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Modulation of macrophage and epithelial cell immune defences by probiotic bacteria: immune stimulation versus suppression

by

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Author Declaration

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Modulation of macrophage and epithelial cell immune defences by probiotic bacteria:immune stimulation versus suppression

Abstract

Probiotic bacteria are live organisms, if consumed in adequate amounts might confer health benefits. These bacteria, such as Lactic acid bacteria (LAB), include a number of strains that have specific health promoting activities, attributed to their immunomodulatory and anti-inflammatory properties. Gut mucosal macrophage subsets play a fundamental role in driving mucosal immune responses. These include, tolerance, associated with an M2-, regulatory macrophage phenotype and inflammatory activation with an M1-like phenotype. The cross-link between mucosal tolerance and inflammatory cytokine suppression, and augmentation of IL-10 production in the gut relate to endotoxin tolerance. Endotoxin tolerance is a context; it could present an example for cell drive through a hypo-responsive state. An example is mucosal inflammatory pathologies, such as Crohn's disease. When tolerance is broken, causing the destruction of gut mucosal tissue. This is where the macrophage phenotype, has been transformed from a regulatory M2- to an inflammatory M1-like phenotype. This is seen as a reaction to both, pathogenic and commensal bacteria. This investigation was aimed at assessing the activities of live probiotic bacteria; *Lactobacillus salivarius* strain MS13 and *Lactobacillus plantarum* strain C28 in the immunomodulation of macrophage subsets in health, inflammation, and endotoxin tolerance. M1- and M2-like macrophages were generated *in vitro* from the THP-1 monocyte cell line by differentiation with PMA and Vitamin D_3, respectively. Additionally, differentiated epithelial cells (Caco-2) were obtained by long term culturing for 21 days. The role of *Lactobacillus* strains C28 and MS13 to modulate epithelial barrier integrity and macrophage-epithelial cell inflammation was investigated. TNFα, IL-1β, IL-18, IL-23, IL-12, IL-6, IL-8, and IL-10 were quantified by ELISA and RT-PCR, whereas TLR-2, TLR-4, Tollip, SOCS3, STAT3 and TRAIL by RT-PCR. This study revealed that, first, live C28 and MS13 stimulated the proinflammatory cytokine by M2-like macrophages as well as the anti-inflammatory cytokine in a homeostatic status; whereas in an inflammatory environment, C28 and MS13 differentially upregulated TNFα and IL-1β by M1 and M2-like macrophages induced by *E.coli* K12-LPS. Both strains

downregulated K12-LPS induced IL-10 by M2-like macrophages. The response of stimulated M1 and M2 macrophages to C28 and MS13, was to differentially induce the gene expression of TLR-2, TLR-4, Tollip, NLRP3, SOCS-3, STAT3 and TRAIL. Second, the repeat-stimulation/tolerisation of M1 and M2 macrophages by live probiotic bacteria revealed, TNFα, IL-1β, IL-23, IL-18, IL-6 and IL-10 were upregulated in M1-like macrophages by C28, whereas MS13 upregulated TNFα, IL-1β, IL-18, and downregulated IL-12, IL-6, and IL-10. On the other hand, the tolerisation of M2-like macrophages by C28 and MS13 resulted in the downregulation of TNFα and IL-12p35 and upregulation of IL-1β, IL-18, IL-23, IL-12, IL-6, and IL-10. These findings were linked with the differential macrophage subset upregulation of TLR-4, NLRP3, STAT-3 and TRAIL gene expression. On the other hand, TLR-2, Tollip and SOCS-3 were downregulated in tolerised macrophage subsets by C28 and MS13. Furthermore, the role of lactobacilli strains C28 and MS13 in the modulation of endotoxin tolerance was to; upregulate TNF-α, IL-18, IL-23 and IL-10 by M1 and M2-like macrophages. This investigation also focused on the induction of the zona-occludin-1 (Zo-1), human β defensin-2 (hBD-2), and cytokine production IL-8 by Caco-2 cells. Trans epithelial electrical resistance (TEER) and RT-PCR measured the main cytokines studied produced by Caco-2, were IL-8, also the epithelial barrier function. Live probiotic C28 and MS13 suppressed the production of IL-8 (in the presence or absence TNFα and IL-1β). Moreover, in the co-culture of Caco-2 with macrophage subsets, MS13 enhanced the expression of hBD-2 and ZO-1. These findings allow for the better understanding of live probiotic roles on macrophage subsets functions and endotoxin tolerisation mechanisms, which may be beneficial for the development of *in vivo* models of probiotic bacteria and therapeutic targeting of inflammatory bowel disease.

