma®) and examined under high power magnification (x40) using an inverted microscope (Olympus CKX31, Olympus Corporation, Tokyo, Japan). Cells inside the central grid were counted **[see Figure 2.1].**

A viable cell count was determined using the following formula: Cell/ml= N x 5 x 10^4

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

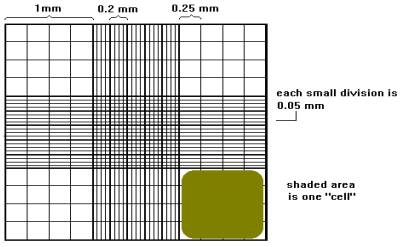

FIGURE 2.1: *Diagrammatic Representation of haemocytometer used to count cells*

2.3.2 MURINE MACROPHAGE CELL LINE J774

The murine macrophage cell line J774A.1 was used in this study and is referred to solely as J774 throughout. The J774 cell line was purchased from the European Collection of Cell Cultures (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 approximately 20 x 10^6 cells. For subculture, cells were split 1 in 10 into 25 ml complete RPMI-1640 in a fresh 75 cm^2 flask. For experiments cells were counted as described [see section 2.2.1].

2.3.3 HUMAN EMBRYONIC KIDNEY CELL LINES HEK293

Human embryonic kidney cell line HEK293 stably transfected with TLR4, CD14 and MD-2, (HEK293-MTC) were a kind gift from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. The HEK293-MTC cell line was cultured with appropriately supplemented complete DMEM media **[see Appendix]**. Cells were cultured in 175 cm^2 flasks as follows; 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 the flasks and cells washed twice with 5 ml ice cold sterile PBS (Invitrogen™). Following this cells were removed from the surface of the flasks by incubating them with 2 ml of 1 X Trypsin solution (Sigma®) for 5 min at 37 °C. 10 ml of complete media was then added to resuspend cells and

cells were spun at 1200 rpm for 5 min. Finally cells were resuspended in media, subcultured or counted for experiments.

2.3.4 PREPARATION OF CELL STOCKS

Cells were grown to a state of sub-confluency and were harvested and counted as previously described in section 2.3.1-2.3.3. 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). These aliquots are placed at -20ºC for 2 hrs and then at -80ºC for 3 hrs before being stored in a liquid nitrogen vessel.

2.3.5 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]**.

2.3.6 ISOLATION OF BONE MARROW-DERIVED DENDRITIC CELLS

2.3.6.1 DAY 1 - BONE MARROW HARVEST

Bone marrow from the tibiae and femurs of BALB/C mice was extracted by flushing RPMI through the bones using a syringe and a 27.5g needle into a sterile petri dish. The bone marrow was then broken up using a syringe and a 19.5g needle and then transferred to a 50ml falcon. Cells were centrifuged for 5 mins at 1200 rpm,

supernatant removed and the bone marrow cells were resuspended in RPMI to allow 1ml of cells per petri dish required. A cell count was preformed using the trypan blue exclusion method to make certain an adequate number of viable cells had been obtained. 9mls of RPMI supplemented with GMCSF and 1ml of cells were added to each petri dish. The cells were incubated at 37°C.

2.3.6.2 DAY 4 – FEEDING CELLS

Petri dishes were tilted slightly so the cell monolayer was easily visible, and using a transfer pipette approximately 6-7mls of media was removed. 10mls of pre-warmed RPMI supplemented with GMCSF was then added gently to each petri dish. Cells were incubated at 37°C.

2.3.6.3 DAY 7 – COUNTING AND PLATING CELLS

To remove semi-detached and therefore immature dendritic cells, the cells were scraped using cell scrapers and media was collected using transfer pipettes and transferred to a 50ml falcon. Each petri dish was examined under the microscope to verify that the majority of the cells were removed. Falcons were spun at 1200rpm for 5mins, supernatant removed and the cells were resuspended in RPMI and then counted using the trypan blue exclusion method. The cell concentration was adjusted with RPMI – generally cells were plated at $1x10^6$ cells/ml.

2.3.7 CULTURE OF THE J558 GMCSF-SECRETING CELL LINE

The mouse gene for GMCSF (granulocyte macrophage colony stimulating factor) was cloned into a mammalian expression vector (Karasuyama, Kudo and Melchers 1990) and transfected into the plasmacytoma line X63-AgS. Cell stocks were kindly donated by Professor Kingston Mills (Trinity College, Dublin).

After removal from liquid nitrogen and rapid thawing, cells were washed in 30mls cRPMI, then resuspended in 5mls of selection medium consisting of 1mg/ml G418 Geneticin (GibcoBRL) in complete RPMI for 2 passages. Cells were seeded at $1x10^6$ cells/ml each time and culture flasks stood upright in the incubator. After the second passage cells were washed twice in cRPMI, counted and seeded in cRPMI at $1x10^6$ cells/ml. When cells reached a medium density they were subsequently seeded at $2.5x10^5$ cells/ml at each passage. Up to and including passage 9, supernatant was collected from the J558 cells and the amount of secreted GMCSF quantified by ELISA (R&D). For bone marrow culture, GMCSF was used at a concentration of 40ng/ml.

2.3.8 MARINE EXTRACTS

Marine extracts were received from UCD and dissolved in DMSO. Stability tests were carried out to ensure the optimum storage temperature for these extracts. The marine extracts were stored at 4ºC and -20ºC and cytokine analysis of the extracts confirmed that they are stable at both 4ºC and -20ºC. Therefore marine extracts were aliquoted and stored at 4ºC. Marine extracts were added on day 7 of culture. On day 7 of culture the cells were treated with a vehicle control (DMSO), or the marine extracts, *Alcyonium digitia* and *Membranipora membranacea* at a range of different dilutions (1:100, 1:1000, 1:10000) 1 hr prior to activation with TLR ligand.

2.3.8.1 FRACTIONATION AND PURIFICATION OF MARINE EXTRACTS

Initial extractions from *Membranipora membranacea* and *Alyconium digitia* crude extracts were carried out based on compound polarity and a series of fractionations were performed with an equal quantity of sample in each fractionation using a different solvent. The solvents chosen were used to represent the spectrum of polarity

from polar to non-polar (pentane, DCM and methanol). The extracts were stirred in these solvents for 24-48 hours before the solvents were filtered and evaporated to leave a mix of fractionated compounds. These mixes were then tested for activity, and the most active fractions underwent a second round of fractionation. This round of fractionation used a new selection of solvents (cyclohexane and ethanol) which were chosen based on the solvent which gave the most active mix in the first round of fractionation. The mixing, filtration, evaporation and testing were carried out again until the highest solvent extraction system – bioactivity relationship has been reached.

Once satisfied with the solvent extraction system, further purification step took place, based on the properties of the compound mixture; column chromatography was chosen as the method to purify the compounds. This purification step led to the isolation of the pure compound INV013. INV013 was then submitted for a large array of spectroscopic tests in order to identify the structure of the compound.

2.3.9 TOLL-LIKE RECEPTOR ACTIVATION

Cells were activated with TLR ligands which are outlined in **Table 2.9**, and incubated for 24 hrs before being used in the relevant assays.

TLR	TLR Ligand	Stock Concentration	Working Concentration
2/1	$PamC_3S_4$	1 mg/ml	1.0 µg/ml
2/6	Zymosan	1 mg/ml	10 µg/ml
3	Poly:(IC)	1mg/ml	10 µg/ml
4	LPS	1 mg/ml	100 ng/ml
5	Flagellin	100 ug/ml	5.0 µg/ml
7	Loxoribine	10 mM	1mM
9	CPG	500uM	2µM

TABLE 2.9: *Concentrations of the TLR ligands used for the activation of dendritic*

2.3.10 ADDITION OF OVA PEPTIDE (323-339)

The OVA peptide sequence is given below and represents a T and B cell epitope of OVA: ISQAVHAAHAEINEAGR [$C_{74}H_{120}N_{26}O_{25}$: MW 1773.9]

1g vials of lyophilized OVA peptide were kept at -20°C and dissolved in 1ml of sterile water to give a 1mg/ml stock which was also kept at -20°C. OVA was added to DCs at 5µg/ml for 24 hrs before use in the co-culture experiments.

2.3.11 CYTOTOXICITY ASSAY FOR MARINE EXTRACT 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 cells into a soluble coloured formazan product. The quantity of formazan product is measured at an absorbance reading of 450nm and is directly proportional to the number of living cells in the culture medium. Bone marrow-derived dendritic cells were cultured for 7 days. On

day 7 cells were collected, counted and plated in a 96- well plate with 100µl per well at $1x10^6$ cells/ml. Marine extracts were then added at different dilutions (1:100, 1:1000, 1:10,000) 1 hr prior to stimulation with LPS (100ng/ml). 24 hrs after the addition of LPS, 20µl of the CellTiter 96® Aqueous One Solution was added to each well of the 96-well plate. The plates were incubated for 2 hrs at 37°C in 5% CO_2 and absorbance read at 490nm. The cell viability of each sample was calculated by treating the absorbance of the vehicle control, DMSO as 100% and comparing the remaining samples to this and expressing results as percentage viability.

The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) is an alternative colorimetric cytotoxicity assays that we used in the study to assess the viability of cells following treatment with marine extracts. The CytoTox 96® Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. The quantity of formazan product is measured at an absorbance reading of 450nm and is directly proportional to the number of living cells in the culture medium. HEK293-MTC cells were counted and plated in a 96-well plate with 100µl per well at $1x10^5$ cells/ml. Marine extracts were then added at different dilutions (1:100, 1:1000, 1:10,000) 1 hr prior to stimulation with LPS (100ng/ml). 24 hrs after the addition of LPS, cells were lysed with 10X lysis solution to Target Cell Maximum LDH Release Control and then centrifuged at 250 x g for 4 mins. 50µl of 1:5000 dilution of LDH positive control was added to separate wells and 50µl of substrate mix was then added to each well. The plate was then incubated for 30 mins in the dark at room temperature. Following this incubation 50µl of stop

solution was added to each well and absorbance read at 490nm. The cell viability of each sample was calculated by treating the absorbance of the vehicle control, DMSO as 100% and comparing the remaining samples to this and expressing results as percentage viability

2.3.12 CD4+ T CELL ISOLATION

2.3.12.1 ERYTHROCYTE LYSIS

(R&D Systems - mouse erythrocyte lysing kit WL2000)

Spleens were removed aseptically and collected in HBSS/10% FCS on ice. A single cell suspension was achieved by pushing each spleen through a cell strainer (40μm, BD falcon). Cells were then washed in HBSS/10% FCS and erythrocytes lysed by adding a 1x solution of M-lyse for approximately 10 minutes at room temperature (2ml of 1x solution per spleen). Following incubation, cells were vortexed for 2 seconds. 40ml of wash buffer was then added and the cells centrifuged and resuspended in 2ml of 1x column wash before being counted and adjusted to $\leq 2x10^8$ cells/ml.

2.3.12.2 $CD4^+$ T CELL ISOLATION

(R&D Systems - Mouse T cell CD4 Subset Column Kit MCD4C-1000)

$\leq 2x10^8$ of cells (in 2ml of column wash) was mixed with 1 vial of monoclonal antibody cocktail (1ml $CD4^+$ enrichment cocktail) and incubated at room temperature for 15 minutes. During this incubation period the column was washed with 10ml of column buffer. After incubation, cells were washed twice with 10mls of 1x column wash and finally resuspended in 1ml column wash. The cells were added to the column and any liquid displaced from the column collected in a sterile falcon tube. Once the cells had moved onto the column, the bottom cap was replaced and the cells

left in the column at room temperature for 10 minutes. This allowed B cells, non-selected T cells and monocytes to bind to the glass beads coated with anti-immunoglobin via both F(ab) and Fc interactions. The column was eluted with 10mls of column buffer. Eluted and therefore CD4 positive T cells were centrifuged (250g for 5 minutes), resuspended in cRPMI, and counted using the trypan blue exclusion method. Cells were adjusted to the required concentration with cRPMI.

2.3.13 DC-T CELL CO-CULTURE

Dendritic cells were grown as previously described for 7 days. At this time (i.e., 24 hrs prior to commencement of the co-culture experiment) cells were treated with the *M.membrancea* for 1 hr prior to activation with OVA peptide (5μg/ml). The cells were incubated for a further 24 hrs at 37^{o}C in 5% $CO_{2.}$ After 24 hrs, DCs were collected and washed twice in sterile PBS/2%FCS to remove any traces of RPMI before being irradiated. DC were irradiated with 40Gy (4000rads) using a gamma irradiator with a Caesium-137 source. Irradiation of DCs is necessary to terminate DC maturation. Cells were then counted and resuspended in cRPMI at a final concentration of $2x10^{5}$cells/ml.

$CD4^{+}$ T cells were isolated from the spleens of OVA transgenic D011.10 mice as described in section 2.3.12. A large portion of the T lymphocytes from these mice express a TCR specific for a peptide within the ovalbumin (OVA) molecule (OVA (323-339)). When this antigen is presented by DCs complexed to MHCII (together with costimulation), these naive $CD4^{+}$ T cells become activated and proliferate (Pompos and Fritsche 2002)

Following purification, OVA transgenic $CD4^+$ T cells were adjusted to $2x10^6$cells/ml. Equal volumes of T cells and DCs were added to a sterile 96-well plate to give a final volume of 200µl/well. Plates were incubated at 37°C in 5% CO_2 for 5 days. On day 5 of the co-culture, plates were centrifugally pulsed to move cells to the bottom of the wells. 200µl of fresh media was added to each well and plates were incubated at 37°C in 5% CO_2 until day 7.

On day 7 of the co-culture, plates were again pulsed before 100µl of media was removed. 100µl of newly harvested OVA-activated DCs pre-treated with *M.membrancea* were added at a concentration of $2x10^5$cells/ml for the second round of T cell stimulation. Recombinant murine IL-2 (Becton Dickinson) was also added at this time (10U/ml). At the end-point of the co-culture experiment (day 10), 200µl of media was removed after plates were pulsed and the supernatant frozen for future cytokine analysis.

2.4 FLOW CYTOMETRY

Flow cytometry is a means of quantitating structural features of cells by optical means. By using up to 4 different fluorescently tagged markers different characteristics can be measured at the same time in one mixture. Cells containing the desired features are measured individually and then aggregated. The FACScan™ system can process thousands of cells in seconds.

2.4.1 CELL SURFACE MARKER STAINING

DCs were cultured for 7 days as previously described in (see section 2.3.6). Cells were then plated at a concentration of $1x10^6$ cells/ml into a 6-well plate (2ml/well). Cells were treated with the marine extract 1 hr prior to stimulation with LPS (100ng/ml) and incubated at 37°C in 5% CO_2 for 24 hrs. Following incubation, cells were scraped using cells scrapers and collected in falcon tubes. An equal volume of FCS was added for 15 minutes to prevent non-specific binding. Tubes were then spun at 1200rpm for 5 mins and cells resuspended in 1ml of FACs buffer **[see Appendix]**. 200µl of cells were added to a 96-well round bottom plate to give approximately 400,000 cells/well. One well per treatment group (i.e., each marine extract treatment +/- LPS) was assigned for each antibody group and one well for each corresponding isotype control group- **[see Table 2.5]**.

Plates were spun at 2000rpm for 10 mins and supernatant carefully removed from the wells. 100µl of the correct antibody and isotype was added to the appropriate wells. Plates were incubated for 30 mins in the dark at 4°C. Following incubation, plates were spun at 2000rpm for 10mins. Cells were washed twice by resuspending in 200µl of FACs buffer and were then fixed in 200µl of 4% formaldehyde/PBS before being

transferred to labelled FACs tubes. Samples were acquired immediately or left overnight at 4°C in the dark. 30,000 events were acquired per sample using a 4-colour FACS Calibur (fluorescence activated cell sorter) Becton Dickson (BD). Data was analysed using CellQuest software and samples gated on $CD11c^+$ cells for DC samples to ensure histograms and dot plots represented pure cell populations.

2.4.2 PHAGOCYTOSIS ASSAY

J774 macrophage were cultured 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. The next day cells were treated with DMSO (vehicle control) or marine extracts 1hr prior to activation with LPS (100ng/ml) or loxoribine (1mM). On the third 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™. Note: latex beads were sourced from Sigma® and were 1 µm in diameter.

2.4.3 CHEMOTAXIS ASSAY

Bone marrow-generated DC were cultured for 7 days as described in [see section 2.3.6]. Cells were counted and plated at a concentration of 1x10^6 cells/ml in a 6 well plate (3mls/well). Marine extract was then added to each required well 1 hr prior to stimulation with LPS (100ng/ml) or loxoribine (1mM). Cells were incubated at 37°C for 24 hrs. Following incubation, cells were removed from wells using a transfer pipette and counted. Transwell® plates were used in accordance with the

manufacturer's instructions; $3x10^5$ cells were added to the insert well in 100µl of media, and 600µl of media supplemented with or without the chemokine CCL19 (100ng/ml) for DCs and GM-CSF (10 ng/ml) and IL-2 (10 BRMP/ml; where 1 BRMP = 40 pg/ml) for macrophages, was added to the bottom chambers- **see Figure 2.2**. Plates were incubated for 5 hrs at 37°C. Cells that had migrated to the bottom chamber were collected and transferred to eppendorf tubes, spun and resuspended in 4% formaldehyde/PBS before being transferred to FACS tubes. Migrated cells were counted for 60 seconds on a BD FACsCalibur.

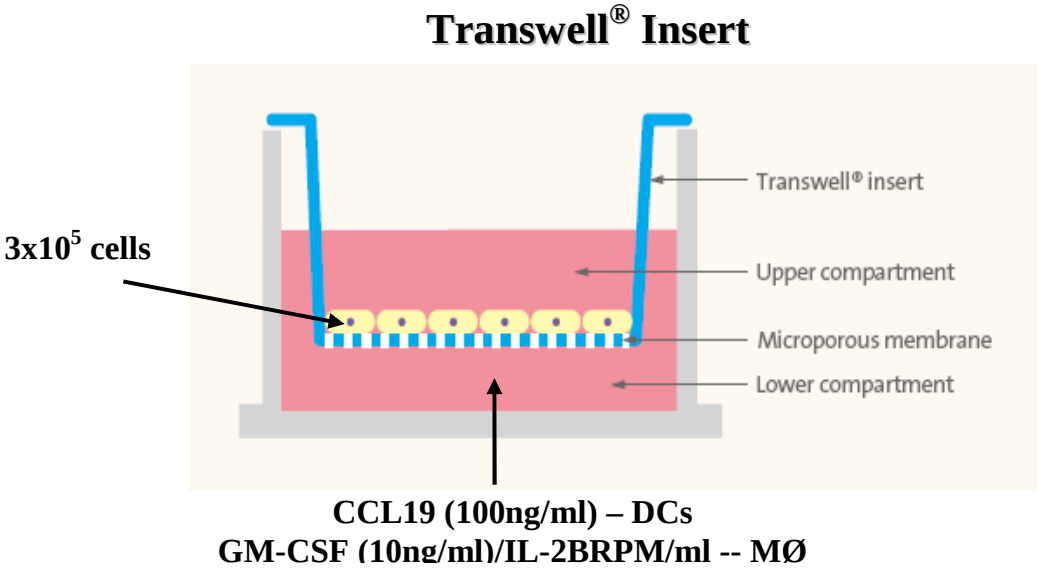

FIGURE 2.2: *Diagrammatic representation of Chemotaxis Assay Transwell™. Schematic taken from Transwell® Permeable Supports Selection and Use Guide, Corning.*

2.5 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

The concentration of cytokines IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, TNF-α, IFN-γ, IFN-β and chemokines MIP-1α, MIP-2, MCP-1 and Rantes in cell supernatants was established using ELISA Duoset kits from R&D Systems in accordance with the manufacturer's instructions. A diagrammatic representation of the principles of a sandwich ELISA are shown in **Figure 2.3**

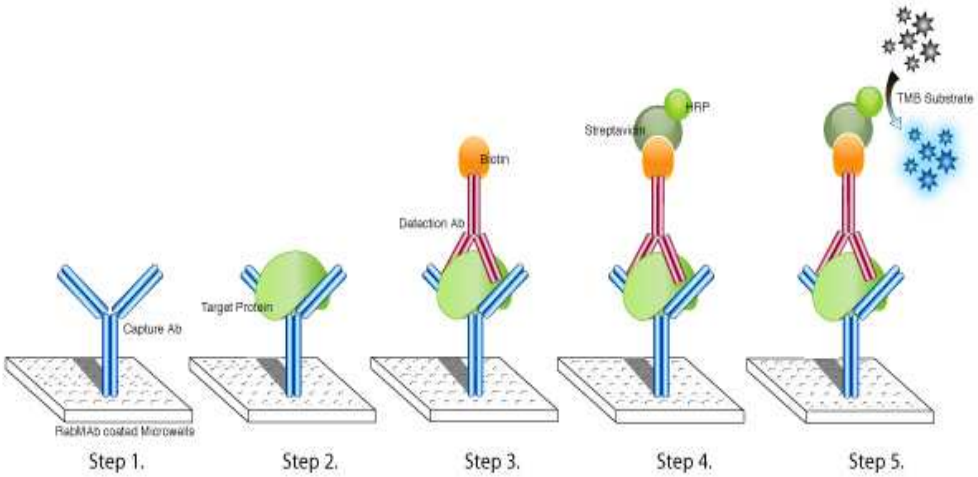

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FIGURE 2.3: *Schematic representation of sandwhich ELISA. Schematic taken from ELISA Kit Technology Principals on www.epitomics.com.*

2.5.1 IL-6, IL-10, IL-12p40, IL-12p70, IL-23, TNF-α ELISA

96- well Nunc™ microtitre plates were coated with 50µl of the appropriate capture antibody diluted to working concentration in PBS **[see Table 2.10]** and incubated overnight at room temperature. Following overnight incubation, plates were washed 3 times with was buffer (PBS/0.05% Tween®) before wells were blocked with 300µl of reagent diluent (PBS/1% BSA) for at minimum of 1 hr at room temperature. After repeating the washing step, 25µl of reagent diluent and 25µl of supernatant or serially diluted standards (top standard serially diluted x6 in reagent diluent- see table 2.10)

were added to wells in duplicate, and plates were incubated overnight at 4°C. The following day plates were washed x3 with wash buffer. 50µl of the appropriate biotinylated detection antibody, diluted in reagent diluent- **[see Table 2.10]**, was added to each well and plates were incubated at room temperature for 2 hrs. Plates were washed x3 with wash buffer and 50µl of streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well. Plates were incubated for 20 mins at room temperature. Finally, wells were washed x3 with wash buffer and 100µl of TMB (Sigma Aldrich) was added to each well and plates were then incubated in the dark. Once the colour had developed sufficiently plates were stopped by adding 25µl of 2N H_2SO_4 per well. Optical densities were read immediately at 450nm on VERSA Amax microplate reader (Molecular devices, CA, USA). The cytokine/chemokine concentrations in the supernatants were determined from the standard curves.

2.5.2 IL-1β

The method above was followed with two variations:

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-12p40, IL-6

Samples were diluted 1:100 in reagent diluent and 50µl of diluted samples and undiluted standards were added to the plates in duplicate. Concentrations of samples were multiplied by the dilution factor once calculated from the standard curve.

2.5.4 TNF-α

Samples were diluted 1:10 in reagent diluent and 50µl of diluted sample and undiluted standards were added to the plates in duplicate. Concentrations of samples were were multiplied by the dilution factor once calculated from the standard curve.

Cytokine	Capture Antibody (µg/ml)	Top Standard (pg/ml)	Detection Antibody (ng/ml)
IL-1β	4.0	1000	400
IL-4	4.0	1000	600
IL-6	2.0	1000	200
IL-8	4.0	2000	20
IL-10	4.0	2000	500
IL-12p40	4.0	2000	400
IL-12p70	4.0	2500	400
IL-17	2.0	1000	400
IL-23	4.0	2500	200
TNF-α	0.8	2000	75
IFN-γ	4.0	2000	800
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
Rantes	2.0	2000	400

TABLE 2.10: *Concentration of standards, capture and detection antibodies used in sandwich ELISA assays.*

2.6 WESTERN BLOT ANALYSIS

2.6.1 PREPARATION OF WHOLE CELL LYSATES

Cells were seeded at 1 x 10^6 cell/ml in a 6-well plate (2 ml/well) and left to rest overnight. Cells were then treated with marine extract 1 hr prior to stimulation with LPS (100ng/ml) or loxoribine (1mM). Following activation, cells were washed with PBS and scraped in 100µl of low stringency lysis buffer-see **Table 2.8**. Protease and phophatase inhibitor 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 then incubated on ice for 30 mins. Following this incubation period samples were centrifuged at 13,000 x g for 5 mins at 4ºC. The protein concentration of the samples were then determined using Bradford assay see section and aliquots containing equal amounts of protein were mixed with 5X SDS sample buffer, before loading on gels samples were boiled at 95 ^{o}C for 5 min to denature proteins.

2.6.2 DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins were fractionated by SDS denaturing polyacrylamide gel electrophoresis (SDS PAGE). Acrylamide gels (12 %) **[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. 20µl 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.

2.6.3 TRANSFER OF PROTEINS TO MEMBRANE

The resolved 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.

2.6.4 IMMUNODETECTION AND DEVELOPMENT

Following transfer, membranes were blocked for non-specific binding by incubation with freshly prepared blocking buffer, 5 % (w/v) non-fat dried skimmed milk/TBS-T **[see Table 2.11]** for 1 h on a slow rocker at room temperature. The membranes were then washed for 5 mins in TBS-T (wash buffer) three times. The membrane was then incubated with appropriate primary antibodies diluted in TBS/5% dried milk or BSA /0.05% Tween 20® (reagent diluent). Membranes were gently agitated with the primary antibodies overnight at 4 ºC. Following overnight incubation, membranes were washed four times for 5 min in wash buffer. Membranes were then incubated with the appropriate secondary antibodies (horseradish peroxidase (HRP) conjugated secondary antibody) **[see Table 2.11]** and incubated with gentle agitation at room temperature for 2 h. Following incubation with secondary antibody, membranes were washed four times for 5 min with washing buffer.

HRP-labelled antibody complexes were visualised using enhanced chemiluminescence (ECL). 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).

2.6.5 STRIPPING AND RE-PROBING MEMBRANES

To reprobe membranes, antibody complexes were removed by incubating membranes in 10 ml 1 X Re-Blot Plus Solution and was made according to manufacturers' instructions (Millipore) for 10 min with gentle agitation. Following this membranes were washed in 5 ml of blocking buffer three times 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 for later use.

1° ANTIBODY	DILUTION	2° ANTIBODY	DILUTION
NFκB p65 **Phospho-IRF3** **Phospho-P38** **IκB** **Total NFκBp65** **Total P38**	1:1000	α-rabbit IgG-HRP	1:2000
Phospho tryosine	1:1000	α-mouse IgG- HRP	1:2000
HA-Mal	1:1000		1:2000
βactin	1:10000		1:20000

TABLE 2.11: *Dilution of primary and secondary antibodies for western blotting.*

2.7 DNA MANIPULATION

2.7.1 DNA TRANSFORMATION INTO BACTERIA

Chemically competent DH5α *E. coli* cells and cloned 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 to ensure the cells were evenly suspended and left for 5 min on ice. The cells were heat shocked in a water bath at 42 ºC for exactly 2 min and then cooled on ice for a further 2 min. As ligation DNA contained an ampicillin cassette for transformation, cells were plated onto selective LB agar plates containing 100µg/ml ampicillin and grown for 16-18 hrs at 37 ºC. Transformed cells were then single colony purified and used to purify plasmids for transfection.

2.7.2 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 cell line. A 500 ml culture typically yielded 1 – 3 mg/ml of plasmid DNA. Glycerol stocks were made by aseptically mixing 930µl bacterial culture and 70µlof DMSO and storing at -80ºC.

2.7.3 TRANSIENT TRANSFECTION USING GENEJUICE®

GeneJuice® (Novagene) transfection reagent is a liposomal based transfection reagent from Novagen and is used for the transfection of HEK293 cells. The ISRE luciferase plasmid, NFκB luciferase plasmid, *Renilla* luciferase plasmid and empty pcDNA3.1 vector (Invitrogen™) were kind gifts from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. For 96 well plate transfections, HEK-MTC cells were seeded at at 4 x 10^5 cell/ml with a total volume of 200µl. Cells were incubated overnight and transfected the following day using geneJuice® transfection reagent according to the manufacturers' instructions. Cells were transfected in triplicate. 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 96-well plate. For both ISRE and NFκB luciferase assays cells were harvested 24hrs following transfection.

2.8 LUCIFERASE REPORTER GENE ASSAY

HEK293-MTC cells were transfected in 96 well plates as described in section 2.7.3. Following 24hr transfection cells were treated with marine extract 1hr prior to stimulation with 100ng/ml of LPS. Media was then aspirated from each well and cells were lysed for 15 min on a rocking platform at room temperature with 50µl of 1 X Passive Lysis Buffer (Promega, Southampton, UK).

2.8.1 MEASUREMENT OF LUCIFERASE ACTIVITY

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 the sample and *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). Firefly luminescence readings were corrected for *Renilla* activity and expressed as fold stimulation over unstimulated empty vetor (EV) control.

2.9 IMMUNOPRECIPATION

HEK293-MTC cells were cultured into 10cm dishes ($1x10^5$ cells/ml) 24hr prior to transfection. Transfections were carried out using Genejuice® Transfection Reagent (Novagen) as previously described in section 2.7.3. 4µg of Mal construct was tranfected and after 24hr transfection cells were treated with marine extract 1 hr prior to stimulation with LPS (100ng/ml). The cells were washed in 5mls of PBS and then scraped in 850µl of low stringency lysis buffer. Cells were then incubated on ice for 30 mins. Following this incubation period samples were centrifuged at 13,000 x g for 5 mins at 4ºC. Protein concentrations of the supernatant were determined by Bradford assay and lysates containing equal protein were added to 30ul of protein A/G sepharose beads. 1ug of relevant antibodies (HA-Mal) was then added and the samples were incubated for 2h at 4ºC with gentle rotation. A portion of the lysate was retained to confirm that the protein of interest was expressed, this was added to sample buffer and boiled for 5 mins. Following the 2 hr incubation the immune complexes were spun at 2300rpm and then washed three times with 1ml lysis buffer. All supernatants were aspirated off and beads were resuspended in 60µl of 5X sample buffer. The samples were boiled for 5 mins and SDS-PAGE analysis were preformed on the precipitated complexes as described in section 2.6.2-2.6.4.

2.10 STATISICAL 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.

The luciferase and immunoprecipitaions experiments in chapter 5 represented by figure 5.23 - 5.25 and figure 5.30, respectively were carried out in conjunction with Dr. Jennifer Dowling in the Biochemistry Group in TCD.

CHAPTER 3

INITIAL SCREENING OF MARINE EXTRACTS FOR ANTI-INFLAMMATORY EFFECTS

3.1 INTRODUCTION

The ocean is a major source of organisms with a diverse range of biological materials which may be a source of novel compounds with promising therapeutic applications**.** To date only a limited portion of the oceans biodiversity has been explored which suggests that there is still enormous potential which has not yet been uncovered. Compounds isolated from marine species have received much attention recently as studies have shown that marine compounds have beneficial effects. These molecules and compounds have come from a variety of marine species including sponges, algae, sea slugs and marine bacteria (Haefner 2003, Schwartsmann, et al. 2003)and have been shown to offer some protection against tumour progression and the development of autoimmune diseases (Jean, et al. 2008, Taddei, et al. 2006). In addition they have been shown to possess anti-viral and anti-bacterial activities (Haefner 2003). The prevalence of inflammatory diseases such as RA is increasing and current treatments for these diseases are not entirely efficacious and are associated with severe side effects. Therefore there is a huge need for new treatments that combat these diseases. This study aimed to screen a range of marine species to identify those compounds with anti-inflammatory activity.

Marine extracts isolated by our collaborators in UCD were screened and active fractions were then further fractionated into numerous compounds. These fractions were then assessed in order to find those containing the anti-inflammatory compounds [see figure 3.1.1].

In order to carry out this study we screened compounds for their effects on DCs. DCs play a key role in the initiation and progression of autoimmunity (Gutcher and Becher

2007). Indeed, the role of DCs in various inflammatory and autoimmune disorders has been well documented including IBD, RA and MS (Gottenberg and Chiocchia 2007, Gutcher and Becher 2007, Blanco, et al. 2008, Karni, et al. 2006)Therefore we hypothesized that DCs may be logical targets of these compounds and that their effects on DC may suppress inflammation and have substantial beneficial effects.

The activation of DCs leads to the up-regulation of co-stimulatory molecules, pattern recognition receptor (PRR) and surface markers including CD40, CD80, CD86, TLR4-MD-2 and MHCII. These surface markers have a pivotal role in the activation of an effective T helper cell response as absence of co-stimulation can result in cell anergy or apoptosis (Kobata, et al. 2000). Alterations in co-stimulatory surface marker expression could modulate the ability of DCs to activate T cells, therefore we examined the effects of the marine extracts on these markers.

While the upregulation of co-stimulatory markers is important, the differentiation of T cells into numerous subsets is partially determined by the cytokines DCs produce i.e. IL-12 induces the generation of T cells to a Th1 phenotype; IL-23, IL-6 and IL-1β are involved in generating a Th17 response; IL-4 drives a Th2 phenotype and IL-10 promotes the induction of type 1 regulatory cells (Agnello, et al. 2003, de Jong, Smits and Kapsenberg 2005, Guermonprez, et al. 2002). Indeed the secretion of IL-10 acts as a potent deactivator of DC pro-inflammatory cytokine production. Consequently, altering the production of these cytokines by DCs may modulate the subsequent adaptive immune response. Therefore we examined the effects of marine extracts on LPS-induced cytokine production from DCs.

Given that impaired expression of chemokines and or/ chemokine receptors can be involved in the development of autoimmune diseases (Borish and Steinke 2003, Cravens and Lipsky 2002), we also assessed whether the extracts effected chemokines such as MCP-1, MIP-1α and MIP-2α. Finally the ability of DCs to migrate to the lymph node homing chemokine CCL19 was also examined.

Chapter 3

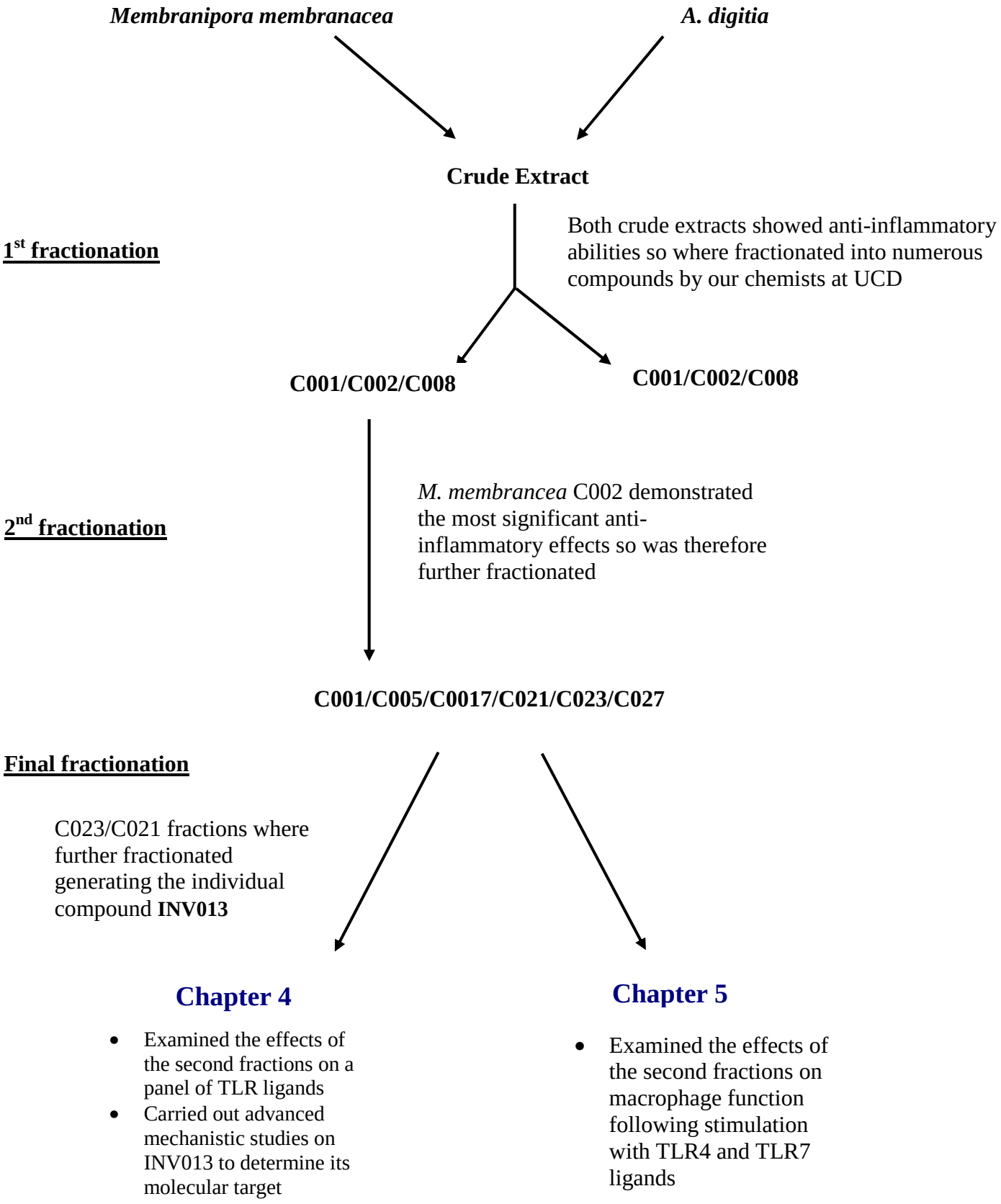


Figure 3.1.1: *Diagrammatic representation of the outline of this project.*

3.2 RESULTS

3.2.1 THE DOSES OF CRUDE MARINE EXTRACTS USED HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY.

We examined the effects of the crude marine extract *Alcyonium digitia* and *Membranipora membranacea* on DCs cytokine production and surface marker expression following stimulation with LPS. Crude extracts that demonstrated anti-inflammatory activity were then sent to our collaborators in UCD where they were fractionated. Initially we examined the toxicity of the crude extracts by exposing DCs to a range of dilutions. Following marine extract treatment the cell viability was assessed using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. The doses of marine extracts (1:100, 1:1,000, 1:10,000) chosen for use in future experiments did not have any significant cytotoxic effect on DC *in vitro* **[Figure 3.1]**

3.2.2 CRUDE MARINE EXTRACTS DOSE-DEPENDENTLY MODULATE LPS-INDUCED CYTOKINE PRODUCTION BY DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), marine extracts; *Alcyonium digitia* or *Membranipora membranacea* at different dilutions (1:100, 1:1000, 1:10,000) for 1 hr prior to stimulation with 100ng/ml LPS (*E.Coli* serotype R515). After 24 hrs supernatants were removed and analysed for levels of IL-12p40 and IL-12p70 **[Figure 3.2]**, IL-23 and IL-6 **[Figure 3.3]**, IL-1β and TNF-α **[Figure 3.4]** and IL-10 **[Figure 3.5]** using specific immunoassays.

The production of the pro-inflammatory cytokines, IL-12p70 and IL-12p40 following LPS activation **[Figure 3.2]** were significantly reduced, in a dose dependant manner in DCs pretreated with *A.digitia* and *M.membrancea* ($p<0.001$) compared to the DMSO control group. A significant reduction was also observed in the pro-inflammatory cytokines, IL-23, IL-6 **[Figure 3.4]** and IL-1β **[Figure 3.4]** following treatment with both marine extracts ($p<0.001$). Furthermore, the levels of TNF-α remained relatively unchanged regardless of marine extract treatment, indicating that the effects of the extracts are specific **[Figure 3.4]**.

In contrast the production of IL-10 post LPS stimulation was significantly increased in the marine extract treated- DC **[Figure 3.5]**.

3.2.3 CRUDE MARINE EXTRACTS MODULATE CELL SURFACE MARKER EXPRESSION ON DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), marine extracts; *A.digitia* or *M.membranacea* at 1:1000 dilution for 1 hr prior to stimulation with 100ng/ml LPS (*E.Coli* serotype R515). Control cells and LPS-stimulated cells were subsequently washed and stained with flurochrome-labelled monoclonal antibodies for various cell surface markers (i.e., CD11c, CD40, CD80, CD86, MHCII, TLR4-MD-2 and CCR5) [see **Table 2.7**] in preparation for cytometric analysis by flow cytometry. .

As expected, stimulation with LPS enhanced the surface marker expression of CD40 **[Figure 3.6]**, CD86 **[Figure 3.7]**, CD80 **[Figure 3.8]**, MHCII **[Figure 3.10]** and

TLR-4-MD-2 **[Figure 3.11]** and decreased the expression of the chemokine receptor CCR5 **[Figure 3.9]**, where control DMSO cells are shown by the filled purple histogram and LPS-treated cells are overlaid with a green line. The marine extracts *A.digitia* and *M.membranacea* did not alter the levels of key surface markers in unstimulated DCs cells, however they were able to suppress the upregulation of some of these surface markers following LPS stimulation. Expression of MHCII **[Figure 3.10]** and TLR4-MD-2 **[Figure 3.11]** were markedly suppressed in *A.digitia*-treated DCs in response to LPS while levels of expression of CD40 **[Figure 3.6]**, CD80 **[Figure 3.7]** and CD86 **[Figure 3.8]** remained relatively unchanged compared to DMSO-treated cells. Whereas *M.membranacea* had no suppressive effect on expression of MHCII **[Figure 3.10]** and TLR4-MD-2 **[Figure 3.11]** but significantly inhibited the expression of CD40 **[Figure 3.6]**, CD86 **[Figure 3.7]** and CD80 **[Figure 3.8]** following LPS stimulation. In addition both extracts blocked the suppression of CCR5 normally seen in mature DC with levels higher than in DMSO-treated cells **[Figure 3.9].**

3.2.4 THE DOSES OF FIRST-ROUND FRACTIONS OF MARINE EXTRACTS USED HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY.

After establishing that crude marine extracts of *A.digitia* and *M.membrancea* had the ability to suppress DC maturation and also modulate cytokine production, these crude extracts were then fractionated. These fractions were then further examined to determine the active fractions. We first assessed their toxicity by exposing DCs to a range of dilutions. After treatment with fractions of marine extract the viability of cells was determined using Cell Titer 96 Aqueous One Solution (Promega, WI, USA)

according to manufacturer's instructions. The doses of fractions of marine extracts selected for use in future experiments did not have any significant cytotoxic effect on DC *in vitro* **[Figure 3.12]**.

3.2.5 FIRST-ROUND FRACTIONS OF MARINE EXTRACTS DOSE-DEPENDENTLY MODULATE LPS-INDUCED CYTOKINE PRODUCTION BY DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), marine extracts; *A. digitia* (C001, C002, C008) or *M. membranacea* (C001, C002, C008) at different dilutions (1:100, 1:1000, 1:10,000) for 1 hr prior to stimulation with 100ng/ml LPS (*E.Coli* serotype R515). After 24 hrs supernatants were removed and assessed for levels of IL-12p40 and IL-12p70 **[Figure 3.13]**, IL-23 and IL-6 **[Figure 3.14]**, IL-1β and TNF-α **[Figure 3.15]** and IL-10 **[Figure 3.16]** using specific immunoassays.

The production of the pro-inflammatory cytokines, IL-12p40, IL-12p70 **[Figure 3.13]**, IL-23 and IL-6 **[Figure 3.14]** were inhibited significantly after exposure to fractions of marine extracts. *A. digitia* (C001, C008) (p<0.001) had the greatest suppressive effect on IL-12p40, IL-12p70 **[Figure 3.13]** and IL-23 **[Figure 3.14]**. The production of these cytokines was also significantly reduced after exposure to *M. membranacea* (C002, C008) (p<0.001). *M. membranacea* C001 (p<0.01) and *A. digitia* C002 (p<0.01) also decreased IL-12p70 **[Figure 3.13]** and IL-23 **[Figure 3.14]** production but to a lesser extent at 1:1000 and 1:10000 dilutions. Furthermore, a significant decrease was observed in the production of IL-1β **[Figure 3.14]** and IL-6 **[Figure 3.15]** with the fractions, *A. digitia* (C001, C008) (p<0.001) and *M.*

membranacea (C002, C008) while *A. digitia* C002 and *M. membranacea* C001 exhibited no effect. In contrast to the other cytokines, which were modulated significantly, levels of TNF-α remained relatively unchanged regardless of treatment with marine extract fractions, suggesting that the effects of the extracts are specific **[Figure 3.15]**.

The production of the anti-inflammatory cytokine, IL-10, was substantially increased in marine treated-DCs following activation with LPS **[Figure 3.16].** *A. digitia* (C001, C008) (p<0.001) and *M. membranacea* (C001, C002, C008) (p<0.001) significantly enhanced IL-10 production at 1:100 dilution however 1:1000 and 1:10,000 dilutions of these marine extracts had no effect following stimulation with LPS.

3.2.6 FIRST-ROUND FRACTIONS OF MARINE EXTRACTS MODULATE LPS-INDUCED CHEMOKINE PRODUCTION BY DCs IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), marine extracts; *A.digitia* (C001, C008) or *M.membranacea* (C002, C008) at different dilutions (1:100, 1:1000, 1:10,000) for 1 hr prior to stimulation with 100ng/ml LPS (*E.Coli* serotype R515). Supernatants were then removed and assessed for levels of MIP-1α, MIP-2 and MCP **[Figure 3.17]** using specific immunoassays.

The production of the pro-inflammatory chemokines, MIP-1α and MCP-1 were significantly suppressed by *A. digitia* C008 (p<0.001) following LPS stimulation, while *A. digitia* C001 exhibited no effect **[Figure 3.17]**. However, *A. digitia* C001 (p<0.001) enhanced MIP-2α expression **[Figure 3.17]**. In contrast, *M.membrancea*

C002 (p<0.001) significantly suppressed the production of MIP-1α and MCP-1 post LPS activation. Again, an increase in the production of MIP-2α was seen following exposure to *M.membrancea* C008 (p<0.001) whereas *M.membrancea* C002 exhibited no alteration **[Figure 3.17]**.

3.2.7 FIRST-ROUND FRACTIONS OF MARINE EXTRACTS INHIBIT DC CHEMOTAXIS

CCL19, also known as MIP-3β, is a ligand for CCR7. Following maturation of DC, CCR7 is up-regulated on the surface of DCs which assists in directing them to T cell areas of draining lymph nodes where they interact with and activate naïve T cells. The chemotaxis of DCs following LPS activation towards the chemokine MIP-3β **[Figure 3.18]** was significantly suppressed by culturing cells with *A. digitia* C008 (p<0.001) and *M. membranacea* C008 (p<0.001) and to a lesser extent with *M. membranacea* C002 (p<0.01) **[Figure 3.18]**. In contrast, *A. digitia* C001 did not have an inhibitory effect on DC migration following LPS activation **[Figure 3.18]**.

3.2.8 FIRST-ROUND FRACTIONS OF MARINE EXTRACTS MODULATE CELL SURFACE MARKER EXPRESSION ON DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), marine extracts; *A. digitia* (C001, C002, C008) or *M. membranacea* (C001, C002, C008) at 1:1000 dilution for 1 hr prior to stimulation with 100ng/ml LPS (*E.Coli* serotype R515). Control cells and LPS-stimulated cells were subsequently washed and stained with flurochrome-labelled monoclonal antibodies for various cell surface markers (CD11c, CD40, CD80, CD86, MHCII,

TLR4-MD-2 and CCR5) [see **Table 2.7**] in preparation for cytometric analysis by flow cytometry. .

As expected, LPS increased the expression of CD40 **[Figure 3.19]**, CD80 **[Figure 3.20]**, CD86 **[Figure 3.21]**, MHCII **[Figure 3.23]** and TLR4-MD-2 **[Figure 3.24]** and decreased the expression of CCR5 **[Figure 3.22]** with control DMSO cells represented by the filled purple histogram and LPS-treated cells overlaid with a green line. The fractions were able to alter the expression of pivotal surface markers. Expression of MHCII **[Figure 3.23]** and TLR4-MD-2 **[Figure 3.24]** were significantly downregulated by the fractionated *A.digitia* marine extract following LPS activation, with C001 and C008 showing the greatest suppression. Furthermore *A.digitia* fractions C001 and C002 slightly decreased the expression of CD86 and CD40, respectively following activation with LPS while no alteration was demonstrated with C008. No modification was seen in the levels of CD80 **[Figure 3.20]** expression with any of the *A.digitia* fractions post stimulation with LPS. In contrast fractionated marine extract *M.membranacea* treated DCs in response to LPS showed relatively no change in surface expression of MHCII **[Figure 3.23]** and TLR4-MD-2 **[Figure 3.24].** However expression of CD40 **[Figure 3.19]**, CD86 and CD80 **[Figure 3.20-3.21]** were significantly suppressed in fractionated *M.membranacea* treated DCs following LPS activation, with fractions C001 and C008 having the greatest effects. Expression of CCR5 was enhanced with *M.membrancea* C008 following stimulation with LPS however no alteration was demonstrated following treatment with *M.membrancea* C001,C002 fraction and also *A.digitia* fractions C001, C002 and C008 **[Figure 3.22]**.

3.2.9 SECOND-ROUND FRACTIONS OF MARINE EXRACT *Membranipora membranacea* HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY

The surface markers CD80 and CD86 are essential in DC maturation and initiating T cell differentiation. Furthermore numerous studies have demonstrated that pro-inflammatory cytokines such as IL-12, IL-6 and IL-1β and chemokines such as MCP-1 and MIP-1α are involved in the progression of numerous inflammatory diseases. Therefore we decided to concentrate on the marine extract *M.membrancea* C002 as this fraction was able to significantly downregulate the expression of these co-stimulatory. In addition *M.membrancea* C002 also modulated the secretion of pro-inflammatory cytokines and chemokines post activation with LPS. Following second-round fractionation of *M.membrancea* C002 we received numerous fractions (C002, C017, C021, C023, and C027) which we examined to determine the active component. Firstly we examined the toxicity of the fractions by using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to manufacturer's instructions. The 1:200 dilutions of marine fractions chosen for use in future experiments did not have any significant cytotoxic effect on DC *in vitro* **[Figure 3.25]**.

3.2.10 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE LPS-INDUCED CYTOKINE PRODUCTION BY DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), fractions of marine extracts; *M. membranacea* (C002, C004, C017, C021, C023 or C027) at 1:200 dilution for 1 hr prior to stimulation with or

without 100ng/ml LPS (*E.Coli* serotype R515). After 24 hrs supernatants were removed and assessed for levels of IL-12p70, IL-12p40, IL-23 **[Figure 3.26]** IL-1β, IL-6, TNF-α and IL-10 **[Figure 3.27]** using specific immunoassays.

The *M.membrancea* fractions (C017, C021, C023 and C027) suppressed the production of IL-12p40 and IL-12p70 following stimulation with LPS ($p<0.001$) while C002 and C004 suppressed IL-12p70 production, they had no effect on IL-12p40 secretion **[Figure 3.26]**. In addition the fractions C017 ($p<0.01$), C021 ($p<0.001$) and C027 ($p<0.01$) were capable of significantly inhibiting the production of IL-23p19 **[Figure 3.26]**.

In contrast levels of TNF-α remained relatively unchanged regardless of the fraction of marine extract used. Fractions, C017, C021, C023 and C027 ($p<0.001$), significantly decreased IL-6 production in LPS stimulated DCs while C002 and C004 showed no effect **[Figure 3.27].** However fractions C002 and C021 ($p<0.05$) were the only fractions capable of inhibiting the production of IL-1β **[Figure 3.27].**

The production of the regulatory cytokine, IL-10 **[Figure 3.27]** was substantially increased in *M.membrancea* fractionated-treated DCs following LPS activation. *M.membrancea* fractions, C002, C017, C021 and C027 ($p<0.001$-$p<0.05$), had the most profound effects on IL-10 production following stimulation of DCs while, C004 and C023 demonstrated no significant effect.

3.2.11 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE CELL SURFACE MARKER EXPRESSION ON DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), second fractions of *M. membranacea* (C021, C023 or C027) at 1:200 dilution for 1 hr prior to stimulation with or without 100ng/ml LPS (*E.Coli* serotype R515) for 24 hrs. Control and LPS-stimulated cells were subsequently stained with flurochrome-labelled monoclonal antibodies for various cell surface markers (CD11c, CD40, CD80, CD86, MHCII and TLR4-MD-2) [see **Table 2.7**] in preparation for cytometric analysis by flow cytometry **[Figure 3.28]**.

As expected LPS stimulation enhanced the surface marker expression of CD40, CD80, CD86 TLR4-MD-2 and MHCII as seen in the first row in **figure 3.28** where DMSO control cells are shown by the filled purple histogram and LPS-treated cells are overlaid with a green line. All the *M.membrancea* fractions including, C021, C023 and C027 were able to significantly reduce the levels of key surface markers CD40, CD86 and MHCII post activation with LPS **[Figure 3.28]**. There was no alteration in the expression of CD80 or TLR4-MD-2 **[Figure 3.28]** again indicating that the marine extracts are acting specifically.

3.2.12 *Membranipora membranacea* MODULATED DCs CAN ALTER CYTOKINE PRODUCTION PROFILES OF $CD4^+$ T CELLS.

The cytokines secreted by DCs and their expression of co-stimulatory molecules are critical for activating T helper cells. Given that fractions from *M.membrancea* can alter DC activation we next assessed whether their effect on DCs had consequences

for T cell activation and cytokine production. DCs were harvested for 7 days as previously described **[see section 2.26].** On day 7 cells were pretreated with marine extract at 1:200 dilution for 1 hr prior to activation with OVA peptide. Following activation DCs were co-incubated with $CD4^+$ T cells isolated from the spleens of D011.10 mice which express a TCR specific for a peptide within the ovalbumin (OVA) molecule (OVA (323-339)). When this OVA antigen is presented by DC complexed to MHCII (together with costimulation), the naive $CD4^+$ T cells become activated and proliferate (Pompos and Fritsche 2002). **Figure 3.29** shows that T cells activated with marine-treated DC produce significantly less IFN-γ ($p<0.001$), less IL-17 ($p<0.001$), and less IL-4 ($p<0.001$) on day 10 of culture.

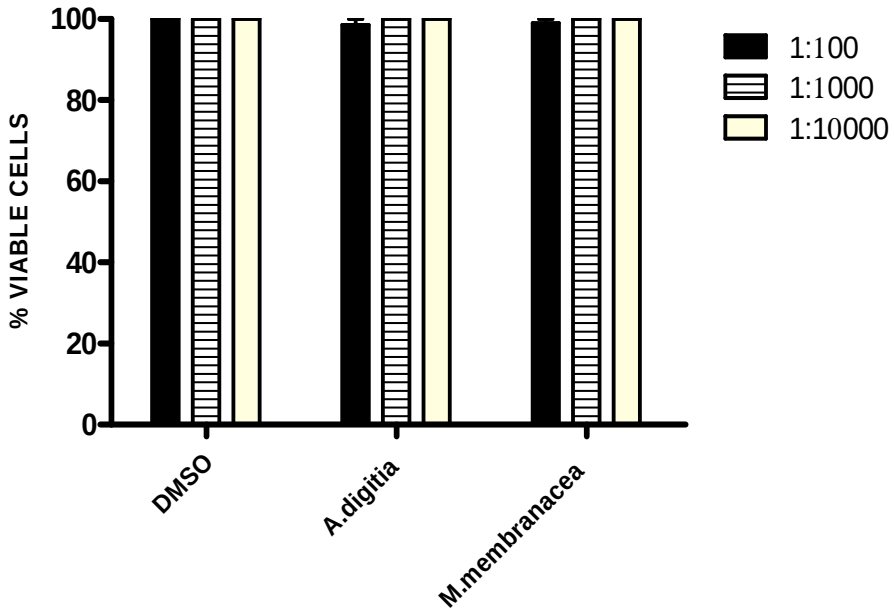


FIGURE 3.1: The concentrations of crude marine extracts used do not significantly affect the viability DCs.
BMDCs were grown for 7 days and then treated with a range of dilutions (1:100, 1:1000, 1:10000) of DMSO (vehicle control), crude *A.digitia* and *M.membranacea* for 24 hrs. Following 24 hr treatment cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of untreated cells.

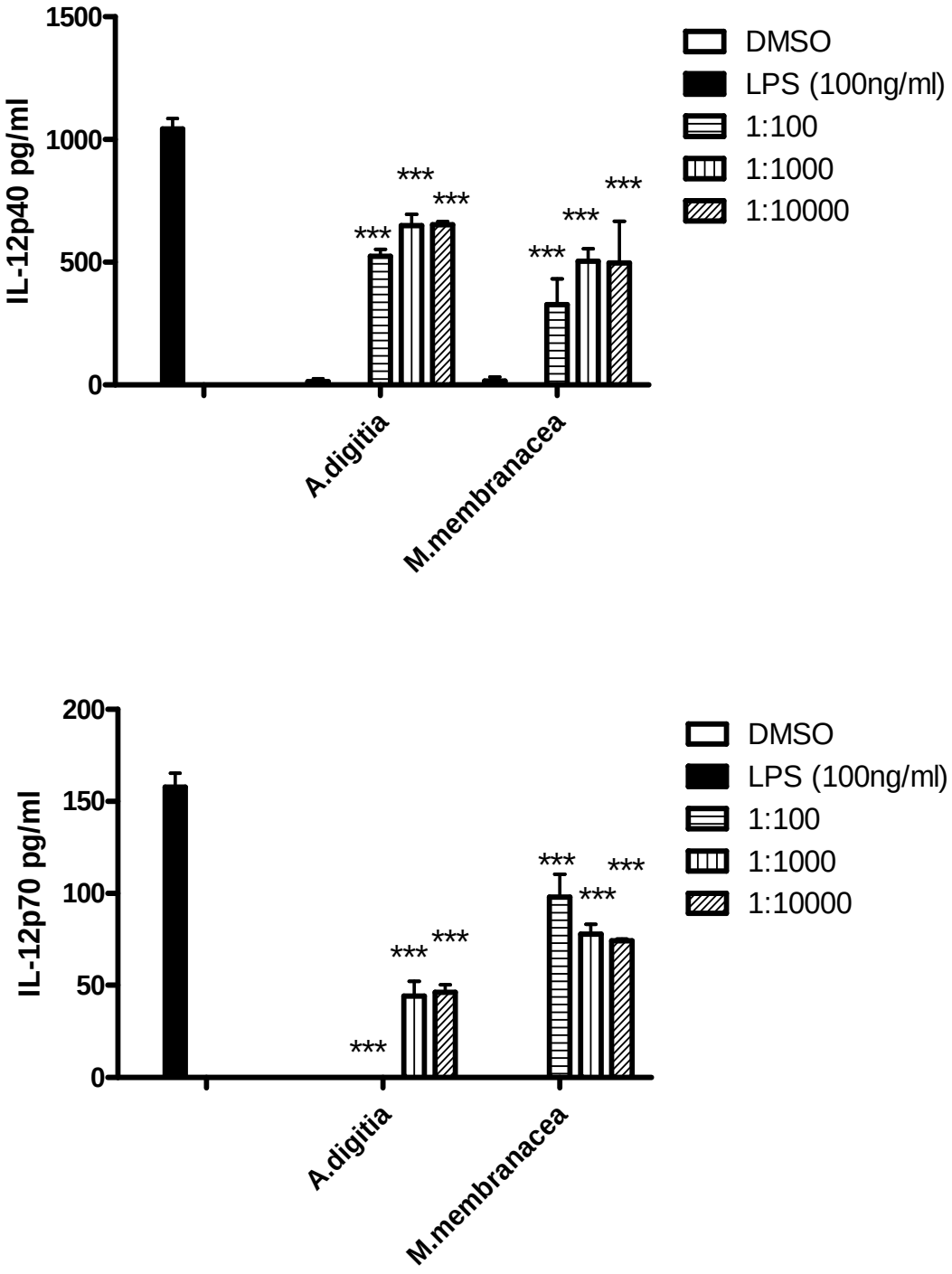


FIGURE 3.2: Marine extracts suppress LPS-induced IL-12p40 and IL-12p70 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), crude marine extracts- *A.digitia* and *M.membranacea* for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-12p40 and IL-12p70 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

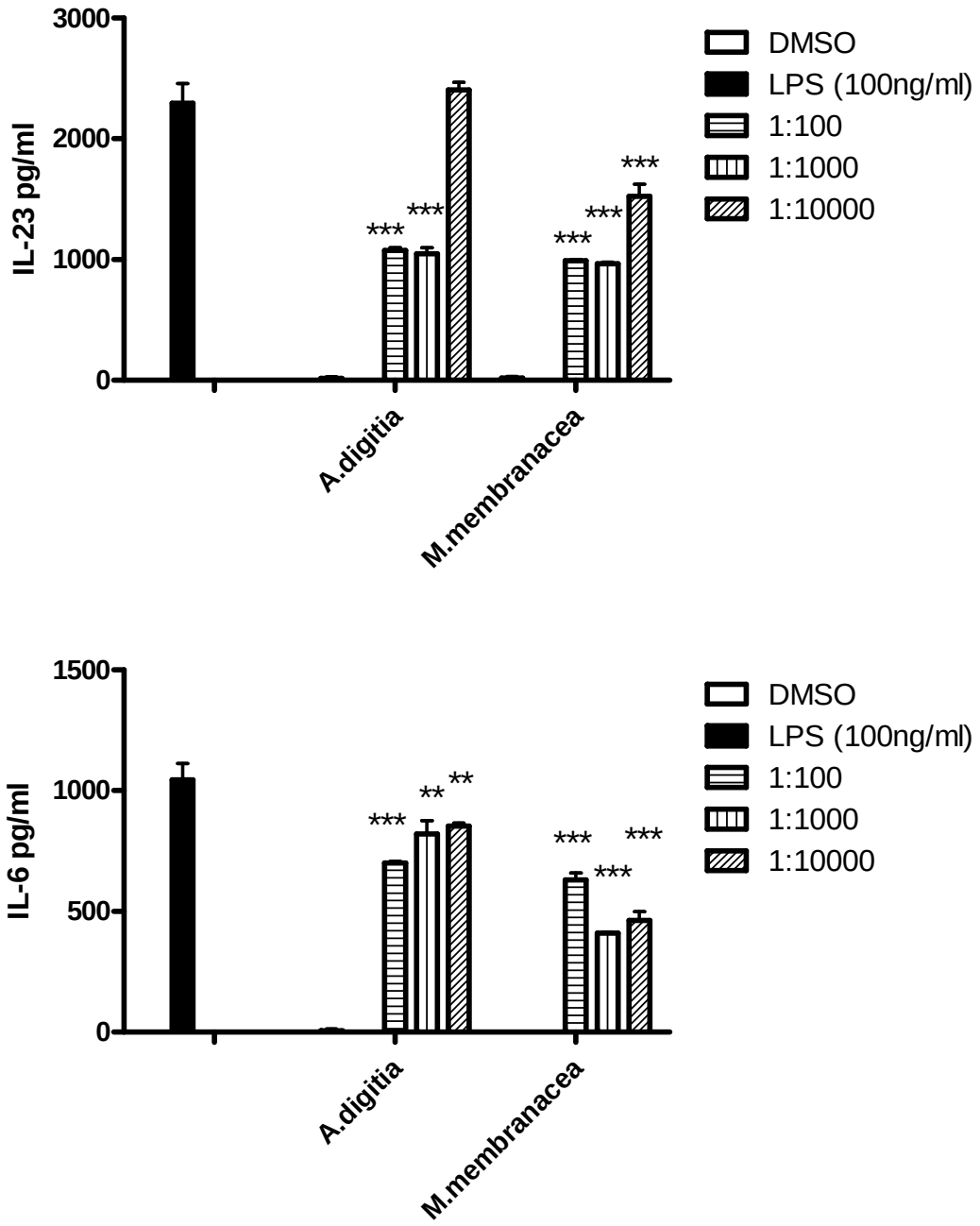
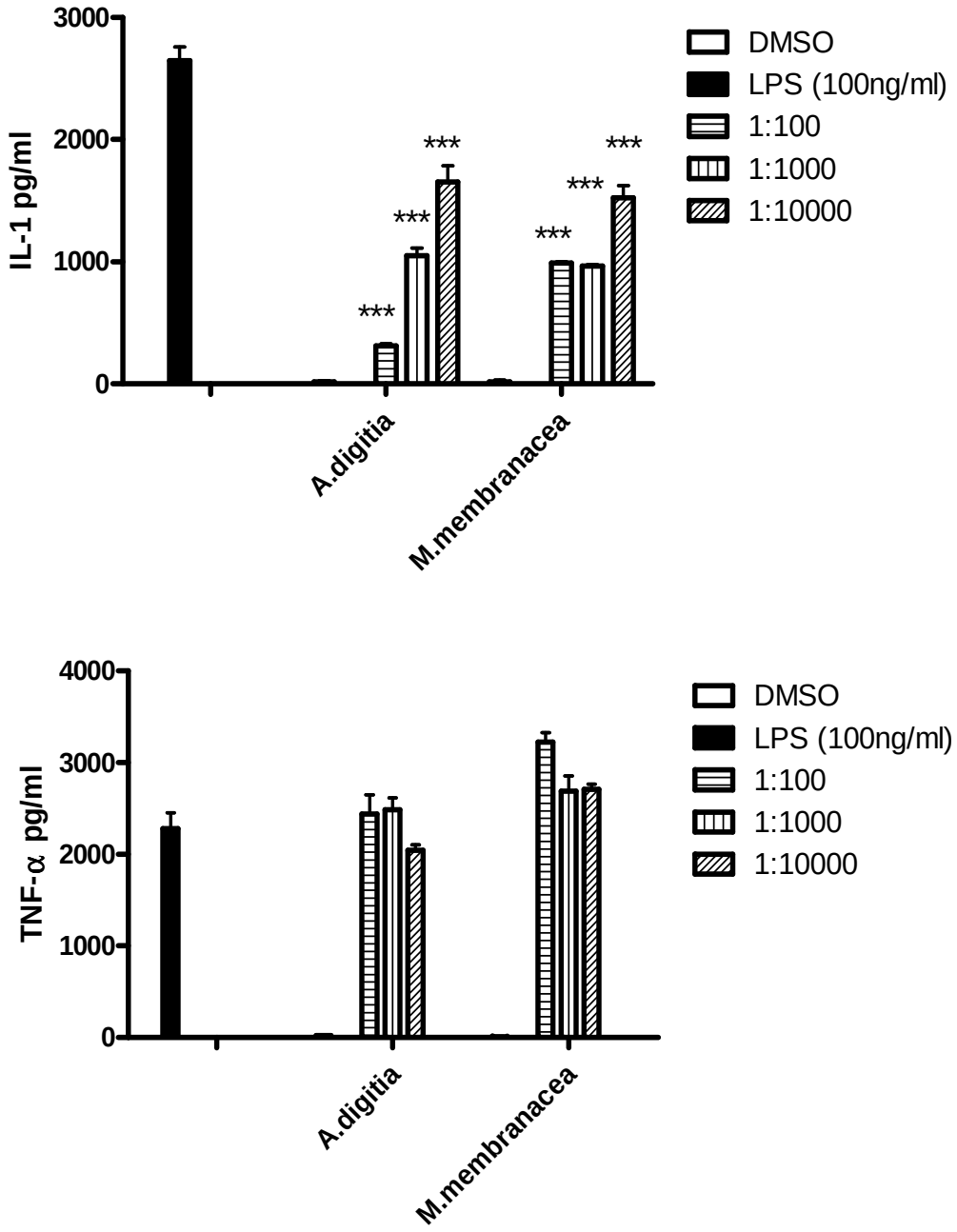


FIGURE 3.3: Marine extracts suppress LPS-induced IL-23 and IL-6 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), crude marine extracts- *A.digitia* and *M.membranacea* for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-23 and IL-6 using specific immunoassays. Results are ± SEM of quadruplicate assays and represent three independent experiments. ***$p<0.001$, **$p<0.01$, *$p<0.05$ comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test



F**IGURE 3.4: Marine extracts suppress LPS-induced IL-1β production but do not alter TNF-α production in DCs.** BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), crude marine extracts- *A.digitia* and *M.membranacea* for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-1β and TNF-α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

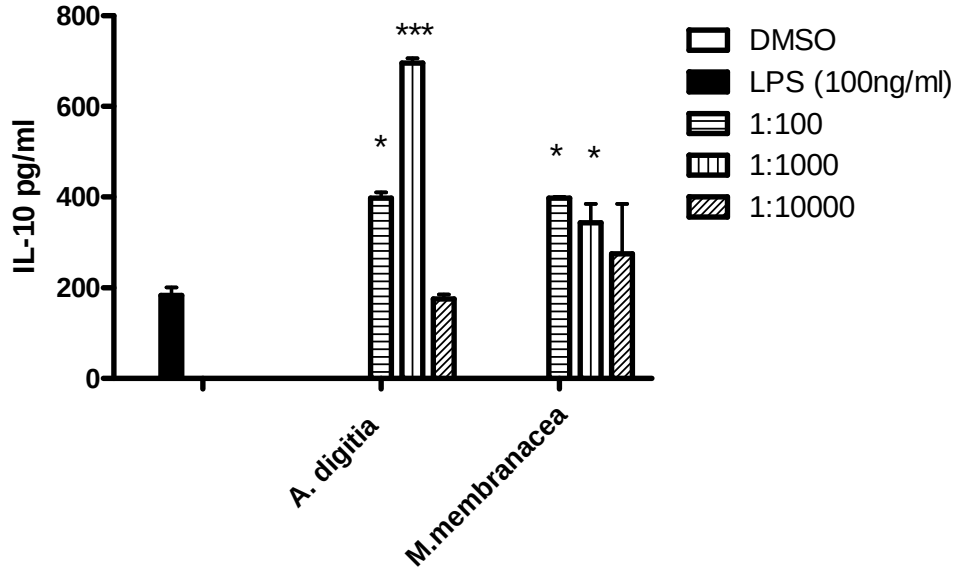


FIGURE 3.5: Marine extracts enhance LPS-induced IL-10 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), crude marine extracts- *A.digitia* and *M.membranacea* for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-10 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***$p<0.001$, **$p<0.01$, *$p<0.05$ comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

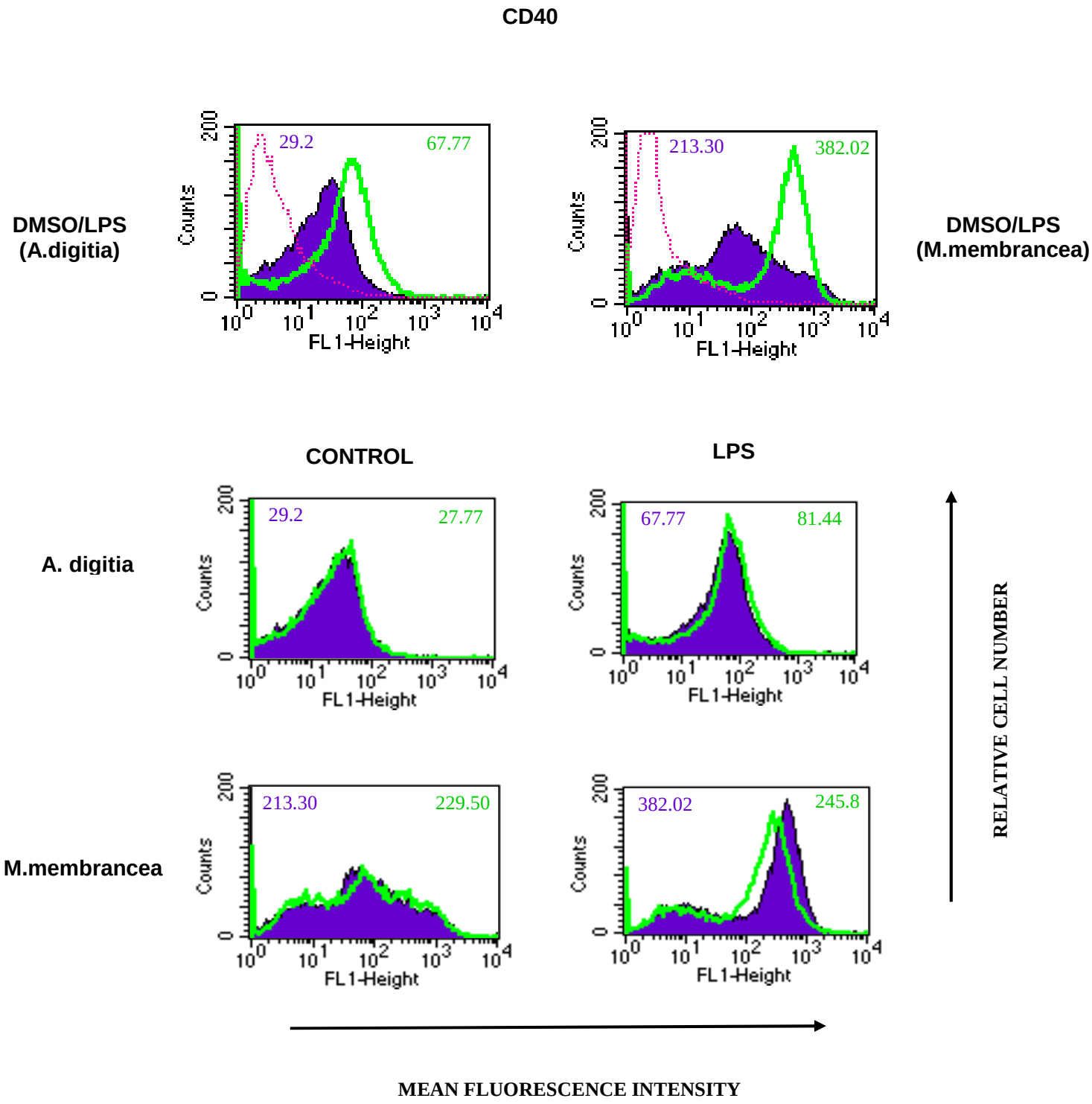


FIGURE 3.6: Marine extracts modulate the expression of CD40 on the surface of DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with crude *A.digitia* and *M.membrancea* for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CD40 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown.**Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom two rows:** DMSO-treated DCs (filled purple histogram) vs. marine extract-treated DCs (green line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top row. Profiles are shown for a single experiment and are representative of 3 experiments.

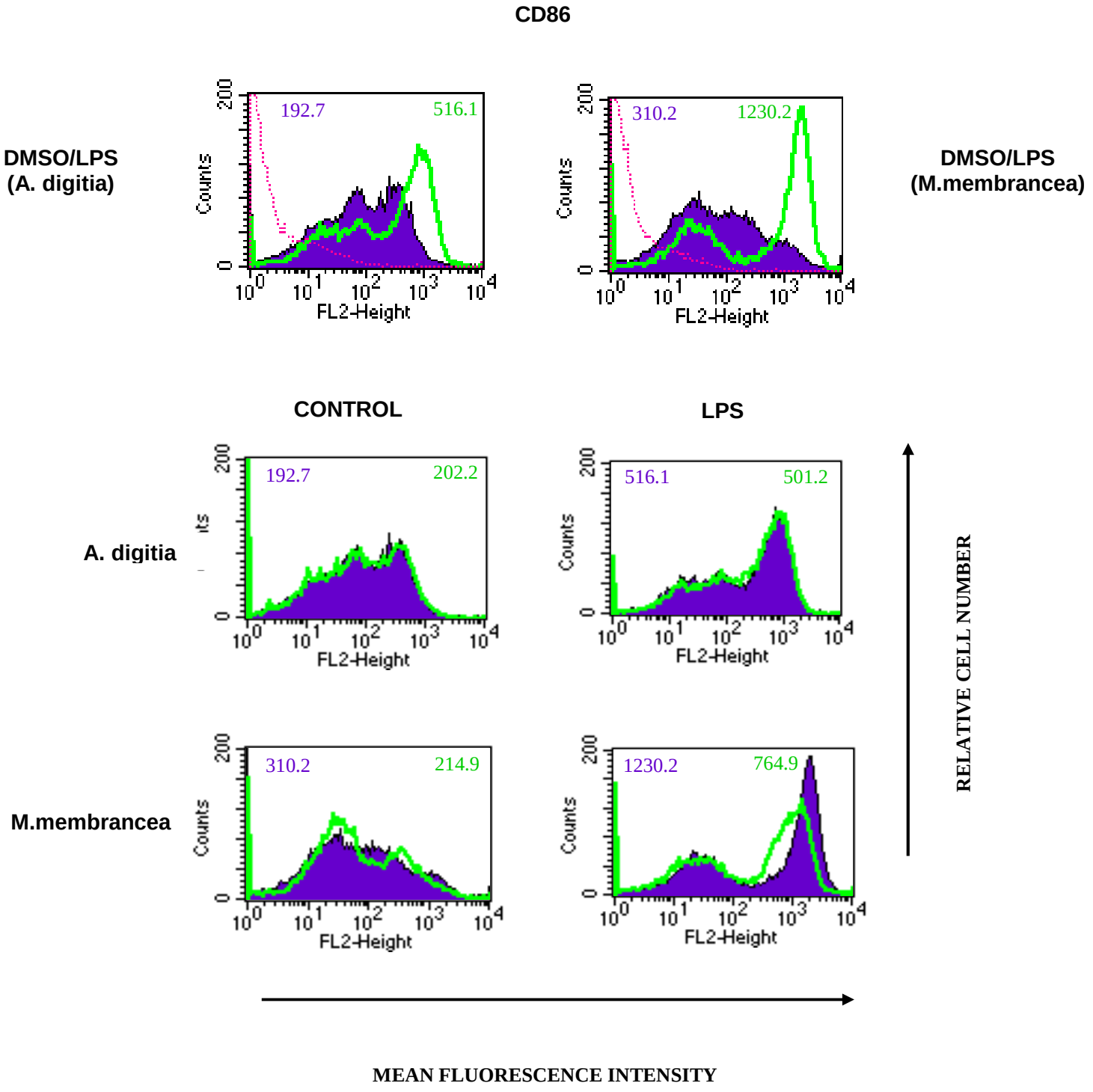


FIGURE 3.7: Marine extracts modulate the expression of CD86 on the surface of DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with crude *A.digitia* and *M.membrancea* for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CD86 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom two rows:** DMSO-treated DCs (filled purple histogram) vs. marine extract-treated DCs (green line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top row. Profiles are shown for a single experiment and are representative of 3 experiments.

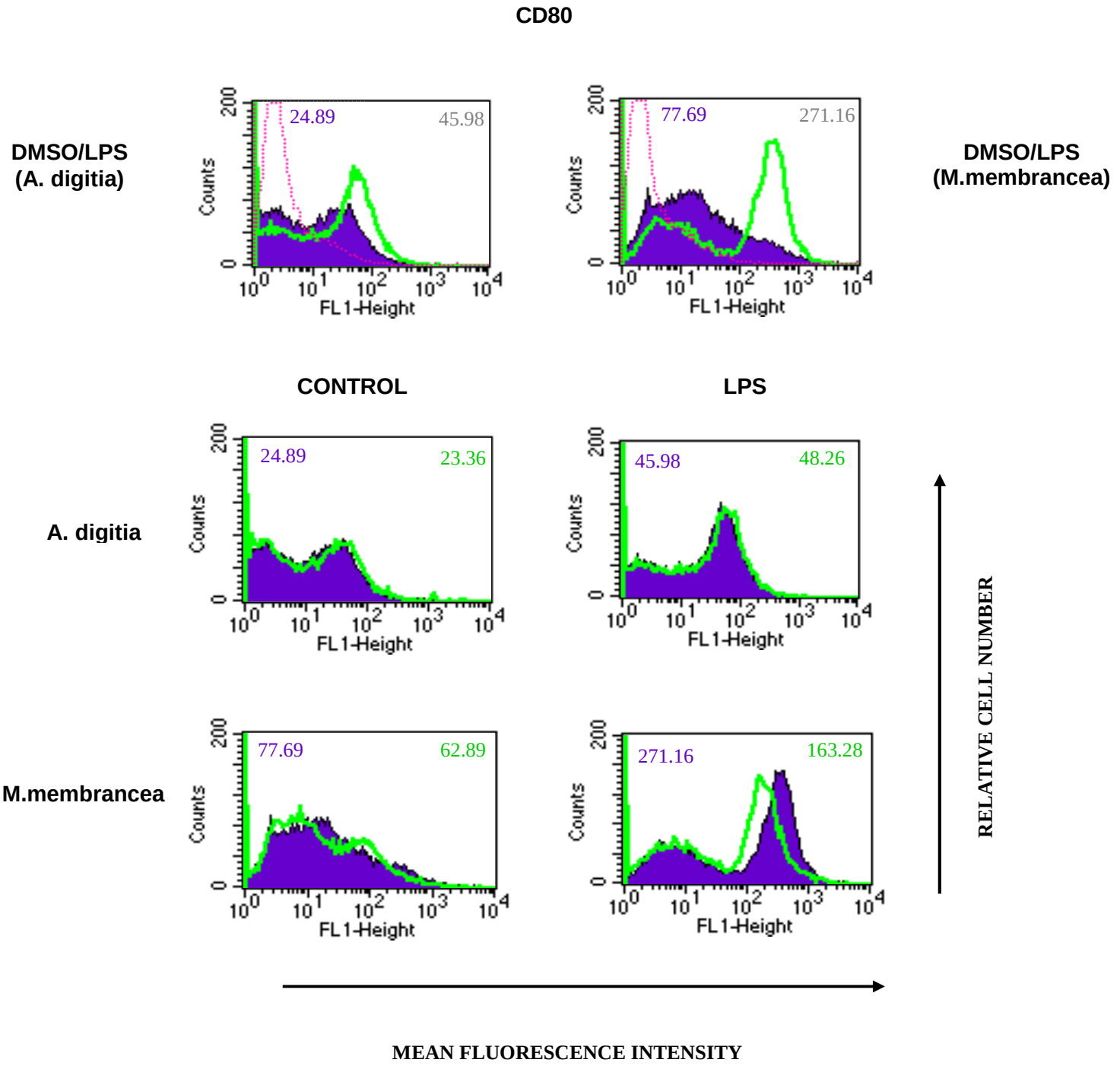


FIGURE 3.8: Marine extracts modulate the expression of CD80 on the surface of DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with crude *A.digitia* and *M.membrancea* for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CD80 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom two rows:** DMSO-treated DCs (filled purple histogram) vs. marine extract-treated DCs (green line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top row. Profiles are shown for a single experiment and are representative of 3 experiments.

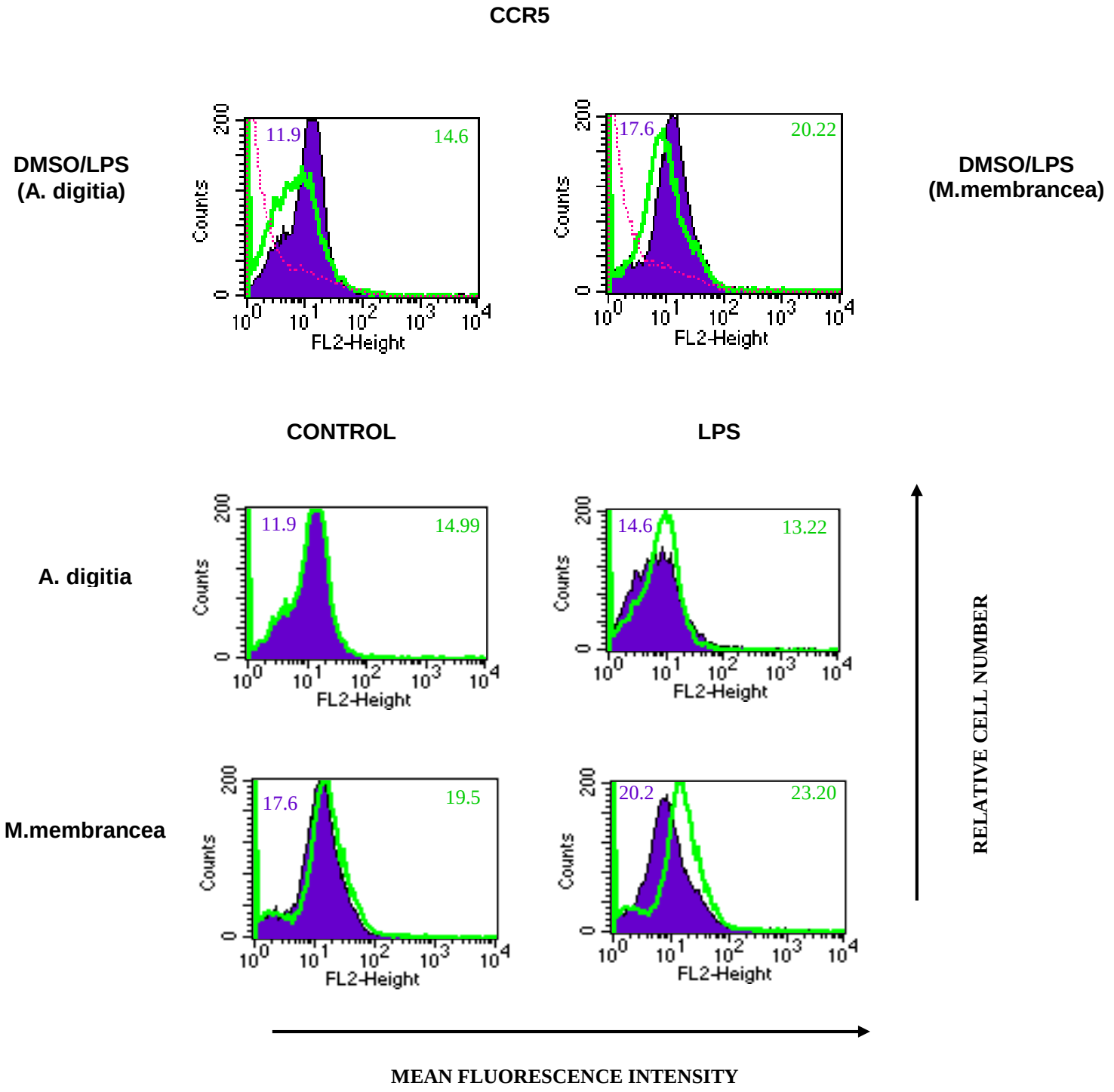


FIGURE 3.9: Marine extracts modulate the expression of CCR5 on the surface of DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with crude *A.digitia* and *M.membrancea* for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CCR5 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom two rows:** DMSO-treated DCs (filled purple histogram) vs. marine extract-treated DCs (green line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top row. Profiles are shown for a single experiment and are representative of 3 experiments.

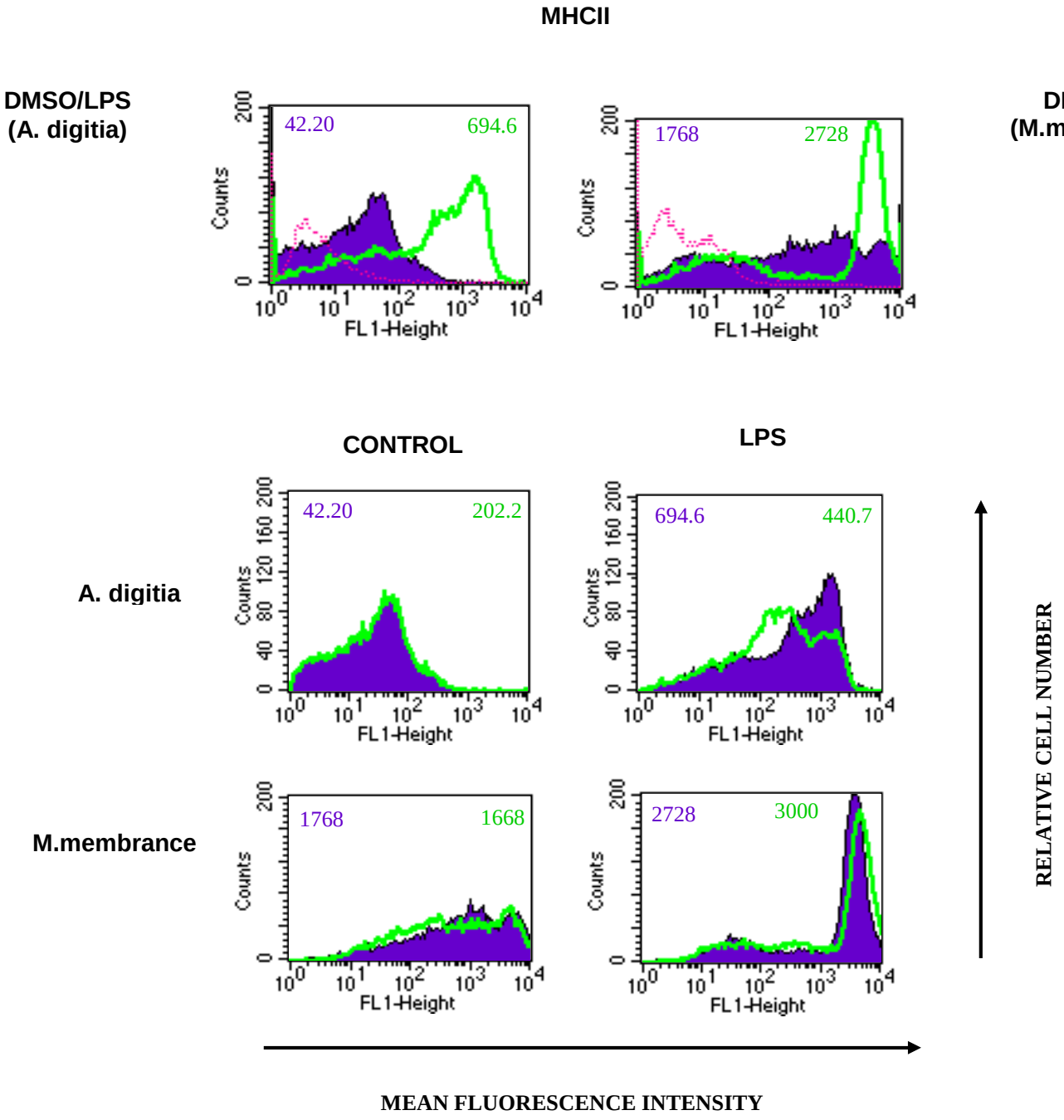


FIGURE 3.10: Marine extracts modulate the expression of MHCII on the surface of DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with crude *A.digitia* and *M.membrancea* for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for MHCII or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom two rows:** DMSO-treated DCs (filled purple histogram) vs. marine extract-treated DCs (green line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top row. Profiles are shown for a single experiment and are representative of 3 experiments.

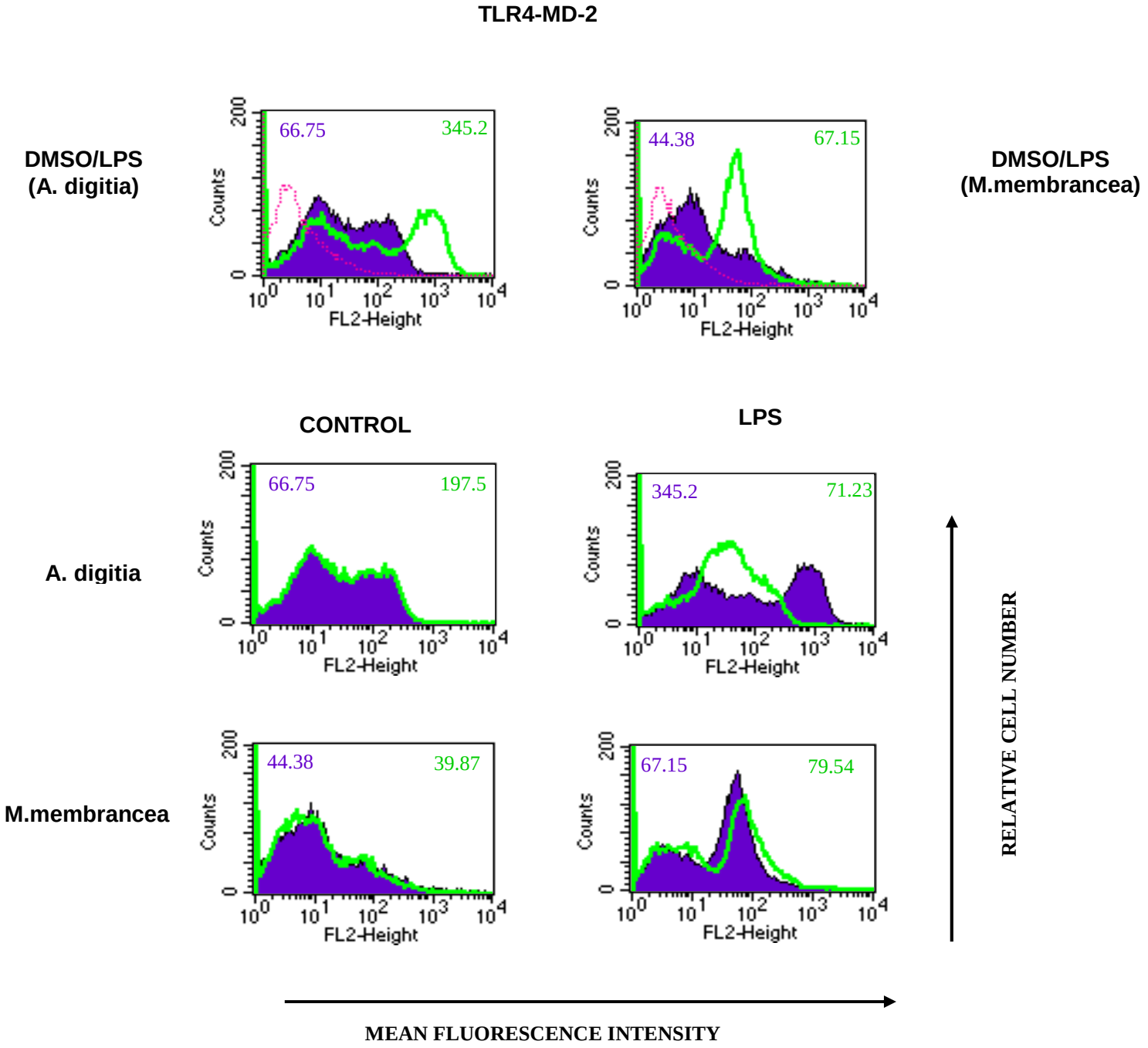


FIGURE 3.11: Marine extracts modulate the expression of TLR4-MD-2 on the surface of DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with crude *A.digitia* and *M.membrancea* for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for TLR4-MD-2 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom two rows:** DMSO-treated DCs (filled purple histogram) vs. marine extract-treated DCs (green line) for unstimulated **[Control]** vs. stimulated **[LPS]** cells. MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top row. Profiles are shown for a single experiment and are representative of 3 experiments.

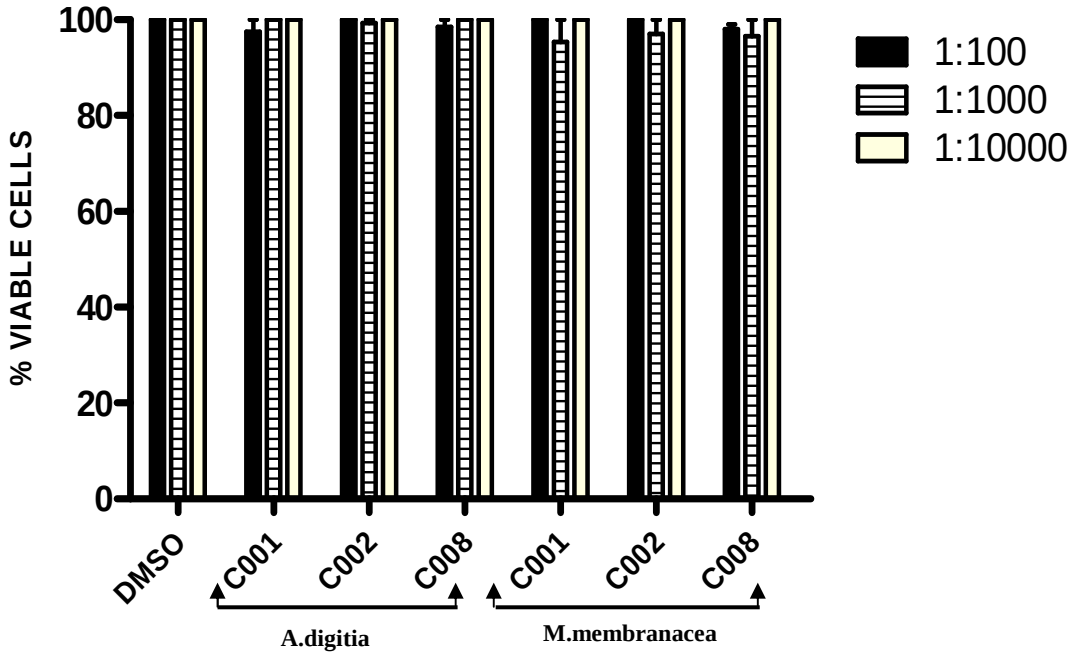


FIGURE 3.12: The concentrations of first-round fractions extracts used do not significantly affect the viability of DCs.
BMDCs were grown for 7 days and then treated with a range of dilutions (1:100, 1:1000, 1:10000) of either DMSO (vehicle control) or *A.digitia* (C008, C009) and *M.membranacea* (C009, C008) for 24hrs. Following 24 hr treatment cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of untreated cells.

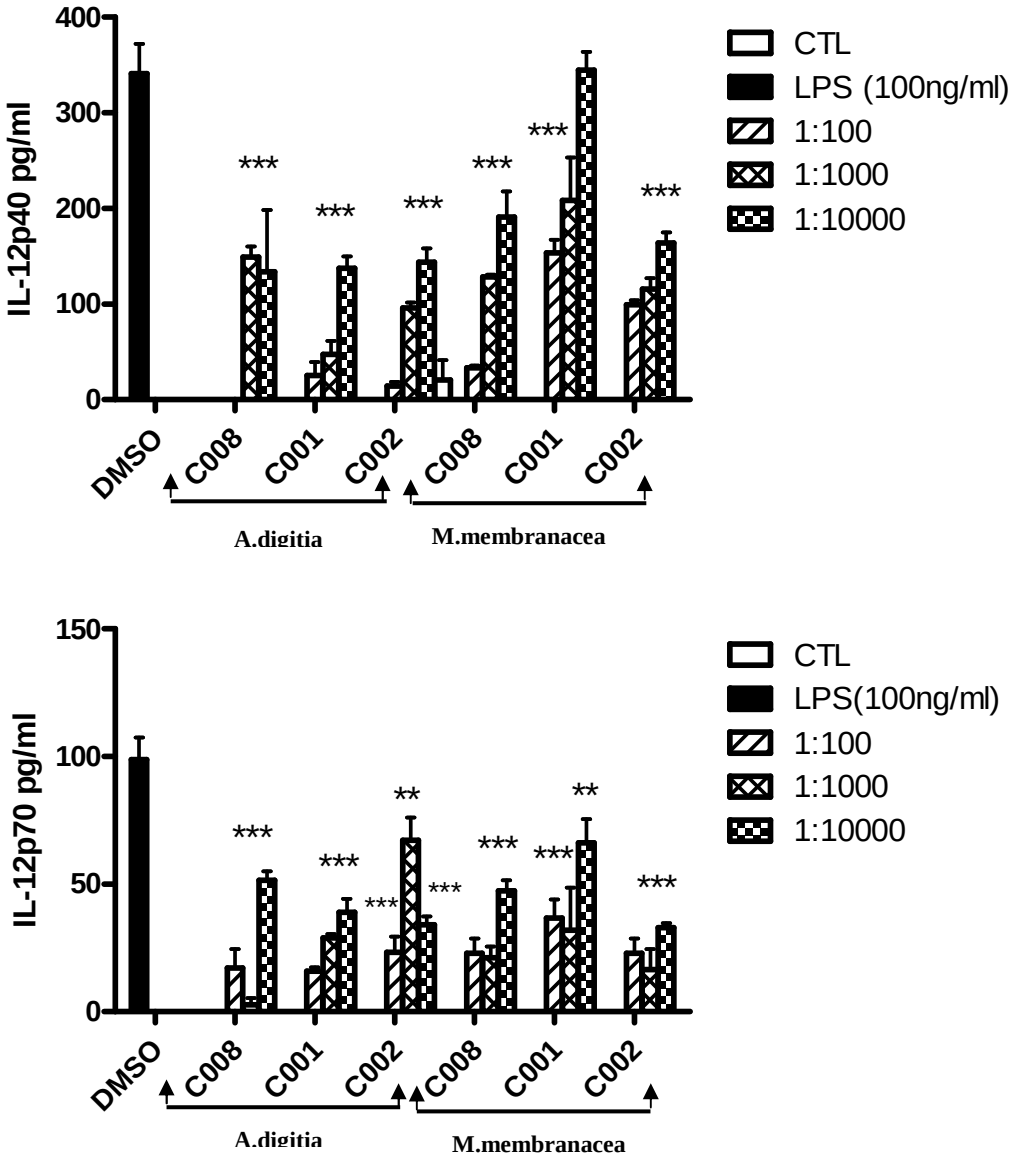


FIGURE 3.13: First-round fractions of marine extracts suppress LPS-induced IL-12p40 and IL-12p70 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), fractions of marine extracts- *A.digitia* (C001, C002, C008) and *M.membranacea* (C001, C002, C008) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-12p40 and IL-12p70 using specific immunoassays. Results are ± SEM of quadruplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

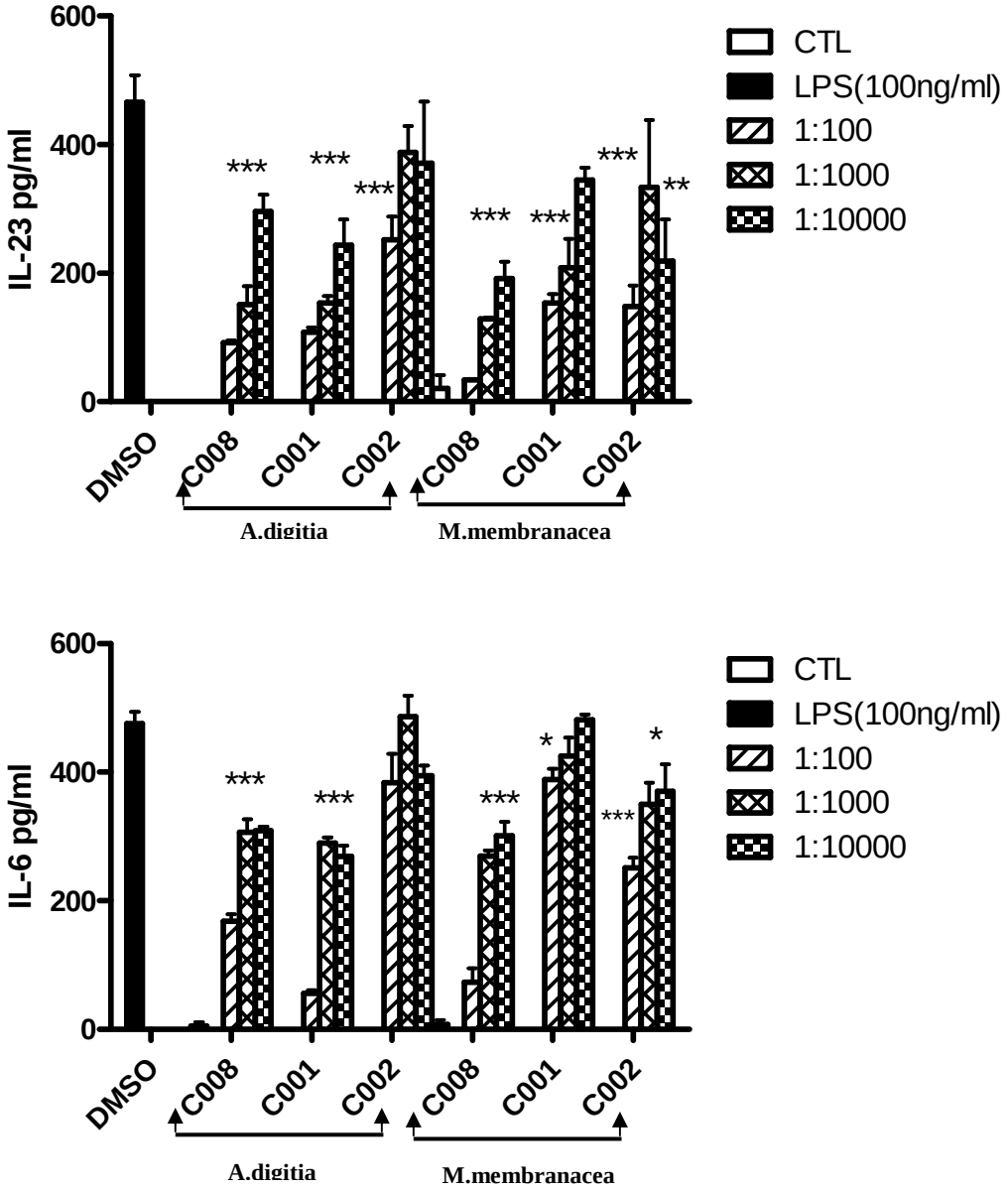


FIGURE 3.14: First-round fractions of marine extracts suppress LPS-induced IL-23 and IL-6 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), fractions of marine extracts- *A.digitia* (C001, C002, C008) and *M.membranacea* (C001, C002, C008) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-23 and IL-6 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