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Abbreviation

Abbreviation	Meaning
Anova	Analysis of variance
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
C'D	Crohn's disease
Co_2	carbon dioxide
DAMP	Damage associated molecular patterns
DeC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco's pbs
DW	Distilled water
ED50	50% effective dose
EDTA	Ethylene-diamine tetra-acetic acid
ELISA	Enzyme linked immune-absorbent assay
FCS	Fetal calf serum
FLAG	Flagellin
GALT	Gut associated lymphoid tissue
GIT	Gastrointestinal tract
GM-CSF	Granulocyte macrophage colony-stimulating factor
HRS	Hours
HBD-2	Human beta defensin -2
Hi	High
HRP	Horseradish peroxidase

IBD	**Inflammatory bowel disease**
IECS	**Intestinal epithelial cells**
IFN-γ	**Interferon gamma**
Ikk	**Inhibitor of κb kinase**
IL	**Interleukin**
iNOS	**Inducible nitric oxide synthase**
IRAK	**IL-1 receptor associated kinase**
IRF	**-IFN-regulatory factor**
Iκb	**Inhibitor of κb**
KDa	**Kilo dalton**
LBP	**Lps binding protein**
LPS	**Lipopolysaccharide**
LTA	**Lipoteichoic acid**
M	**Molar**
M cells	**Micro fold cells**
M1 subset	**Macrophage (pro-inflammatory mφ)**
M2 subset	**Macrophage (anti-inflammatory mφ)**
MAPK	**Mitogen-activated protein kinases**
MCP-1	**Macrophage chemoattractant protein-1**
M-CSF	**Macrophage colony stimulating factor**
MDP	**Muramyl dipeptide**
Mg/ml	**Microgram per millilitre**
mg	**Milligram**
MHC	**Major histocompatibility complex**
Min	**Minute**
Myd88	**Myeloid differentiation factor 88**
NCBI	**National centre of biotechnology institute**
NIBSC	**National institute for biological standards and control**
NF-κb	**Nuclear factor kappa b**
ng	**Nanogram**

NODs	**Nucleotide oligomerisation domains**
NOs	**Nitric oxide synthase**
OD	**Optical density**
PAMPS	**Pathogen associated molecular patterns**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PG	**Peptidoglycan**
Pi3k	**Phosphatidylinositide 3-kinases**
PMA	**Phorbol-12-myristate acetate**
PPs	**Peyers' patches**
PRRS	**Pattern recognition receptors**
R10	**Rpmi supplemented with 10% v/v foetal calf serum (fcs)**
RPMI	**Roswell park memorial institute**
Rt- PCR	**Reverse transcriptase polymerase chain reaction**
RT	**Room temperature**
SDS-page	**Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis**
SE	**standard error**
sirRNA	**Small interfering rna**
SIGGER	**Suppressor of cytokine signaling**
STAT	**Signal transducers and activators of transcription**
TGF-β	**Transforming growth factor beta**
TAMs	**Tumor associated macrophages**
TEER	**Transepithelial electrical resistance**
Th	**T helper cell**
TLR	**Toll-like receptor**
TNF	**Tumour necrosis factor alpha**
Tjs	**Tight junctions**
Tlr	**Toll inhibitory protein**
Traf6	**TNF receptor associated factor 6**
Uc	**Ulcerative colitis**

Uv	**Ultra violet**
V/v	**Volume by volume**
Wb	**western blot**
W/v	**weight by volume**

Chapter 1:

General introduction and literature review

1.1 Introduction

The symptoms of gastrointestinal disorders range from mild to severe. Some conditions could affect health status (e.g. homeostasis) of the gut, such infectious diseases, cancer and autoimmune diseases, as well as medicines, such as antibiotics and those that suppress the immune system.These changes in the gut environment have resulted in growing interest in therapies that influence these changes of the gut, such as probiotic bacteria. Probiotics are live beneficial bacteria that confer an accurate health benefit on the host, in combination with prebiotics, (indigestible dietary fiber/carbohydrate). Interestingly, probiotics colonies the intestine and can use their influence on the mucosal tissue locally (Hill *et al.*, 2014; Hungin *et al.*, 2013). Rationales for probiotic consumption by individuals both, healthy and sick include the improvement of gastrointestinal (GI) symptoms (Guyonnet *et al.*, 2009; Hungin *et al.*, 2013), and strengthening the immune system (Fukushima *et al.*, 1998; Rolfe, 2000; Schley & Field, 2002). They are relevant for alleviating the pathology of intestinal disorders and eliminating invading pathogens (Panigrahi *et al.*, 2017). However, the efficacy of probiotics in treating infectious or current conditions for example cardio-metabolic pro-inflammatory bowel disease are still under discussion (Lamb *et al.*, 2019; Rondanelli *et al.*, 2017), while some research has even reported probiotics to be associated morbidity and mortality (Besselink *et al.*, 2008; Rijkers *et al.*, 2011). Up to 60 % of US healthcare workers prescribed probiotics to their patients (Draper, Ley & Parsonnet, 2017). In fact, dysbiosis of the microbiota in the gastrointestinal tract was considered as contributing factors in the development of certain gastrointestinal diseases and malignancies (Sobhani *et al.*, 2013). Probiotic microorganisms, such as lactic acid bacteria (LAB), exert a number of strain-specific, health-promoting activities attributed to their im-

munomodulatory, anti-inflammatory and anti-carcinogenic properties that underlie gastrointestinal inflammation and a predisposition to cancer (Tiptiri-Kourpeti et al., 2016). Previous research assessed different strains of probiotics to modulate or activate the macrophage immune response, some can activate the immune system and others can suppress it. The variety in function is determined by macrophage differentiation, activation, signalling and pre-programming in distinct monocyte subsets. This diversity in macrophage subsets and functionality is also reflected in mucosal pathologies associated with chronic inflammation (Crohn's disease, chronic periodontitis) and immunosuppression detected in solid tumours such as (oral squamous cell carcinoma) (Lumerman, Freedman & Kerpel, 1995).

1.2: The History of probiotic bacteria

The word probiotic means in Greek [for life], Lilley and Stillwell first described in 1965 that material secreted from one microorganism can stimulate the growth of another. The probiotic works opposite to the antibiotics actions. This was followed by Speriti (1971), who described the tissue extract, which stimulated microbial growth. Fuller (1992) described the probiotic as live microorganisms and their beneficial effect on host animal by improving the intestinal microbial environment. These live microorganisms are heterogenetic species (Bauer et al., 2002). Probiotic bacteria can be classified into three categories based on; 1) the type of interaction in host gut, 2) Probiotics can affect pathogenic microorganisms, 3) it may act as a trap to other pathogenic bacteria in the gut because of their abilities to aggregate with pathogenic bacteria and prevent their translocation to the circulation (Kmet and Lucchini, 1999).

1.2.1: Immunomodulatory effects of probiotic bacteria

Metchnikoff (1907) proposed evidence that intestinal bacteria have a vital role in the maintenance of health, when he recorded the impact of lactic acid bacteria present in fermented milk products on longevity in humans. Actually, the probiotic area grew widely in people and animal appliance therapy when Shirota and Kellogg in 1930s and German nutritionists in 1950s used probiotics in the treatment of several diseases. In 2001, FAO/WHO termed probiotics as 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). Probiotics can affect pathogenic microorganisms, they may act as a trap to Butyrate regulation of distinct macrophage subsets (Foey, 2011): opposing effects on M1 and M2-macrophages, pathogenic bacteria in the gut because of their abilities to aggregate with pathogenic bacteria, and prevent their translocation to the circulation (Kmet and Lucchi-

ni,1999). Probiotics can enhance the function of intestine barrier of epithelial layer. According to Habil et al., (2014) probiotic strains differentially regulate hBD-2 mRNA expression and protein secretion, modulation being dictated by inflammatory stimulus and resulting cytokine environment. Studies showed the treatment of membrane bound epithelial cells with probiotic bacteria can exhibit anti-inflammatory effects by suppression of tumor Necrosis Factor (TNF)-α (Habil, 2013, Natividad and Verdu 2013). Probiotic bacteria are safe for human consumption that can potentially be used as a live vector for oral or mucosal adjuvant. Furthermore, these can affect the modulation of the mucosal immune system Furthermore; the gut microbiota fulfils important physiological and metabolic functions including the maintenance of the gut-associated immune system (Gawkowski and Chikindas, 2012, Purchiaroni *et al*., 2013).