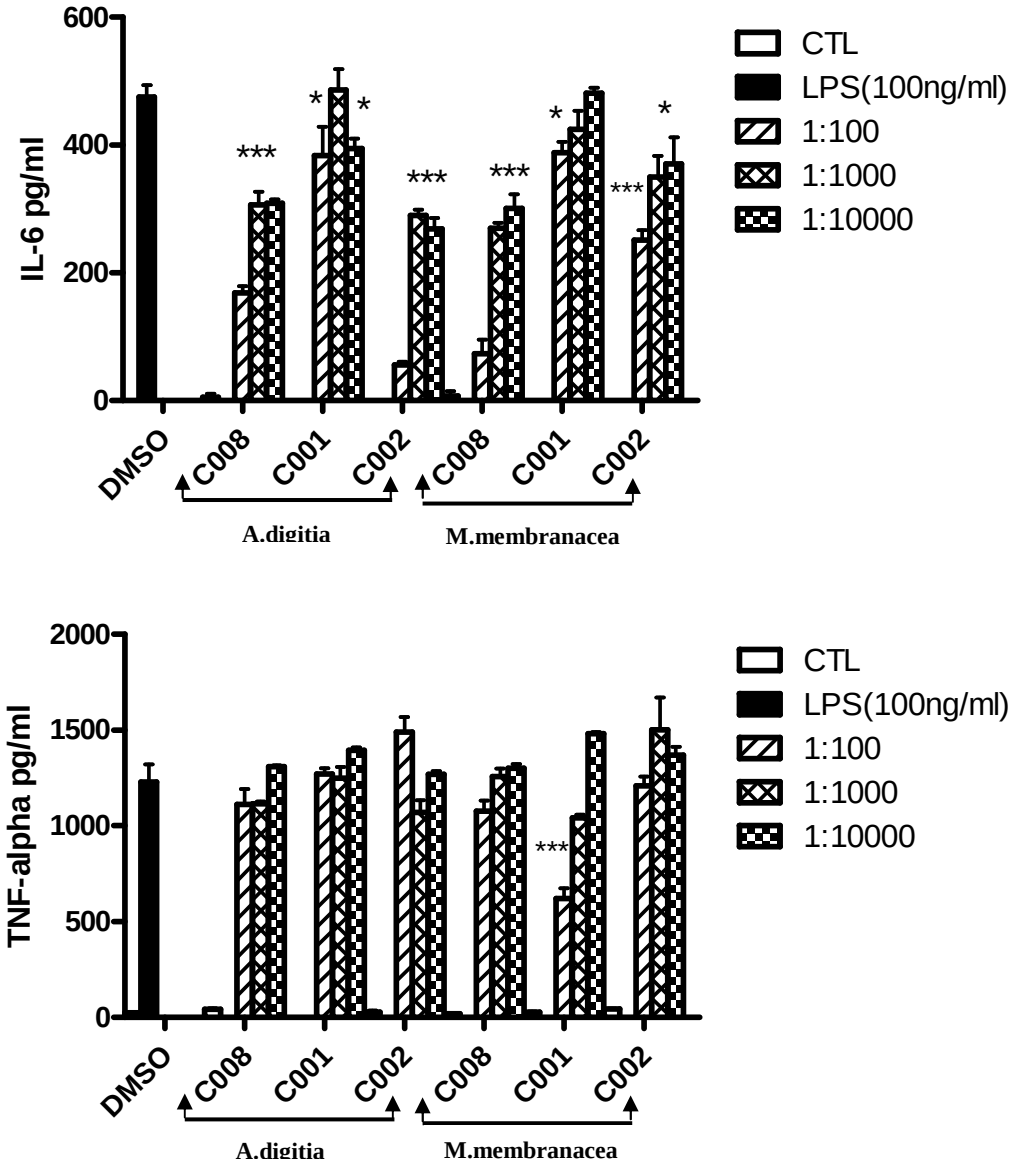


FIGURE 3.15: First-round fractions of marine extracts suppress LPS-induced IL-1β production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), fractions of marine extracts- *A.digitia* (C001, C002, C008) and *M.membranacea* (C001, C002, C008) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-1β and TNF-α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

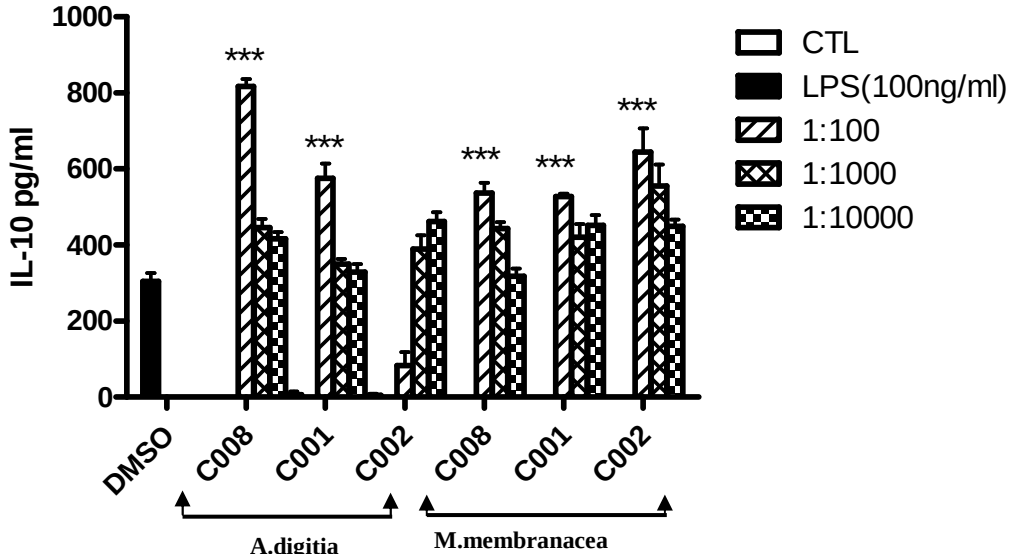


FIGURE 3.16: First-round fractions of marine extracts enhance LPS-induced IL-10 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), fractions of marine extracts- *A.digitia* (C001, C002, C008) and *M.membranacea* (C001, C002, C008) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-10 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

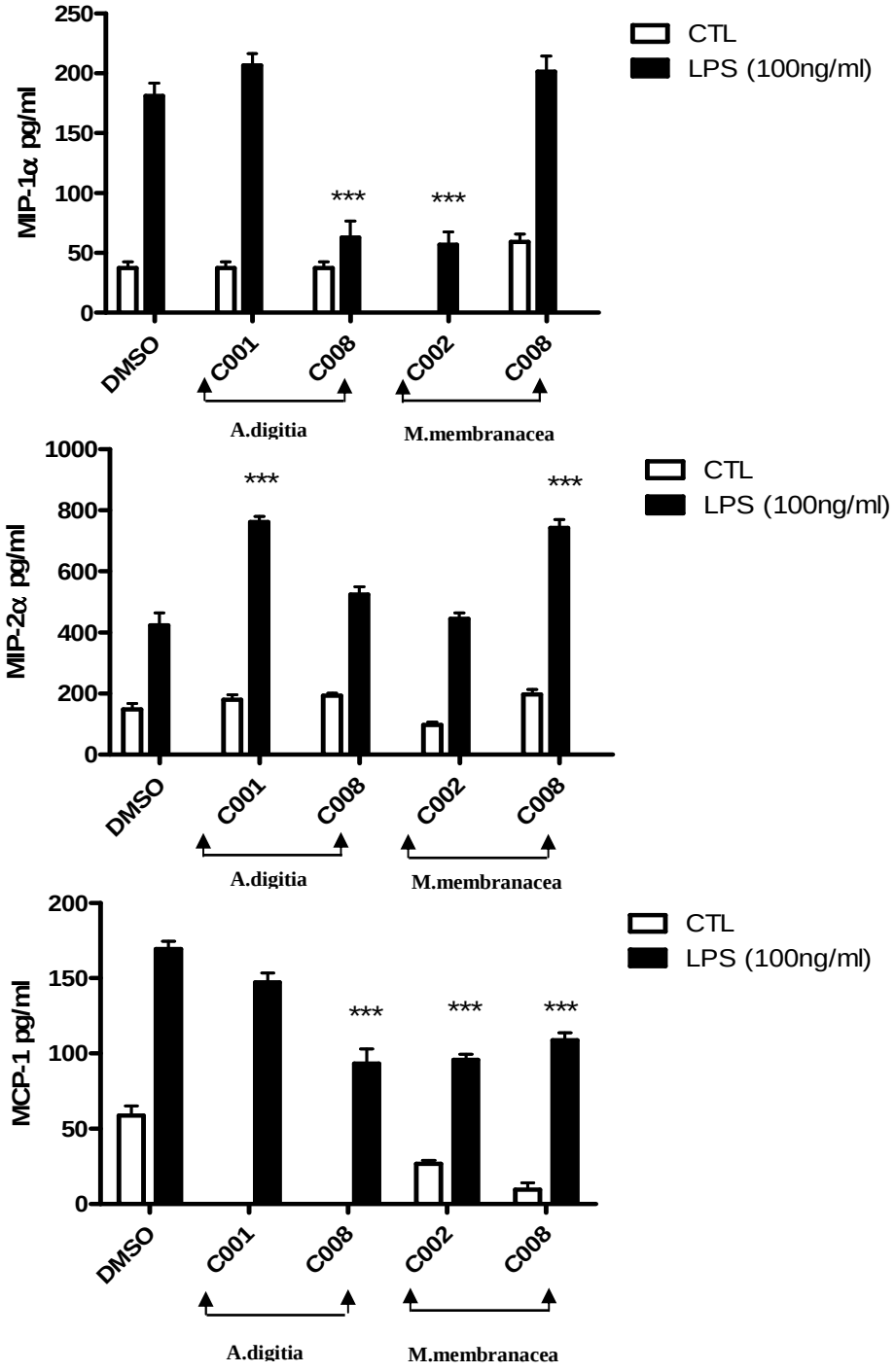


FIGURE 3.17: First-round fractions of marine extracts modulate LPS-induced chemokine production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), fractions of marine extracts- *A.digitia* (C001, C008) and *M.membranacea* (C002, C008) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of MIP-1α, MIP-2α and MCP-1 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

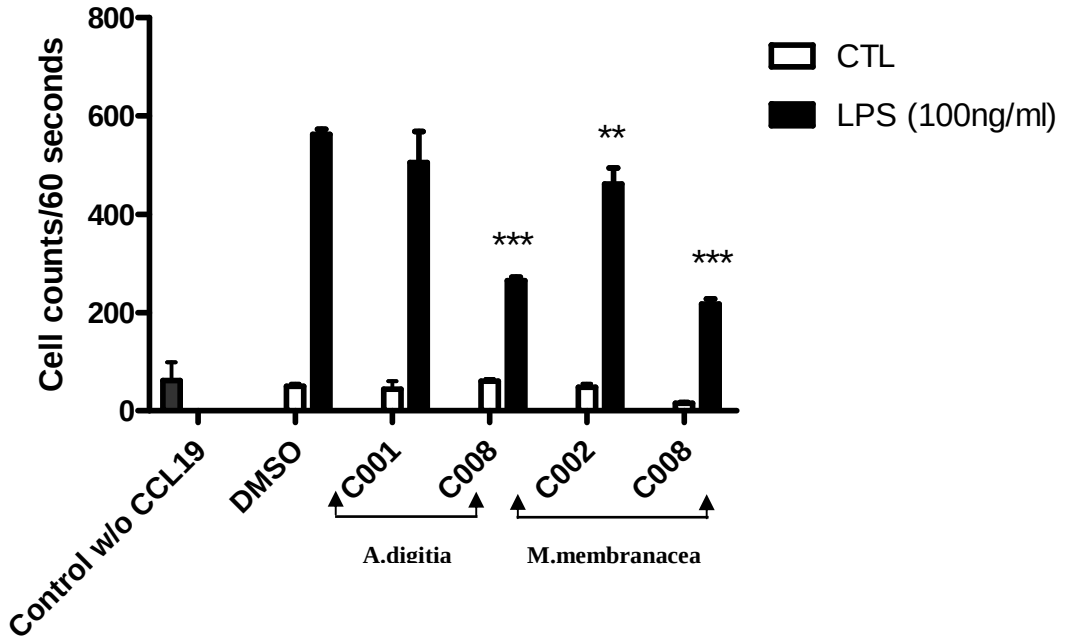


FIGURE 3.18: Fractions of marine extracts modulate the migration of DCs towards the chemokine MIP-3β (CCL19). DC were cultured in the presence *A.digitia* (C001, C008) and *M.membranacea* (C002, C008) and stimulated with LPS (100ng/ml) before $3x10^5$ cells were placed in the upper chamber of a Transwell plate (8.0μm). Media containing recombinant CCL19 (100ng/ml) was added to the lower chamber and plates were incubated for 5 hrs at 37ºC. To determine the number of migrated cells, media from the bottom well was collected and events (cells) for 1 min were counted on a BD FACsCalibur™.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

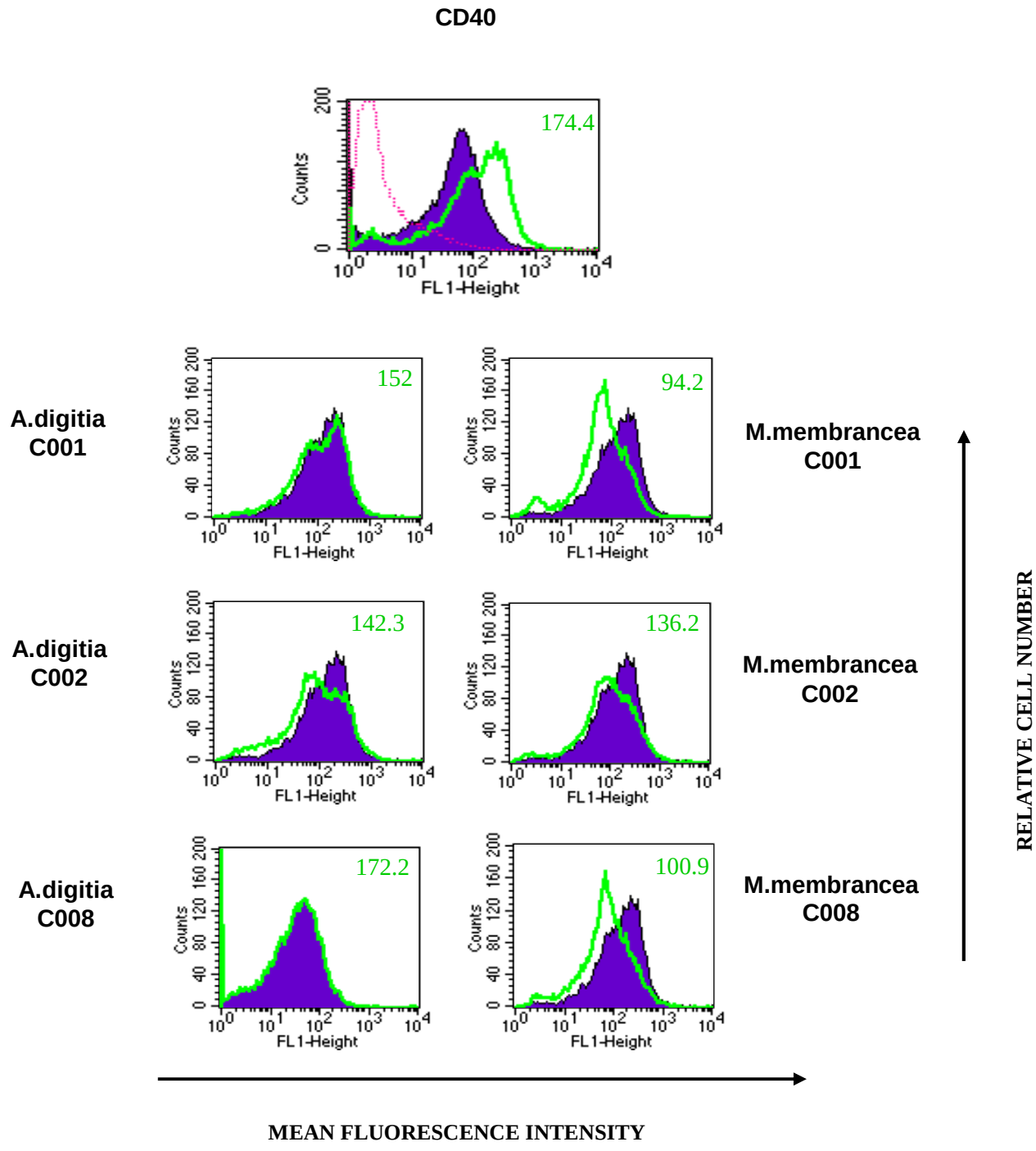


FIGURE 3.19: First-round fractions of marine extracts modulate the expression of CD40 on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with fractions of *A.digitia* (C001, C002, C008) and *M.membrancea* (C001, C002, C008) for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CD40 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottow three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments.

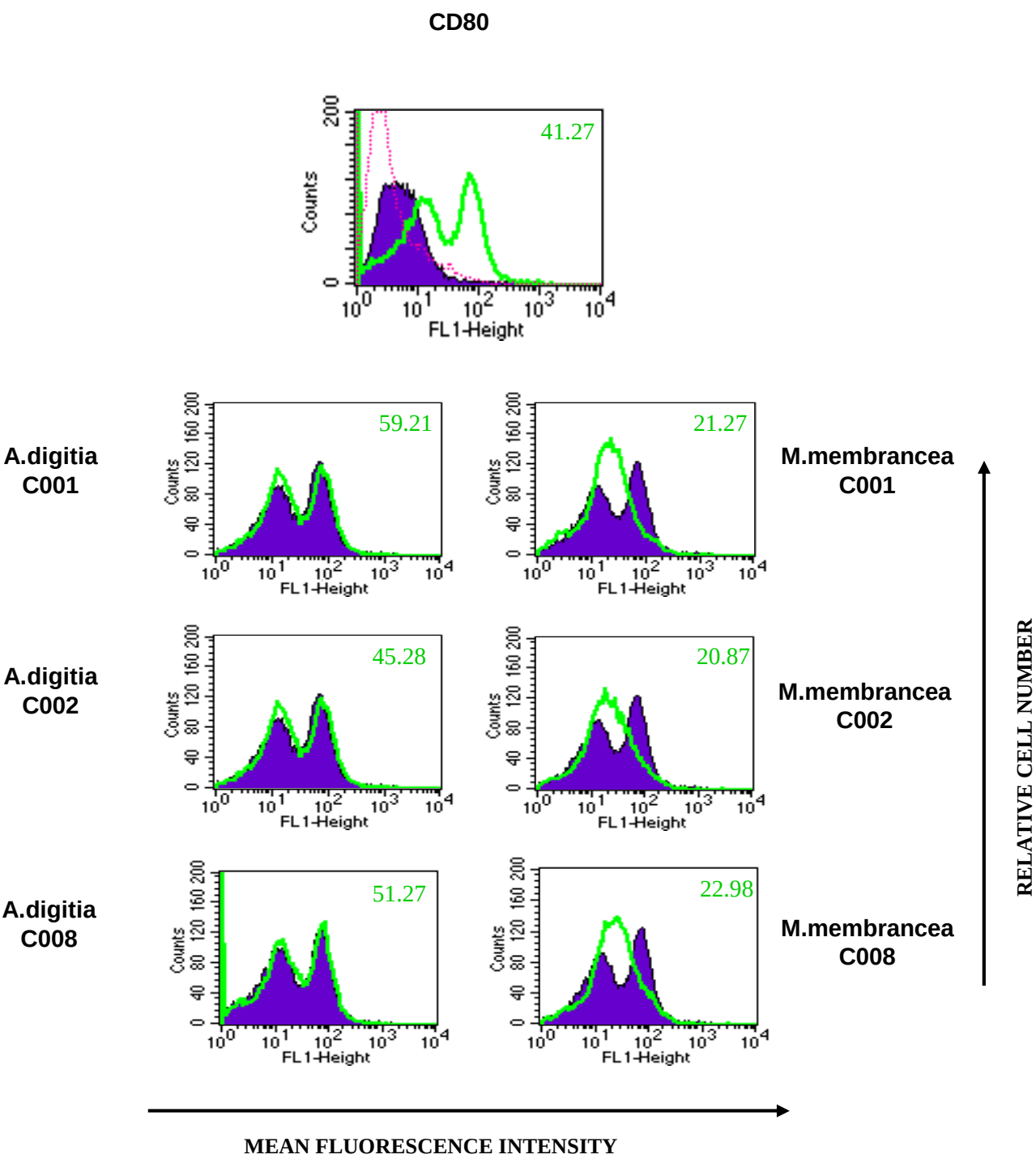


FIGURE 3.20: First-round of fractions of marine extracts modulate the expression of CD80 on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with fractions of *A.digitia* (C001, C002, C008) and *M.membrancea* (C001, C002, C008) for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, 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. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottow three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments..

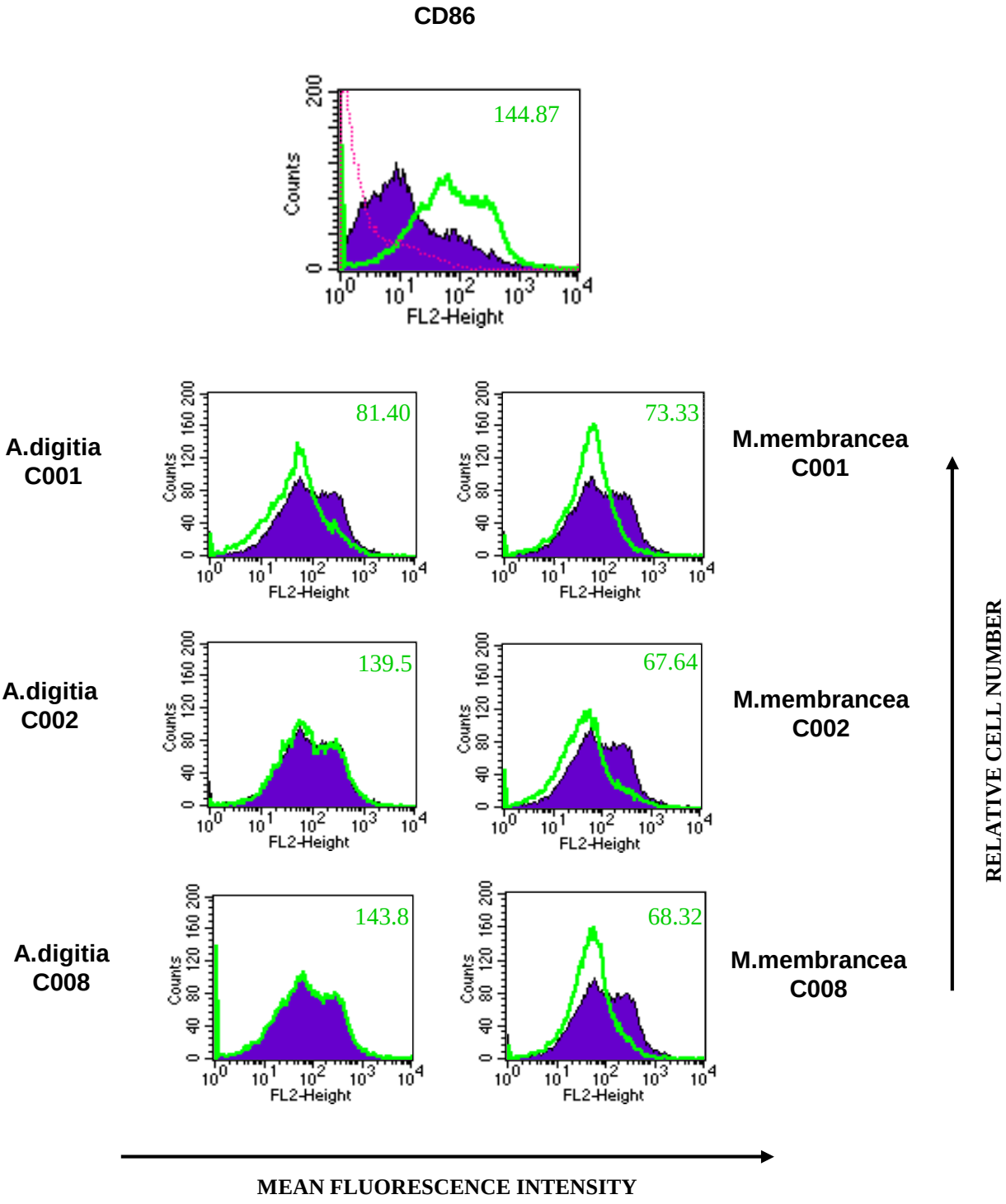


FIGURE 3.21: First-round fractions of marine extracts modulate the expression of CD86 on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with fractions of *A.digitia* (C001, C002, C008) and *M.membrancea* (C001, C002, C008) for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CD86 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottow three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments.

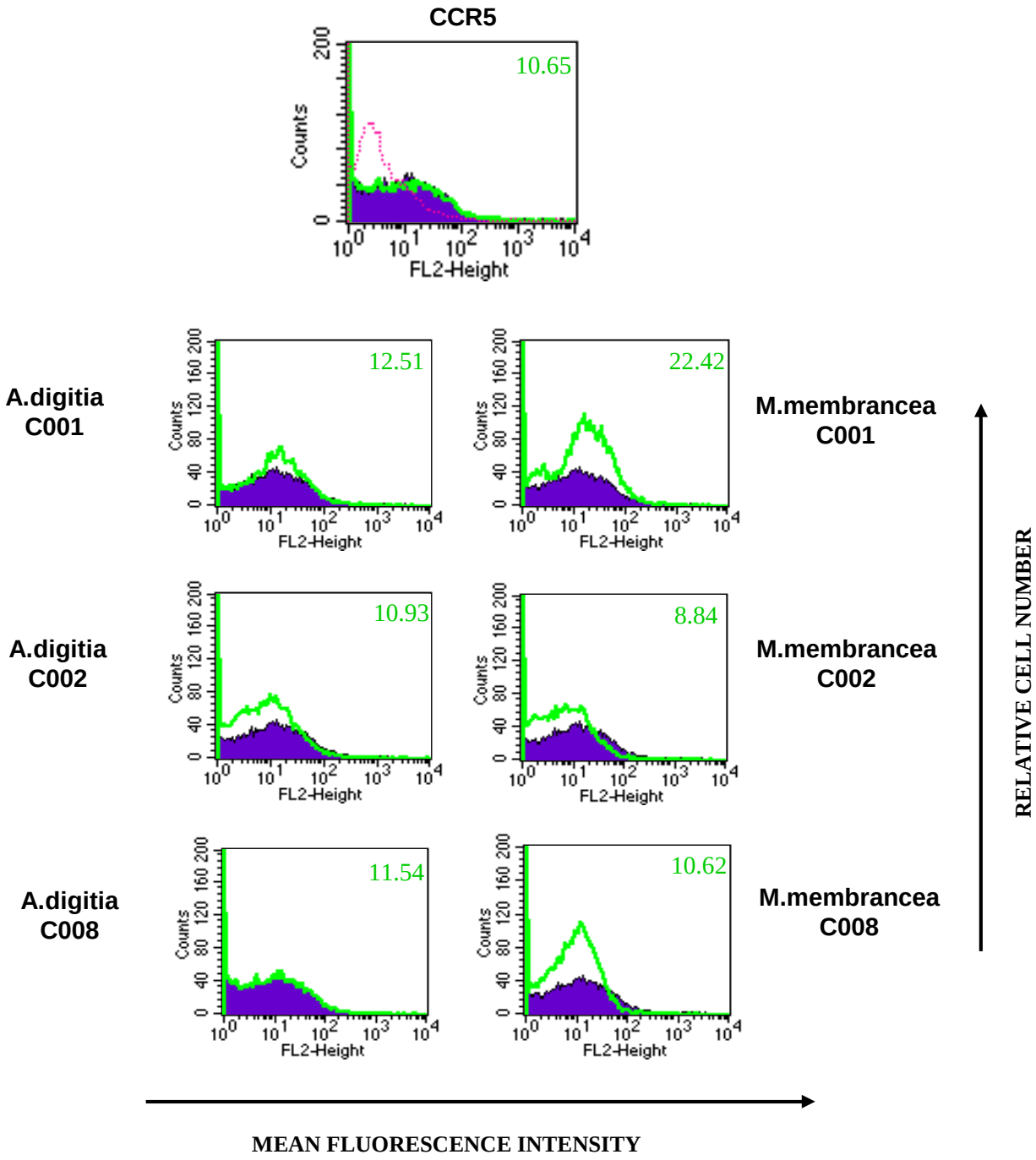


FIGURE 3.22: First-round fractions of marine extracts modulate the expression of CCR5 on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with fractions of *A.digitia* (C001, C002, C008) and *M.membrancea* (C001, C002, C008) for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for CCR5 or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottow three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments.

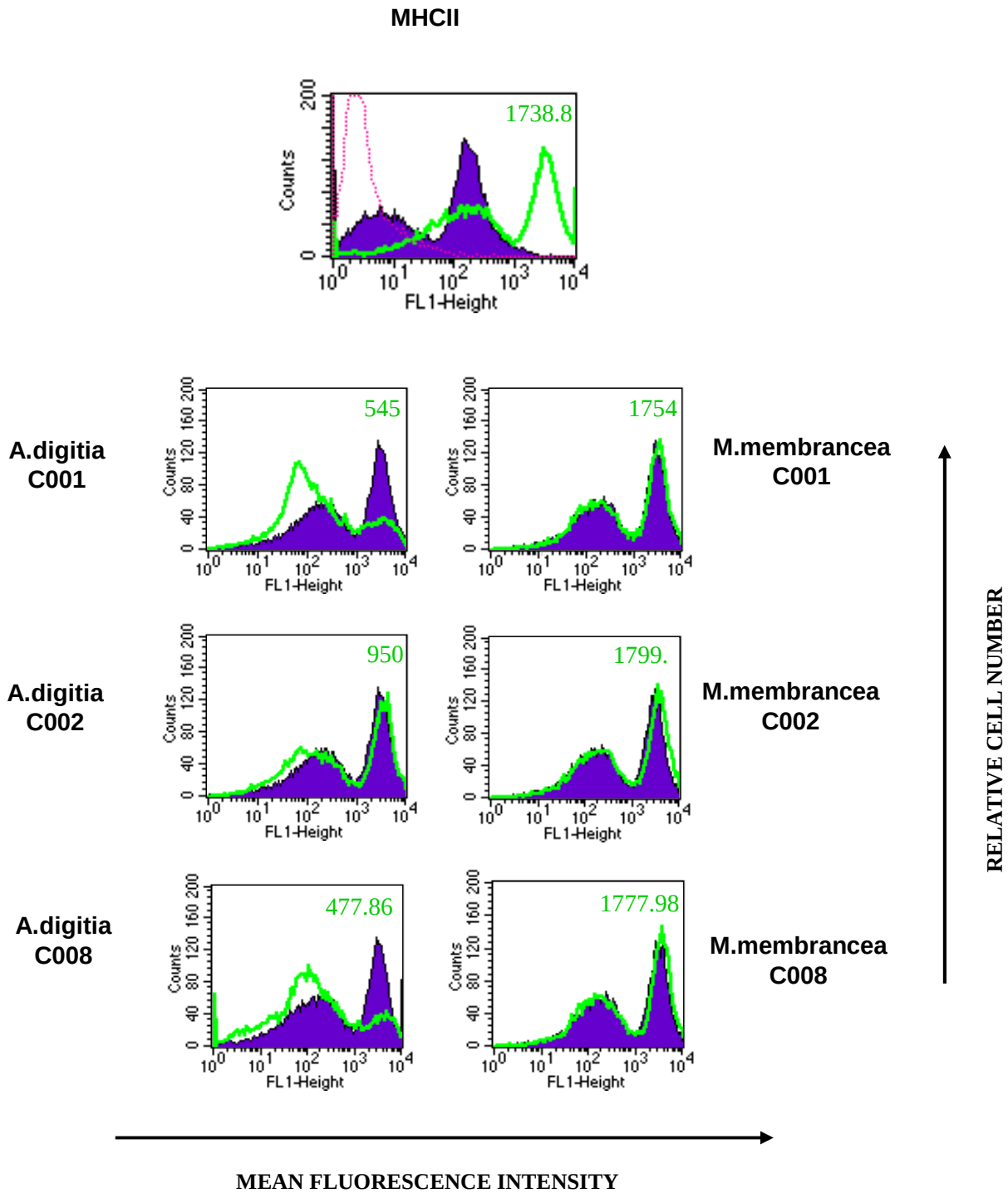


FIGURE 3.23: First-round fractions of marine extracts modulate the expression of MHCII on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with fractions of *A.digitia* (C001, C002, C008) and *M.membrancea* (C001, C002, C008) for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with antibody specific for MHCII or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottow three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments.

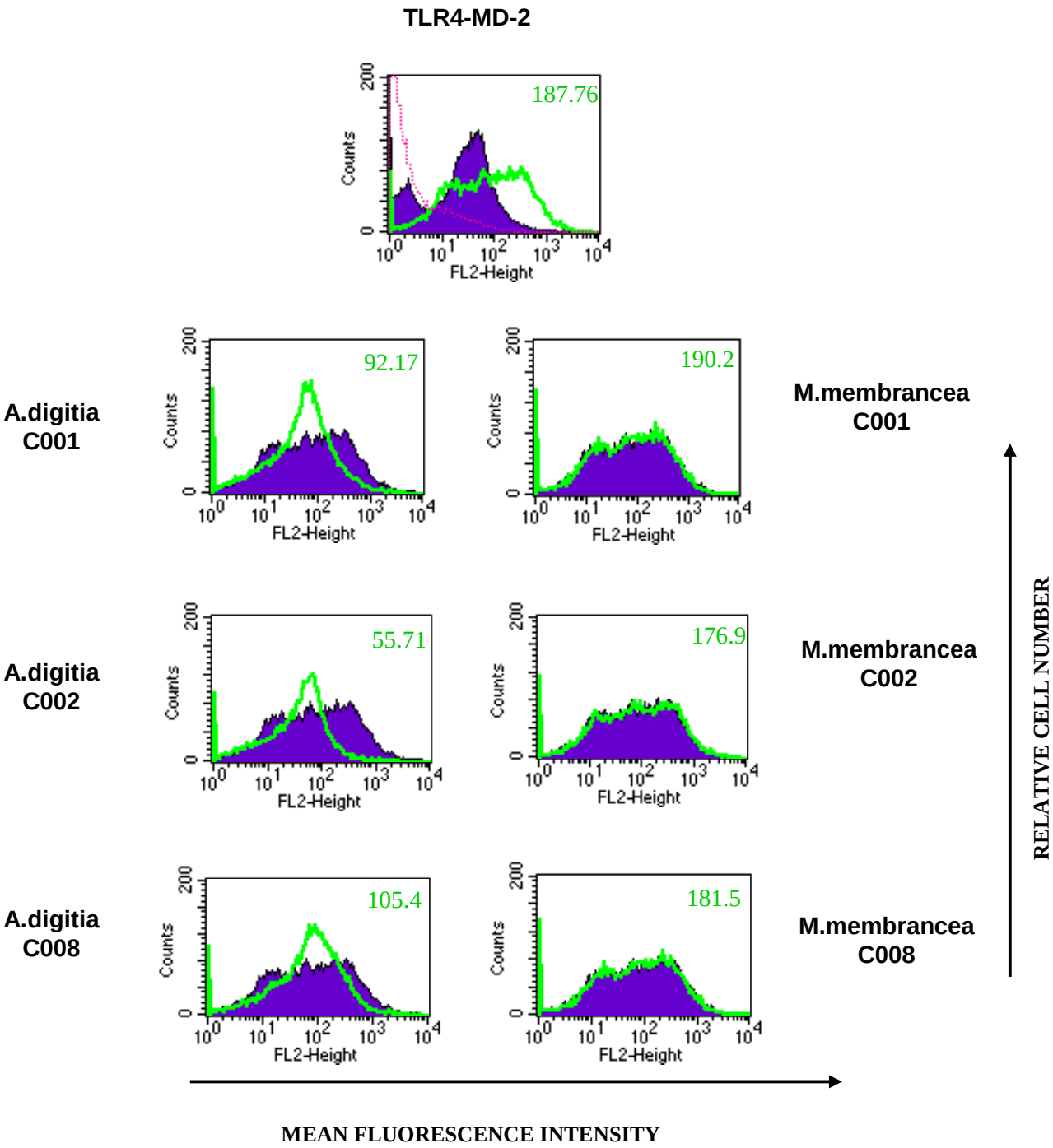


FIGURE 3.23: First-round fractions of marine extracts modulate the expression of TLR4-MD-2 on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with fractions of *A.digitia* (C001, C002, C008) and *M.membrancea* (C001, C002, C008) for 1 hr prior to stimulation with LPS (100ng/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 and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottow three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments.

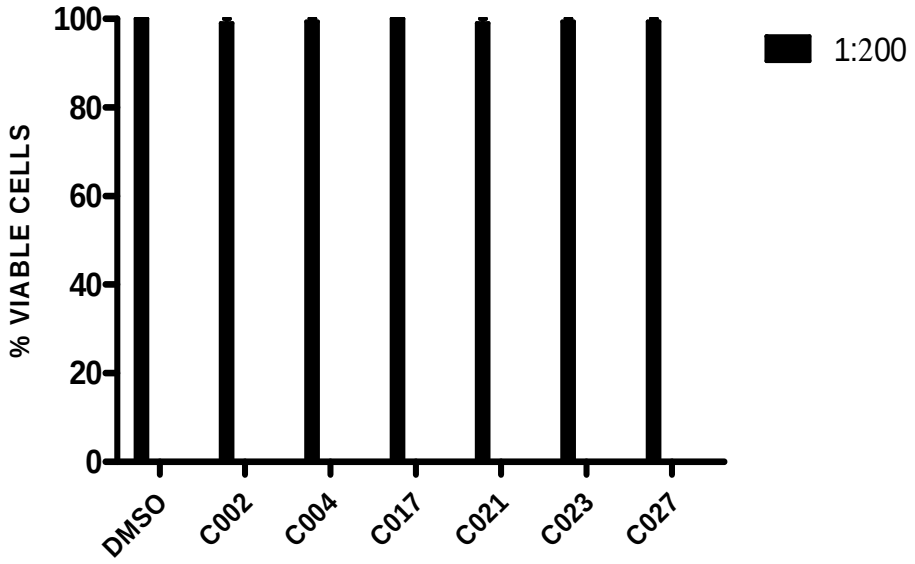


FIGURE 3.25: The concentration of second-round *M.membrancea* fractions used did not significantly affect the viability of DCs.
An assay was carried out in DCs to assess the possible toxicity of the fractions of marine extracts used**:** BMDCs were grown for 7 days and then treated with a 1:200 dilution of either DMSO (vehicle control) or *M.membranacea* (C002, C004, C017, C021, C023, C027) for 24hrs. Following 24 hr treatment cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of untreated cells.

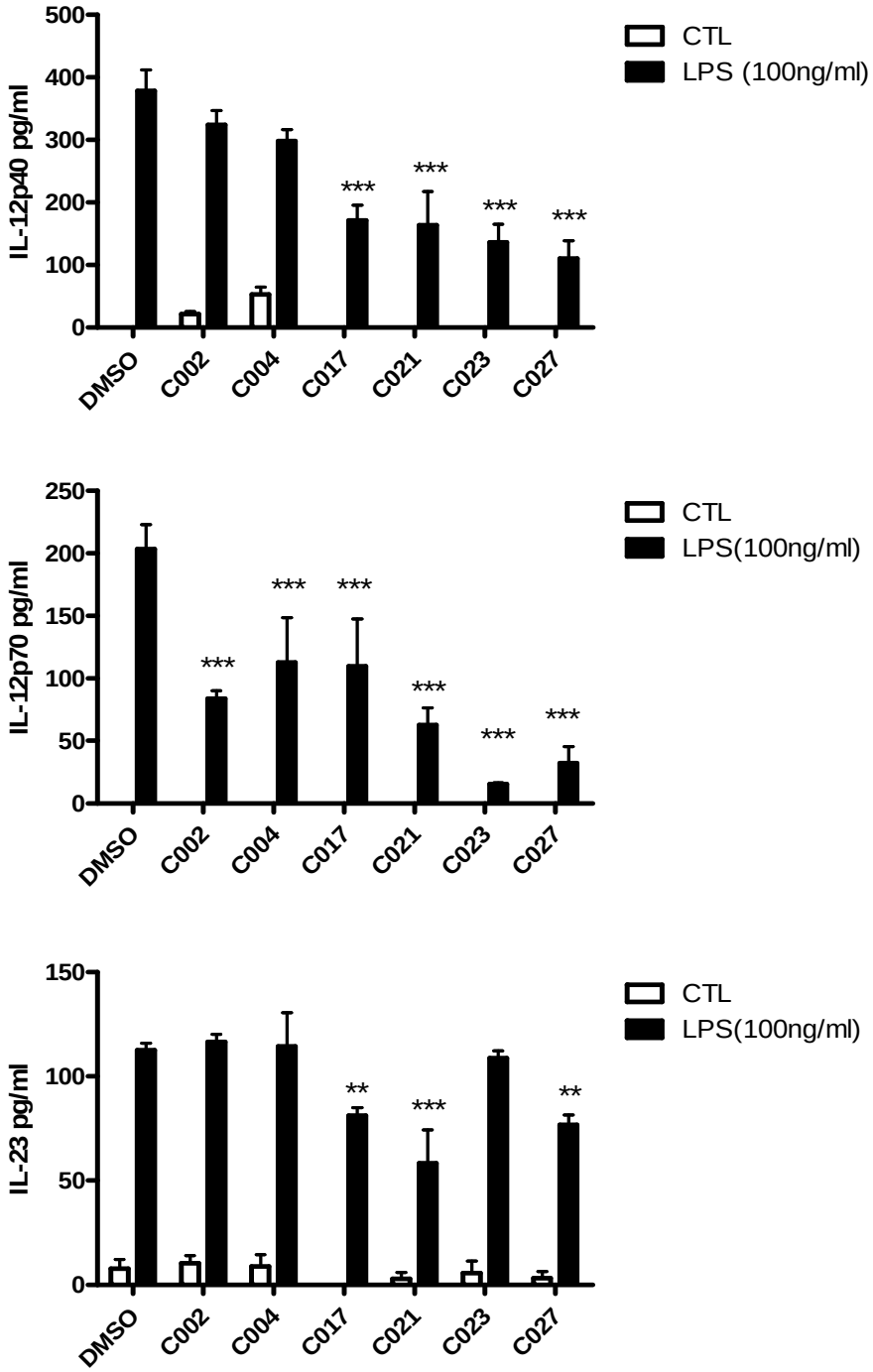


FIGURE 3.26: Second-round *M.membrancea* fractions suppress LPS-induced IL-12p40, IL-12p70 and IL-23 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C002, C004, C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-12p40, IL-12p70 and IL-23 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

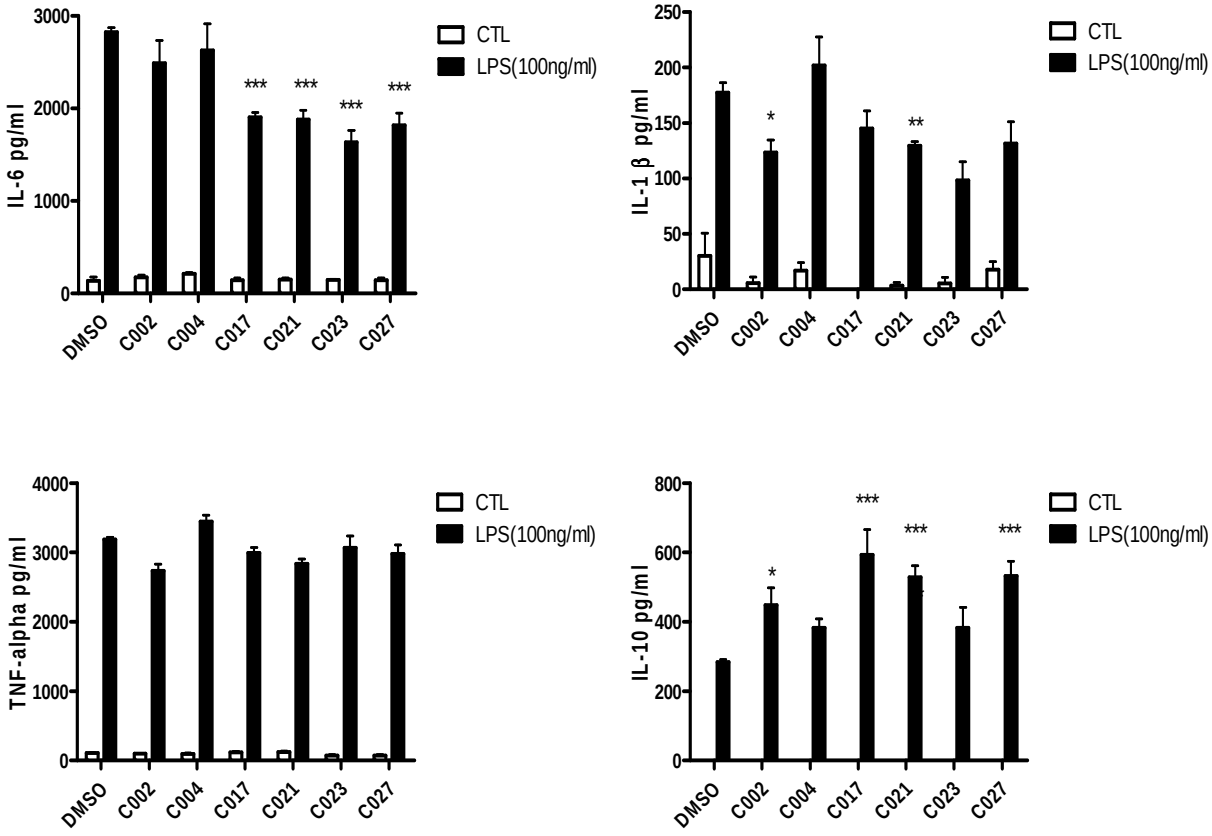


FIGURE 3.27: Second-round *M.membrancea* fractions suppress LPS-induced IL-6, IL-1β production and enhance IL-10 production in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C002, C004, C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS. Supernatants were recovered after 24 hrs and assessed for levels of IL-6, IL-1β, TNF-α and IL-10 using specific immunoassays. Results are ± SEM of quadruplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs marine extract/LPS groups as determined by one-way ANOVA test

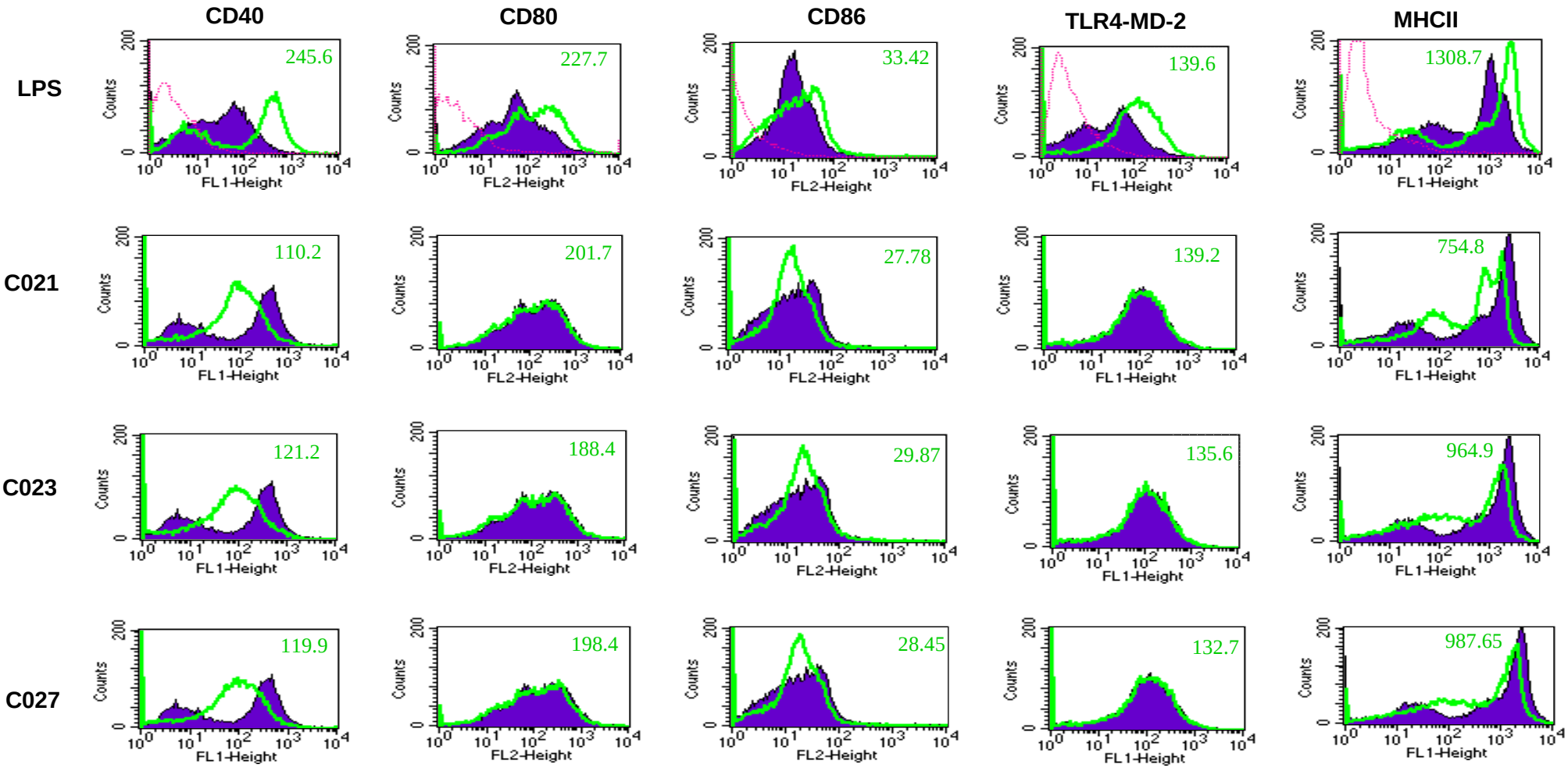


FIGURE 3.28: Second-round *M.membrancea* fractions modulate the expression of CD40, CD86 and MHCII on DCs. BMDC were differentiated in the presence of GMCSF for 7days and then treated with second *M.membrancea* fractions (C021, C023, C027) for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with the specific antibodies or with an isotype matched control. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled purple histogram) vs LPS stimulated DCs (green line) and isotype control (pink dotted line). **Bottom three rows:** DMSO-treated DCs (filled purple histogram) vs. LPS induced marine extract-treated DCs (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel. Profiles are shown for a single experiment and are representative of 3 experiments.

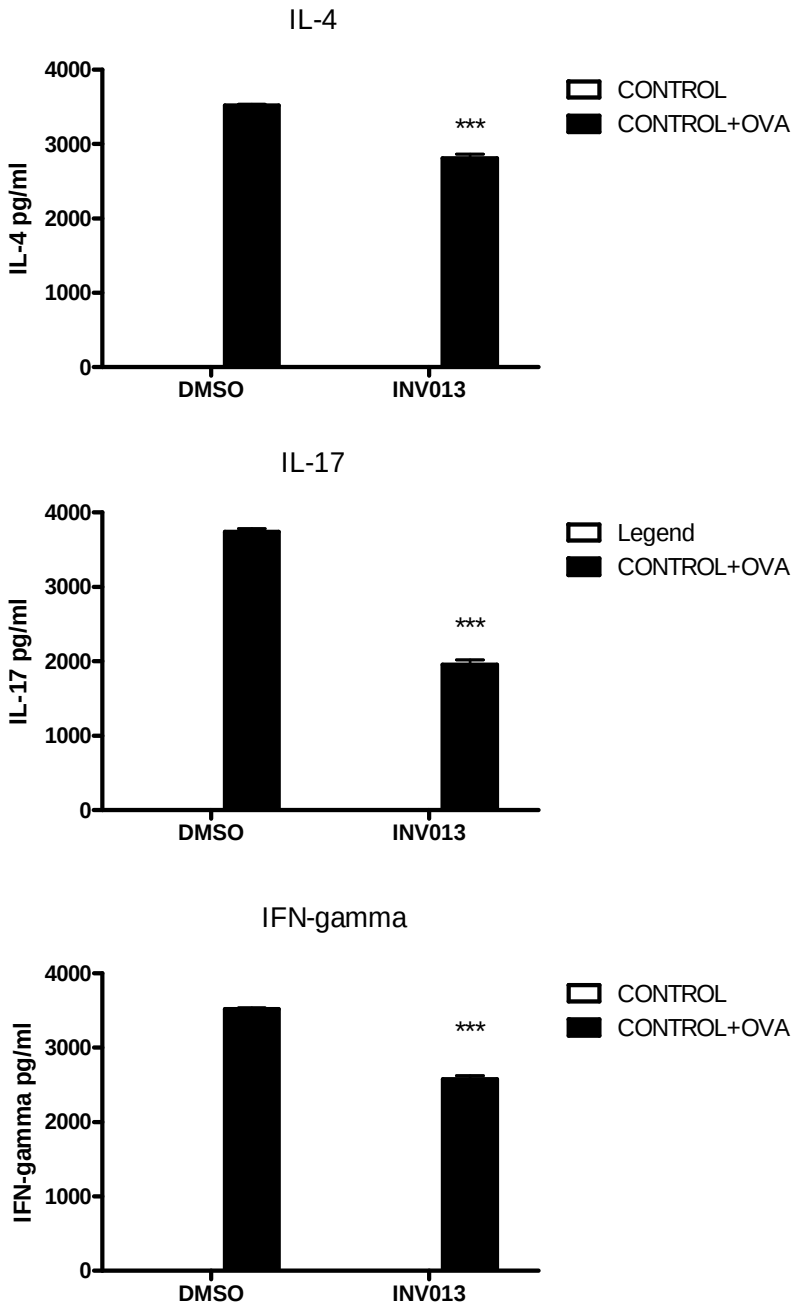


FIGURE 3.29: Crude extract of *M.membrancea*-modulated DCs can inhibit subsequent T cell cytokine production. DCs were treated with DMSO (control), or marine extract *M.*membrancea for 1 hr prior to activation with OVA peptide (5μg/ml). After 24 hrs DCs ($2x10^5$/ml) were added to $CD4^+$ T cells ($2x10^6$/ml) purified from the spleens of OVA D011.10 transgenic mice. Fresh *M.membrancea*-treated pre-activated DCs were added on day 7 plus rIL-2 (10U/ml), and supernatants removed on day 10. Samples were analysed for levels of IL-4, IL-17 and IFNγ using specific immunoassays.

Results are ± 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

3.3 DISCUSSION

Autoimmune diseases are responsible for significant ill-health and morbidity worldwide and several findings have demonstrated that DCs are key players in both the initiation and progression of autoimmunity. Studies have shown DCs are present in high numbers in the synovial fluid of patients with RA and high levels of circulating DCs secreting pro-inflammatory cytokines are associated with MS (Drakesmith, Chain and Beverley 2000). Additionally, numerous studies have demonstrated that DCs play an early and fundamental role in the pathogenesis of IBD (Stagg, et al. 2003, Ikeda, et al. 2001). Consequently, altering DC responses possess tremendous therapeutic potential. In this study we carried out a screening process of different marine extracts. Firstly we examined the effects of crude marine extracts *A.digitia* and *M.membrancea* on LPS-stimulated DCs. Those that showed to have anti-inflammatory activity were then sent to our collaborators in UCD where they were fractionated. These fractions were then screened in order to determine the active ones. From these fractions we decided to fractionate the marine extract *M.membrancea* further as it was able to suppress pro-inflammatory cytokines and chemokines and also downregulate DC surface markers CD80 and CD86 which are essential for T cell activation and differentiation.

Results here demonstrate that the crude marine extracts and their first round fractions of *A.digitia* and *M.membranace* (C001, C002, C008) and second round fractions of *M.membrancea* (C017, C021, C023, C027) strongly inhibit the production of important pro-inflammatory cytokines IL-12p40, IL-12p70 and IL-23. IL-12 is a key cytokine that links innate and adaptive immunity as its targets include DCs, T cells and NK cells. IL-12 has pleiotrophic functions, and one of its fundamental functions

is to differentiate naïve T cells into a Th1 phenotype. In addition it has an essential role in maintaining the balance between Th1 and Th2 responses *in vivo* (Brahmachari and Pahan 2008, Caprioli, Pallone and Monteleone 2011). Several studies have addressed the role of IL-12 in autoimmune diseases such as RA, IBD and MS by using IL-12 deficient mice and overproduction is important in inflammatory states such as septic shock (Adorini 1999b, Adorini, et al. 1997). For instance, the expression of IL-12p40 is enhanced in inflamed colonic tissue from patients with IBD (Ng, et al. 2010). Furthermore, a study by Smith and workers showed decreased production of IL-12p40 in endotoxemic mice following treatment with cannabinoid agonist, tetrahydracannabinal (THC) (Smith, Terminelli and Denhardt 2000). Therefore targeting this cytokine may have therapeutic advantage. Given that the crude marine extracts and their fractions are able to impede the secretion of IL-12 suggests that they may be beneficial in disease.

IL-23 is a heterodimeric cytokine that is also a member of the IL-12 family of cytokines. It is composed of a p40 subunit and a unique p19 subunit. Similar to IL-12, formation of biologically active IL-23 requires synthesis of both p19 and p40 subunits within the same cell. IL-23 binds to the IL-23R on $CD4^+$ T cells promoting their development into Th17 cells, characterized by the secretion of IL-17 and IL-6 (Langrish, et al. 2004, Bettelli, Oukka and Kuchroo 2007). IL-23 appears to be an essential player in numerous chronic inflammatory diseases such as IBD and collagen induced arthritis (Yago, et al. 2007). IL-23 is produced by DCs and promotes the expansion and survival of already differentiated Th17 cells but it is not involved in Th17 initial differentiation. Naïve T cells cultured in the presence of TGF-β and IL-6 are polarized to Th17 cells and also allows for the up-regulation of IL-12R (Kikly, et

al. 2007). Studies have shown that IL-1β and TNF-α have a synergistic effect on IL-23 mediated IL-17 production in a model of EAE (Brahmachari and Pahan 2008). Our study demonstrates a robust reduction in the secretion of IL-23 following treatment with crude extracts and both rounds of fractionated marine extracts. Numerous studies have demonstrated that inhibiting the production of IL-23 can have therapeutic advantage. For example, a study by Xiao and colleagues showed that the vitamin A metabolite retinoic acid (RA) resolved EAE by decreasing the production of IL-23 and enforcing the generation of T regulatory cells therefore inhibiting the development of Th17 cells (Xiao, et al. 2008). Our findings highlight for the first time the effects of marine extracts on IL-23 production. Furthermore a significant downregulation was also observed in the production of IL-6 and IL-1β in crude and fractionated treated DCs post activation with LPS. This may point to a consequential downstream effect of marine extract-treated DCs on Th17 cell development or maintenance. In addition, it suggests that these marine compounds may have therapeutic potential in the treatment of inflammatory diseases including RA and IBD. Other compounds isolated from marine species have been primarily demonstrated to reduce the secretion of IL-1β and IL-6. For instance, extracts isolated from *Staphylococcus arlettae* and *Planococcus maritimus* showed a robust reduction in the secretion of IL-1β in PMBCs (Krishnaveni and Jayachandran 2009). No alteration was observed in TNF-α production in both crude and fractionated marine treated DCs which indicates that the extracts are acting specifically.

In contrast to the above mentioned cytokines, IL-10 is considered an anti-inflammatory or regulatory cytokine. It down-regulates the production of pro-inflammatory cytokines including IL-1, IL-6, IL-12 and TNF-α from APCs. IL-10 can

also act directly on CD4 T cells, inhibiting proliferation and production of IL-2, IFN-γ and IL-4 (Asadullah, et al. 1998, Asadullah, Sterry and Volk 2003) . Indeed, ablation of IL-10 leads to the overproduction of pro-inflammatory cytokines and the development of chronic diseases; this has been studied extensively in IL-10 deficient mouse models (Conti, et al. 2003). Primarily, an increase in IL-10 can inhibit Th1 cell differentiation or direct cells to a regulatory response (Asadullah, Sterry and Volk 2003). The crude marine extracts and their fractions robustly enhance the expression of IL-10. Indeed a crude extract isolated from the carribean sponge *Agrococcus jenensis* has already shown to significantly upregulate IL-10 production in macrophages (Tabares, et al. 2011). Interestingly, IL-10 can suppress IL-12 production and Loscher et al has shown that the decrease in IL-12 induced by the n-6 polyunsaturated fatty acid, CLA is dependant on IL-10 (Loscher, et al. 2005). Further to its effects on cytokine production, IL-10 can also modulate surface marker expression by inhibiting the complete maturation of DC by downregulating the expression of CD80 and CD86 (de Jong, Smits and Kapsenberg 2005). In addition, IL-10 has the ability to prevent the peptide-MHCII complexes translocating to the DC plasma membrane (Banchereau, et al. 2000) all of which lead to impaired T helper cell responses. In contrast, IL-12 causes the upregulation of MHCII, CD80 and CD86 expression on APC (Bettelli and Kuchroo 2005). The changes seen in the production of IL-10 and IL-12 in this study with the marine fractions could also explain the recorded alterations in cell surface marker expression.

The engagement of CD80, CD86 on APCs with CD28 on T cells initiates T cell proliferation and differentiation and also cytokine production. CD80 and CD86 play important pathogenic roles in T-cell responses. For instance Odobasic and colleagues

demonstrated that monoclonal antibodies for CD80 and CD86 significantly decreased the production of IL-17 in the synvioum of mice therefore suppressing the development of arthritis (Odobasic, et al. 2008). In addition the interactions between CD40 and CD40L are bi-directional as it not only activates DCs but also enhances T cell activation. Ligation of CD40 with CD40L also triggers the production of extremely high levels of IL-12 (Danese, Sans and Fiocchi 2004, Kato, Yamane and Nariuchi 1997). Predictably, over expression of these surface markers have been associated in the pathology of tissues from patients with IBD, RA and MS (Kobata, et al. 2000, Toubi and Shoenfeld 2004, Polese, et al. 2003). Altering these markers on the surface would have detrimental consequences for T cells responses, ultimately indicating that co-stimulatory molecules are potential targets for treating inflammatory diseases.

In the present study, it was demonstrated that treating DCs with crude marine extract *M.membranacea* significantly downregulated the surface marker expression of CD40, CD80 and CD86 in LPS-induced DCs. It is possible that the strong suppression of CD40 may contribute to the notable reduction in IL-12 secretion in treated marine extracts post LPS activation. A study by Walker and workers demonstrated that the inhibiton of IL-12 by neuroblastoma (NB)-derived gangliosides in DCs is mediated by deficient CD40 expression and signalling. Furthermore the decrease in expression of CD86 may attribute to the enhancement in IL-10 production in LPS induced treated cells (Walker, Redlinger and Barksdale 2005). A significant reduction in the expression of CD40, CD80 and CD86 was still observed in *M.membrancea* treated DCs from both rounds of fractionation, indicating that these fractions are active and have tremendous anti-inflammatory potential. A study by Platten and workers

demonstrated that catabolites of the amino acid tryptophan suppressed the expression of CD40, CD80 and CD86 reversing paralysis in mice with EAE (Platten, et al. 2005). The treatment with crude marine extract, *A.digitia*, showed a significant reduction in the expression of TLR4-MD-2 following LPS activation. Since TLR4 is required for the initiation of intracellular signalling pathways, ultimately leading to the activation of transcription factors, NFκB and IRF3, and subsequent secretion of pro-inflammatory cytokines and IFN's, this suggests that *A.ditgitia* extracts may affect TLR4 signalling. The expression of MHCII was also distinctly suppressed by crude and fractionated *A.digitia* extracts following LPS stimulation. DCs are capable of activating naïve T cells specifically because they express MHCII for antigen presentation along with the necessary co-stimulatory surface molecules for T cell activation. Since marine extracts *A.digitia* decrease MHCII suggests this treatment would have downstream affects on T cell response.

The recruitment of leukocytes in response to chemokines is paramount in clearing infection, however elevated levels of chemokines are also implicated in inflammatory disease (Godessart and Kunkel 2001). The most active fractions for *A.digitia*, C002 and C008, and for *M.membranacea*, C002 and C008, were investigated further in this study by assessing their modulatory effects on numerous cytokines including MCP-1, MIP-1α and MIP-2. In addition the chemotaxis of DCs towards the chemokine CCL19 was also assessed.

Effects on chemokine secretion was specific for each fraction but the most potent effects were with, *A.digitia* fraction C008 and *M.membrancea* fraction C002 which significantly suppressed production of MIP-1α and MCP post stimulation with LPS.

MIP-1α acts as a robust chemoattractant to a variety of leukocytes and elevated levels of this chemokine have been shown in inflammatory disorders including RA and IBD (Pender, et al. 2005). Furthermore increased levels of MIP-1α have been demonstrated in biopsy samples of asthmatic patients (Alam, et al. 1996). Correspondingly, MCP-1 is implicated in MS and RA (González-Escribano, et al. 2003). Indeed triptolide, a compound isolated from the vine plant Tripterygium wilfordii Hook F (TWHF) was shown to significantly inhibit MCP-1 and MIP-1α in rats induced with adjuvant arthritis (AA) (Wang, et al. 2006). A decrease in the chemotaxis of DC to CCL19 was also demonstrated with these *M.membrancea* fractions and it would also be reasonable to assume that the decrease in DC chemotaxis would have serious consequences for T cell responses considering the DC must migrate to the lymph nodes in order to activate naïve T cells and initiate adaptive immunity. A variety of compounds have been generated that antagonize chemokine receptor function, some of which are currently undergoing clinical trials (Zidek, Anzenbacher and Kmonickova 2009a). A study by Yang and workers reported that a compound purified from the marine sponge *Ircinia sp* downregulated the expression of the receptor for CCL19 (Yang, et al. 2003).

Numerous molecules have been described that have demonstrated cytokine, chemokine and surface marker alteration similar to the results obtained in our study. For instance, Kong and collagues demonstrated that docosahexaenoic acid (DHA) a long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) from fish oils suppressed the production of pro-inflammatory cytokines IL-12p70 and IL-23 and increased the production of the regulatory cytokine IL-10. In addition DHA downregulated the expression of CD40, CD80, CD86 and MHCII in bone marrow derived DC following

stimulation with LPS (Kong, et al. 2010). Furthermore findings by Panther and workers showed that treatment of DCs with adenosine simultaneously showed a downregulation in the production of IL-12 and an enhancement in IL-10 secretion following activation with LPS. In addition a decrease in the pro-inflammatory chemokine, IP-10 was observed (Panther, et al. 2003). However the authors reported an increase in the surface marker expression of CD40, CD80, CD86 and MHCII in immature and mature DCs.

Since the marine extracts were able to modulate LPS induced DC cytokine production and surface marker expression which is vital for DC maturation, and T helper cell activation, we decided to see if the marine extract *M.membrancea,* had an overall effect on subsequent T helper cell responses. Results demonstrated that *M.membrancea*-treated DCs had consequences for the resulting adaptive immune response. This was demonstrated when *M.membrancea* -modified DCs suppressed the production of IFN-γ, IL-17 and IL-4 by $CD4^+$ T cells in a DC-T cell co-culture model suggesting that *M.membrancea*-altered DCs can destabilise Th1, Th2 and Th17 responses by reducing IFN-γ, IL-4 and IL-17 production respectively. Levels of IFN-γ were significantly suppressed. IFN-γ is a pro-inflammatory cytokine and is the signature cytokine of Th1 dominated autoimmune processes. The involvement of IFN-γ in the development of autoimmune diseases has been well documented over the years. One of the vital roles of IFN-γ is to activate numerous cells in the immune system including macrophages and DCs and also induce the production of IL-12 (Watford, et al. 2003). However the role of IFN-γ in autoimmunity is continuously questioned as the ablation of IFN-γ either enhances or inhibits collagen induced arthritis in mice and also augments disease severity in EAE mice models. In contrast,

certain molecules have demonstrated therapeutic potential by altering IFN-γ production. For instance, Peroxisome proliferator-activated receptor-γ (PPARγ) ligands inhibit IFN-γ production and this was demonstrated in numerous studies to ameliorate RA (Giaginis, Giagini and Theocharis 2009). IL-17 is the cytokine that is responsible for the progression of EAE where as IFN-γ had a protective effect. The presence of *M.membrancea* robustly decreased the secretion of IL-17. IL-17 is the principal cytokine produced by Th17 cells and plays a pathogenic role in numerous autoimmune diseases including MS and RA which was previously accredited to the dysregulated Th1 response (Pernis 2009). Therefore this makes Th17 cells and their producing cytokine IL-17 possible therapeutic targets. For example, treatment with the synthetic glucocorticod, methylprednisolone (MP) reduced the production of IL-17 in cells isolated from the CNS of rats with EAE thus ameliorating the disease (Miljkovic, et al. 2009).

In conclusion our findings highlight for the first time distinct effects of extracts and fractions from the marine species *A.digita* and *M.membrancea* on cytokine, chemokine and cell surface marker expression in dendritic cells. These effects point to their potential as anti-inflammatory compounds. Furthermore, their ability to target cytokines such as IL-12, IL-23 and IL-6, known to be involved in inflammatory diseases further supports this. One of the main objectives of this study was to determine which fraction showed to have the most potent anti-inflammatory properties and fractions that demonstrated the greatest bioactivity were then sent to UCD to begin the final purification step in order to determine the identity of the active compound that possess these anti-inflammatory effects.

CHAPTER 4

THE EFFECTS OF SECOND FRACTIONS OF *M.MEMBRANCEA* ON MACROPHAGE FUNCTION

4.1 INTRODUCTION

Macrophage, (MØ) execute many roles within innate immunity and their activation is fundamental for the initiation of host defence and in directing adaptive immune responses. MØ are prodigious phagocytic cells that have the ability to recognise, engulf and destroy invading pathogens (Geissmann, et al. 2010). They are actively involved in the clearance of infection and without this crucial function the host would not survive. While the innate immune response generated against a pathogen is essential in controlling infection, a persistent inflammatory reaction can actually do more harm than the pathogen itself. The role of MØ in various inflammatory disorders has been well documented including; IBD, RA, schistosmiasis and atherosclerosis (Zhang and Mosser 2008). In order to develop new therapeutics for inflammatory diseases it is important to determine their effects on the cells that initiate the immune responses. Since we demonstrated the second fractions of *M.membrancea* to have potent anti-inflammatory effects on activated DCs we therefore decided to examine their effects on MØ, to determine if they affected them in a similar way. Furthermore, given their role in pathogen clearance we wanted to see if this was affected by the fractions. We examined the effects of the fractions on MØ following stimulation with a bacterial ligand via TLR4 and a viral ligand via TLR7. TLR7 is an anti-viral receptor that carry's out its function in intracellular compartments in the cell leading to the activation of the transcription factors NFκB and IRF7. It recognises ssRNA from viruses such as HIV and influenza (Hemmi, et al. 2002). Contrastingly, TLR4 is found on the surface of cells and recognises components found on the outer membrane of gram negative bacteria such as *Escherichia coli* and *Salmonella Minnesota* (Yan 2006).

There are a number of parameters of MØ activation which the marine extracts may affect. Activation signifies a change in the secretary profile and morphology of MØ. Continued stimulation of MØ in the presence of infection leads to increased production of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 through the activation of NFκB (Geissmann, et al. 2010). These cytokines affect organs such as the brain and liver which are far removed from the site of infection. IL-1 acts on the brain and is involved in the regulation of sleep, appetite and fever (Apte, et al. 2006), while IL-6 plays an important role in the acute phase response (Maggio, et al. 2005). Over production of IL-6 and TNF-α has been extensively documented in RA, Crohn's disease (CD), MS and in many other autoimmune diseases and consequently inhibition or blockade of these cytokines has proved efficacious in treating many of these disorders (Palladino, et al. 2003, Fonseca, et al. 2009). Differentiation of T cells into various subsets is partially determined by the cytokines that MØ produce. MØ secrete the pro-inflammatory cytokines IL-12 and IL-23 which are involved in Th1 and Th17 polarization respectively (Jager, et al. 2009, Bettelli and Kuchroo 2005). Therefore, the effect of the *M.membrancea* on cytokine release from MØ following stimulation with TLR4 and TLR7 ligands was assessed to evaluate any beneficial effects.

In addition, activation of MØ initiates the secretion of various chemokines which trigger the migration of monocytes into injured tissue, thereby initiating a central step during infection. These chemokines include MIP-1α/CCLS, MIP-2/CXCL2 and MCP (Zidek, Anzenbacher and Kmonickova 2009b). Chemokines are essential mediators of inflammation and are important for controlling infection and ablation of these chemokines causes reduced ability to clear infections such as *Listeria monocytogenes*

(Heesen, et al. 2006). 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). Numerous therapies are now being developed to combat these chemokine-mediated diseases (Viola and Luster 2008). As a result we have examined the effects of the second fractions of *M.membrancea* on MØ chemokine production following stimulation with LPS and loxoribine to evaluate any advantageous changes. Furthermore, the response of cells to chemokines and subsequent migration to sites of inflammation remains crucial during an immune response, therefore we have also assessed whether the fractions affect the chemotaxis of MØ in response to TLR4 and TLR7 stimuli.

Phagocytosis is a hallmark function of MØ in the resolution of inflammation (Aderem 2003). In addition to eliminating microbial pathogens, phagocytosis triggers the secretion of pro-inflammatory cytokines and chemokines and activates antigen processing and presentation by up-regulating key surface markers including MHCII, CD40, CD80 and CD86 leading to subsequent adaptive immune responses (Kang, et al. 2008). Phagocytosis is vital in clearing infection and disruption to this process can lead to systemic inflammatory diseases such as malaria (Urban and Roberts 2002). As key phagocytes, the rate of phagocytosis by MØ was investigated following exposure to *M.membrancea* in order to determine if the extracts were able to alter susceptibility to infection following activation with a bacterial pathogen mediated through TLR4 or a viral infection mediated through TLR7.

4.2 RESULTS

4.2.1 SECOND-ROUND FRACTIONS OF MARINE EXRACT *Membranipora membranacea* HAVE NO SIGNIFICANT EFFECT ON CELL VIABILITY IN J774 MACROPHAGES.

Since *M.membrancea* fractions (C017, C021, C023 and C027) demonstrated significant anti-inflammatory effects we decided to determine their effects on macrophages function following stimulation with two different TLR ligands. Firstly we examined the toxicity of the fractions by using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to manufacturer's instructions. The 1:200 dilutions of marine fractions chosen for use in future experiments did not have any significant cytotoxic on J774 macrophage *in vitro* **[Figure 4.1]**.

4.2.2 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE CYTOKINE PRODUCTION BY J774 MACROPHAGES FOLLOWING STIMULATION WITH TLR4 LIGAND ONLY IN VITRO

J774 macrophages were plated at a concentration of 1 x 10^6 cell/ml and pretreated with DMSO (vehicle control) or *M.membrancea* marine fractions (C017, C021, C023 or C027) at 1:200 dilution for 1 hr prior to activation with 100 ng/ml LPS (*E.Coli* serotype R515) or loxoribine (1mM). After 24 hrs, supernatants were removed and assessed for levels of IL-12p40, IL-23p19 **[Figure 4.2]**, IL-6 , IL-1β **[Figure 4.3]**, IL-10 and TNF-α **[Figure 4.4]** and using specific immunoassays.

The production of the pro-inflammatory cytokines IL-12p40 and to a lesser extent IL-23 were both decreased in the supernatant of marine fractionated-treated macrophages following LPS stimulation compared to the DMSO control group. All marine fractions were able to significantly decrease the production of IL-12p40 ($p<0.001$) **[Figure 4.2]**. IL-23, IL-6 and IL-1β production was also significantly decreased by all fractions ($p<0.05$-$p<0.001$) **[Figure 4.2-4.4]**. Similar to what we have seen in DCs, the marine fractions did not alter the production of TNF-α demonstrating that the marine extract is acting specifically **[Figure 4.4]**. The production of IL-10 was increased in marine-treated macrophage post activation with LPS with C027 ($p<0.01$) having the most profound effect on IL-10 production and fractions C021 and C023 also increasing IL-10 but to a lesser extent ($p<0.05$). The marine fraction, C017, had no effect on IL-10 production following LPS stimulation **[Figure 4.4]**.

In contrast to LPS stimulation, the *M.membrancea* second-round fractions had no effect on cytokine production when macrophages were activated with the TLR7 agonist, loxoribine **[Figure 4.2-4.4].**

4.2.3 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE CHEMOKINE PRODUCTION BY J774 MACROPHAGES FOLLOWING STIMULATION WITH TLR4 LIGAND ONLY IN VITRO

J774 macrophages were plated at a concentration of 1 x 10^6 cell/ml and pretreated with DMSO (vehicle control) or *M.membrancea* marine fractions (C017, C021, C023 or C027) at 1:200 dilution for 1 hr prior to activation with 100 ng/ml LPS (*E.Coli* serotype R515) or loxoribine (1mM). After 24 hrs, supernatants were removed and

assessed for levels of MIP-1α, MIP-2α **[Figure 4.5]** and MCP-1 **[Figure 4.6]** using specific immunoassays.

The production of MIP-2α was not significantly affected by marine fractionated-treated macrophage regardless of whether cells were activated with LPS or loxoribine **[Figure 4.5]**. *M.membrancea* fractions C021, C023 and C027 were able to downregulate the production of MIP-1α and MCP-1 following activation with LPS **[Figure 4.5-4.6].** In contrast, no alteration in the production of any of the chemokines was demonstrated following loxoribine stimulation **[Figure 4.5-4.6]**

4.2.4 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* INHIBIT MØ CHEMOTAXIS

GM-CSF and IL-2 are frequently used in macrophage chemotaxis studies and have also been employed here (Stagg, et al. 2004, Perri, Annabi and Galipeau 2007). IL-2 is a ligand for CXCR2, which is largely expressed on macrophage and is involved in the recruitment of macrophage to sites of inflammation (Boisvert, et al. 1998). GM-CSF (granulocyte-macrophage colony stimulating factor) is a cytokine that initiates the activation of monocytes and macrophages (Hasskamp, Elias and Zapas 2006). The migration of J774 macrophages towards the chemokines IL-2 and GM-CSF was significantly upregulated when cells were activated with LPS or loxoribine. The LPS-induced chemotaxis was significantly inhibited by culturing cells with marine fractions C021, and C027 ($p<0.001$) **[Figure 4.7].** In contrast the marine extracts had no effect on loxoribine-induced chemotaxis **[Figure 4.7].**

4.2.5 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE THE RATE OF PHAGOCYTOSIS IN J774 MACROPAHGE *IN VITRO*

J774 macrophages were plated at 2.5 x 10^5 cell/well and left to rest overnight. To assess the rate of phagocytosis in stimulated cells, cells were treated with *M.membrancea* fractions (C021, C023 or C027) for 1 hr before stimulation with LPS (100ng/ml) or loxoribine (1mM) for 24 hrs before addition of fluorescently labelled latex beads (Sigma®). On the following day, 2.5 x 10^6 fluorescently labelled latex beads were added to the required wells for 2, 4, 6, 12, 20 and 24 hr. 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™ to measure the fluorescence of cells from bead uptake. The rate of phagocytosis increased over time in LPS and loxoribine stimulated macrophages where LPS or loxoribine-stimulated cells are shown by the filled purple histogram and marine treated-LPS or loxoribine-activated cells with the addition of latex beads are overlaid with a green line (top row) **[Figure 4.8-4.9]**.

Fractions, C021 and C023 and to a lesser extent C027 decreased phagocytosis at 2 h, 4 h, 6 h and 12 hrs after LPS stimulation (bottom three rows) **[Figure 4.8].** In contrast phagocytosis remained relatively unchanged in *M.membrancea*-treated macrophages post loxoribine activation with compared to control **[Figure 4.9]**. Confirmation that latex beads were phagocytosed following stimulation with LPS can be observed in figure 4.9a.

4.2.6 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE CELL SURFACE MARKER EXPRESSION ON J774 MACROPHAGES IN VITRO

J774 macrophages were plated at a concentration of 1 x 10^6 cell/ml and pretreated with a 1:200 dilution of *M.membrancea* fractions (C021, C023 or C027) 1 hr before activating with 100 ng/ml LPS (*E.Coli* serotype R515) or loxoribine (1mM) for 24 hrs. Cells were subsequently washed and stained with fluorochrome-labelled monoclonal antibodies for numerous cell surface markers (i.e., CD40, CD80, CD86, TLR4-MD-2 and MHCII) [See **Table 2.7**] in preparation for cytometric analysis by flow cytometry **[Figure 4.10-4.11]**.

As expected, LPS and loxoribine enhanced the surface marker expression of CD40, CD80, CD86, TLR4-MD-2 and MHCII as seen in the top row of **Figure 4.10-4.11** where control DMSO cells are shown by the filled purple histogram and LPS or loxoribine-treated cells are overlaid with a green line.

Marine fractions decreased the levels of key surface markers in J774 macrophages activated with LPS however no reduction in surface marker expression was demonstrated post activation with loxoribine **[Figure 4.10-4.11]**. *M.membrancea* fractions C023 and C027 had similar effects on most of the cell surface markers analysed; following LPS stimulation these fractions reduced the expression of CD40, CD80, MHCII and CD86 expression. Similarly fraction C021 was able to downregulate the surface expression of CD40, but to a greater extent than the other fractions. Furthermore, expression of TLR4-MD-2 was also markedly suppressed in C021-treated macrophages in response to LPS however levels of TLR4-MD-2

remained relatively unchanged in both C023- and C027-treated macrophage **[Figure 4.10]**.

4.2.7 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* INHIBITS PHOSPHORYLATION OF NFκB-P65 BY TLR4 LIGAND ONLY

J774 macrophages were plated at a concentration of 1 x 10^6 cell/ml and pretreated with DMSO (vehicle control) or *M.membrancea* fraction C027 at 1:200 dilution. The cells were then stimulated with LPS or loxoribine for a number of time-points (0, 5, 15, 30 and 45 mins) and lysates were generated as described in [section 2.6]. Lysates were then run on SDS gels, transferred onto nitrocellulose membranes and immunoblotted for phospho-NFκB-p65 and phosphor-IRF3 [Figure 4.12-4.14].

As shown in figure 4.12 NFκB-p65 was phosphorylated rapidly with maximum phosphorylation occurring at 15 mins in LPS-stimulated cells. In cells pretreated with the *M.membrancea* fraction C027 this phosphorylation of p65 was significantly reduced at 15, 30 and 45 mins following activation with LPS compared to the control which can be seen in all three phospho-NFκB-p65 blots [Figure 4.12]. Densiometric analysis of the blots revealed that the changes at 5, 15 and 30 mins were statistically significant ($p<0.001$) [Figure 4.12]. Contrastingly, there was no alteration in the phosphorylation of NFκB-p65 in *M.membrancea* C027-treated cells post stimulation with loxoribine at any time point [Figure 4.13]. The phosphorylation of IRF3 was not modulated in cells treated with *M.membrancea* fraction C027 compared to control cells [Figure 4.14]. Again densiometric analysis demonstrated there was no significant change in phospho-IRF3 [Figure 4.14].

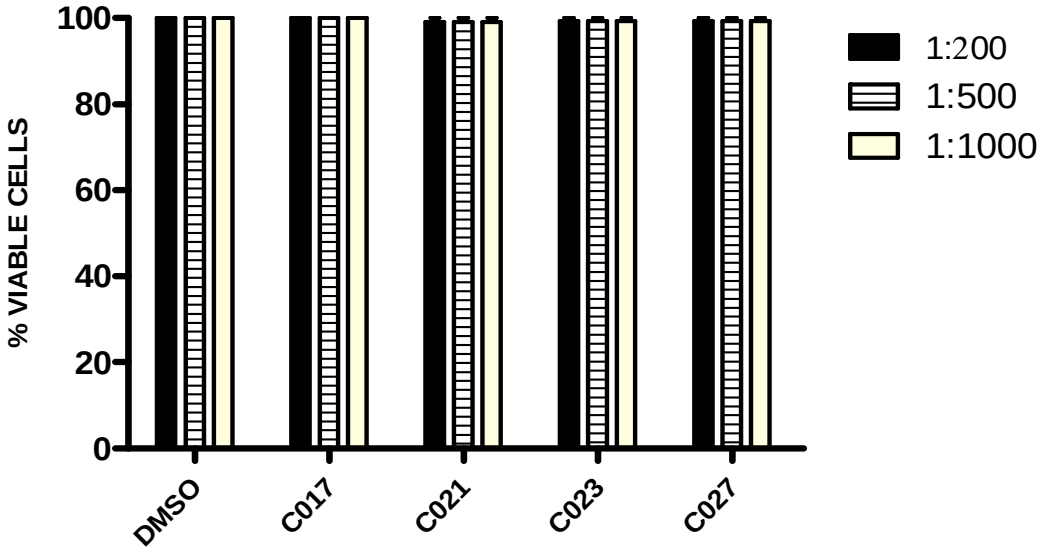


FIGURE 4.1: The concentration of second-round *M.membrancea* fractions used did not significantly affect the viability of J774 macrophages

Macrophage were pretreated with the specified dilutions (1:200, 1:500 and 1:1000) of either DMSO (vehicle control) or *M.membrancea* fractions (C002, C004, C017, C021, C023 and C027) for 1 hr prior to stimulation with LPS (100ng/ml) for 24 hrs. Following 24hr activation cellular viability was assessed using an MTS assay (CellTiter 96® AQueous One Solution (Promega)). Results are expressed as a percentage of untreated cells

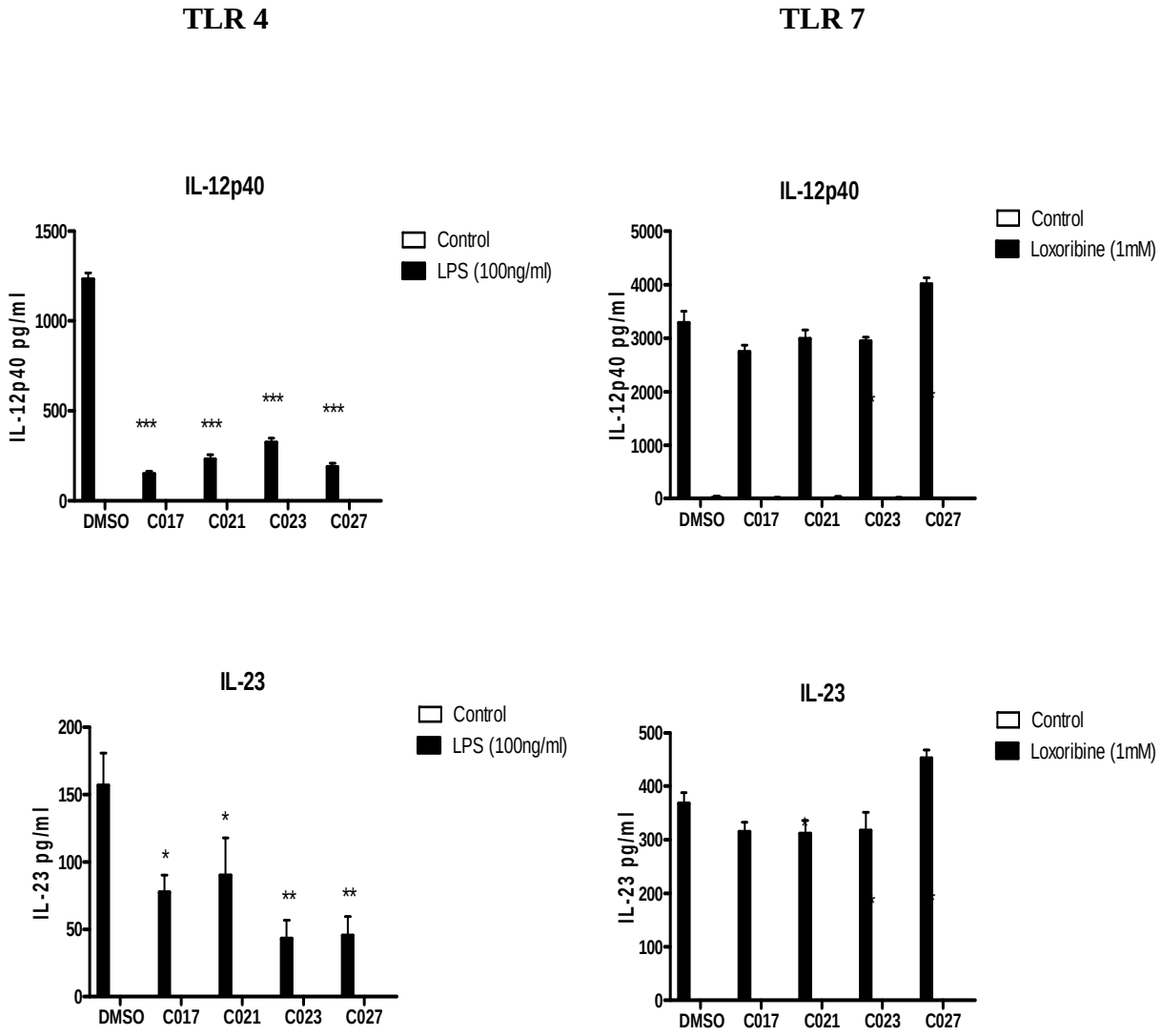


FIGURE 4.2: Second-round *M.membrancea* fractions suppress LPS-induced IL-12p40 and IL-23 production in J774 macrophages. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS or loxoribine (1mM). Supernatants were recovered after 24 hs and assessed for levels of IL-12p40 and IL-23 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***$p<0.001$, **$p<0.01$, *$p<0.05$ comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

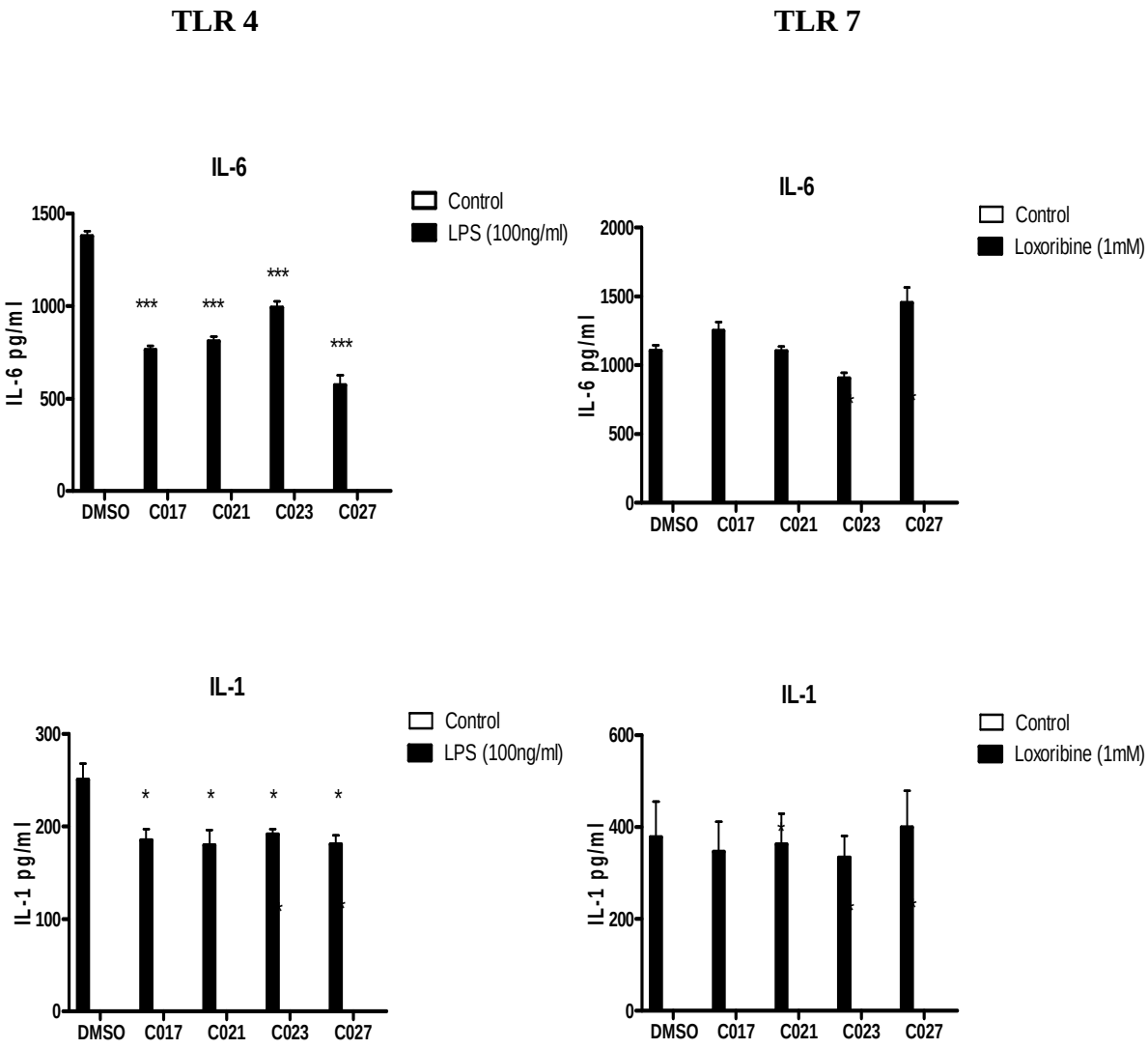


FIGURE 4.3: Second-round *M.membrancea* fractions suppress LPS-induced IL-6 and IL-1 production in J774 macrophages. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS or loxoribine (1mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-6 and IL-1β using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

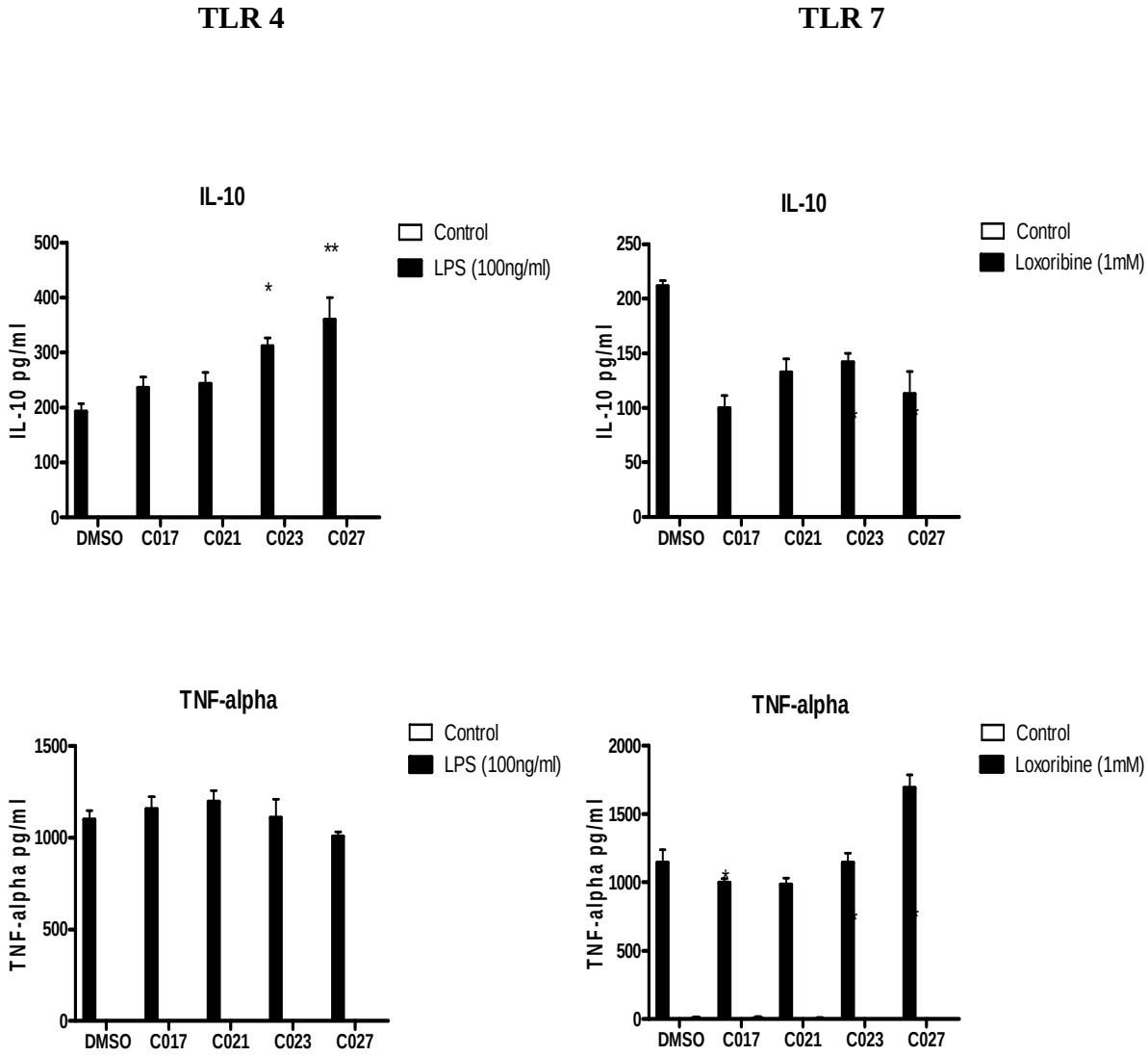


FIGURE 4.4: Second-round *M.membrancea* fractions enhance LPS-induced IL-10 production in J774 macrophages. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS or loxoribine (1mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-10 and TNF-α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

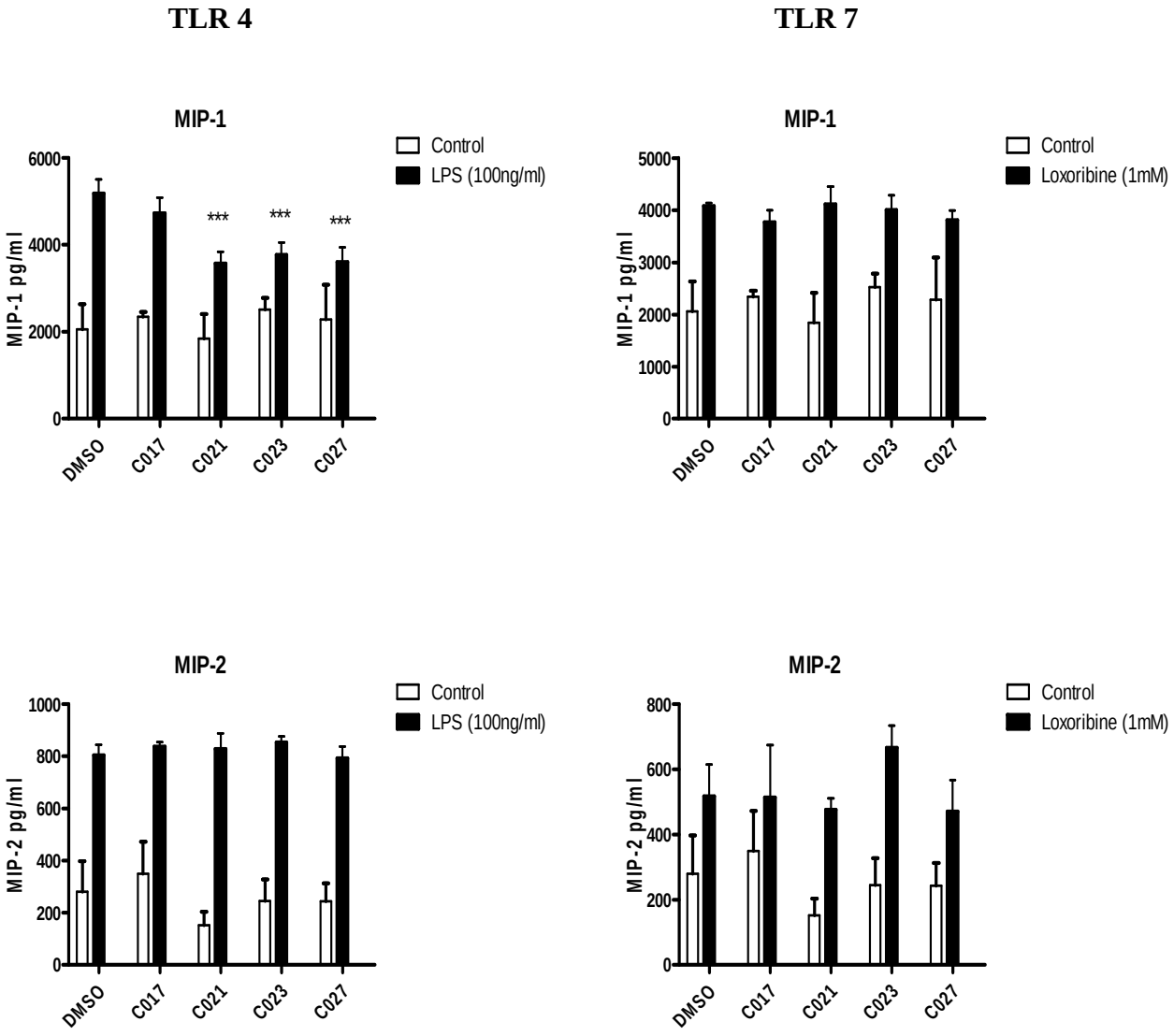


FIGURE 4.5: Second-round *M.membrancea* fractions suppress LPS-induced MIP-1α production in J774 macrophages. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS or loxoribine (1mM). Supernatants were recovered after 24 hrs and assessed for levels of MIP-1α and MIP-2α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

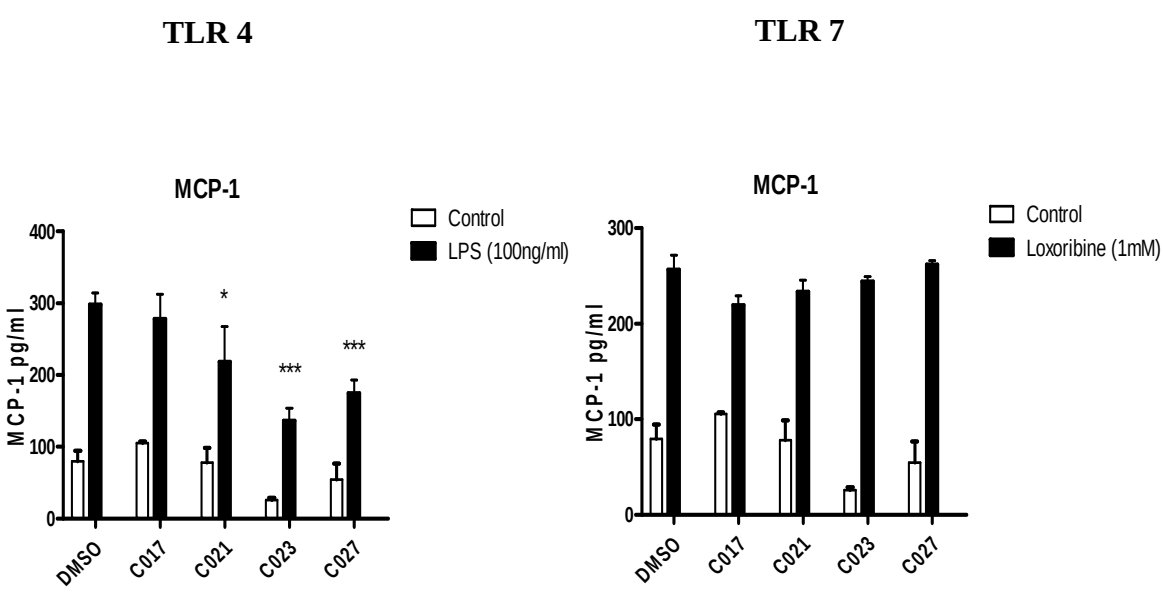


FIGURE 4.6: Second-round *M.membrancea* fractions suppress LPS-induced MCP-1 production in J774 macrophages. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS or loxoribine (1mM). Supernatants were recovered after 24 hrs and assessed for levels of MCP-1 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

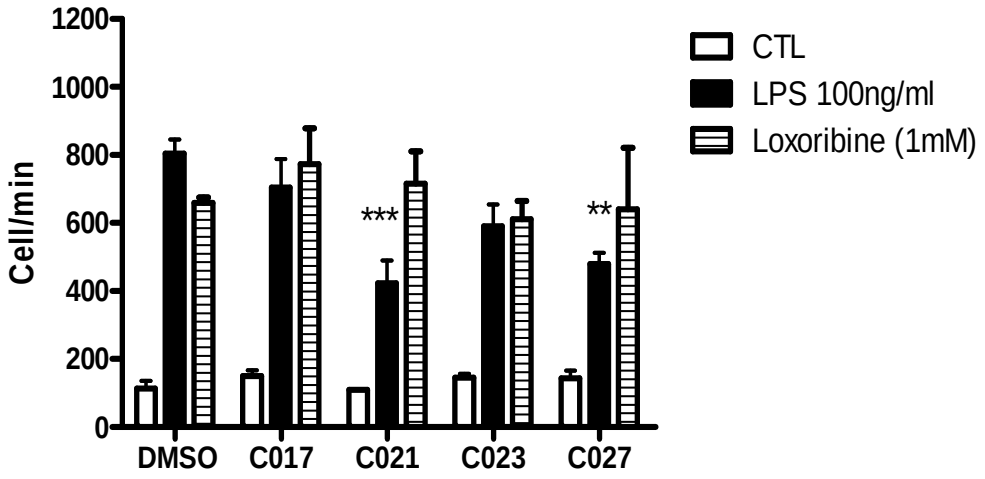


FIGURE 4.7: Second-round *M.membrancea* fractions modulate the migration of J774 macrophages in response to LPS. MØ were cultured with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with 100ng/ml of LPS or loxoribine (1mM). 3 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 hrs 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™.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***$p<0.001$, **$p<0.01$, *$p<0.05$ comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

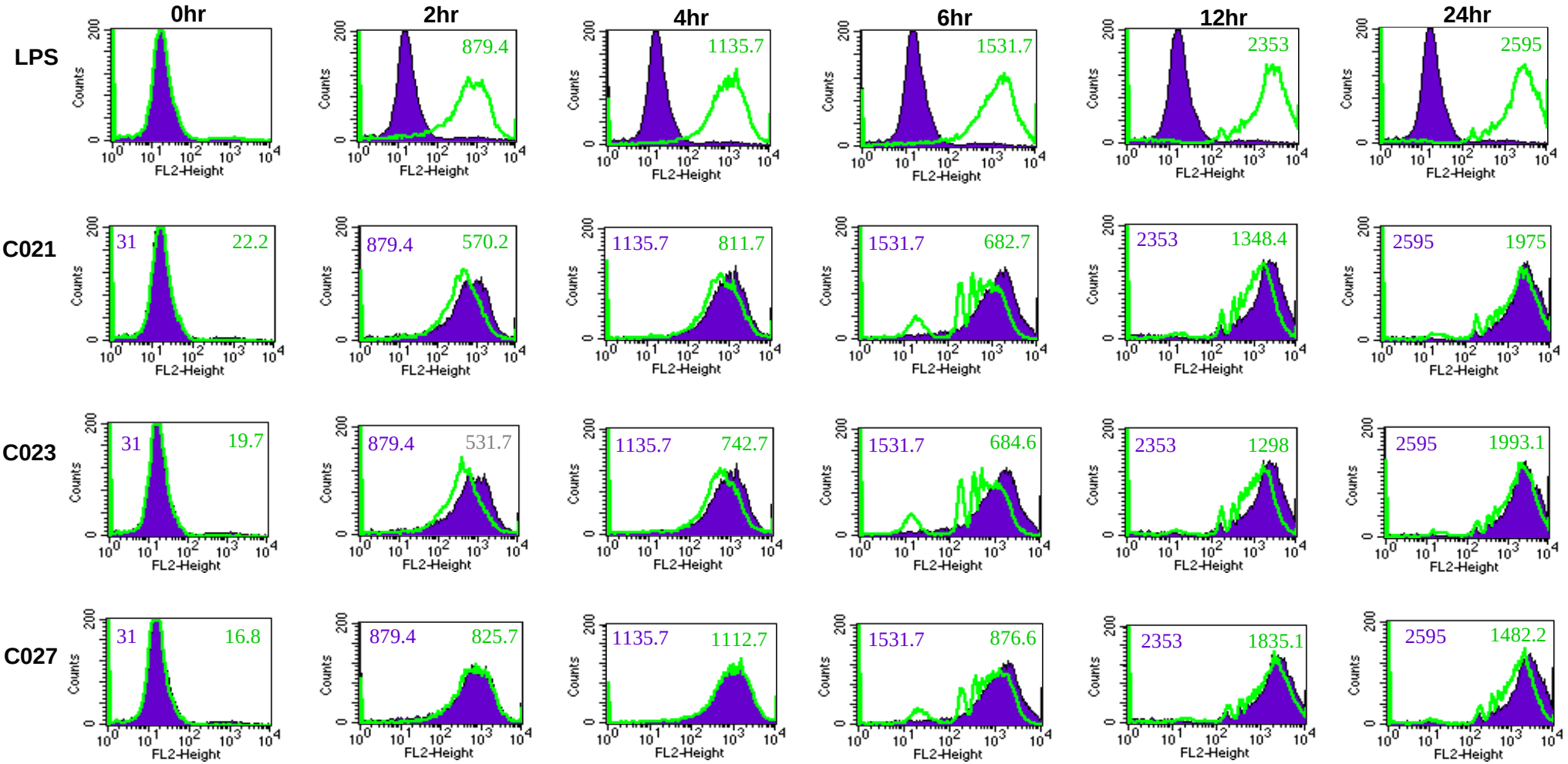


FIGURE 4.8: Second-round *M.membrancea* fractions modulate the rate of phagocytosis in J774 macrophage following LPS activation. Cells were plated at 5 x 10^5 cell/well in a 6-well plate and left to rest overnight. The next day, cells were treated with DMSO or second fractions of *M.membrancea* (C017, C021, C023 and C027) for 1 hr prior to stimulation with LPS (100ng/m). 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 MØ was assessed using a BD FACSCalibur™. **Top row:** LPS-stimulated MØ (filled purple histogram) vs. LPS-stimulated MØ with the addition of latex beads (green line). **Bottom three rows:** LPS-stimulated MØ with the addition of latex beads (filled purple histogram) vs. marine extract-treated MØ with the addition of latex beads (green line).

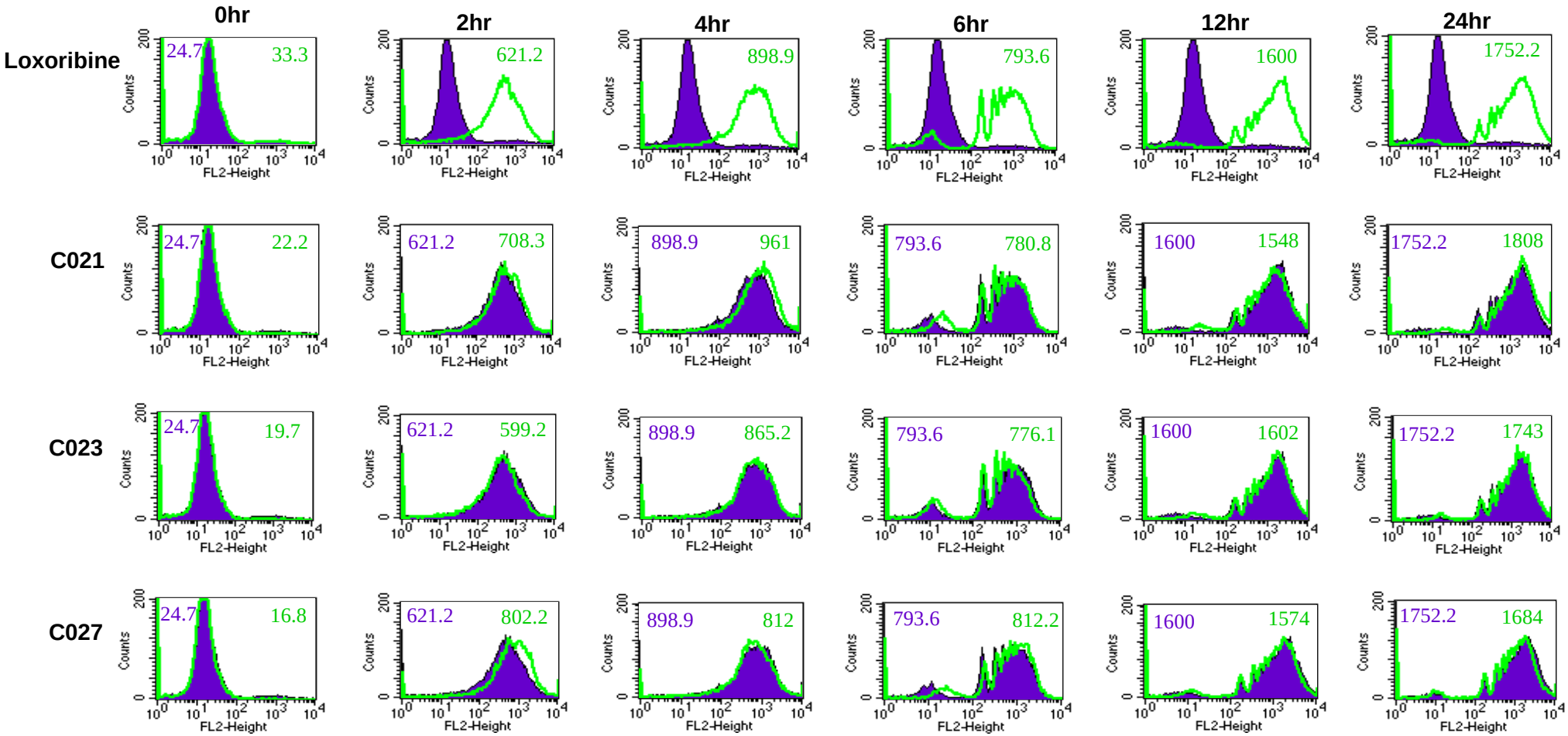


FIGURE 4.9: Second-round *M.membrancea* fractions modulate the rate of phagocytosis in J774 macrophage following Loxoribine activation. MØ were plated at 5 x 10^5 cell/well in a 6-well plate and left to rest overnight. The next day, cells were treated with DMSO or second fractions of *M.membrancea* (C017, C021, C023 and C027) 1 hr prior to stimulation with loxoribine (1mM). 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 MØ was assessed using a BD FACSCalibur™. **Top row:** loxoribine-stimulated MØ (filled purple histogram) vs. loxoribine -stimulated MØ with the addition of latex beads (green line). **Bottom three rows:** loxoribine-stimulated MØ with the addition of latex beads (filled purple histogram) vs. marine extract-treated MØ with the addition of latex beads (green line).

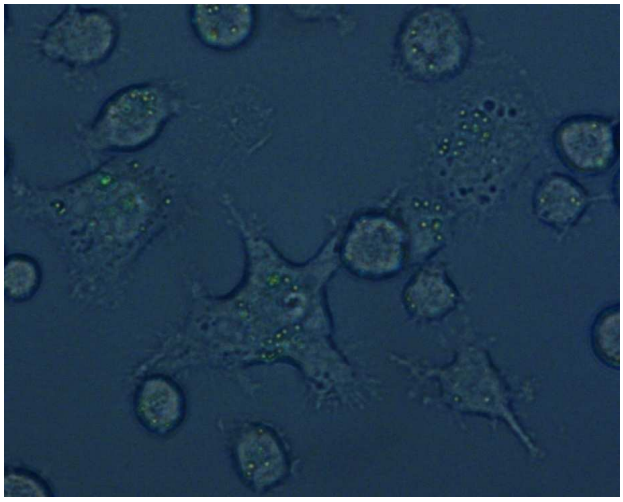


FIGURE 4.9a: Confirmation that latex beads were phagocytosed following stimulation with LPS (100ng/ml) by fluorescent imaging (60X).

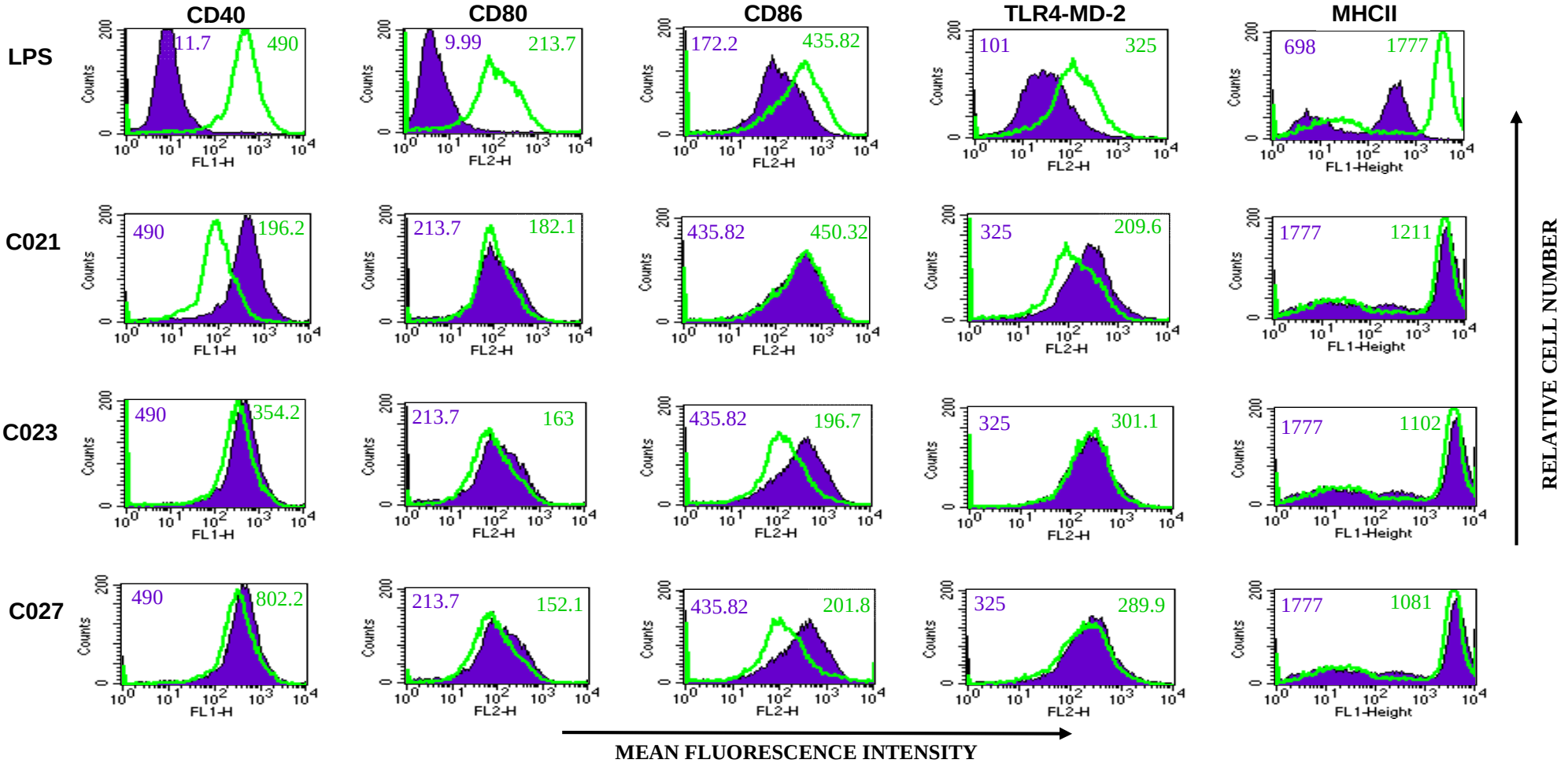


FIGURE 4.10: Second-round *M.membrancea* fractions modulate the expression of CD40, CD80, CD86 and MHCII on J774 Macrophages following LPS stimulation. MØ were treated with second fractions of *M.membrancea* (C021, C023, C027) for 1 hr prior to stimulation with LPS (100ng/ml). Subsequently, cells were washed and stained with specific antibodies. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated MØ (filled histogram) vs LPS stimulated MØ (green line). **Bottom three rows:** LPS-stimulated MØ (filled purple histogram) vs. LPS induced marine extract-treated MØ (green line). MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel.

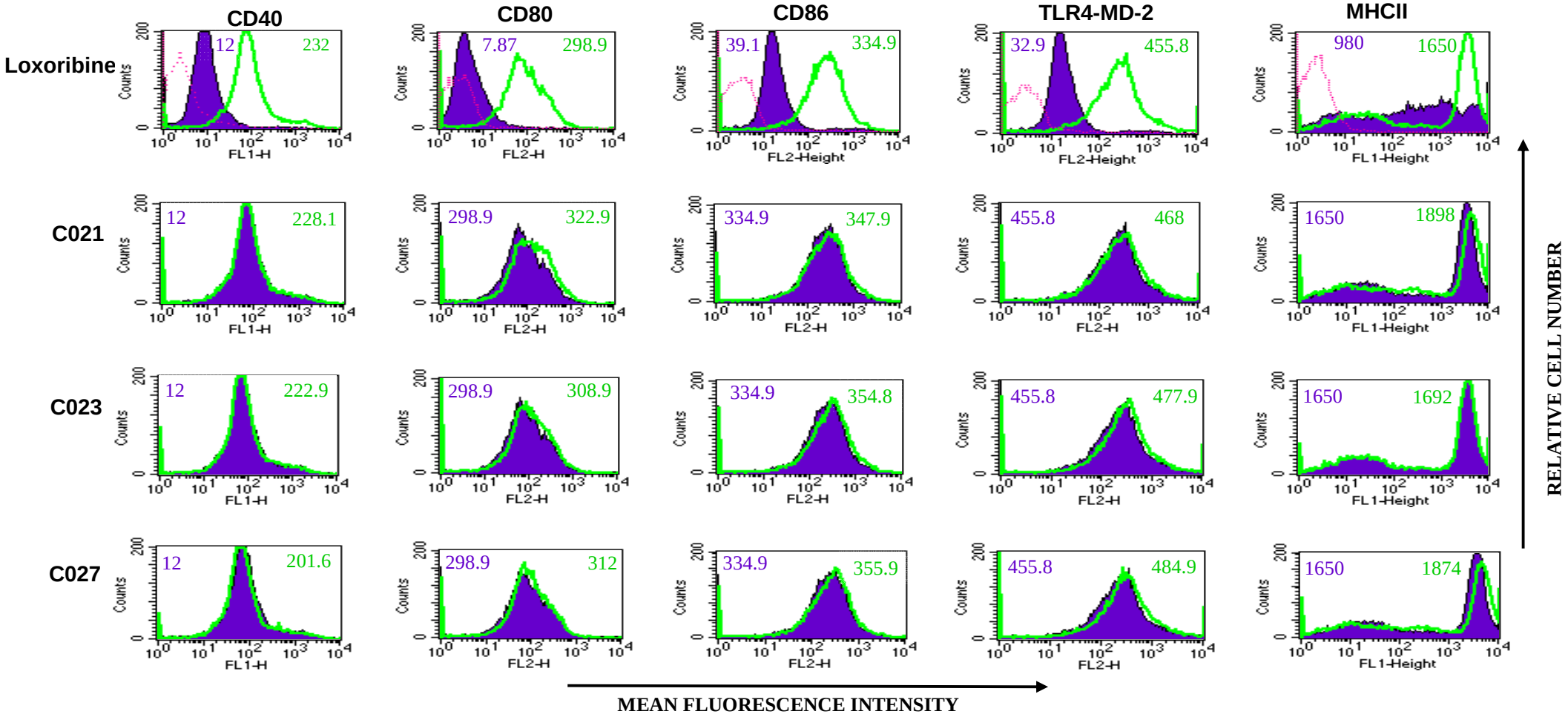


FIGURE 4.11: Second-round *M.membrancea* fractions modulate the expression of CD40, CD80, CD86 and MHCII on J774 Macrophages following Loxoribine activation. MØ were treated with DMSO or second fractions of *M.membrancea* (C021, C023, C027) for 1 hr prior to stimulation with loxoribine (1mM). Subsequently, cells were washed and stained with specific antibodies. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated MØ (filled histogram) vs loxoribine stimulated MØ (green line). **Bottom three rows:** Loxoribine-stimulated MØ (filled purple histogram) vs. loxoribine induced marine extract-treated MØ (green line).MFI values for marine treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel.

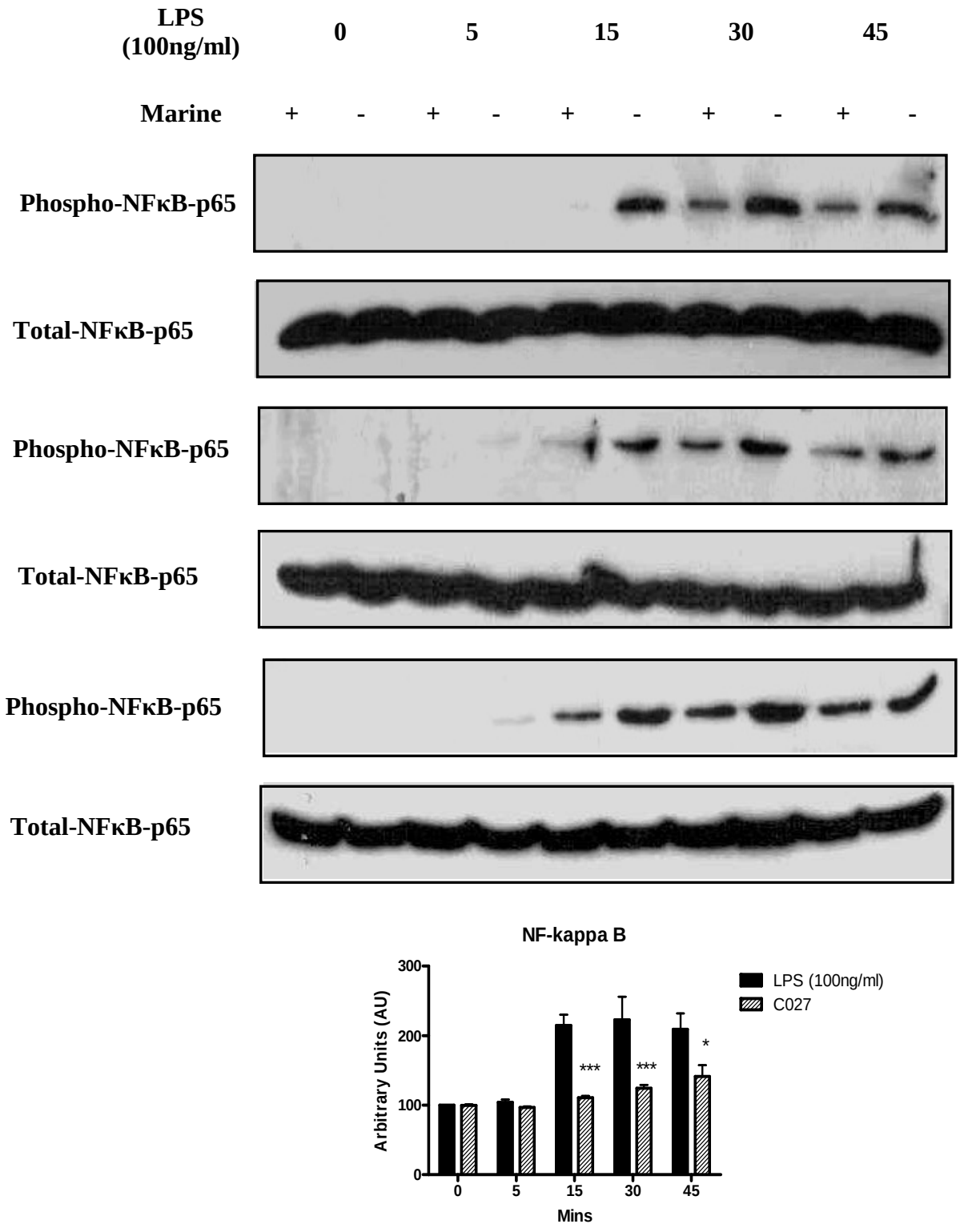


FIGURE 4.12: Second-round *M.membrancea* fraction (C023) suppresses phosphorylation of NFκB-p65 in J774 macrophages following LPS activation. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), *M.membranacea* fraction C023 for 1 hr prior to stimulation with 100ng/ml of LPS over a time-course, after which cells were lysed and immunoblotted for phospho-NFκB-p65. Total cellular levels of total NFκB-p65 were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of phospho-NFκB-p65 expression in arbitrary units (AU) is shown.

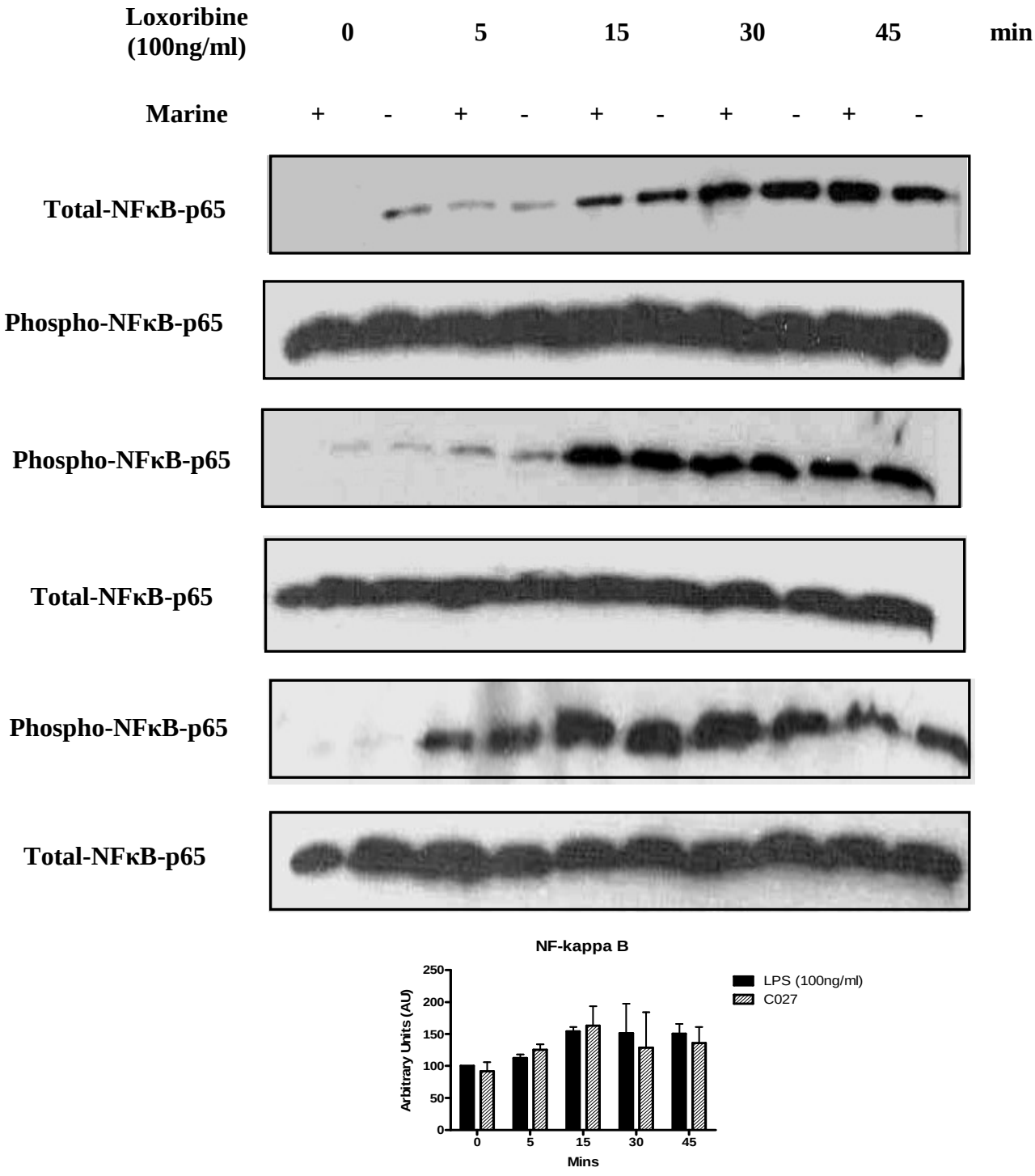


FIGURE 4.13: Second-round *M.membrancea* fraction (C023) does not suppress the phosphorylation of NFκB-p65 in J774 macrophages following loxoribine stimulation. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), *M.membranacea* fraction C023 for 1 hr prior to stimulation with 100ng/ml of loxoribine over a time-course, after which celsl were lysed and immunoblotted for phospho-NFκB-p65. Total cellular levels of total NFκB-p65 were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of phospho-NFκB-p65 expression in arbitrary units (AU) is shown.

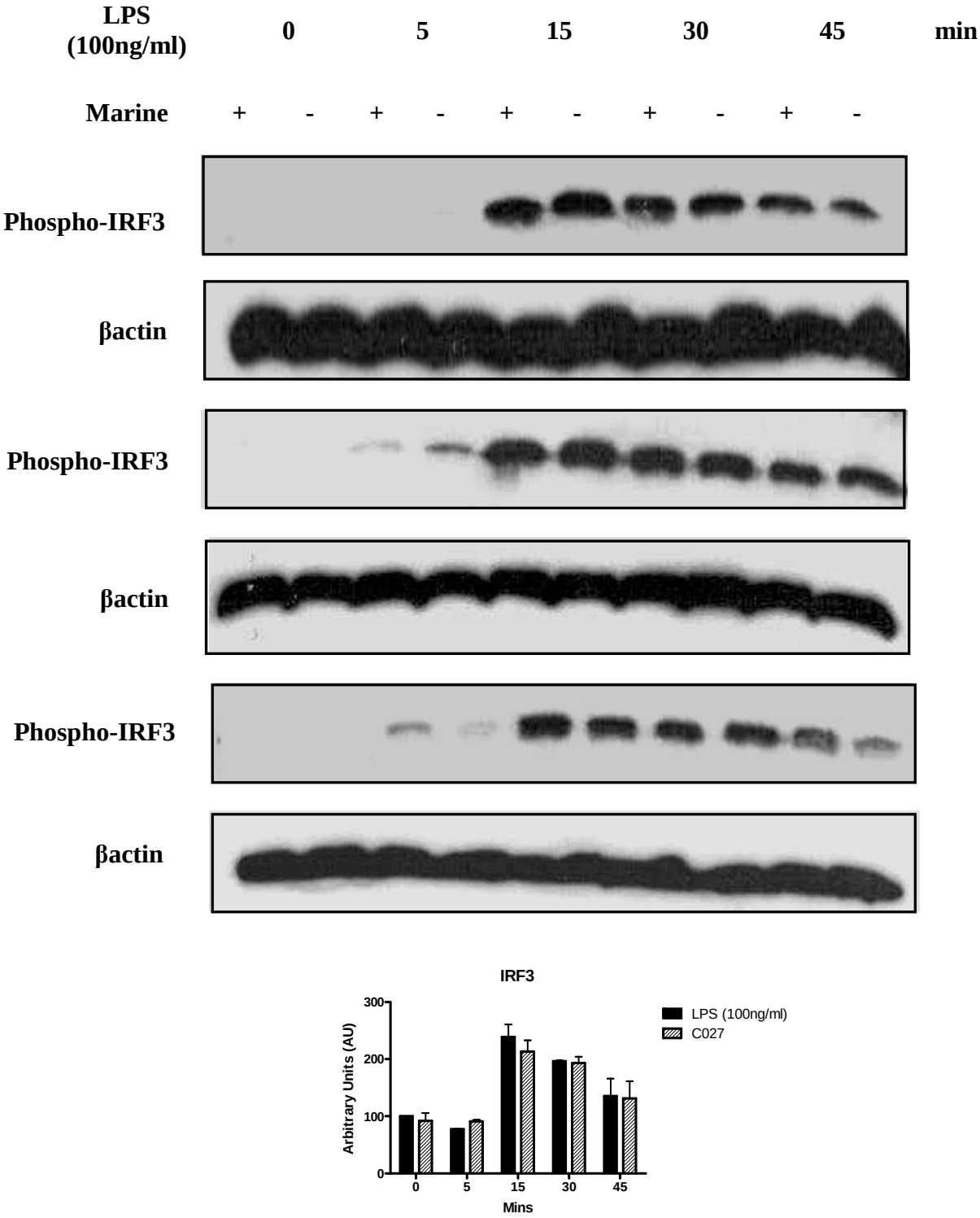


FIGURE 4.14: *M.membrancea* fraction (C023) does not suppress the phosphorylation of IRF3 in J774 macrophages. J774 macrophages were plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), *M.membranacea* fraction C023 for 1 hr prior to stimulation with 100ng/ml of LPS over a time-course, after which cells were lysed and immunoblotted for phospho-IRF3. Total cellular levels of βactin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of phospho-IRF3 expression in arbitrary units (AU) is shown.

4.3 DISCUSSION

The findings of this study demonstrate that the second-round fractions of *M.membrancea* demonstrate anti-inflammatory properties when MØ are stimulated with the TLR4 ligand, LPS but not with the TLR7 ligand, loxoribine. Numerous studies have shown that over activation of macrophages play a major role in mediating chronic mucosal inflammation which has been seen in patients with ulcerative colitis and Crohn's disease (Xavier and Podolsky 2007). Additionally, aggravated macrophage activation results in extensive tissue damage associated with autoimmune diseases such as RA (Feldmann and Maini 2008) and with infection, such as schistosmiasis (Wynn, et al. 2004). Therefore, altering macrophage function may hold great therapeutic potential. The parameters examined in this study have extensive implications and importance in both macrophage activation in inflammatory disease and their involvement in the normal immune response to infection. Furthermore, this comparative study has revealed that the marine fractions only suppress inflammation when cells are activated through certain TLRs.

It has been extensively shown in numerous autoimmune diseases, such as RA, that the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 synergistically mediate synovitis and destruction of bone cartilage and that IL-1 and TNF-α trigger the secretion of IL-6 by synovial cells (Szekanecz and Koch 2007). A study by Hata and colleagues demonstrated that the synovial fluid of SKG mice contained high amounts of IL-6, TNF-α and IL-1 and that genetic deletion in IL-6 completely suppressed the development of arthritis in these mice (Hata, et al. 2004). In addition, enhanced IL-1 production has been implicated in the development and progression of atherosclerosis

(Andersson, Libby and Hansson 2010). As a result considerable effort has been placed on developing therapeutic targets that can modulate the activities of these cytokines. Anti-TNF-α and IL-1 receptor antagonist (IL-1ra) drugs have been shown to ameliorate RA. For instance**,** treatment with anakinra, a recombinant form of human IL-1ra has been shown to significantly reduce levels of IL-1 and improve the progression of RA (Fleischmann,R.M, 2003). Results from our study provide evidence that the second-round fractions of *M.membrancea* robustly inhibits the production of the pro-inflammatory cytokine, IL-6 and a slight reduction in IL-1β following stimulation with LPS. Anti-IL-6 receptor antibodies have been developed as a therapeutic agent for these diseases, with some efficacy revealed in clinical trials. The effects of *M.membrancea* on the production of these cytokines have not been reported to date and suggests that it might be useful in diseases such as RA. Interestingly, two marine compounds bolinaquinone and petrosaspongiolide M isolated from *Dysidea sp* and *Petrosaspongia nigra*, respectively, have been shown to significantly reduce the levels of IL-1β in mice with crohns diseases (Busserolles, et al. 2005)

IL-12 and IL-23 are closely related cytokines and are heterodimers made up of a common p40 subunit complexed to unique p35 (IL-12) or p19 (IL-23) subunits, respectively. The primary function of IL-12 and IL-23 is to direct newly activated T helper cells to a Th1 and Th17 phenotype, respectively (Bettelli and Kuchroo 2005). Marked production of IL-12 and IL-23 by macrophages are largely implicated in autoimmune diseases including; MS, IBS and RA and targeting these cytokine may hold therapeutic potential (Choy and Panayi 2001, Li, et al. 2007, Uhlig, et al. 2006). IL-12 is a potent inducer of IFN-γ which directs the induction of classically activated

macrophage by inactivating feedback inhibitory mechanisms, such as those mediated by IL-10 (Trinchieri 2003a). Furthermore, a study by Elliott and colleagues demonstrated that ustekinumab reduced the severity of plaque psoriasis by inhibiting IL-12 and IL-23 mediated cellular responses (Elliott, et al. 2009). Results here provide evidence that the second fractions of *M.membrancea* significantly inhibit IL-12p40 and IL-23 in macrophages. To the best of our knowledge this is the first report of alterations in these cytokines by a marine extract.

The 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 Th cell subsets (Lu, Wang and Linsley 1997). Overexpression of these surface markers has been reported in the inflamed tissue of patients with RA and IBD signifying that costimulatory markers are potential therapeutic targets for treating inflammatory disorders (Maerten, Liu and Ceuppens 2003). In the present study, we found that culturing macrophages with second-round fractions of *M.membrancea* decreased the surface expression of CD80, but more significantly, CD40 expression, following stimulation with the TLR4 ligands however no alteration was observed following TLR7 simulation. This suggests that they may be useful in inflammatory diseases. The expression of MHCII was also markedly down-regulated by *M.membrancea* after LPS activation. MHCII is essential for antigen processing and presentation which allows macrophages to initiate an adaptive immune response by interacting with T cells in the lymph nodes (Villadangos 2001). Many viruses have been shown to reduce MHC class II expression to avoid protective immune response such as *Chlamydia trachomatis* (Zhong, Fan and Liu 1999). However, abnormal expression of MHCII is linked to the development of autoimmune and infectious diseases such as RA (Guardiola and

Maffei 1993). A study by Alisky and colleagues demonstrated that the gluccorticoid, dexamethasome decreased levels of MHCII on activated microglia and this inhibition of class II expression may be one mechanism utilized by glucocorticoids in the suppression of neuroinflammatory disease (Alisky 2006). Our results demonstrate that *M.membrancea* significantly downregulate MHCII again indicating their potential in the treatment of a range of inflammatory diseases.

Chemokines initiate the recruitment and activation of macrophages leading to enhanced inherent protective responses to invading pathogens. Macrophage infiltration is of paramount importance during innate host defence. In a model of pulmonary *pseudomonas aeruginosa* infection, inhibition of MIP-2 impaired bacterial clearance (Tsai, et al. 2000). In addition, depletion in MIP-1α levels significantly decreases clearance of *Cryptococcus neoforms* (Doyle and Murphy 1997). The interplay between macrophage and bacteria leads to the clearance of infection. This highlights the benefits of these chemokines in antibacterial, antifungal and anti-viral host defence. Notably, impaired bacterial clearance and increased mortality in mouse models of bacterial pneumonia have been reported (Tang, et al. 1996). However, dysregulated expression of chemokines causes a mass influx of leukocytes to the site of inflammation leading to inflammatory diseases and infections such as RA, MS and meningococcal disease (Godessart and Kunkel 2001, Mellergard, et al. 2010, Halstensen, et al. 1993). Therefore impeding the production of chemokines and their receptors may have therapeutic potential. This study has assessed the modulatory effects of *M.membrancea* on MIP-1α, MIP-2 and MCP-1 following stimulation with bacterial and viral TLR ligands. The chemokines, MIP-1α and MCP-1 were significantly reduced following treatment with second fractions of *M.membrancea*

post TLR4 activation however no alteration was demonstration in chemokine production following stimulation with the viral ligand, loxoribine. MIP-1α production is enhanced in mice with pulmonary fibrosis by the secretion of IL-6 and anti-IL-6 antibodies have been demonstrated to reduce MIP-1α expression thus ameliorating the disease (Smith, et al. 1998). Furthermore, MCP-1 mediates the trans-endothelial migration of leukocytes across the blood-brain barrier into the CNS initiating neuroinflammatory diseases including Alzheimer's disease and Parkinson disease (Reale, Greig and Kamal 2009). Reduced levels of IL-6 in *M.membrancea* treated macrophage following LPS activation reported here may account for the decrease in MIP-1α. Similarly, MCP-1 is regulated by IL-1 and TNF-α (Adams and Lloyd 1997) therefore *M.membrancea* reduced IL-1 production may partly be responsible for the significant reduction in MCP-1 post LPS stimulation. In addition, the chemotaxis of cells have been implicated in the pathogenesis of inflammatory diseases. For instance, there is a significant enhancement in the migration of T cells in patients with systemic lupus erythematosus (SLE) (Li, et al. 2007). Therefore the ability of the second-round fractions of *M.membrancea* to inhibit chemotaxis demonstrates that they have great anti-inflammatory potential. While the decrease in chemokines and chemotaxis may be beneficial in disease, it is also important to note that these chemokines are central to cell recruitment during infection and therefore may suppress the normal host immune response to infection and thus delay clearance. For instance, genetic deletion of MCP-1 in mice significantly reduced the recruitment of monocytes and therefore impaired the response to infection by the intracellular bacterium *Mycobacterium tuberculosis* (Lu, et al. 1998)

Phagocytosis is a vital role of all macrophages and is fundamental in the ingestion and clearing of pathogens (Aderem 2003). To date no studies have investigated the role of *M.membrancea* on phagocytosis activity. Here we report a suppressed ability of LPS-stimulated macrophage to phagocytose as a result of *M.membrancea* treatment however no alteration in the ability to phagocytosis following loxoribine stimulation was observed. Numerous diseases have resulted from a decrease in the ability of macrophages to phagocytose (Allen and Aderem 1996). However, phagocytosis is crucial in the host in controlling infection and it has been well documented that a reduction in phagocytosis can cause increased susceptibility to infection. A study by Morran and workers demonstrated that phagocytosis is significantly reduced in diabetic NOD mice stimulated with LPS under hyperglycemic conditions causing infections to flourish (Morran, et al. 2009). Results reported in our study suggest that *M.membrancea* can cause increased susceptibility to infection, particularly to those pathogens who utilize TLR4 however not to pathogens that utilize the viral receptor, TLR7. Studies have shown that NFκB is involved in the regulation of phagocytosis and deletion of the p65 subunit by siRNA inhibited LPS induced phagocytosis (Suzuki and Umezawa 2006). A reduction in phosphorylation of NFκB-p65 was demonstrated in our study in *M.membrancea* treated macrophages following stimulation with only LPS which may account for the decreased ability of these cells to phagocytose. This overall study demonstrates that the second-fractions of *M.membrancea* have the ability to suppress effects downstream of the bacterial ligand, TLR4 and not the viral ligand, TLR7 which led us to explore their effects on a wider range of TLRs.

CHAPTER 5

ELUCIDATION OF THE MECHANISM OF THE PURIFIED MARINE FRACTION, INV013

5.1 INTRODUCTION

In chapter 3 we described the suppression of cytokine and chemokine production and the down-regulation in cell surface marker expression by crude marine extracts, *A.digitia and M.membranace,* and also by the sub-fractions of these extracts following stimulation of DCs with LPS. The fractions of *M.membrancea,* shown to have the most potent anti-inflammatory effect were further fractionated and in this chapter we set out to elucidate the mechanisms through which the marine extracts exert their anti-inflammatory effects. Initially we screened the fractions *M.membrancea* C017, C021, C023, C027 to determine which fraction was the most active. Our collaborating chemists at UCD then isolated the individual marine compound (INV013) by using column chromatography and spectroscopy. INV013 was then used to determine the mechanism of action of the marine compound and identify the molecular target. Our marine extracts were able to alter the effects of DCs following activation with LPS however they did not modulate the function of immature DCs, this finding and the fundamental role of TLR4 in signalling in DC activation led us to investigate the effects of the marine extracts on TLR signalling pathways.

LPS is the most widely studied and extensively used TLR ligand and it is an effective activator of, TLR4 (Sandor and Buc 2005, O'Neill 2007). TLR4 is the only TLR capable of activating both the MyD88 dependent and MyD88 independent pathways leading to the induction of the transcription factors NF-κB and IRF-3, respectively. This leads to the production of pro-inflammatory cytokines, such as IL-6, IL-12 and TNF-α, via NFκB activation and type 1 interferons (IFN) via IRF3 activation (Takeda and Akira 2007). TLR2 can heterodimerize with TLR1 and respond to triacylated lipoproteins such as Pam3CSk4 and peptidoglycan (PGN) or it can dimerize with

TLR6 and respond to diacylated lipoproteins such as zymosan (West, Koblansky and Ghosh 2006). TLR2 and TLR4 are distinct from other TLRs in that they are the only TLR complexes that utilize the adaptor protein Mal in association with MyD88 to initiate downstream signalling which leads to NFκB activation and the release of pro-inflammatory cytokines such as IL-12, IL-6 and IL-1β (Sheedy and O'Neill 2007).

The remainder of the TLRs do not utilise the adaptor Mal. These include, TLR5 which is activated by flagellin and activation of this receptor mobilizes the nuclear factor NFκB and stimulates pro-inflammatory production (de C Ventura, et al. 2008). TLR3 recognizes double stranded RNA (dsRNA), such as Poly:(IC), and the stimulation of this receptor induces the activation of IRF-3 through the exclusive signalling pathway independent of MyD88 utilizing a sole adaptor, TRIF (Sandor and Buc 2005). TLR7 and TLR8 both detect viral single stranded RNA (ssRNA) such as loxoribine and TLR9 detects unmethylated CpG (CpG) motifs from bacterial and viral DNA. These TLRs trigger inflammatory cytokine secretion and IFN secretion through MyD88.

In this chapter we examined the effects of second-round fractions of *M.membrancea* (C017, C021, C023, and C027) and the individual compound, INV013, on DCs activated with a range of TLR ligands. We also investigated their effects on NFκB and IRF3 activation which are downstream of TLR activation. Furthermore, we carried out advanced mechanistic studies on INV013 by examining its potential to target TLR adapter molecules.

5.2 RESULTS

5.2.1 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* MODULATE CYTOKINE PRODUCTION BY DENDRITIC CELLS FOLLOWING STIMULATION WITH TLR2 AND TLR4 LIGANDS ONLY IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), fractions of marine extracts; *M. membranacea* (C017, C021, C023 or C027) at 1:200 dilution for 1 hr prior to stimulation with TLR ligands, Pam_3CSK_4 (5µg/ml), Zymosan (10µg/ml), PGN (10µg/ml), Poly:(IC) (10µg/ml), Flagellin (5µg/ml), Loxoribine (5µg/ml) or CpG (2µM). After 24 hrs supernatants were removed and assessed for levels of IL-12p40 **[Figure 5.1-5.2]**, IL-12p70 **[Figure 5.3-5.4]**, IL-23 **[Figure 5.5-5.6]**, IL-6 **[Figure 5.7-5.8]**, IL-1β **[Figure 5.9-5.10]**, TNF-α **[Figure 5.11-5.12]**, and IL-10 **[Figure 5.13]** using specific immunoassays.

The secretion of the pro-inflammatory cytokines IL-12p40, IL-12p70 and IL-23 were significantly suppressed in the second-round fractions of *M.membrancea* extracts (C017, C021, C023 and C027) following stimulation with LPS, Pam_3CSK_4, Zymosan and PGN **[Figure 5.1-5.5]** compared to the control group ($p<0.001$). In marine extract treated-DCs stimulated with PGN and LPS a significant reduction was also observed in the secretion of the pro-inflammatory cytokines IL-6 ($p<0.05$-$p<0.001$). In addition, a reduction was demonstrated in IL-1β post PGN activation ($p<0.001$) **[Figure 5.7-5.9]** however there was increased levels of IL-1β when DCs were treated with fractions, C017 and C027 following activation with zymosan activation **[Figure 5.9]**.

The levels of TNF-α remained relatively unchanged regardless of the marine fraction used or the TLR ligand used **[Figure 5.11-5.12]**. Exposure of DCs to the *M.membrancea* second-fractions (C017, C021, C023, C027) resulted in a small increase in IL-10 with LPS ($p<0.001$) or Pam_3CSK_4 ($p<0.01$). In contrast, the fractions decreased IL-10 production when cells were activated with PGN **[Figure 5.13]**. Furthermore, no change was observed in any cytokine expression when DCs were activated through TLR3, TLR5, TLR7 and TLR9 with Poly:(IC), flagellin, loxoribine and CpG, respectively **[Figure 5.2-5.14]**.

5.2.2 THE DOSE RESPONSE OF THE PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* USED HAVE NO SIGNFICIANT EFFECT ON CELL VIABILITY.

Since the second fractions of *M.membrancea* C023 and C027 demonstrated the most significant anti-inflammatory effects our collaborators at UCD carried out further fractionations. From this we received a pure compound which we coded INV013. With this pure compound we carried out numerous experiments to determine its molecular target [see figure 3.1.1]. Initially we examined the toxicity of INV013 by exposing DCs to a range of dilutions. Following INV013 treatment the cell viability was assessed using Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. The doses of INV013 (1:100, 1:200, 1:500, 1:1,000, 1:10,000) chosen for use in future experiments did not have any significant cytotoxic effect on DCs *in vitro* **[Figure 5.15]**

5.2.3 PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* MODULATES CYTOKINE PRODUCTION BY DENDRITIC CELLS FOLLOWING STIMULATION WITH TLR2 AND TLR4 LIGANDS ONLY IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control), purified marine compound, INV013 at 1:200 dilution for 1 hr prior to stimulation with TLR ligands, LPS (100ng/ml), Zymosan (10µg/ml), PGN (10µg/ml), Poly:(IC) (10µg/ml), Flagellin (5µg/ml), Loxoribine (5µg/ml) and CpG (2µM). After 24 hrs supernatants were removed and assessed for levels of IL-12p40, IL-12p70 **[Figure 5.16]**, IL-23, IL-6 **[Figure 5.17]**, IL-1β, TNF-α **[Figure 5.18]**, IL-8, Rantes and IFN-β **[Figure 5.19]** using specific immunoassays.

Treatment with INV013 significantly inhibited the production of the IL-12 family of cytokines IL-12p40, IL-12p70 and IL-23 following activation with the TLR4 ligand LPS (100ng/ml) and the TLR2 ligands PGN (10µg/ml) and Zymosan (10µg/ml) (p<0.001) **[Figure 5.16-5.17]**. Furthermore INV013-treated DCs were able to decrease the secretion of the pro-inflammatory cytokines, IL-6 and IL-1β post stimulation with LPS and PGN (p<0.001) and to a lesser extent post activation with zymosan (p<0.05) **[Figure 5.17-5.18]**. The levels of TNF-α, Rantes and IFN-β remained unchanged regardless of how cells were activated **[Figure 5.18-5.19]**. Furthermore, a notable decrease was observed in the secretion of the pro-inflammatory cytokine IL-8 in INV013-treated DCs post activation with LPS and PGN (p<0.001) **[Figure 5.19].** In contrast, no alteration in the profile of any cytokine was demonstrated following stimulation with the TLR ligands: Poly:(IC), Flagellin, Loxoribine and CpG **[Figure**

5.16-5.19]. Therefore the pure compound, INV013, only exerts its anti-inflammatory effects following TLR2 and TLR4 ligation giving us a clear indication that the molecular target of INV013 is downstream of these TLR receptors. Furthermore our observation that INV013 blocked cytokines downstream of NFκB (IL-8) but not IRF3 (IFN-β and Rantes) indicates that its target may be involved in the NFκB signalling pathway.

5.2.4 PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* MODULATES CELL SURFACE MARKER EXPRESSION IN DENDRITIC CELLS IN VITRO

BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 10^6 cell/ml and pretreated with either DMSO (vehicle control) or the purified compound, INV013 at 1:200 dilution for 1 hr prior to stimulation with or without 100ng/ml LPS (*E.Coli* serotype R515), PGN (5µg/ml) or flagellin (5µg/ml) for 24 hrs. Control cells and LPS, PGN and flagellin-stimulated cells were subsequently stained with flourochrome-labelled monoclonal antibodies for various cell surface markers (CD11c, CD40, CD80, CD86, MHCII and TLR4-MD-2) [see **Table 2.7**] in preparation for cytometric analysis by flow cytometry **[Figure 5.20-5.22]**.

Stimulation of DCs with LPS, PGN and flagellin enhanced the expression of CD40, CD80, CD86, TLR4-MD-2 and MHCII (top row) and the purified compound, INV013 was able to inhibit some of these LPS and PGN-induced changes (bottom row) **[Figure 5.20-5.22]**. Following, LPS and PGN stimulation INV013 significantly downregulated CD40 and MHCII expression. A small decrease was also observed in the expression of surface marker CD86 and there was no change in TLR-4-MD-2. In

addition, a significant downregulation in CD80 expression was demonstrated post PGN activation **[Figure 5.20-5.21]**. Contrastingly, INV013 did not change the expression of any surface markers following activation with flagellin **[Figure 5.22]**. Furthermore, there was no significant effect of INV013 on expression of these surface markers in resting DCs **[Figure 5.20-5.22]**.

5.2.5 SECOND-ROUND FRACTIONS OF MARINE EXTRACT *Membranipora membranacea* INHIBITS THE ACTIVATION OF NFκB BUT NOT IRF3 FOLLOWING TLR4 LIGATION

Given that our extracts were able to block cytokines downstream of NFκB but not IRF3 we next examined the effect of *M.membrancea* second-round fractions on the activation of these transcription factors. HEK 293 cells stably expressing TLR4-CD14-MD-2 (HEK-MTC) were plated at 1 x 105 cell/ml 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. Following transfection the cells were pretreated with second fractionated *M.membrancea* extracts (C017, C021, C023 or C027) for 1 hr prior to stimulation with LPS for 6 hrs and activation of NFκB and IRF3 were assessed. In addition supernatants were removed and assessed for levels of downstream readouts, IL-8 and Rantes respectively, using specific immunoassays **[Figure 5.23].**

In cells treated with second-round fractions of *M.membrancea* (C017, C021, C023 or C027) NFκB activation was significantly inhibited and subsequent IL-8 production was also reduced following stimulation with LPS ($p<0.001$) **[Figure 5.23].** However,

no modulation was observed in IRF3 activation and subsequent Rantes secretion compared to control group **[Figure 5.23]**

5.2.6 PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* DOSE DEPENDENTLY INHIBITS THE ACTIVATION OF NFκB BUT NOT IRF3 FOLLOWING TLR4 LIGATION

We next examined the effect of the purified compound INV013 on activation of NFκB and IRF3. HEK 293 cells stably expressing TLR4-CD14-MD-2 (HEK-MTC) were plated at 1 x 105 cell/ml 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. Following transfection the cells were pretreated with the purified compound, INV013 at a range of doses (1:50, 1:100, 1:200, 1:500 1:1000, 1:10,000) for 1 hr prior to stimulation with LPS for 6 hrs and activation of NFκB and IRF3 were assessed. In addition supernatants were removed and assessed for levels of downstream readouts IL-8 and Rantes respectively using specific immunoassays **[Figure 5.24].**

As shown in figure 5.24 activation of NFκB was significantly inhibited in a dose dependant manner and subsequent IL-8 production was also significantly reduced in INV013 treated- HEK-MTC cells ($p<0.001$) post stimulation with LPS. In contrast, no alteration was demonstrated in the activation of IRF-3 or subsequent Rantes secretion compared to the control **[Figure 5.24].**

5.2.7 PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* INHIBITS MAL-DRIVEN NFKB IN HEK-MTC CELLS.

Given that INV013 specifically suppresses the effects of TLR4 and TLR2 ligands and the fact that TLR4 and TLR2 utilize Mal, we next examined whether INV013 could block Mal-driven NFκB activation. HEK 293 cells stably expressing TLR4-CD14-MD-2 (HEK-MTC) were plated at 1 x 10^5 cell/ml and transiently transfected with NFκB luciferase reporter plasmid and co-transfected with reporter plasmid encoding Mal at different concentrations (20ng and 50ng) or co-transfected with TRIF, which served as a control. Following transfection the cells were pretreated with either DMSO (vehicle control) or purified compound, INV013 at 1:200 dilutions for 1 hr prior to LPS activation. Control cells, which were not transfected with either Mal or TRIF, were activated with LPS (100ng/ml) for 6 hrs **[Figure 5.25]**.

As expected, a significant reduction was observed in the activation of NFκB in INV013-treated cells following stimulation with LPS (p<0.001) **[Figure 5.25]**. Exposure of HEK-MTC cells to INV013 was able to significantly block Mal-induced NFκB activation at both of the Mal concentrations employed (p<0.001). Furthermore, INV013 treatment exerted no effect on TRIF-driven induction of NFκB as expected **[Figure 5.25]**.

5.2.8 PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* INHIBITS LPS INDUCED PHOSPHORYLATION OF NFκB-P65, DEGRADATION OF IκBα AND PHOSPHORYLATION OF P38 BUT NOT PHOSPHORYLATION OF IRF3

Given that Mal is essential for the TLR4 activated pathway leading to the phosphorylation of NFκB-p65 on serine 536 (Doyle, Jefferies and O'Neill 2005), we decided to investigate the effects of INV013 on the phosphorylation of NFκB-p65 using western blotting techniques. BMDC isolated from the bone marrow of BALB/c mice were differentiated in the presence of GMCSF for 7 days and plated at 1 x 106 cell/ml and pretreated with either DMSO (vehicle control), and the purified compound, INV013 at 1:200 dilution. The cells were then stimulated with LPS over a time course (0, 5, 15, 30 and 45 mins) and lysates were generated as described in [section 2.6.1]. Lysates were then run on SDS gels, transferred onto nitrocellulose membranes and immunoblotted for phospho-NFκB-p65, IκBα, phospho-p38 and phospho-IRF3 using specific antibodies. Total NFκB, β-actin and total p38 were used a controls **[Figure 5.22-5.25].**

As shown in figure 5.26 NFκB-p65 was phosphorylated following LPS stimulation with maximum phosphorylation occurring at 15 mins in control cells. In cells pretreated with the purified marine compound, INV013, this phosphorylation of p65 was significantly reduced at 0, 5, 15 and 30 mins following activation with LPS compared to the control phospho-NFκB-p65 blots. Densitometric analysis of the blots revealed that the changes at 5, 15 and 30 mins were statistically significant ($p<0.001$) **[Figure 5.26].**

Interestingly, when the samples were immunoblotted for IκB degradation, it was observed that treatment with INV013 caused a delay in IκB degradation at 0, 5, 15 and 30 mins when compared with the degradation in the control samples. Again densitometric analysis demonstrated this delay in degradation of IκBα be to statistically significant (p<0.001) **[Figure 5.27].** Furthermore, we decided to examine p38, a member of the MAP Kinase family, which is phosphorylated downstream of Mal (Piao, et al. 2008). LPS strongly induced p38 phosphorylation where the effect of LPS was evident at 15 mins. INV013 was able to inhibit the phosphorylation of p38 in response to LPS at all time-points. Densitometric analysis of the blots revealed that the changes at 5, 15 and 30 mins were statistically significant (p<0.001) **[Figure 5.28].** In addition, we immunoblotted for phospho-IRF3 and as expected no alteration in the phosphorylation of IRF3 was observed in INV013-treated DCs post LPS activation. Again densitometric analysis demonstrated there was no significant change in phospho-IRF3 **[Figure 5.29].**

5.2.9 PURIFIED MARINE COMPOUND, INV013, ISOLATED FROM *Membranipora membranacea* INTERFERS WITH THE TRYOSINE PHOSPHORYLATION OF OVEREXPRESSED MAL IN HEK-MTC CELLS.

Given that Mal undergoes tyrosine phosphorylation during TLR2 and TLR4 signalling and that this phosphorylation is fundamental for the activation of NFκB we next examined whether INV013 could modulate the phosphorylation of Mal. HEK 293 cells stably expressing TLR4-CD14-MD-2 (HEK-MTC) were plated 1 x 10^5 cell/ml and transiently transfected with a reporter plasmid encoding Mal (4μg/dish). Following transfection, the cells were pretreated with either DMSO (vehicle control)

or purified compound, INV013 at 1:200 dilutions for 1 hr prior to LPS activation. The cells were then stimulated with LPS for a number of time-points (5, 15, 30 mins) and lysates were generated as described in [section 2.6.1]. Lysates were immunoprecipitated (IP) with HA-Mal antibody using A/G sepharose beads as described in section [section 2.8]. Lysates were then run on SDS gels, transferred onto PVDF membranes and immunoblotted (IB) for α-phospho-tyrosine. To examine total levels of Mal proteins, whole cell lysates were immunblotted with HA-Mal antibody.

Figure 5.30 demonstrates that immunopreciptiation of Mal with HA-Mal and immunblot analysis with α-phospho-tyrosine-specific antibody showed a constitutive Mal tyrosine phosphorylation. In cells pretreated with the purified marine compound, INV013, tyrosine phosphorylation of overexpressed Mal was significantly reduced **[Figure 5.30]**. Furthermore, INV013-treated cells downregulated the tyrosine phosphorylation of Mal at 5, 15 and 30 mins following activation with LPS compared to the control Densitometric analysis showed that these reductions were statistically significant ($p<0.05$ $p<0.01$-) **[Figure 5.30]**

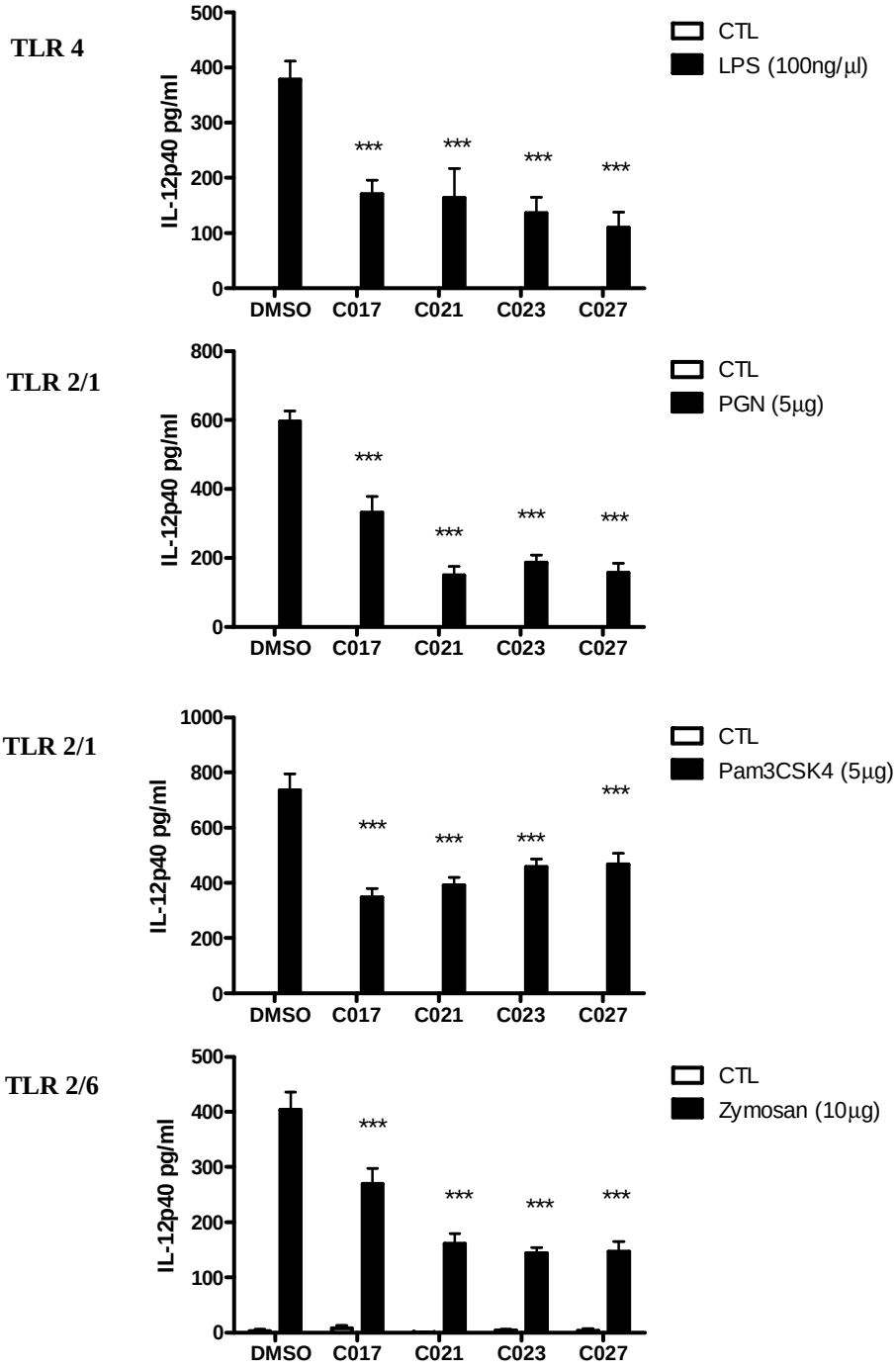


FIGURE 5.1: Second-round *M.membrancea* fractions suppress IL-12p40 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of IL-12p40 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

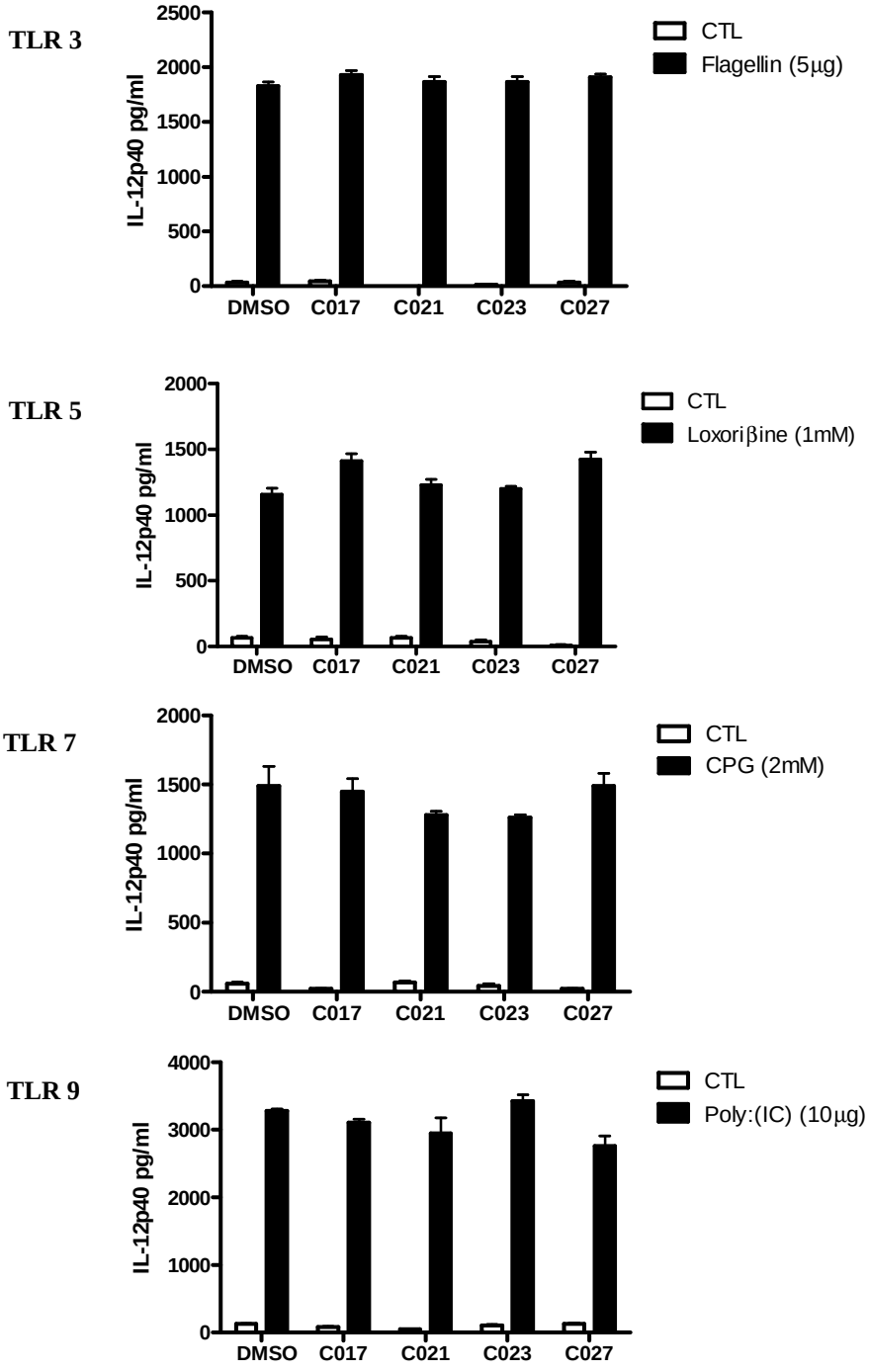


FIGURE 5.2: Second-round *M.membrancea* fractions do not suppress IL-12p40 production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10μg), flagellin (5μg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-12p40 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***$p<0.001$, **$p<0.01$, *$p<0.05$ comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

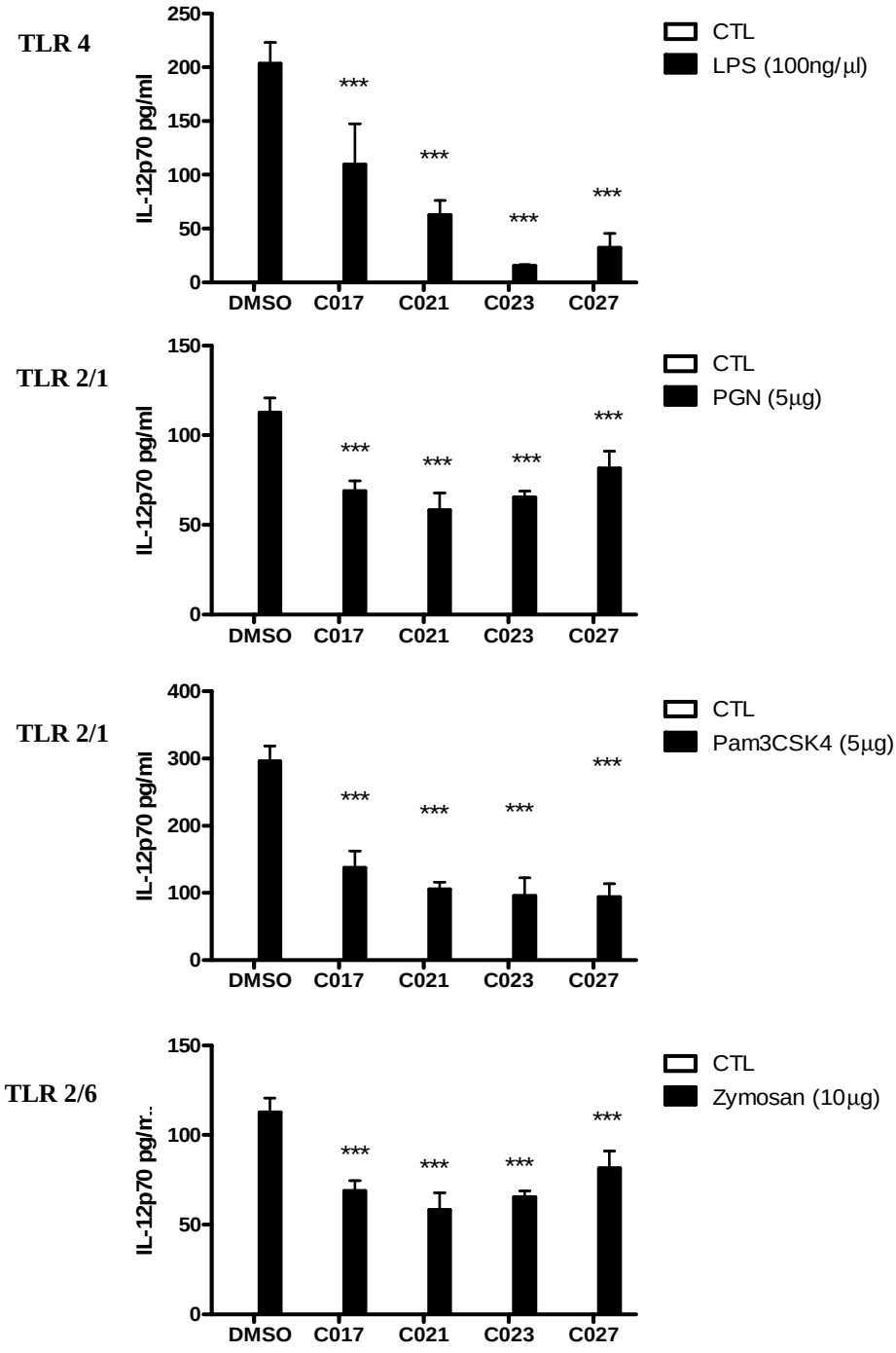


FIGURE 5.3: Second-round *M.membrancea* fractions suppress IL-12p70 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of IL-12p70 using specific immunoassays. Results are ± SEM of quadruplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

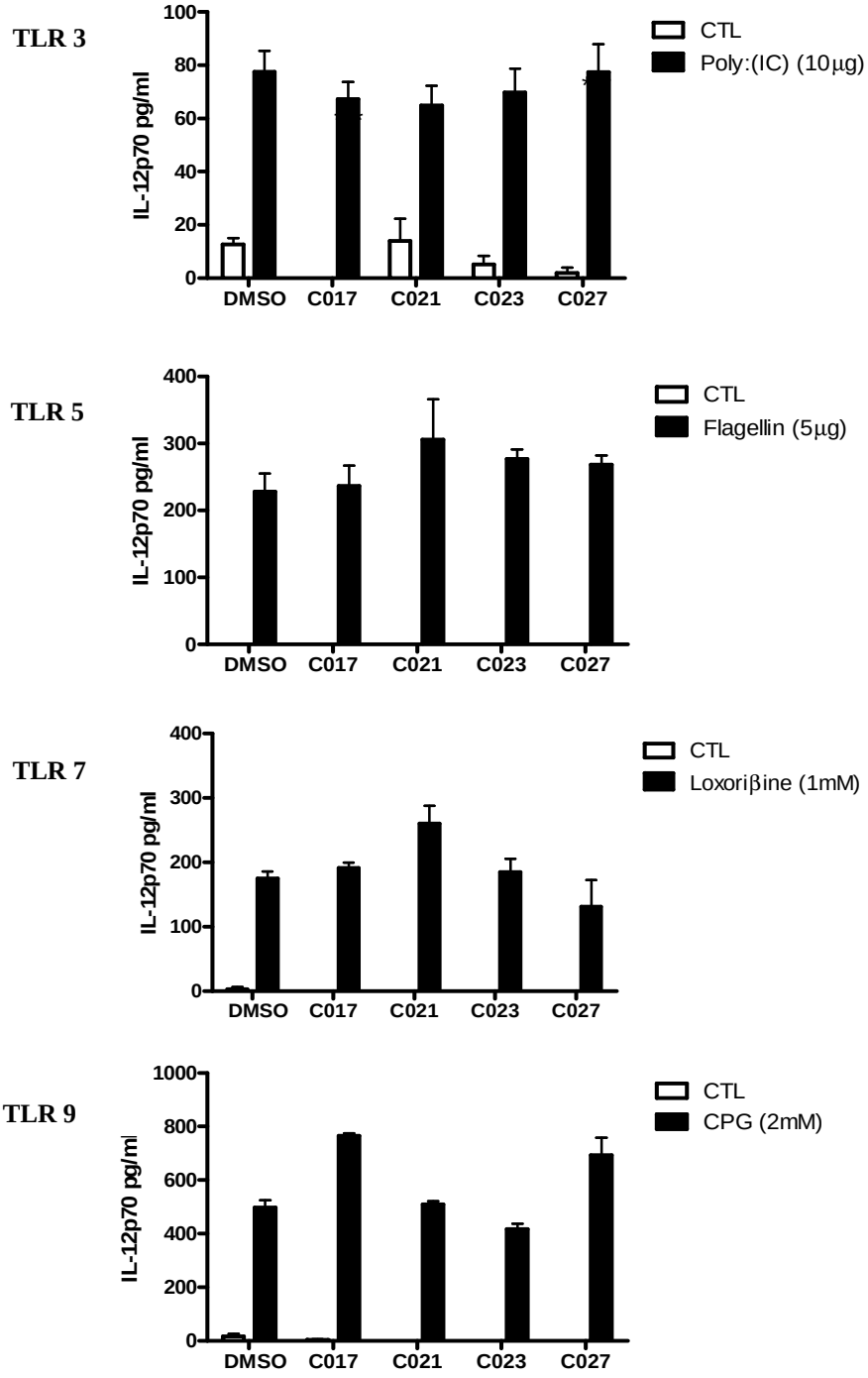


FIGURE 5.4: Second-round *M.membrancea* fractions do not suppress IL-12p70 production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10µg), flagellin (5µg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-12p70 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

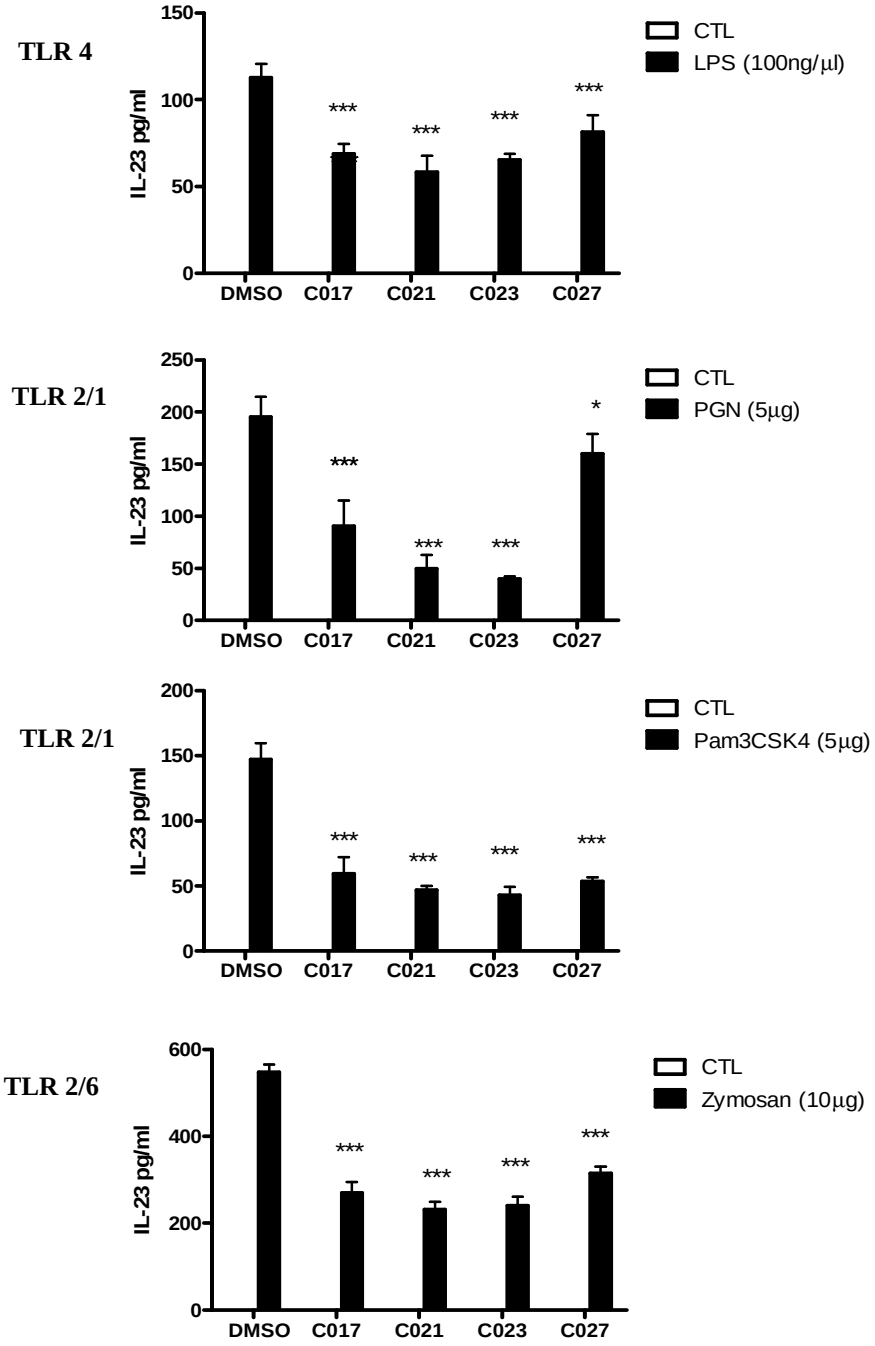


FIGURE 5.5: Second-round *M.membrancea* fractions suppress IL-23 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of IL-23 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

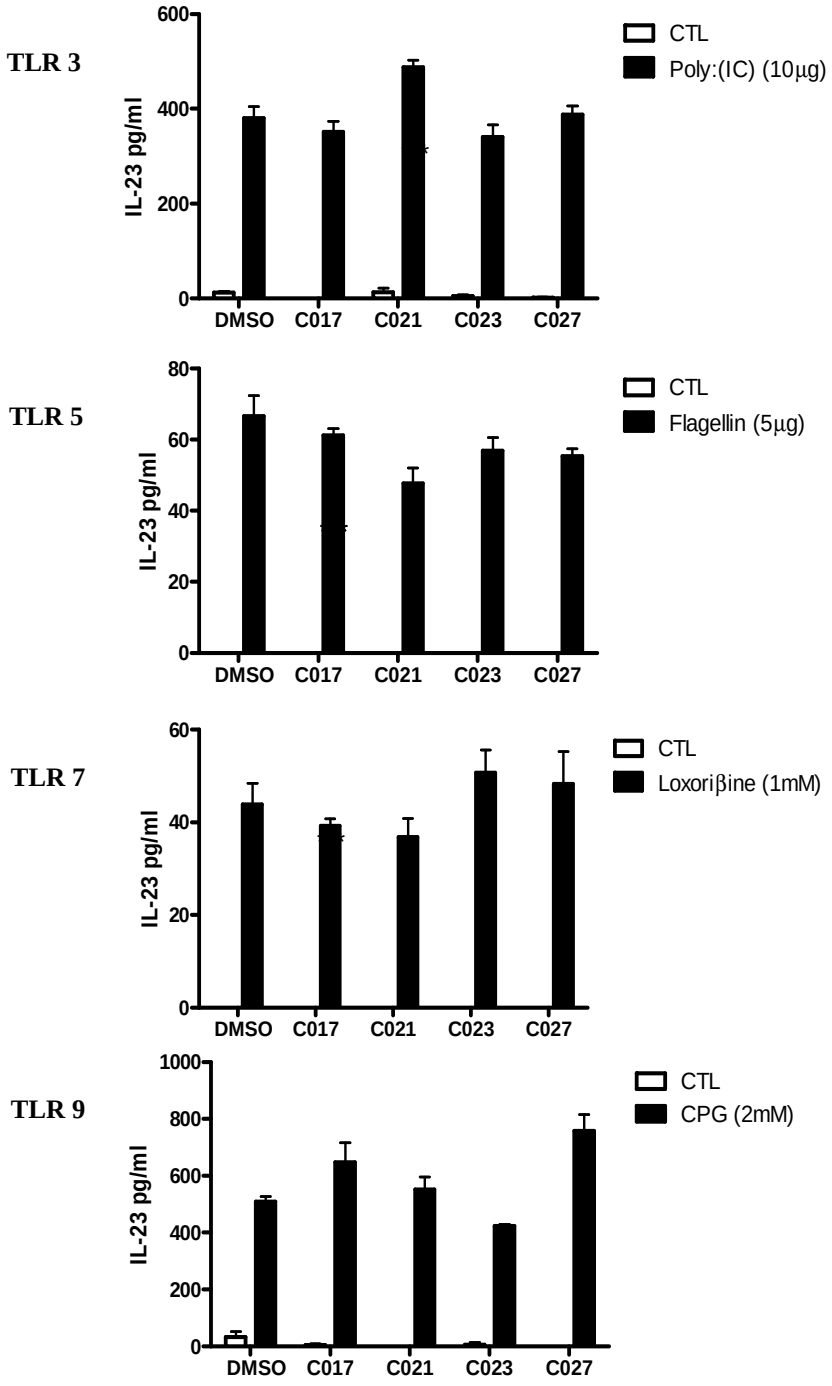


FIGURE 5.6: Second-round *M.membrancea* fractions do not suppress IL-23 production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10µg), flagellin (5µg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-23 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

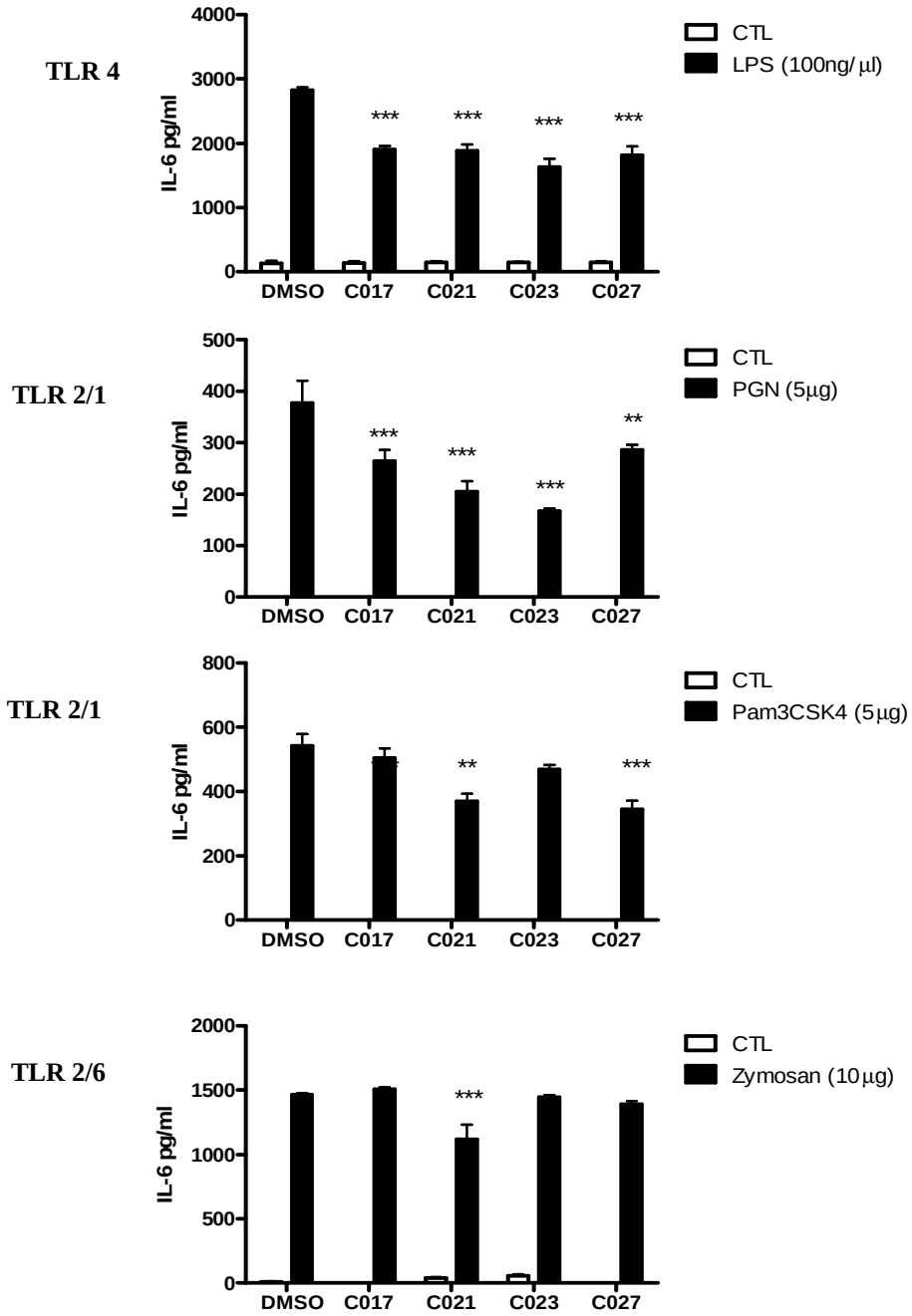


FIGURE 5.7: Second-round *M.membrancea* fractions suppress IL-6 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of IL-6 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

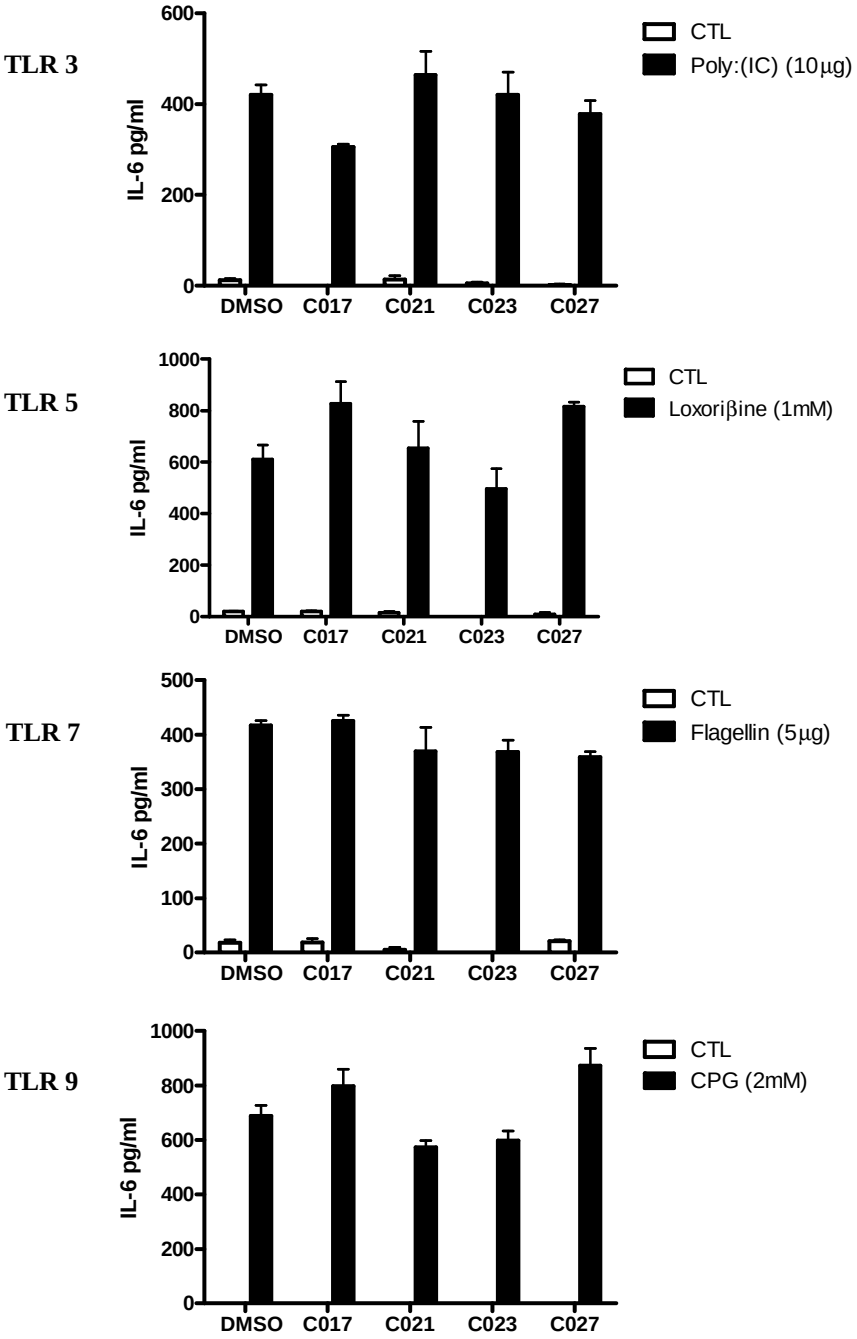


FIGURE 5.8: Second-round *M.membrancea* fractions do not suppress IL-6 production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10µg), flagellin (5µg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-6 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

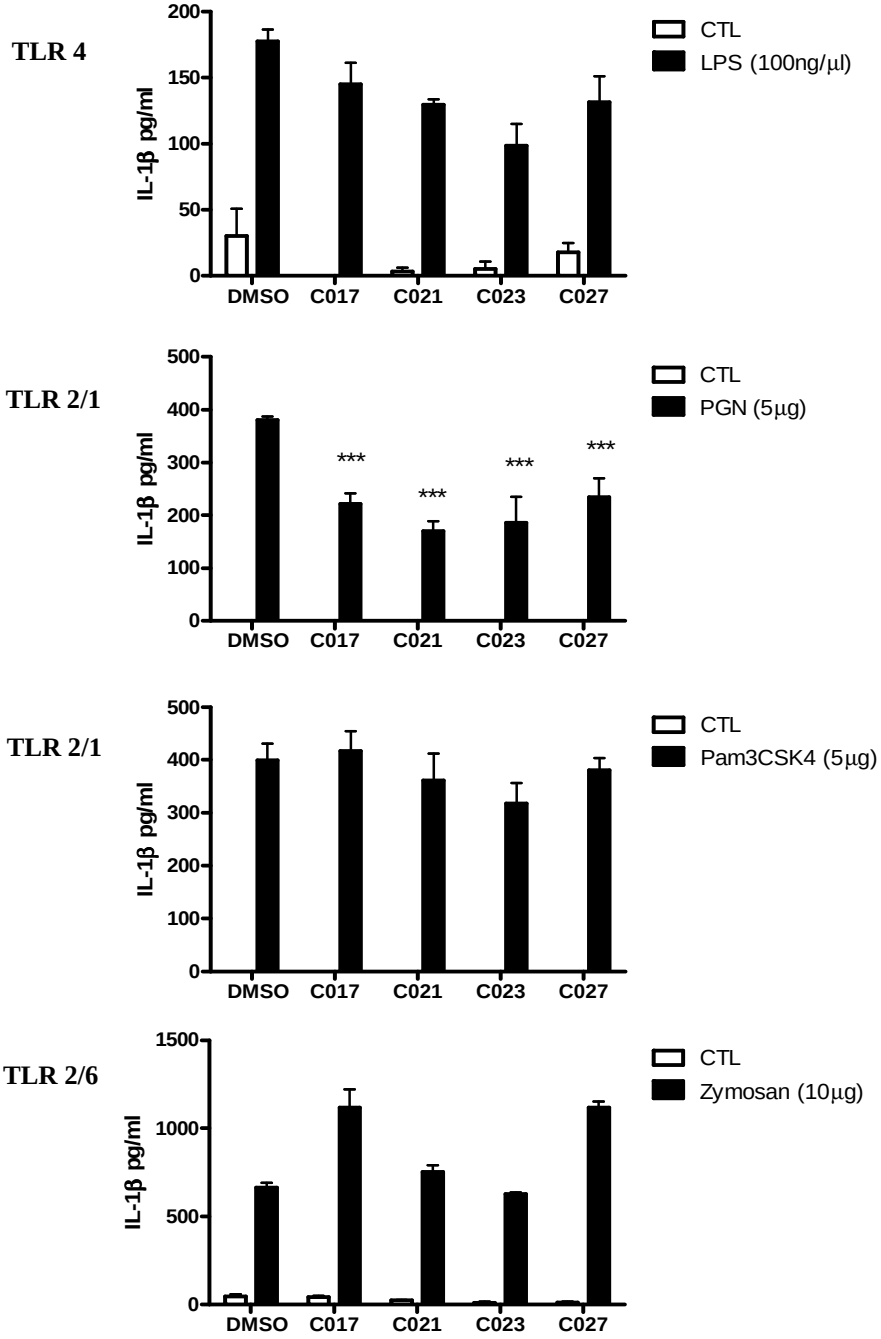


FIGURE 5.9: Second-round *M.membrancea* fractions suppress IL-1β production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of IL-1β using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

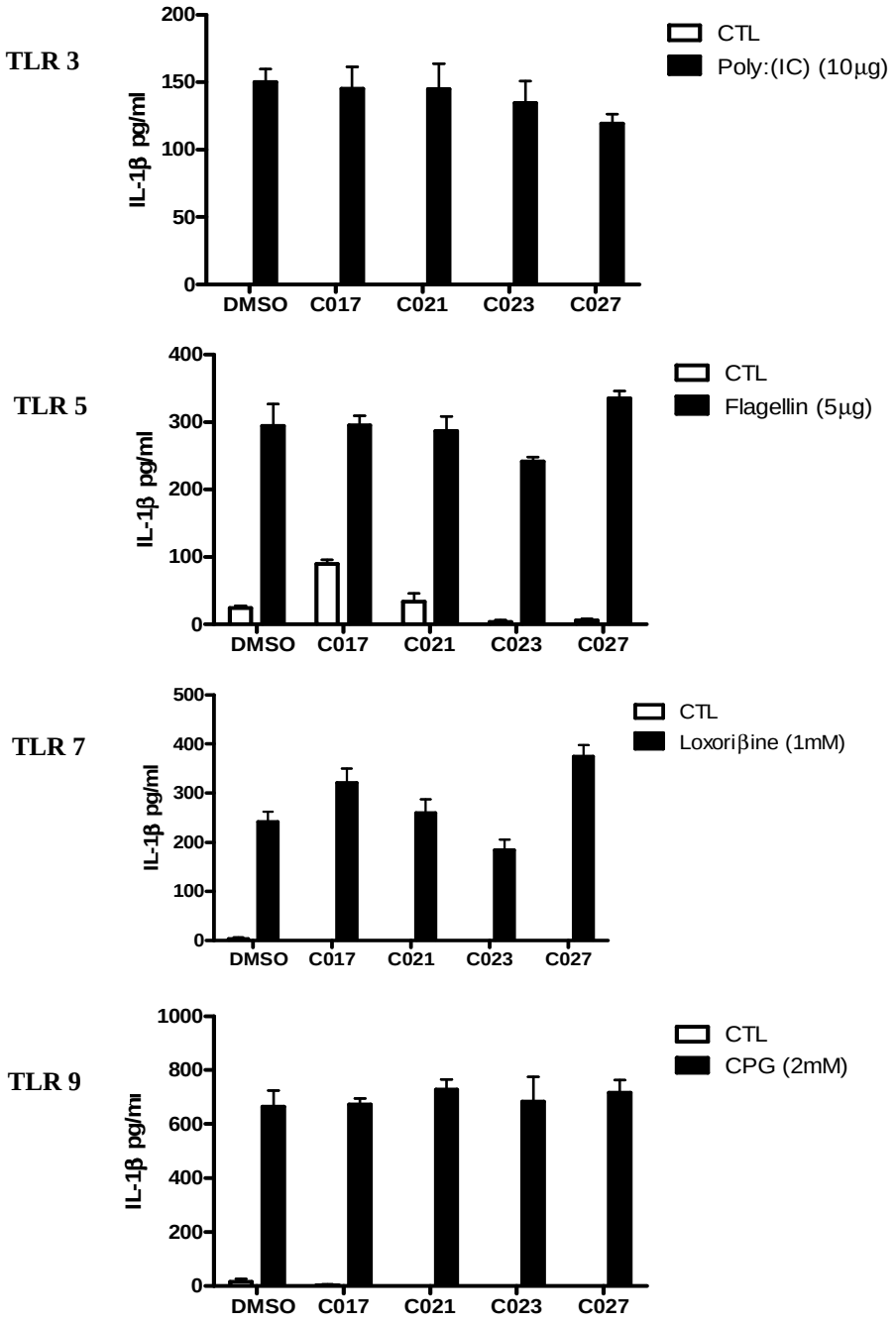


FIGURE 5.10: Second-round *M.membrancea* fractions do not suppress IL-1β production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10µg), flagellin (5µg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-1β using specific immunoassays.Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

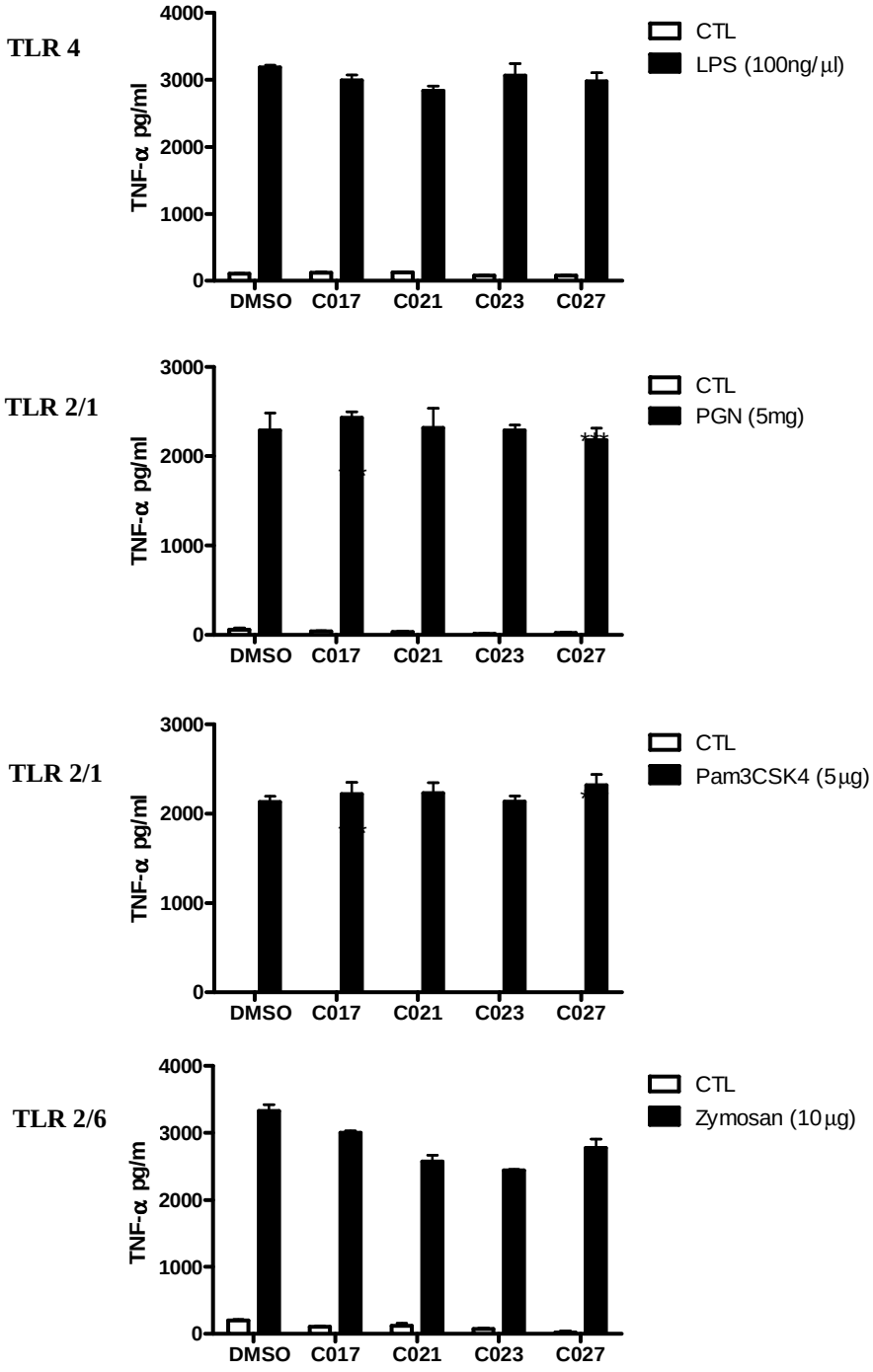


FIGURE 5.11: Second-round *M.membrancea* fractions do not suppress TNF-α production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of TNF-α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

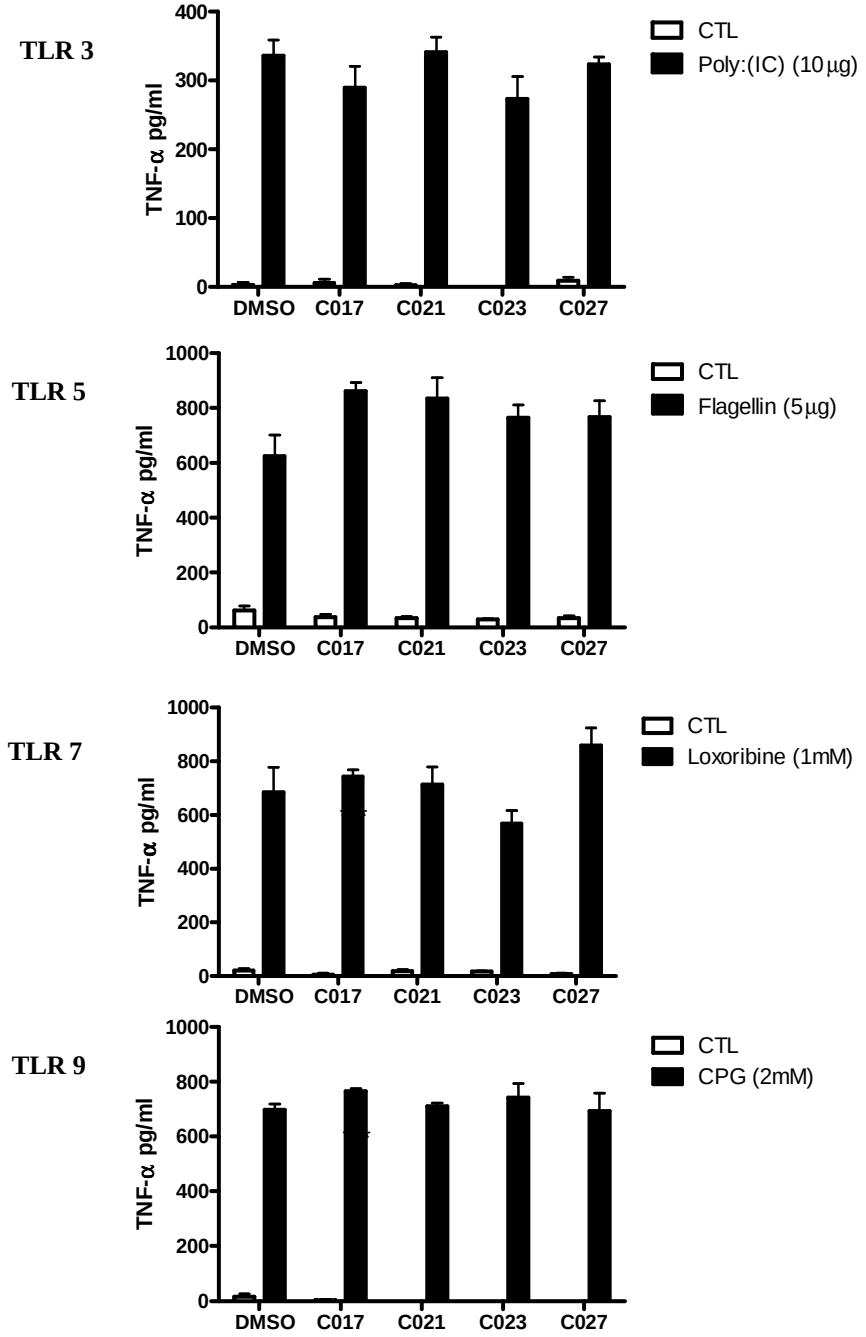


FIGURE 5.12: Second-round *M.membrancea* fractions do not suppress TNF-α production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10μg), flagellin (5μg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of TNF-α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

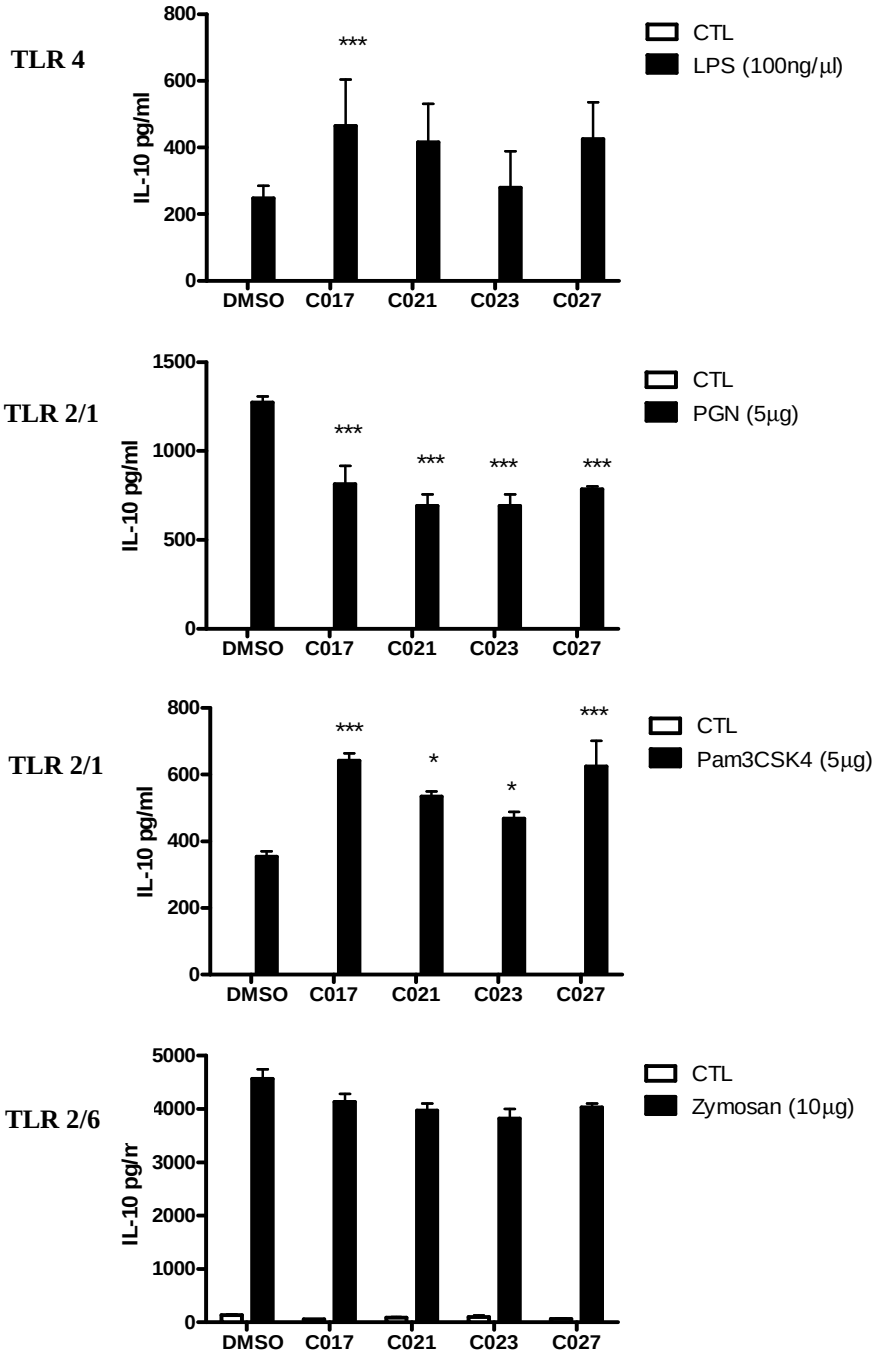


FIGURE 5.13: Second-round *M.membrancea* fractions enhance IL-10 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either LPS (100ng/ml), PGN (5µg), Pam_3CSK_4 (5µg) or zymosan (10µg). Supernatants were recovered after 24 hrs and assessed for levels of IL-10 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

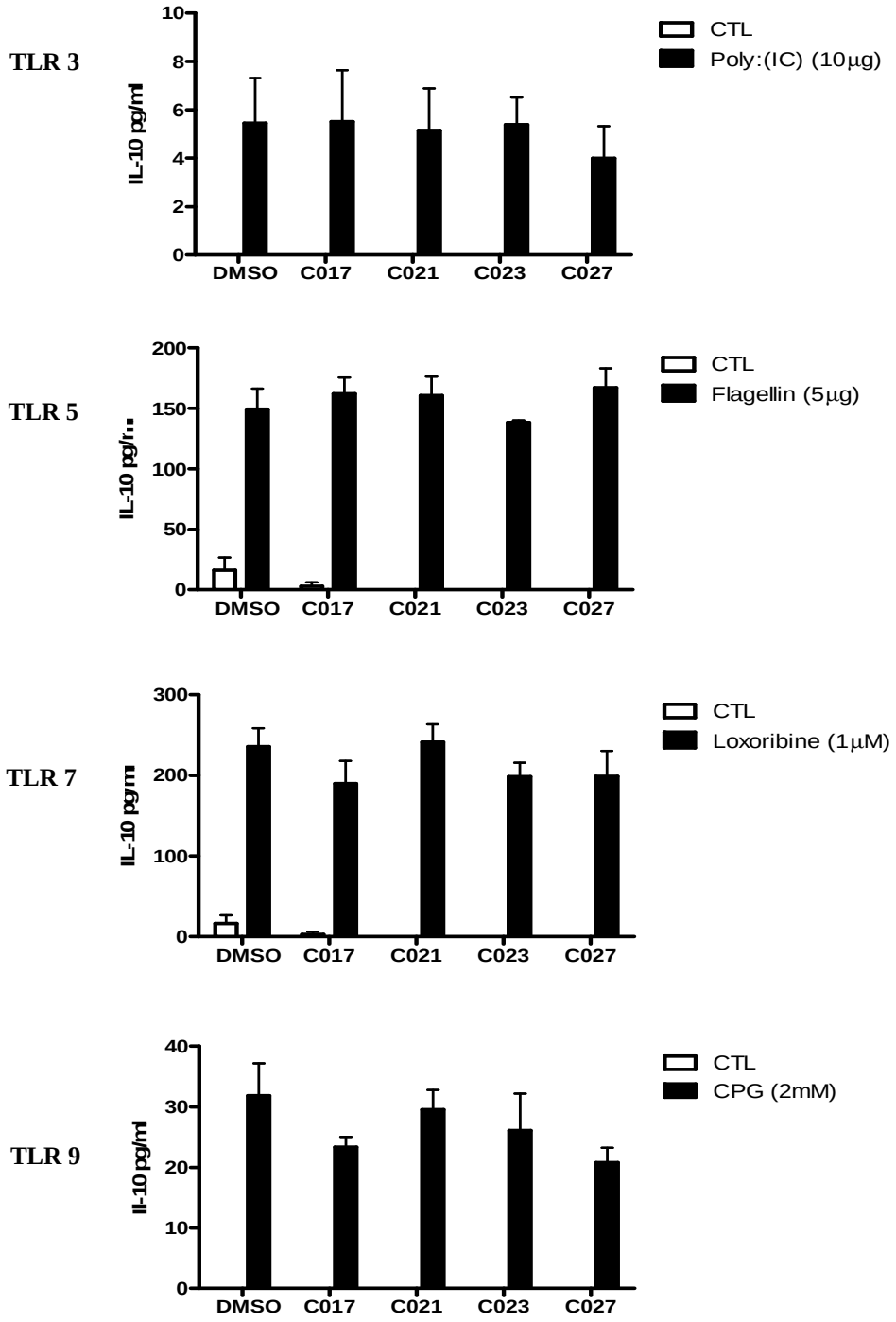


FIGURE 5.14: Seond-round *M.membrancea* fractions do not enhance IL-10 production in DCs following stimulation with TLR3, TLR5, TLR7 and TLR9 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control), second fractions of *M.membranacea* (C017, C021, C023, C027) for 1 hr prior to stimulation with either poly:(IC) (10µg), flagellin (5µg), loxoribine (1mM) or CPG (2mM). Supernatants were recovered after 24 hrs and assessed for levels of IL-10 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

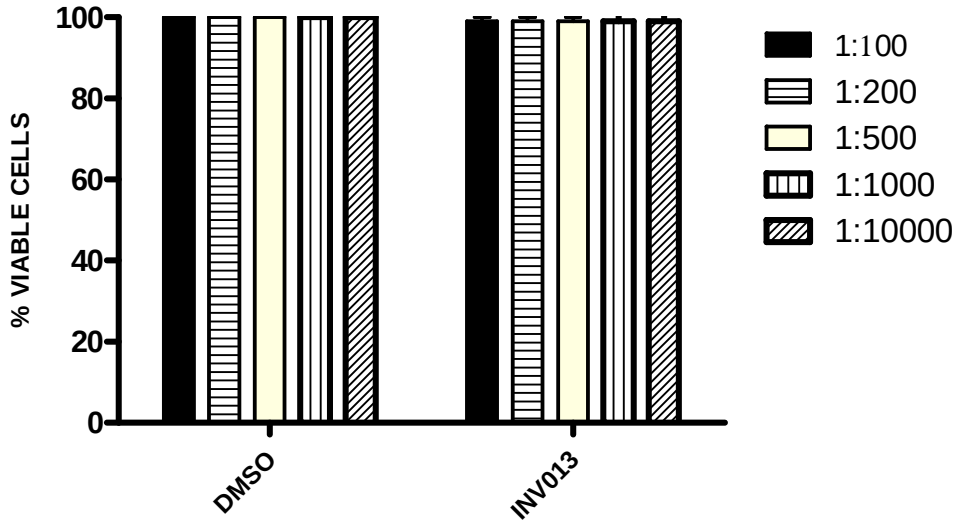


FIGURE 5.15: The concentrations of the purified marine compound, INV013, used do not significantly affect the viability of dendritic cells (DCs).
BMDCs were grown for 7 days and then treated with either a range of dilutions (1:100, 1:200, 1:500, 1:1000, 1:10000) of DMSO (vehicle control) or purified compound, INV013 for 24 hrs. Following 24 hr treatment cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution – Promega, WI, USA). Results are expressed as a percentage of untreated cells.

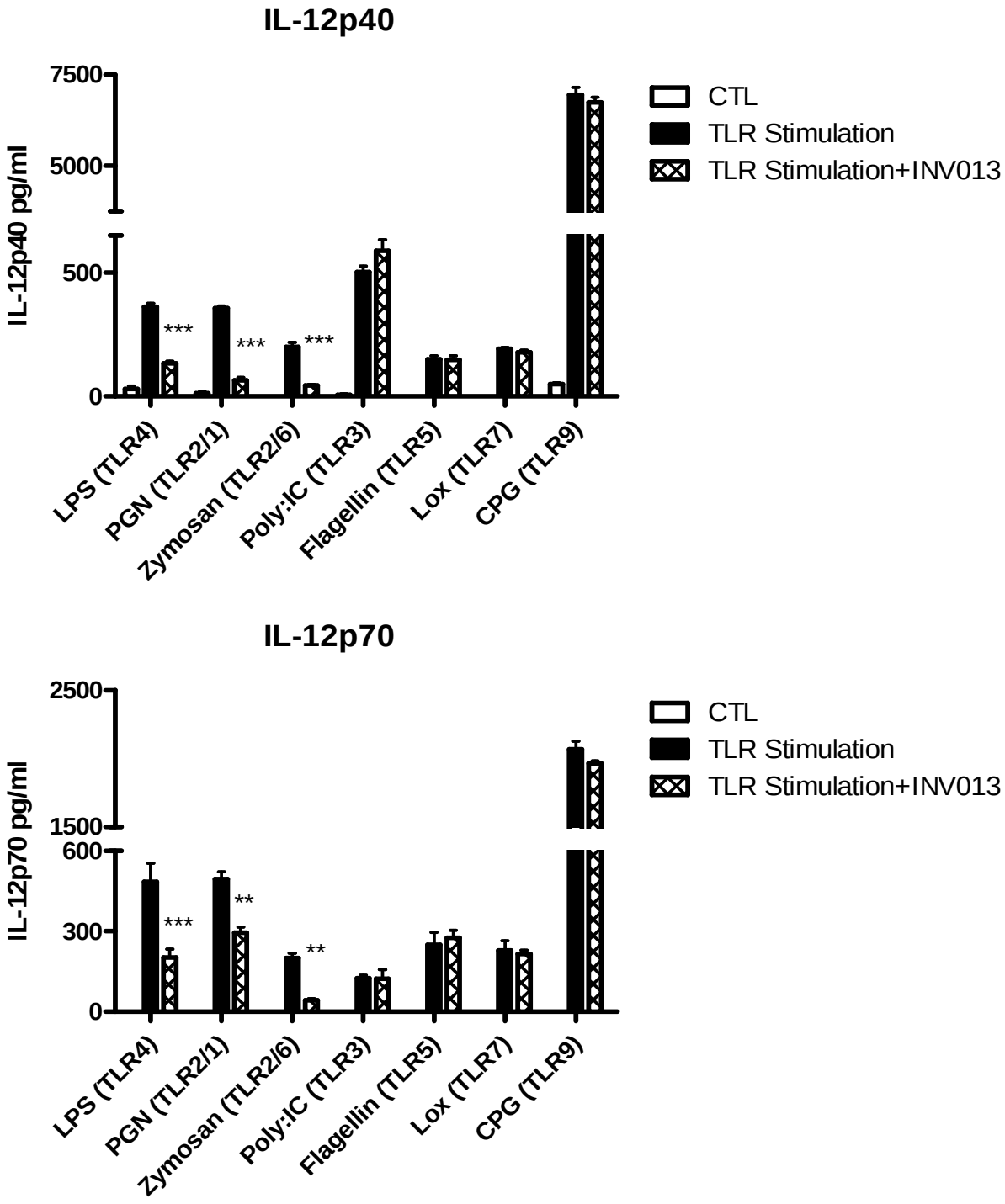


FIGURE 5.16: The purified marine compound, INV013, suppresses IL-12p40 and IL-12p70 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 for 1 hr prior to stimulation with numerous TLR ligands. Supernatants were recovered after 24 hrs and assessed for levels of IL-12p40 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

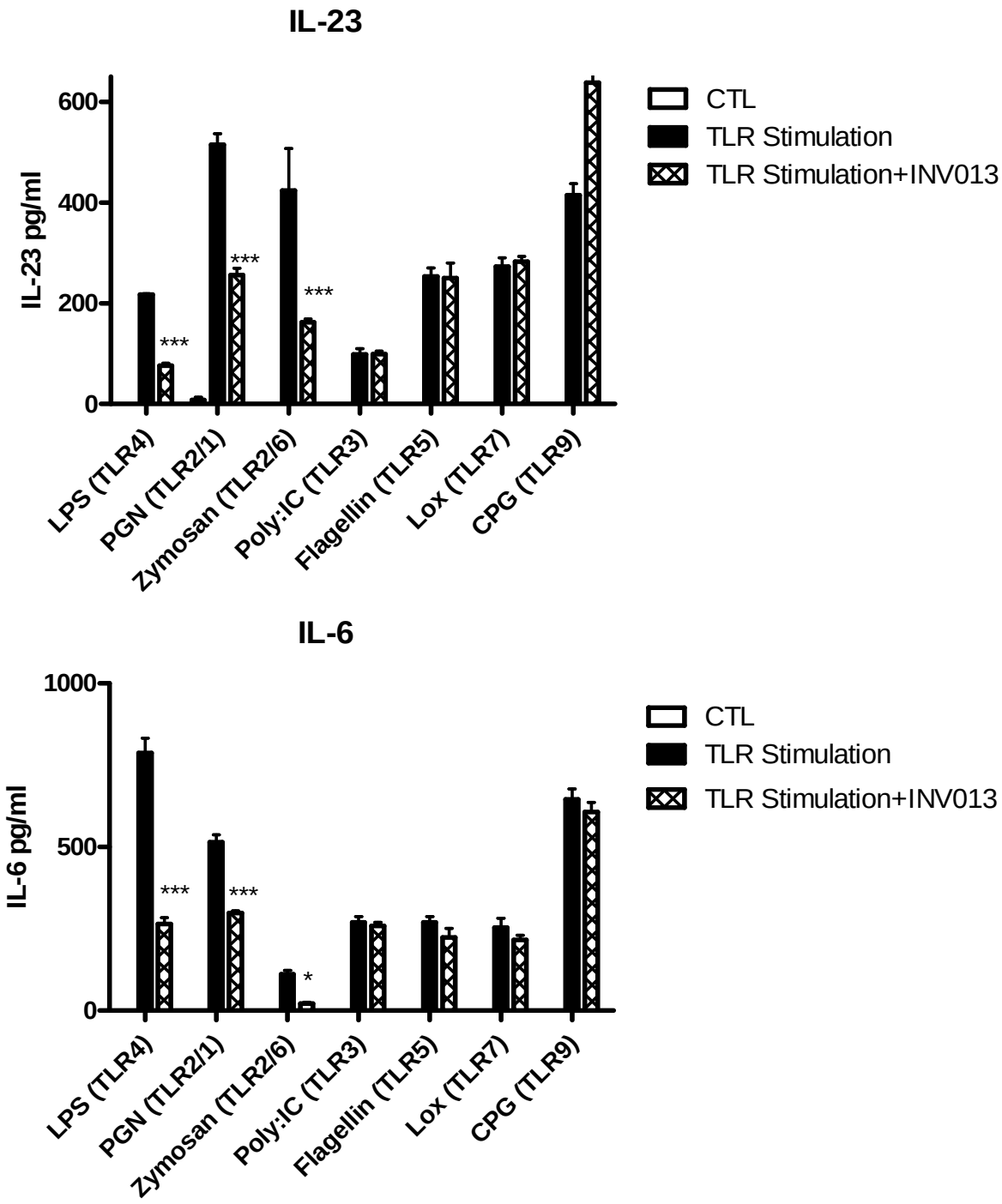


FIGURE 5.17: The purified marine compound, INV013, suppresses IL-23 and IL-6 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 for 1 hr prior to stimulation with numerous TLR ligands. Supernatants were recovered after 24 hrs and assessed for levels of IL-23 and IL-6 using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***$p<0.001$, **$p<0.01$, *$p<0.05$ comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

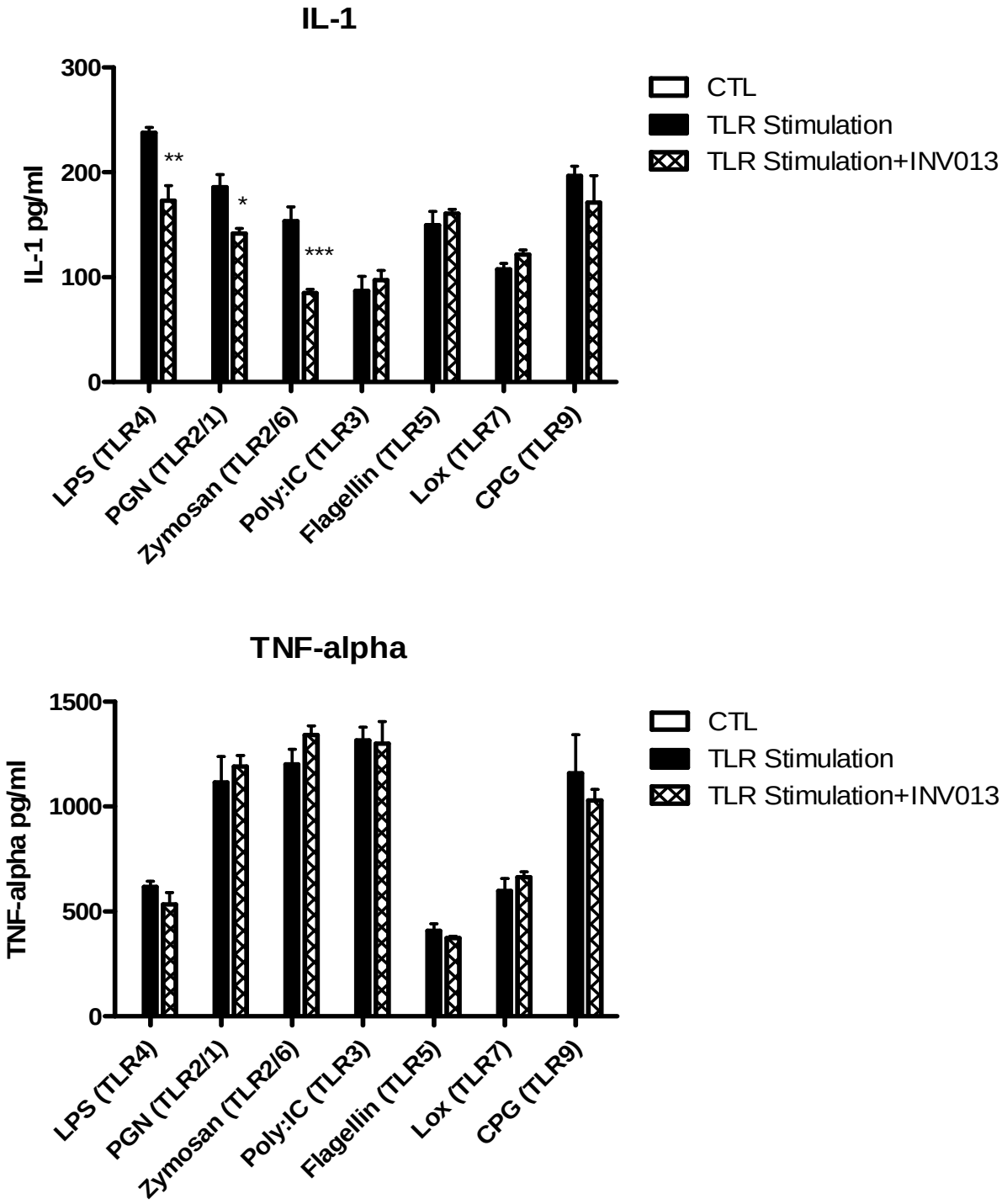


FIGURE 5.18: The purified marine compound, INV013, suppresses IL-1β production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 for 1 hr prior to stimulation with numerous TLR ligands. Supernatants were recovered after 24 hrs and assessed for levels of IL-1β and TNF-α using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

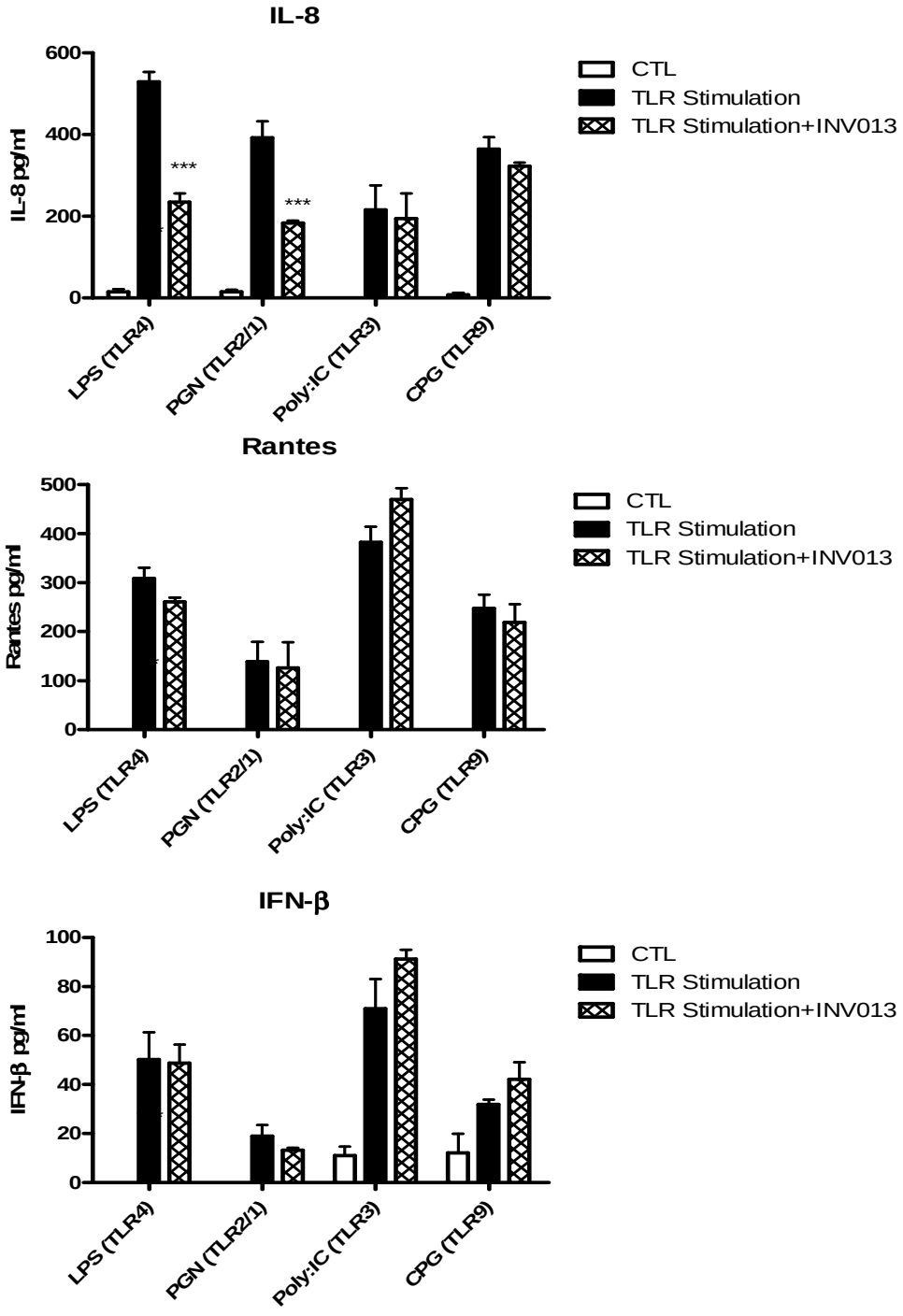


FIGURE 5.19: The purified marine compound, INV013, suppresses IL-8 production in DCs following stimulation with TLR2 and TLR4 ligands. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 1 hr prior to stimulation with numerous TLR ligands.. Supernatants were recovered after 24 hrs and assessed for levels of IL-8, Rantes and IFN-β using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 comparing TLR ligand-activated group in the presence/absence of marine fractions as determined by one-way ANOVA test

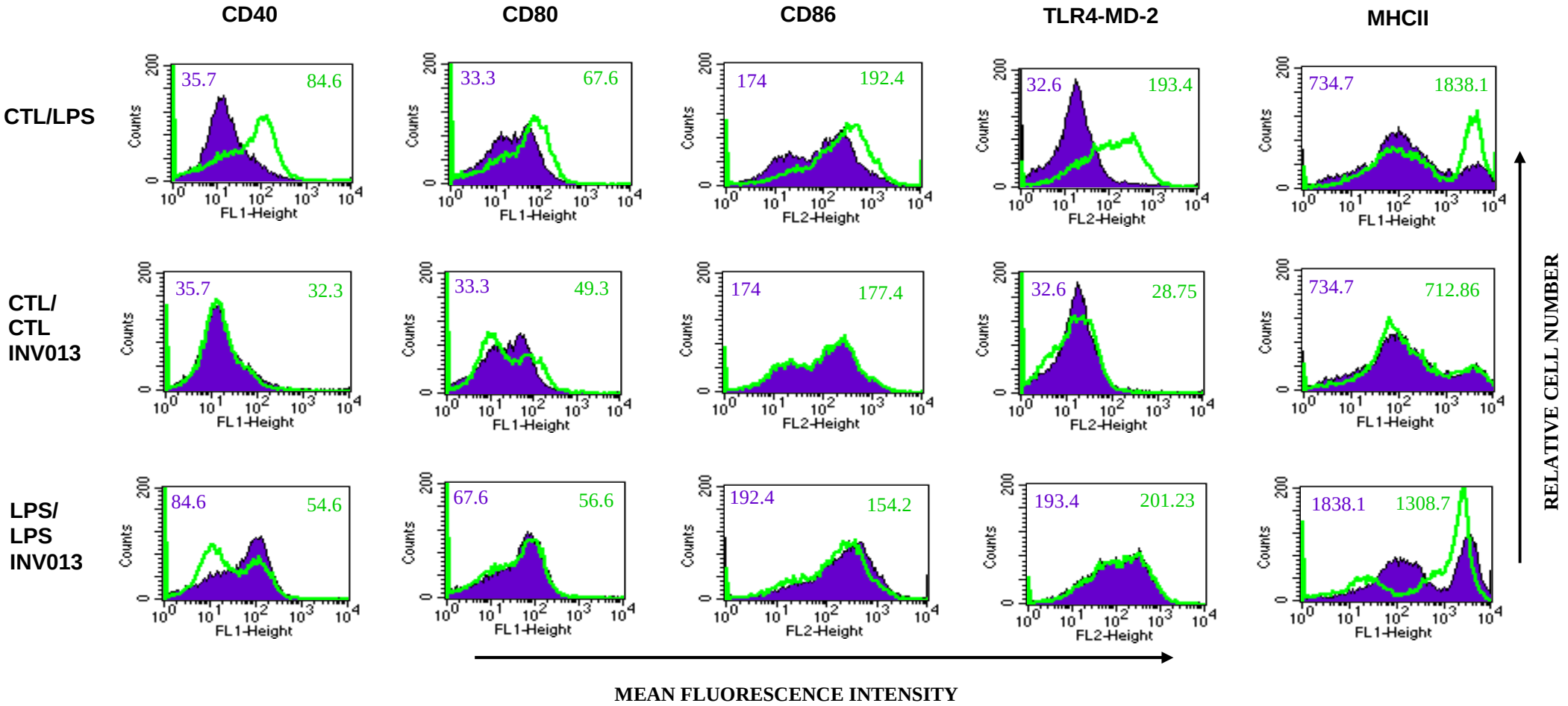


FIGURE 5.20: The purified marine compound, INV013, modulates the expression of CD40, CD86 and MHCII on the surface of DCs following stimulation with TLR4 ligand. BMDC were differentiated in the presence of GMCSF for 7 days and then treated with DMSO or INV013 for 1 hr prior to stimulation with LPS (100ng/ml).Subsequently, cells were washed and stained with specific antibodies. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled histogram) and LPS-stimulated DCs (green line). **Middle row:** DMSO-treated DCs vs INV013-treated DCs. **Bottom row:** LPS-stimulated DCs (filled purple histogram) vs. LPS induced INV013-treated DCs (green line). MFI values for INV013-treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel.

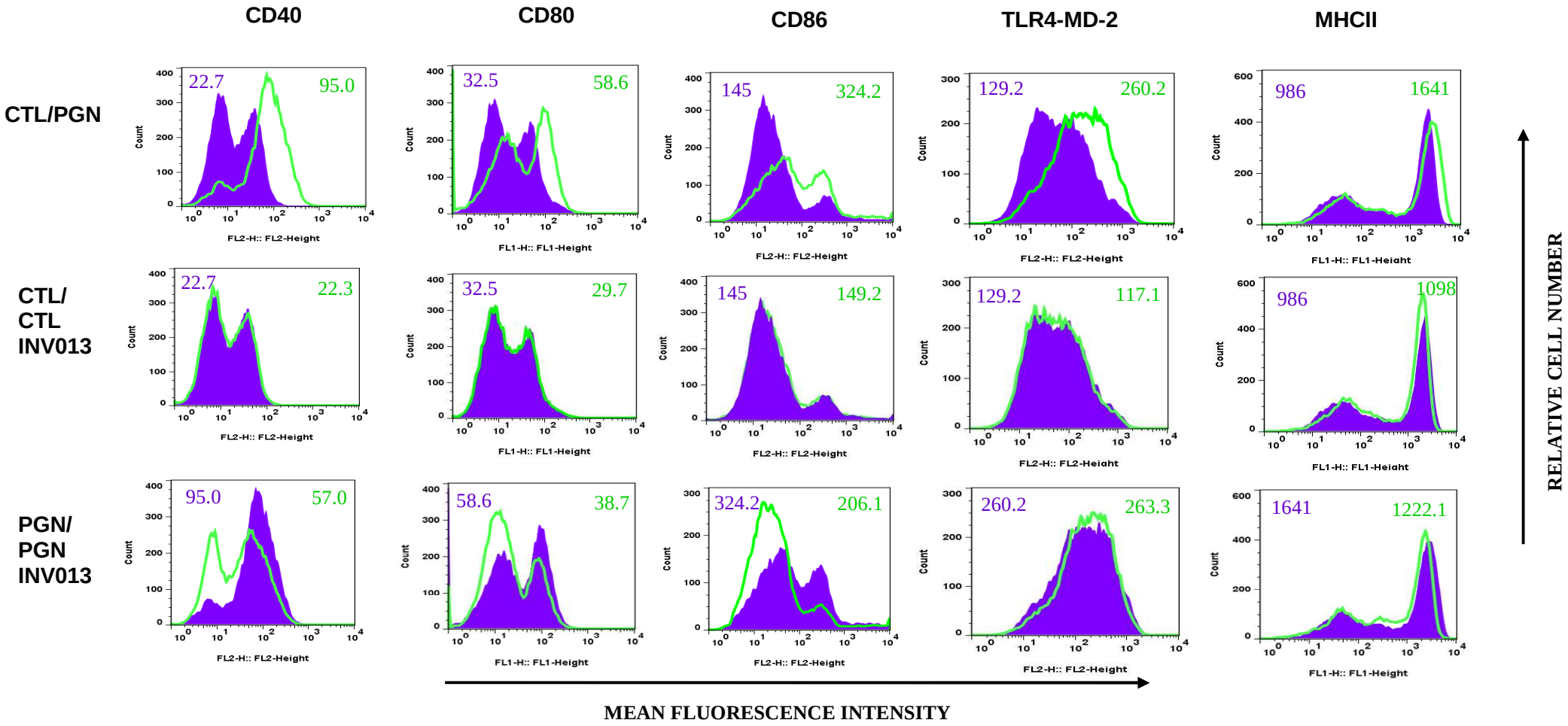


FIGURE 5.21: The purified marine compound, INV013, modulates the expression of CD40, CD80, CD86 and MHCII on the surface of DCs following stimulation with TLR2 ligand. BMDC were differentiated in the presence of GMCSF for 7 days and then treated with DMSO or INV013 for 1 hr prior to stimulation with PGN (5µg).Subsequently, cells were washed and stained with specific antibodies. Results of flow cytometric analysis and corresponding MFI values are shown. **Top row:** DMSO-treated DCs (filled histogram) and PGN-stimulated DCs (green line). **Middle row:** DMSO-treated DCs vs INV013-treated DCs. **Bottom row:** PGN-stimulated DCs (filled purple histogram) vs. PGN induced INV013-treated DCs (green line). MFI values for INV013-treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel.

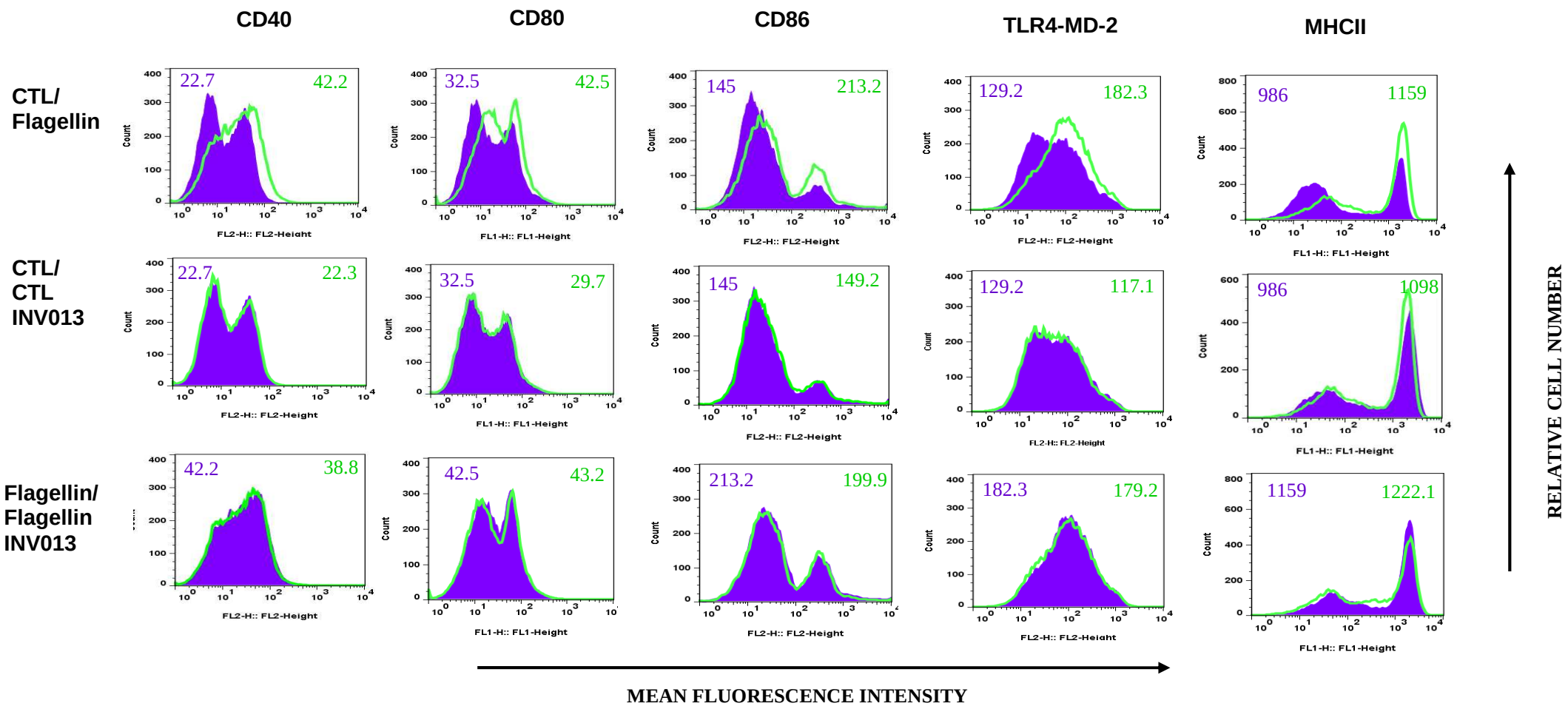


FIGURE 5.22: The purified marine compound, INV013, does not modulate the expression any of on the surface markers of DCs following TLR5 stimulation. BMDC were differentiated in the presence of GMCSF for 7 days and then treated with DMSO or INV013 for 1 hr prior to stimulation with flagellin (100ng/ml).Subsequently, cells were washed and stained with specific antibodies. Results of flow cytometric analysis and corresponding MFI values are shown **Top row:** DMSO-treated DCs (filled histogram) and flagellin-stimulated DCs (green line). **Middle row:** DMSO-treated DCs vs INV013-treated DCs. **Bottom row:** flagellin-stimulated DCs (filled purple histogram) vs. flagellin induced INV013-treated DCs (green line). MFI values for INV013-treated cells are also displayed on all histograms for comparison of those of DMSO groups indicated in the top panel.

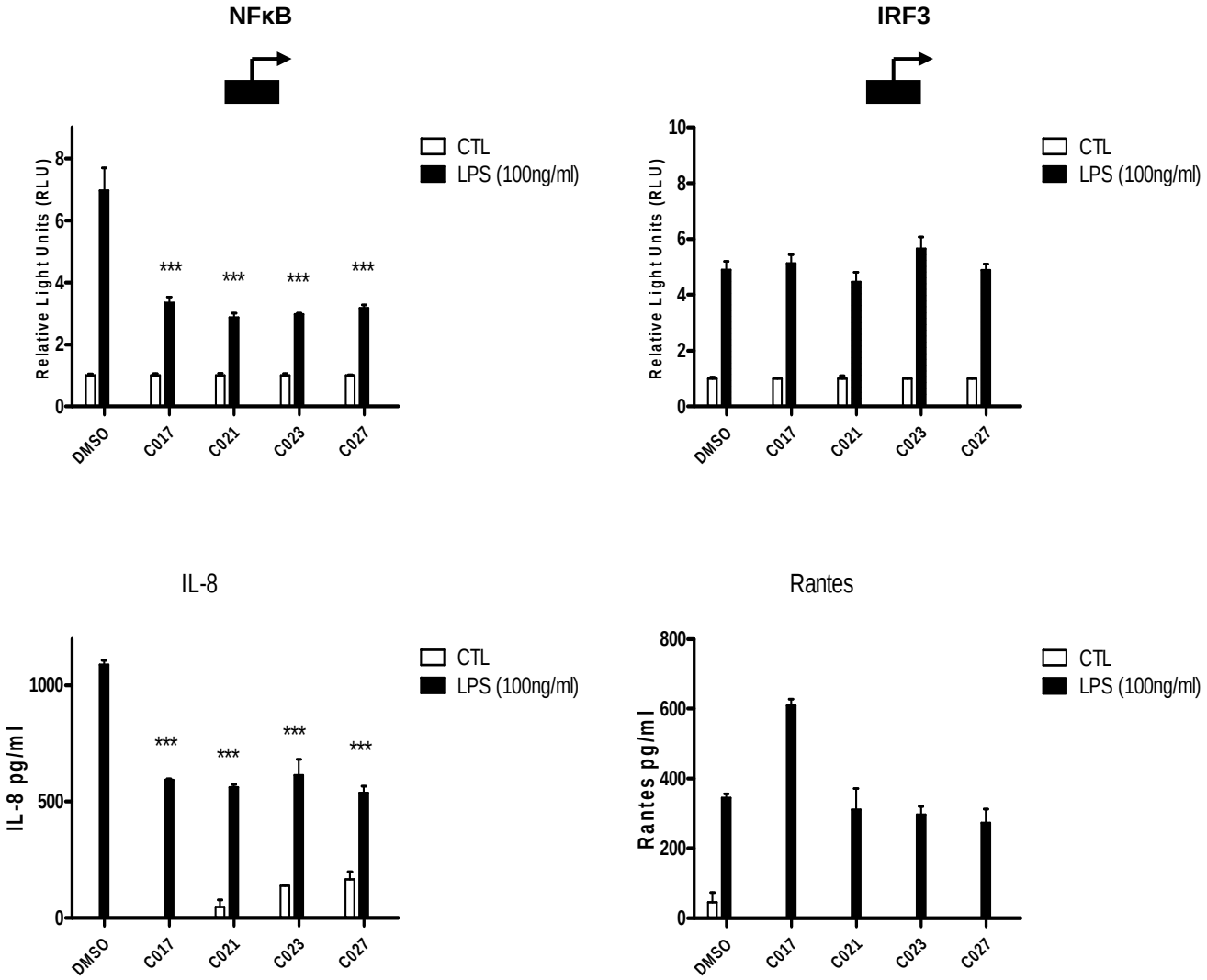


FIGURE 5.23: Second-round *M.membrancea* fractions suppress activation of NFκB but not IRF3 activation downstream of TLR4. HEK-MTC cells were transiently transfected with either an NFκB or ISRE luciferase reporter plasmid for 24hrs. Subsequently, HEK-MTC cells were treated with either DMSO (vehicle control) or second fractions of *M.membrancea* fractions (C017, C021, C023, C027) for 1hr prior to LPS activation. Induction of both transcription factors was assessed following 6 hr stimulation with LPS (100ng/ml). Supernatants were also recovered and levels for IL-8 and Rantes were measured using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 vs DMSO/LPS vs marine extract/LPS control determined by one-way ANOVA

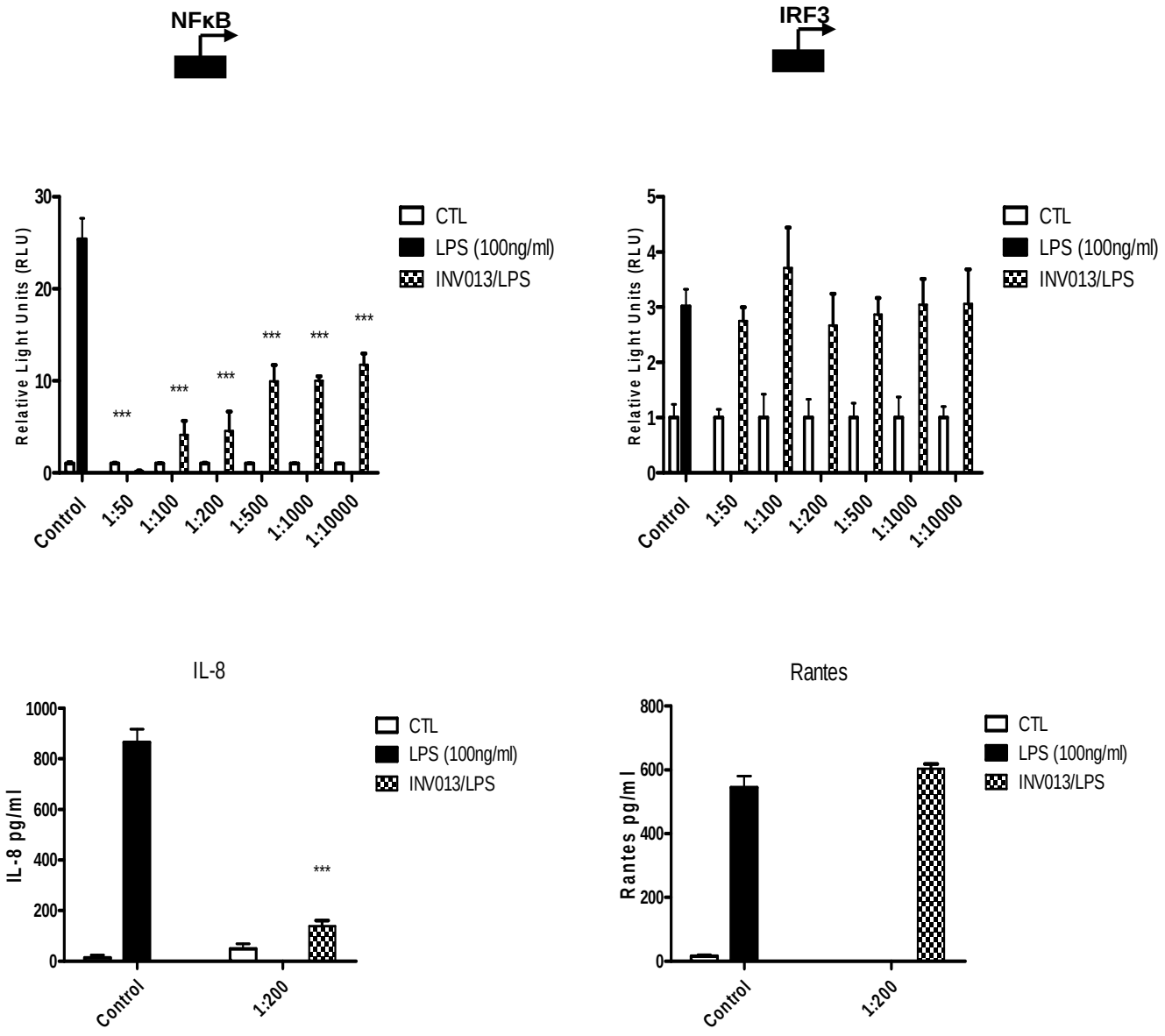


FIGURE 5.24: The purified marine compound, INV013, suppresses activation of NFκB but not IRF3 activation downstream of TLR4 in a dose dependant manner. HEK-MTC cells were transiently transfected with either an NFκB, ISRE luciferase reporter plasmid for 24hrs. Subsequently, HEK-MTC cells were treated with either DMSO (vehicle control) or INV013. Induction of both transcription factors was assessed following 6 hr stimulation with LPS (100ng/ml). Supernatants were also recovered and levels for IL-8 and Rantes were measured using specific immunoassays.
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 vs DMSO/LPS vs INV013/LPS control determined by one-way ANOVA

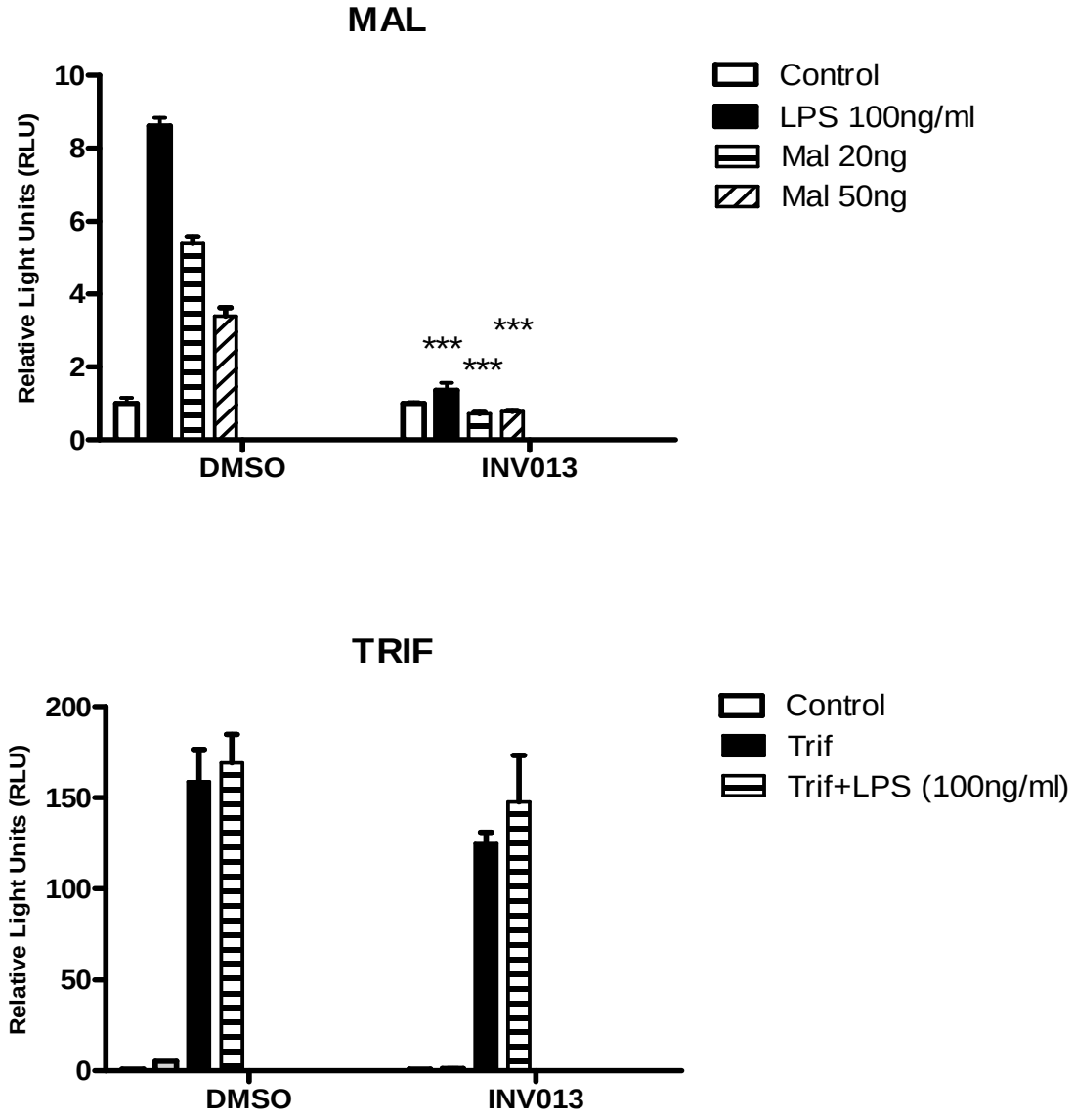
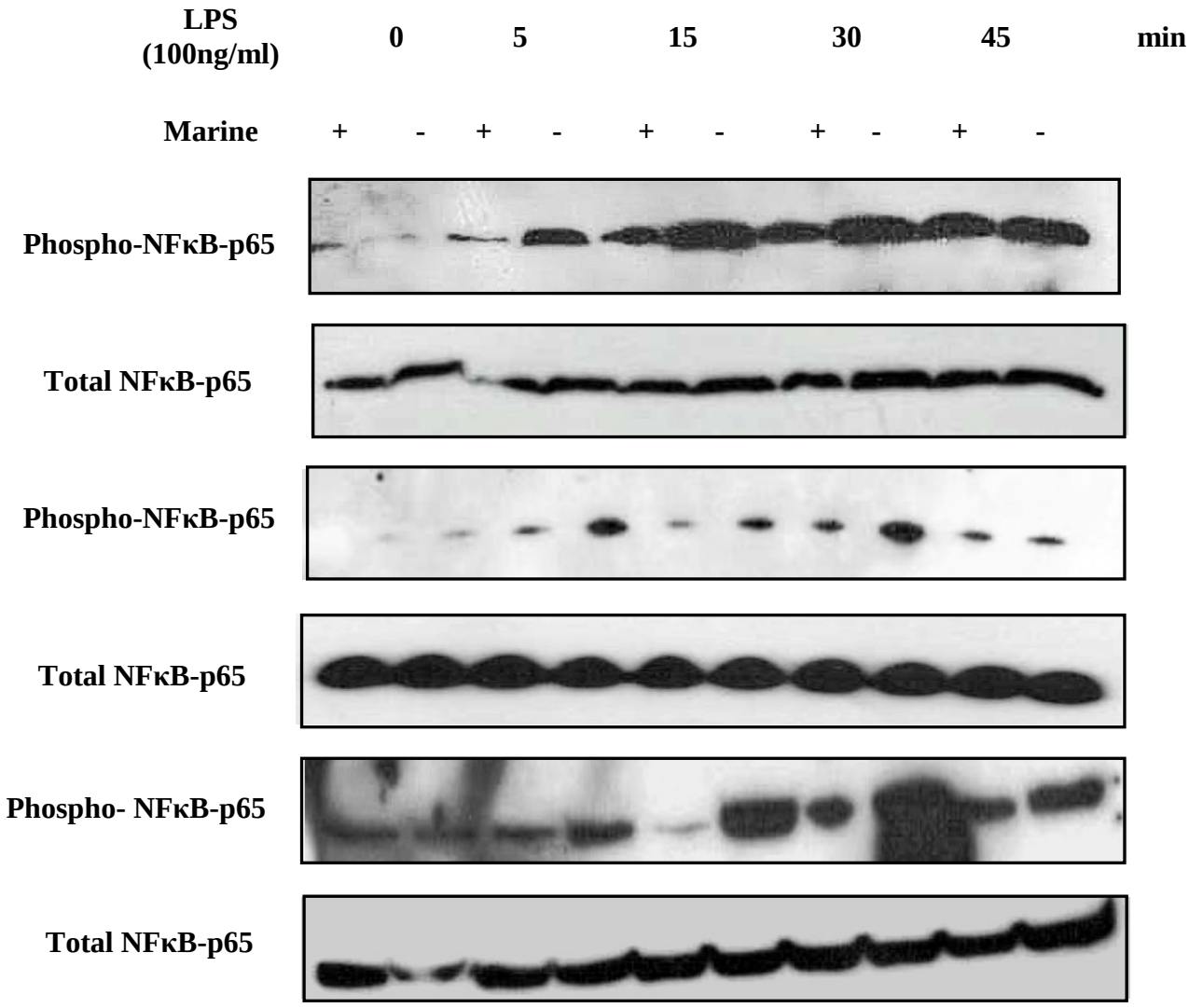


FIGURE 5.25: The purified marine compound, INV013, blocks Mal-driven activation of NFκB. HEK-MTC cells were transiently transfected with either an NFκB, Mal (20ng. 50ng), TRIF luciferase reporter plasmid for 24hrs. Cells were then cultured with DMSO (vehicle control) or INV013 for 1hr. Activation of NFκB was assessed following 6 hr stimulation with LPS (100ng/ml).
Results are ± SEM of quadruplicate assays and represent three independent experiments.
***p<0.001, **p<0.01, *p<0.05 vs DMSO/Mal vs marine extract/Mal vehicle control determined by one-way ANOVA



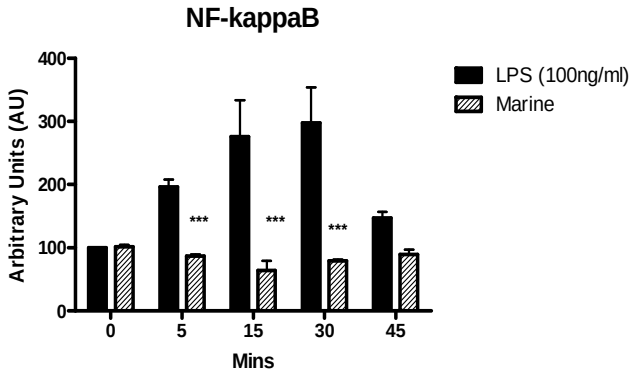
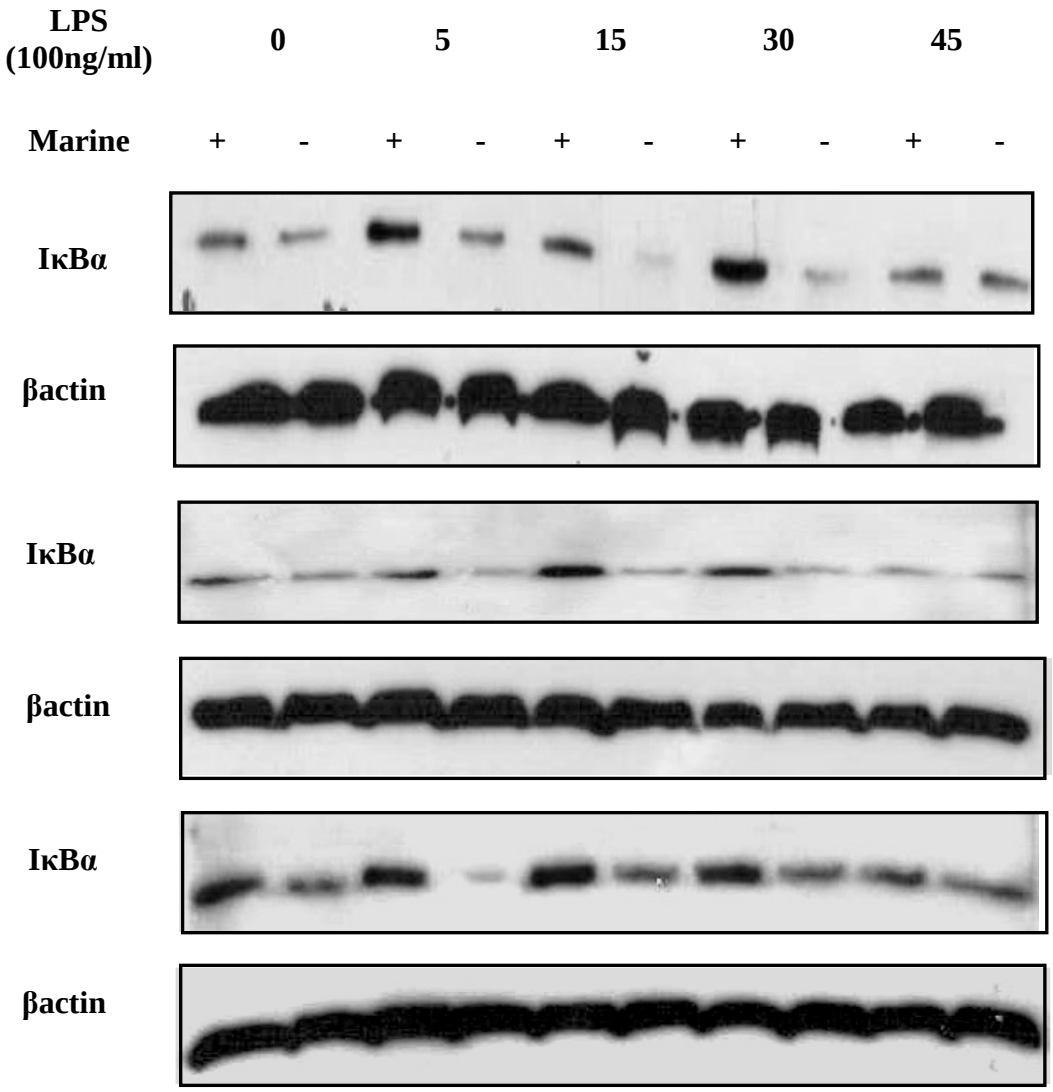


FIGURE 5.26: The purified marine compound, INV013, suppresses phosphorylation of NFκBp65 in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 (1:200 dilution) 1 hr prior to stimulation with 100ng/ml of LPS over a time-course after which cells were lysed and immunoblotted for phospho-NFκBp65. Total cellular levels of total NFκBp65 were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of phosphor-NFκBp65 expression in arbitrary units (AU) is shown.



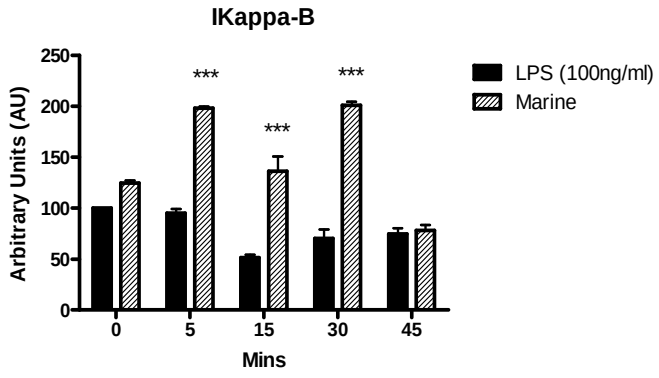
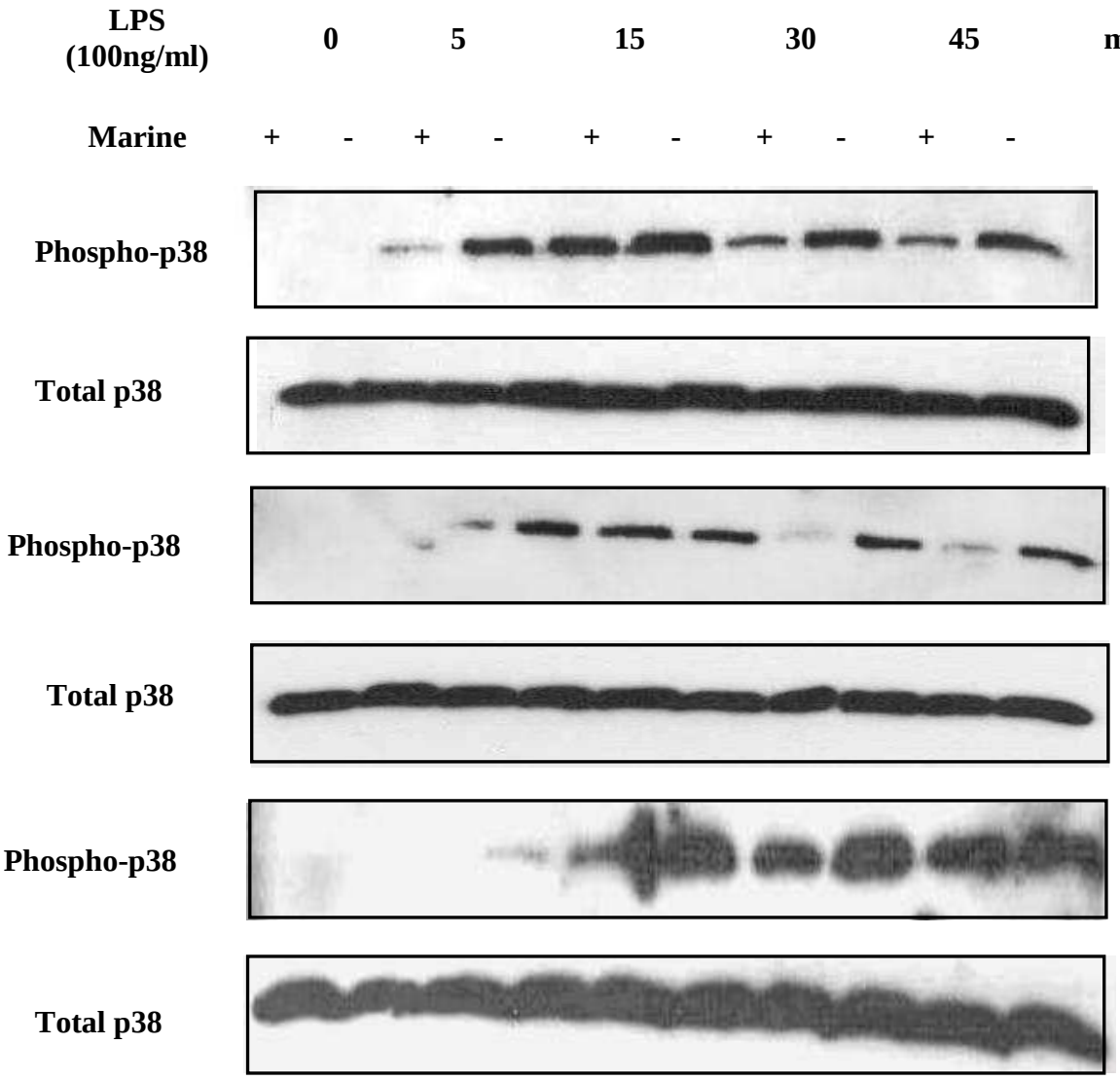


FIGURE 5.27: The purified marine compound, INV013, suppresses the degradation of IκBα in DC. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 (1:200 dilution) 1 hr prior to stimulation with 100ng/ml of LPS over a time-course, after which cell were lysated and immunoblotted for IκBα. Total cellular levels of βactin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of IκBα expression in arbitrary units (AU) is shown.



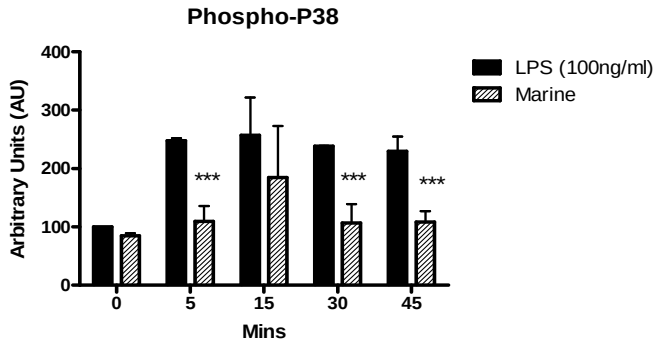


FIGURE 5.28: The purified marine compound, INV013, suppresses phosphorylation of p38 in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 (1:200 dilution) 1 hr prior to stimulation with 100ng/ml of LPS over a time-course, after which cells were lysed and immunoblotted for phospho-p38. Total cellular levels of total p38 were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of phospho-p38 expression in arbitrary units (AU) is shown.

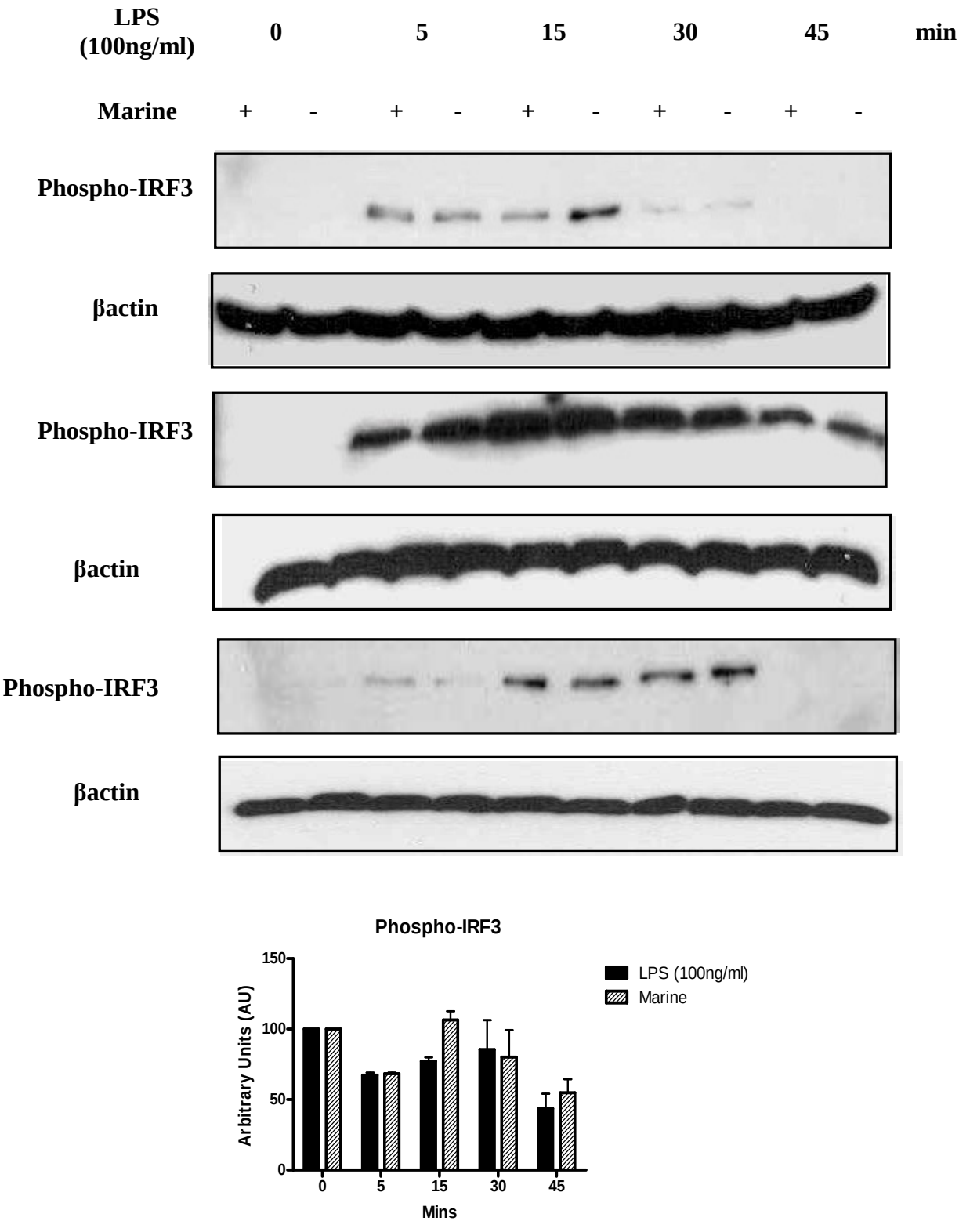


FIGURE 5.29: The purified marine compound, INV013, doesn't suppress phosphorylation of IRF3 in DCs. BMDCs were differentiated in the presence of GMCSF for 7 days, plated at ($1x10^6$ cells/ml) and then treated with either DMSO (vehicle control) or INV013 (1:200 dilution) 1 hr prior to stimulation with 100ng/ml of LPS over a time-course, after which cells were lysed and immunoblotted for phospho-IRF3. Total cellular levels of βactin were used as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of phospho-IRF3 expression in arbitrary units (AU) is shown.

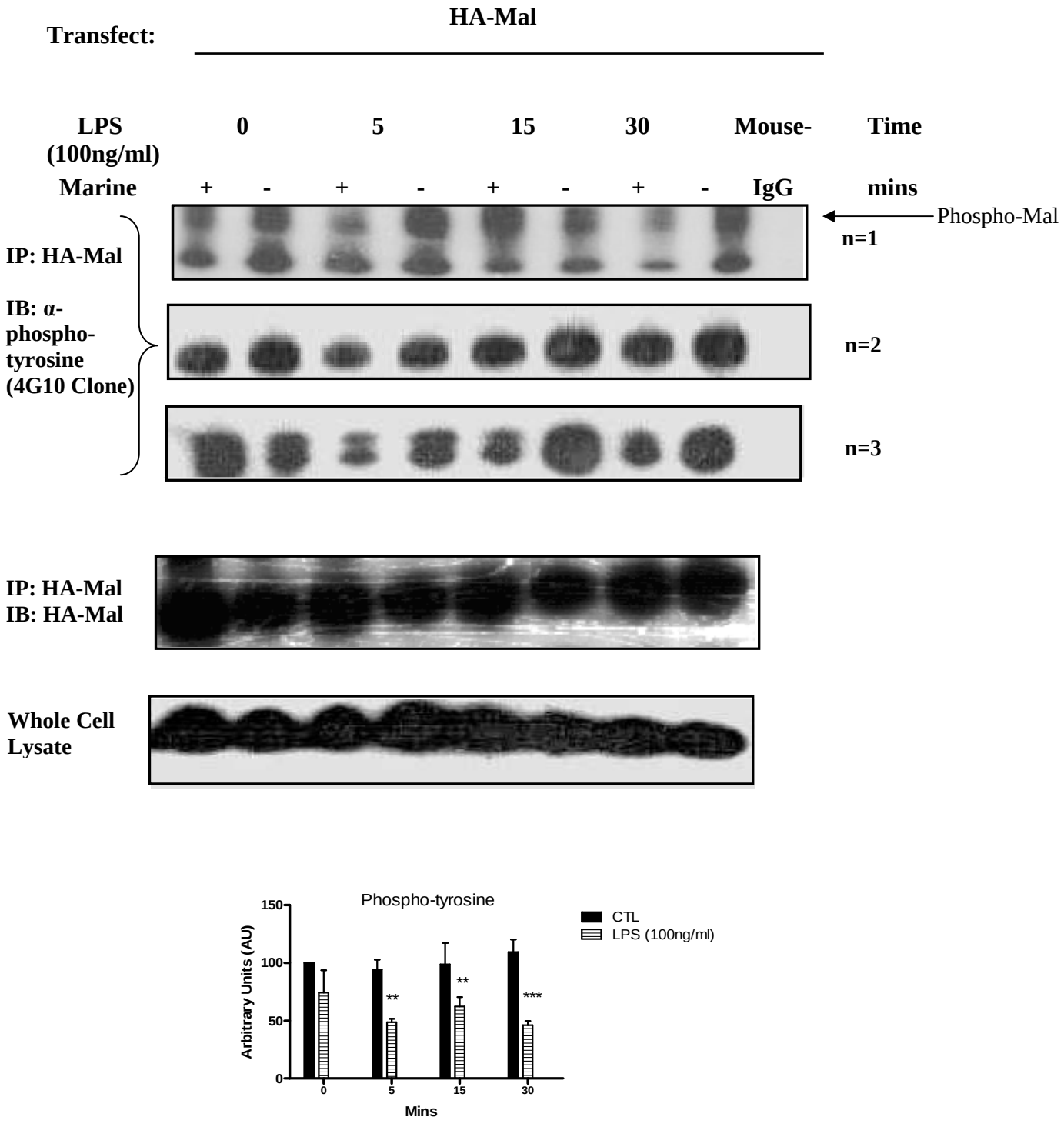


FIGURE 5.30: The purified marine compound, INV013, suppresses Mal-tyrosine phosphorylation in HEK-MTCs. HEK-MTC cells were transiently transfected with HA-Mal (4µg) for 24hrs. Subsequently, HEK-MTC cells were cultured with DMSO (vehicle control) or INV013 for 1hr prior to stimulation with 100ng/ml of LPS over various time-points as indicated, after which cells were lysed, immunoprecipitaed with HA-Mal and immunoblotted with α-phospho-tyrosine antibody. Whole cell lysates were immunoblotted with HA-Mal as a loading control. Densitometric analysis was conducted on immunoblots and graphical representation of α-phospho-tyrosine expression in arbitrary units (AU) is shown.

5.3 DISCUSSSION

The focus of this study was to investigate the possible mechanism underlying the anti-inflammatory effects of *M.membrancea* on DCs reported in chapter 3. The ability of these fractions to suppress macrophage function following activation through TLR4 and not through TLR7 led our study to examine the possible mechanisms underlying the *M.membrancea* extracts by activating DCs with a panel of TLR ligands. In this study we have demonstrated that the pure compound of *M.membrancea* (INV013) exerts its anti-inflammatory effects by suppressing the phosphorylation of Mal, the downstream adaptor which is unique to the TLR2 and TLR4 signalling pathways.

TLRs sense invasion of pathogens by detecting microbial components that are conserved among micro-organisms thus triggering the activation of innate immunity. They are considered a link between innate and adaptive immunity due to their promotion of pro-inflammatory cytokine production, surface maker expression and activation of T cells. On engagement with ligands, TLRs recruit specific adaptor molecules that propagate downstream signalling (McCoy, et al. 2008). Two predominant TLR signalling pathways have been identified, the MyD88 dependant and the MyD88 independent pathways. Although different TLRs share the same pathways, there are dissimilarities in patterns of the inflammatory response to TLR ligands (Akashi-Takamura and Miyake 2008). Roles for TLRs have emerged in sepsis (Lorenz, et al. 2002), RA (Seibl, et al. 2003), asthma (Eder, et al. 2004) and atherosclerosis (Kiechl, Wiedermann and Willeit 2003). Numerous studies have reported that interfering with TLR signalling can improve inflammatory diseases (O'Neill, Bryant and Doyle 2009) . For example, a study by Posadas and colleagues

demonstrated that mannoproteins protects intestinal tissue against *Salmonella triphymorium* infection in rats by impeding TLR5 expression (Posadas, et al. 2010)

Results here demonstrate that treatment of DCs with second-round fractions of *M.membracea* (C017,C021,C023,C027) significantly suppresses the production of the IL-12 family of cytokines (IL-12p40, IL-12p70 and IL-23) following activation with ligands for TLR4 (LPS), TLR2/1 (PGN, Pam_3CSK_4) and ligands for TLR2/6 (zymosan). In contrast, the *M.membranea* fractions had no effect when cells were activated with ligands for TLR5 (flagellin), TLR3 (Poly:(IC)), TLR7 (loxoribine) and TLR9 (CpG). This result demonstrated that our compound is exerting its anti-inflammatory properties downstream of TLR2 and TLR4 pathway but does not affect TLR signalling downstream of any other TLR. TLR2 and TLR4 are highly expressed in the synovial tissue of patients with RA and are associated with high levels IL-12 (Radstake, et al. 2004). Additionally, a study by Gomariz and workers demonstrated an up-regulation of TLR2 and TLR4 in a mouse model of Crohn's disease and treatment with Vasoactive intestinal peptide (VIP) induced a decrease in these receptors ameliorating the disease (Gomariz, et al. 2005). We found C023 and C027 to have the most potent anti-inflammatory effects so we therefore chose these fractions to be further fractionated in order to carry out advanced mechanistic studies. We received a purified compound from our collaborators in UCD termed INV013. This pure compound demonstrated similar properties to the second fractions of *M.membrancea,* downregulating the production of pro-inflammatory cytokines IL-12p40, IL-12p70, IL-23, IL-6 and IL-1β following stimulation with TLR4 and TLR2 ligands however not with the other TLR ligands. A number of studies have reported molecules that specifically suppress TLR2 and TLR4, activation. For instance, the

compound rabeximod has been shown to suppress arthritis severity in mice by exerting its anti-inflammatory effects downstream of TLR2 and TLR4 however it's molecular target has not been identified (Hultqvist, et al. 2010). Therefore we decided to examine the effects of INV013 on the pathways downstream of these TLRs.

TLR2 signalling activates the MyD88 dependant pathway leading to activation of NFκB however TLR4 signalling is unique in its ability to activate both NFκB and IRF3 via MyD88 dependent and independent pathways, respectively (Akira and Takeda 2004a, Akashi-Takamura and Miyake 2008). The data generated in this study demonstrates that second-round fractions of *M.membrancea* and the purified compound, INV013 all suppress NFκB activation and subsequent IL-8 production. This wasn't surprising as many of the cytokines that *M.membrancea* fractions had suppressed are know to be NFκB dependant such as IL-12, IL-1β and IL-6 (Atreya, Atreya and Neurath 2008). Furthermore, our data demonstrates that INV013 has no effect on IRF-3 activation post LPS stimulation. The activation of IRF-3 leads to the secretion of IFN-β and Rantes (Yoneyama, Suhara and Fujita 2002). Our results also demonstrate no alteration in the production of these cytokines in INV013-treated cells following TLR4 stimulation indicating further that this compound mediates its affects through the MyD88 dependant pathway.

Upon activation, NFκB is released from its inhibitory protein IκBα, leading to the phosphorylation of NFκB on serine residues. This phosphorylation is required for transactivation of gene expression because mutating these serine residues greatly impairs NFκB-dependant gene transcription (Schmitz, Bacher and Kracht 2001). Our data demonstrates that culturing DCs in the presence of INV013 inhibits this

phosphorylation of p65 post LPS stimulation. A report by (Moussaieff, et al. 2007) demonstrated that the compound incensole acetate isolated from *Boswellia* resin, inhibited inflammation in the inflamed paw model in mice by suppressing the phosphorylation of NFκBp65. Furthermore, numerous marine compounds have been reported to date as NFκB inhibitors (Folmer, et al. 2008). For example, hymenialdisine, a compound isolated from the marine sponges *Axinella verrucosa* and *Acanthella aurantiaca,* inhibited IL-8 production in U937 cells by blocking DNA binding of NFκB following stimulation with LPS (Breton and Chabot-Fletcher 1997). Similarly, Cycloprodigiosin hydrochloride (cPrG·HCl) obtained from a marine bacterium *Pseudoalteromonas denitrificans* suppresses NFκB-dependent gene expression through the inhibition of transcriptional activation (Kamata, et al. 2001). We also examined the signalling molecule IκBα that leads to NFκB activation. Our data demonstrates that the degradation of IκBα was interrupted in INV013-treated DCs post LPS activation. A number of marine compounds have been shown to interfere with this signalling molecule. For example, Scytonemin a marine natural product isolated from cyanobacteria inhibits IκBα degradation (Shah, et al. 2002). Of significant note is that the phosphorylation of the p65 subunit of NFκB at serine 536 in response to LPS requires the adaptor Mal (Gray, et al. 2006). We next examined the effect of INV013 on Mal driven NFκB activation and showed that it is inhibited indicating that our extract is mediating its anti-inflammatory effects through Mal. A study by Medvedev and colleagues demonstrated that overexpression of Mal in human embryonic kidney 293T cells led to activation of p38, NFκB and IL-8 expression and mutations in the phosphorylation of Mal reduces the ability to mediate IκBα degradation, p38 phosphorylation and NFκB reporter activation (Piao, et al. 2008). Given that p38 is phosphorylated downstream of Mal we examined the effects

of INV013 on p38 phosphorylation. Our findings support our hypothesis that INV013 targets Mal. Numerous reports showed a significant reduction in the phosphorylation of p38 in Mal deficient cells (Yamamoto, et al. 2003b). Additionally, activation of the p38 pathway has been shown to be involved in IL-12p40 promoter activity and cytokine release in DCs. Our study demonstrated that treatment of DCs with INV013 significantly reduced the phosphorylation of p38.

It has been well documented that inhibiting Mal impedes the production of pro-inflammatory cytokine following stimulation with TLR2 and TLR4 ligands (Yamamoto,M. 2010). The reduced levels (Kikuchi, et al. 2003) of pro-inflammatory cytokines in *M.membrancea* treated DCs following TLR4 and TLR2 activation reported here may be due to inhibition of Mal, given that the marine extract only exerts there effects when DCs are activated by TLR2 or TLR4 ligand. Additionally, it has been shown that inhibition of Mal alters the expression of cell surface markers. A study by Akira and workers demonstrated that enhancement of MHCII following LPS activation was severely impaired in Mal-deficient splenocytes (Yamamoto, et al. 2002). Interestingly we showed a significant reduction in MHCII expression in INV013-treated DCs post TLR2 and TLR4 activation however there was no alteration following TLR5 stimulation.

To date no specific compounds inhibiting Mal have been identified. However, a Mal inhibitory peptide that specifically interferes with Mal signalling by blocking TIR-TIR domain interactions has been described and is commonly used in experiments to examine the effects of Mal on TLR signalling (Horng, et al. 2002a, Doyle, et al. 2003).

The final question of this study concerns how INV013 inhibits Mal signalling. A study by Gray and workers demonstrated that upon LPS stimulation of macrophages, Mal is tyrosine phosphorylated by Bruton's tyrosine kinase (Btk) at residues 86 and 187 following TLR2 and TLR4 activation (Gray, et al. 2006). It has been reported in numerous studies that tyrosine phosphorylation of Mal is essential in TLR2 and TLR4 signalling (Piao, et al. 2008). Furthermore, it was shown that this tyrosine phosphorylation of Mal is important for the translocation of NFκB into the nucleus via the phosphorlaytion of the p65 subunit (Doyle, Jefferies and O'Neill 2005). To confirm that INV013 exerts its effect on Mal, we carried out immunoprecipitation with HEK-MTC cells over expressing Mal. Our findings demonstrated a significant reduction in the tyrosine phosphorylation of Mal in INV013-cultured DCs post LPS stimulation. Numerous diseases such as diabetes, RA and cardiovascular disease are linked to both TLR2 and TLR4 therefore interfering with the tyrosine phosphorylation of Mal is an attractive target for these diseases (Underhill, et al. 1999, Park, et al. 2004, Frantz, Ertl and Bauersachs 2007). In addition, a mutation in Mal where the serine at position 180 is mutated to leucine, conferred protection against disease including pneumonia, malaria and bacteraemia (Khor, et al. 2007).

Results presented in this chapter confirm that our purified marine compound, INV013, exerts its anti-inflammatory effects on the TLR adaptor, Mal, specifically by inhibiting its tyrosine phosphorylation. A schematic illustrating our proposed model for INV013-mediated effects on TLR2 and TLR4 signalling is presented in figure 5.30

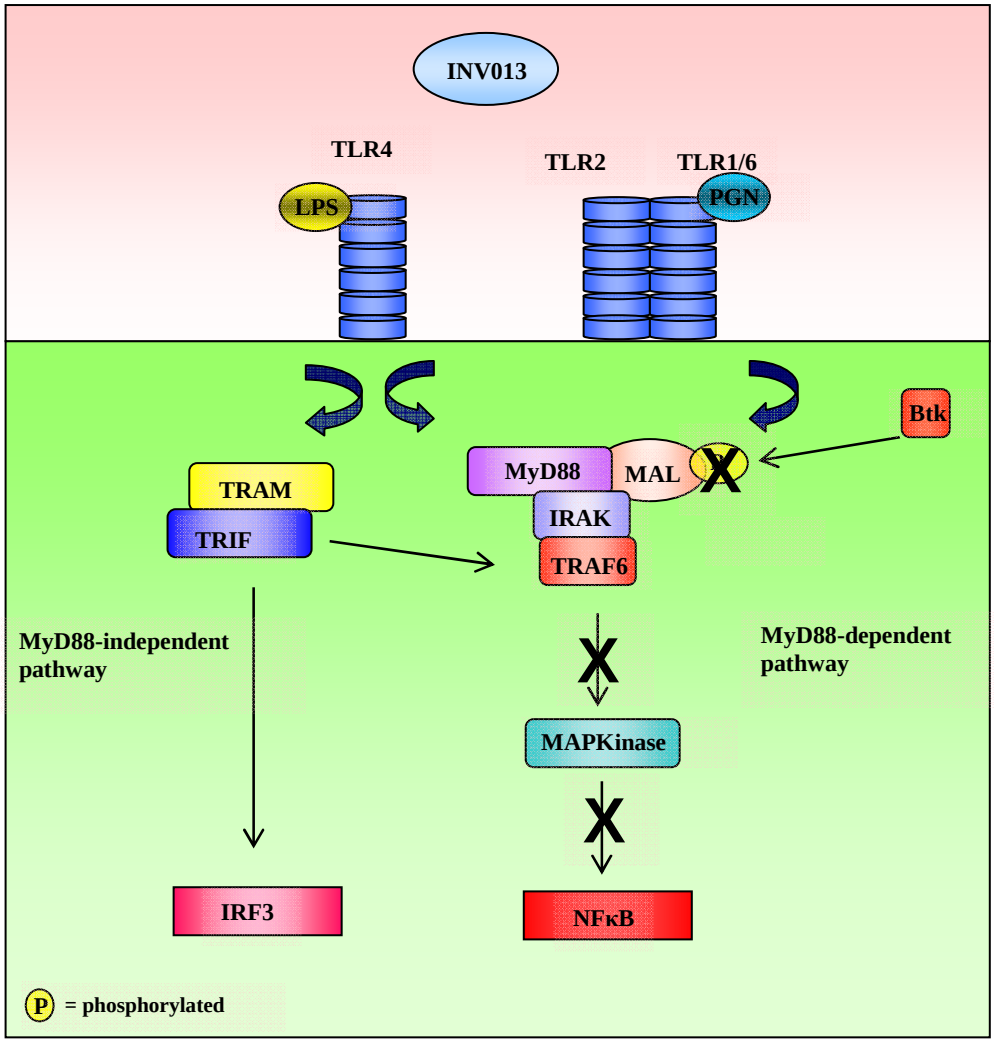


FIGURE 5.30: Illustration of our proposed model of INV013-mediated effects on TLR4 and TLR2 signalling. INV013 inhibits MAP kinase and NFκB activation by suppressing the phosphorylation of the adaptor MAL following TLR2 and TLR4 stimulation.

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

Inflammation is a crucial process initiated by the innate cells of the immune system and dysregulation at any stage of cellular activation can have detrimental effects. Inflammation is part of the normal host response that protects us from invading pathogens and infectious micro-organisms in general circumstances (Calder 2006). However, excessive and prolonged inflammation is harmful to the host and in some cases fatal, leading to extensive tissue damage, uncontrolled infection and acute and chronic diseases (O'Neill, Bryant and Doyle 2009). Numerous inflammatory diseases have been associated with this sustained inflammatory response such as, IBD, MS, RA and atherosclerosis (Andreakos, Foxwell and Feldmann 2004). Biological agents such as steroids have been developed to treat these diseases however a large proportion of patients are unresponsive to treatments and can have severe side effects (Hultqvist, et al. 2010). Therefore, novel compounds with better therapeutic efficacy are required. Within many inflammatory cascades or pathways there are often pivotal molecular targets that, when antagonized or neutralized, block the output of the pathway. For instance, resveratrol, a polyphenol found in grapes, has been shown to exert its anti-inflammatory effects by interacting with the adaptor TBK-1 which is downstream of TLR3 and TLR4 (Youn, et al. 2005). Furthermore, advances in our understanding of the inflammatory process have led to the development of therapies which selectively inhibit pro-inflammatory mediators, such as cytokines, chemokines and cell surface markers. The ocean represents a rich resource for the development of therapeutic drugs and a lot of pharmacologically important substances have been isolated with novel anti-microbial, anti-tumour and anti-inflammatory properties (Bhadury and Wright 2004).

The main strategy for this study involved screening numerous marine species in order to identify a compound that can be used as a therapeutic drug for the treatment of inflammatory diseases. A range of marine species were harvested from the Irish Sea by National University of Ireland, Galway (NUIG) and subsequently given to our collaborating chemists at UCD where they generated the crude marine extracts isolated from marine algae, *Alcyonium digitia* and the marine sponge, *Membranipora membrancea*. These crude marine extracts were examined to see if they had any anti-inflammatory properties and those that were active were sent to UCD were they were fractionated. *A.digitia* and *M.membrancea* first-round fractions were then screened and *M.membrancea* fractions were chosen to be further fractionated (second-round fractions) as they demonstrated to have the most potent anti-inflammatory properties. This overall process allowed for the isolation of a novel purified marine compound from *M.membrancea* (INV013) that has demonstrated to have potent anti-inflammatory capabilities [see figure 3.1.1]. In order to examine the activity of these marine extracts we used parameters that were relevant to the pathogenesis of inflammatory diseases. For instance we examined their effects of the production of the pro-inflammatory cytokines IL-12 and IL-23 as numerous studies have demonstrated their detrimental effect in inflammatory disease such as IBD and MS (Gee, et al. 2009, Wakashin, et al. 2009). Additionally, we focused on examining their effects on dendritic cells and macrophages as they are the main immune cells implicated in inflammatory disease. Given the success of the fractionation and the identification of the anti-inflammatory compound (INV013) we also undertook to identify the molecular target.

The anti-inflammatory activity of the compounds was evident in a number of cell types. DCs are considered to be the most potent antigen presenting cells possessing the unique ability to activate naïve T cells. In this study we found that DCs exposed to the marine extracts produced significantly less IL-12 and IL-23 following LPS stimulation. Since IL-12 and IL-23 have such significant roles in initiating and maintaining inflammation in EAE, RA and IBD (Bettelli and Kuchroo 2005, Stockinger, Veldhoen and Martin 2007), their inhibition has proved beneficial in treating these inflammatory disorders (Zhang, et al. 2007). For instance, in several rodent models of IBD, treatment with anti-IL-12 antibody prevents the development of colitis (Blumberg, Saubermann and Strober 1999). As well as suppressing pro-inflammatory IL-12 and IL-23 production, treatment with the marine extracts enhanced the production of IL-10 from DCs *in vitro*. IL-10 is known for its regulatory and anti-inflammatory properties and administration of IL-10 to patients with Crohn's disease has been reported to reduce bowel inflammation (Mocellin, et al. 2004).

Since IL-12 and IL-23 are involved in initiating Th1 and Th17 cell development respectively, and because IL-10 promotes the differentiation of T regulatory cells, the marine-induced suppression of IL-12 and IL-23 and enhancement of IL-10 suggests that the marine extracts may have consequential effects for T cell responses, therefore we investigated the down-stream effects of *M.membrancea* on adaptive immunity. Numerous autoimmune diseases such as IBD have been documented as predominantly Th1 mediated disorders associated with high levels of IFN-γ production. Our data demonstrates a significant reduction in IFN-γ production from a DC-T cell co-culture model following treatment with *M.membrancea*. Given the

widely accepted pro-inflammatory role of IFN-γ, inhibition of this cytokine may have tremendous therapeutic potential in inflammatory diseases. The compound *Bosweilla carterii* isolated from the plant *Burseraceae*, has shown some efficacy in treating patients with arthritis by inhibiting IFN-γ (Chevrier, et al. 2005). Furthermore, fontolizumab, a monoclonal antibody directed against human IFN-γ, has been demonstrated to have tremendous therapeutic effects in patients with Chron's disease (Hommes, et al. 2006).

Th17 cells are another subset of T cells that have been implicated in a number of inflammatory disorders and their presence has been linked to some of the pathology previously attributed to a disproportionate Th1 response (Wong, et al. 2008, Komiyama, et al. 2006, Paradowska, et al. 2007). We found that IL-17 was significantly reduced by *M.membrancea* in a DC-T cell co-culture model. Numerous studies have evaluated the benefit of neutralising IL-17 to alleviate inflammation. For instance the active form of vitamin D_3 (1,25-dihydroxyvitamin D_3) suppresses the production of IL-17 from Th17 cells and ameliorates the onset of the EAE (Chang, et al. 2010). This overall result from the DC-T cell co-culture demonstrates that the inhibition of the cytokines from DCs by our marine extracts has a subsequent effect on T helper cell responses. The significant decrease in cytokine production by DCs and T cell response reported in the study demonstrates the potential for the marine compound as a therapeutic drug for a range of inflammatory diseases.

Furthermore our results indicated that the marine compounds may have an overall effect on T helper cell responses by inhibiting key surface markers found on DCs. In order to activate a naïve T cell, dendritic cells present antigen coupled to MHCII and

express co-stimulatory markers CD40, CD80 and CD86 that interact with corresponding receptors on T cells (Lu, Wang and Linsley 1997). Enhanced expression of these surface markers has been implicated in disease. The over-expression of CD80 molecules has been reported in the inflamed tissues of patients suffering from MS and IBD (Kobata, et al. 2000) and numerous researchers have also reported increased expression of CD40 and CD40L in the inflamed ileum of CD sufferers (Battaglia, et al. 1999), therefore targeting co-stimulatory molecules can have therapeutic potential in inflammatory diseases. Examination of surface marker profiles in marine-treated DC showed a collective decrease in CD80, CD40 and MHCII expression. The reduction in T cell cytokines observed in our study may be due to the decreases in these markers as it would impair the ability of DCs to present antigen to T cells and activate them. Inhibiting the expression of CD80 using a CD80-CAP (competitive antagonist peptide) has been shown to suppress established inflammation in TNBS-induced colitis (Eri, et al. 2008). Furthermore, a study by Danese and workers demonstrated that the use of anti-CD40L antibodies in TNBS-induced colitis effectively prevented mucosal inflammation and decreased IFN-γ production by lamina propria T helper cells (Danese, Sans and Fiocchi 2004). The significant down-regulation of these surface markers in our study further demonstrates the potential for the marine compound as a therapeutic drug in a range of inflammatory diseases.

Macrophages, 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. TNF-α, IL-6 and IL-1β are the principle cytokines produced by these cells and are highly implicated in the pathogenesis of numerous

inflammatory diseases, in particular RA (Kinne, et al. 2000). In RA, TNF-α is mainly produced by MØ in the synovial membrane and is the proximal cytokine in the inflammatory response (Feldmann and Maini 1999). In addition, IL-1 gene expression is found predominantly in $CD14^+$ MØ and acts in sequence after TNF-α and mediates articular damage in arthritis (Kinne, et al. 2000). Furthermore, IL-6 is highly elevated in the synovial fluid of patients with RA and is implicated in the promotion of osteoclasts (Kinne, et al. 2000). Numerous drugs that target these specific cytokines have been developed and have demonstrated great effects in ameliorating diseases. For example, Golimua is a human monoclonal antibody directed against TNF-α and is widely used in the treatment of patients with RA (Smolen, et al. 2009). Our results, demonstrate a significant reduction in IL-6 but there is no alteration in the levels of TNF-α in macrophages following LPS stimulation. Given that blockade of TNF-α has been shown to effectively treat RA, this may suggest that our marine extract would not be very effective in this disease. However, MØ do secrete IL-12 which plays a major role in interacting with activated Th1 cells enhancing the production of IFN-γ. Largely on the basis of animal studies, RA has been considered a Th1 cell-mediated disease as the synovial fluid contains high levels of IFN-γ (Adorini 1999a). Retinoids are used in the treatment of patients with RA and in MØ they have shown a significant reduction in IL-12 secretion (Nozaki, et al. 2005). Therefore, the inhibition of IL-12 by MØ in our study demonstrates that *M.membrancea* can still have great effects on a wide range of diseases including RA. Regardless of the effects of the marine extracts on MØ, their effect on DCs is so potent and specific for the generation of Th1 and Th17 cells which are known to mediate a range of inflammatory diseases, that the effects of *M.membrancea* on DCs alone may be sufficient to treat the diseases.

If the effect of *M.membrancea* on MØ is less important than DCs then is it beneficial to turn off a wide range of MØ functions? In this study we have found distinct effects of our marine extracts on macrophage function. We have demonstrated that the effects of the second-round fractions of *M.membrancea* on MØ may modulate parameters which suggest increased susceptibility to infection. The ability of macrophage to phagocytose, a fundamental event in bacterial clearance and homeostasis was decreased following treatment with marine fractions of *M.membrancea*. Furthermore, we found *M.membrancea* fractions also significantly reduced chemotaxis and the production of the chemokines MIP-1α and MCP-1. The infiltration of leukocytes to the site of infection is crucial in mounting an immune response. Furthermore, depleted levels of MCP-1 have been broadly implicated in impaired bacterial clearance (Matsukawa, et al. 1999). In addition chemokines have a crucial anti-viral role (Melchjorsen, Sorensen and Paludan 2003). Numerous studies have reported that MIP-1α knockout mice have a reduced ability to clear virus infections such as influenza (Cook, et al. 1995). Although phagocytosis, migration and the production of chemokines by macrophages are essential in the host in controlling infections they have also been implicated in the pathogenesis of numerous inflammatory diseases including IBD and MS (Murdoch and Finn 2000, Reale, Greig and Kamal 2009, Adams and Lloyd 1997). It has been well documented that enhanced levels of MIP-1α and MCP-1 are greatly involved in the promotion of MS by mediating an influx of macrophages and T cells into the CNS (Simpson, et al. 1998). Furthermore, enhanced levels of MCP-1 and MIP-1α have been shown in patients with active lupus nephritis, idiopathic pulmonary fibrosis, atherosclerosis, ischemia and RA (Adams and Lloyd 1997). Although inhibiting these chemokines can have great therapeutic potential they

can also cause increased susceptibility to infection. Numerous studies have demonstrated that MIP-1 knockout mice are resistance to EAE however have increased susceptibility to *T. gondii* infection compared to wild type mice (Power 2003). Our study shows that second-fractions of *M.membrancea* may only increase susceptibility to infection to bacterial pathogens via TLR4 but not to viral pathogens via TLR7.

Since the fractions of *M.membrancea* and the purified marine compound demonstrated significant anti-inflammatory effects we therefore decided to elucidate the mechanism underlying their actions. TLRs are important at initiating innate immunity and recognising diverse microbial products. Upon recognition of a ligand, TLRs recruit specific adaptor molecules that initiate downstream signalling. Although TLRs induce common signalling pathways, there is specificity in recruitment of TLR adaptors. Therefore the first step in our mechanistic study was to examine if the marine extracts of *M.membrancea* had any specific effects on these TLR pathways by activating DCs with a panel of TLR ligands. Our data demonstrated that the fractions of *M.membrancea* and its purified compound demonstrate significant anti-inflammatory effects by downregulating pro-inflammatory cytokines and surface marker expression following stimulation with TLR2 and TLR4 ligands but not when cells were activated with other TLR ligands. The TLR2 and TLR4 signalling pathways are unique as they are the only TLRs capable of recruiting the TIR adaptor, Mal. Numerous studies have shown that Mal signals exclusively through the MyD88-dependant pathway leading to the activation of MAP kinases and the transcription factor NFκB (Verstak, et al. 2009, Horng, et al. 2002b). Firstly we showed that INV013 decreased NFκB activation but not IRF3 activation, indicating that the

marine extract is exerting its effect through the MyD88-dependant pathway. We then showed that it significantly reduced Mal driven NFκB activation in HEK-MTC cells. Furthermore, the phosphorylation of p38 and NFκBp65 was greatly impaired in INV013-treated DCs following LPS stimulation. Finally, we showed that INV013 significantly reduced the tyrosine phosphorylation of Mal, demonstrating that our marine extract exerts its anti-inflammatory abilities by targeting this specific adaptor. It has been reported that Mal-deficient mice are resistant to the toxic effects of LPS, with defective induction of IL-6, IL-1β and IL-12, decreased activation of NFκB and MAP kinases (Horng, Barton and Medzhitov 2001a). The recruitment of neutrophils to the lungs is also abrogated in Mal-deficient mice reducing the severity of LPS-induced lung inflammation in this model (Togbe, et al. 2006). This also correlates with our study as we saw a significant reduction in chemokines and migration in LPS-induced DCs following treatment with INV013. It has been well documented that Mal plays a major role in diseases such as RA, IBD and acute lung injury. A study by Sacre and workers demonstrated that RA synovial cell cultures release a TLR ligand that stimulates human macrophages in a Mal-dependent manner, contributing to the destructive processes of RA (Sacre, et al. 2007). Furthermore, a variant in Mal (S180L) showed to be protective against diseases such as systemic lupus erythematosus (Castiblanco, et al. 2008). Since our purified marine compound, INV013 has the ability to target this specific adaptor it may have therapeutic advantage in a wide range of diseases. To date numerous anti-inflammatory drugs have been identified that have specific targets allowing them to have major benefits in specific inflammatory diseases (Simmons 2006). For instance, the anti-inflammatory drug, Celebrax, specifically targets the enzyme COX-2 and is widely used in the treatment of patients with RA. Furthermore, the drug canakinumab specifically blocks

the production of IL-1β and is currently being tested in clinical trials for treating patients with type 1 and type 2 diabetes (Dinarello 2010). The compound, TAK-242 (resatorvid) has been shown to selectively bind to TLR4 and subsequently disrupt the interaction of TLR4 with adaptor molecules Mal and TRAM thereby inhibiting TLR4 signal transduction (Matsunaga, et al. 2011). A wide range of molecules have been shown to interfere with TLR2 and TLR4 signalling. The oxidised 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine (OxPAPC), has been demonstrated to inhibit LPS (TLR4) and Pam_3CSK_4 (TLR2) signalling but not other TLRs which can be used in the treatment of TLR-mediated inflammatory diseases such as RA (Erridge, et al. 2008a). However only one drug to date has been identified to specifically interact with Mal and has been used in numerous studies (Radin, et al. 2008).

Steroids such as glucocorticoids are currently being used to treat inflammatory diseases but have demonstrated serious side effects, one of which is decreased ability to mount an immune response to infection-immunosuppressant. Therefore by finding a drug that has a specific target that has fewer side effects may be more beneficial (McCoy, et al. 2008). The major advantage of our marine compound is that it is specific and may only suppress the ability to mount a normal immune response to gram-positive and gram-negative bacteria whereas the ability to mount a normal immune response to most other pathogens would not be affected.

As the novel compound has demonstrated tremendous potential for the development of an anti-inflammatory drug, an invention disclosure has been filed. The identification of the structure is currently being carried out by our collaborators at UCD.

Future Work

- Given that we have already generated a significant amount of data *in vitro*, future work will concentrate on examining the effects of INV013 in *in vivo* models. Firstly we will assess the effects of INV013 on Th1 and Th17 responses *in vivo* using an adoptive transfer model. Following this we will undertake a study using two murine models of IBD to determine whether administration of INV013 results in suppression of Th1 and Th17 response and thus inhibition of disease. If INV013 demonstrates significant anti-inflammatory effects in both these studies it will allow the translation of this research into a therapeutic, therefore permitting us to patent this compound.
- The mRNA expression of cytokines following treatment with INV013 will be assessed by real time-PCR to support the decreased cytokine production presented in chapter 3, 4 and 5.
- Exam alternative effects that INV013 may have on the adadptor Mal; does it interfere with caspase-1 binding? What effect does it have on PKC-δ activity?
- Examine the effects on INV013 on the promoter regions of cytokines in the nucleus such as IL-12p40, IL-12p70, TNF-α by chromatin immunoprecipitation (CHIP).
- Once the structure has been identified future work will concentrate on developing analogues of the structure and screening them to see if potency can be improved.

CHAPTER 7

APPENDICES

7.1 APPENDIX

CELL CULTURE MEDIA

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

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

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

LOW STRINGENCY LYSIS BUFFER:

1M HEPES (pH 7.9)	11.92g into 50ml
1M $MgCl_2$	74.55g into 50ml
1M KCl	3.73g into 50ml
1M NaCl	2.92g into 50ml
0.5M PMSF	0.3484g into 4ml acetone
0.5M EDTA	9.306g into 50ml
Glycerol	25% = 12.5ml into 50ml 20% = 10ml into 50ml

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

CHAPTER 8

BIBLIOGRAPHY

BIBLIOGRAPHY

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