Lactobacilli are found in small numbers in the small intestine of adults but some may come from fermented foods or the oral cavity, which is home to a few autochthonous species. In adult faeces only a slight component of the microbiota ranging from 0.01 to 0.6% of total counts were found (Lebeer et al., 2008) . While in the new born, lactobacilli are found in faeces in variable amounts ranging from 10^5 to 10^8 CFU/g with *L.salivarius*, *L. rhamnosus*, and *L. paracasei* being common species (Lebeer et al., 2008).

The lactobacilli have assumed significance in considering acute infectious diarrhoea and in the prevention of antibiotic-associated diarrhoea in human clinical trials (Sazawal *et al.*, 2006). The treatment with probiotic lactobacilli of and in anticipation of allergic diseases and in the treatment of allergic rhinitis/asthma has been reviewed recently (Kalliomaki *et al.*,2010; Vliagoftis *et al.*, 2008). Several medical studies on allergy have been conducted with *Lactobacillus rhamnosus* GG (LGG) that revealed that they inhibit atopic eczema and dermatitis

(Kalliomaki *et al.*, 2010;Kalliomaki *et al.*, 2003). Following findings on the usage of LGG in the treatment of atopic eczema, suggested a therapeutic effect (Isolauri *et al.*, 2000; Majamaa & Isolauri, 1997; Viljanen *et al.*, 2005). In general there is promising evidence that specific lactobacilli probiotics are respected with the protection and treatment of diverse diseases, but their successful application would benefit greatly from a better understanding of the mechanisms of probiotic action in clinical studies (Wells et al., 2010)**.**

1.2.2: Probiotic Modulation of Intestinal Epithelial Cells

Regarding gut homeostasis, there is a balance between epithelial cell proliferation and apoptosis, permitting this dynamic cellular barrier to concurrently replace itself, protect from infectious pathogenic agents and to die off prior to cellular transformation resulting from long-term exposure to carcinogenic agents present in intestinal/digest-associated water. Intestinal epithelial cells (IECs) represent the physical barrier that maintains the isolation between luminal microbes, digest, and the mucosal immune system (Peterson & Artis, 2014). Modulation of function IEC by probiotics and commensals by different ways, has an indirect impact on microbial biofilms (Vastano *et al.*, 2016) and direct effects on IECs through augmentation of barrier function by enhancing tight junctions and mucin production (Zyrek *et al.*, 2007); antimicrobial peptides (AMPs) and heat shock protein production (Liu *et al.*, 2017; Schlee *et al.*, 2008); modulation of pro-inflammatory and immunoregulatory cytokines (IL-10 and IL-6) (Schulze-Tanzil, 2011) and interference with pathogenesis (Chen *et al.*, 2006; Resta-Lenert & Barrett, 2003). The functions of the intact epithelial barrier rely on intracellular signalling cascades, on which probiotics exert their effects. An increasing number of both *in vitro* and *in vivo* studies are elucidating these effects.

1.2.3: Intestinal epithelial barrier function

Mucosal surfaces beneath the epithelial cells and between epithelial cells form a barrier, which produced pathways between external milieus and the internal environment. However, mucosae are also responsible for interchange of material like food absorption and secretion, which involve a selectively porous barrier (Beck, 2015; Peatman & Beck, 2015). Turner (2009a) reported that in the presence of an integral epithelium, mucosal penetrability is mainly determined by tight junction barrier function. Intestinal epithelial cells mediate interactions between the mucosal immune system and luminal materials (Aviello & Knaus, 2017). Further investigation of pathways that integrate mucosal barrier function, or dysfunction, and immune regulation will give a good understanding of the mechanisms underlying these complex interactions and provide a rational basis for the development of more effective and targeted therapeutic interventions (Seeleyet al., 2015). A single layer of enterocyte and tight junctions (intercellular multiportion complexes) form the intestinal epithelial barrier that monitors the transport of molecules through transcellular and para-cellular pathways. A dysfunctional or “leaky” intestinal tight junction barrier permits amplified permeation of luminal antigens, endotoxins, and bacteria into the bloodstream. Various substances and environments have been shown to influence the maintenance of the intestinal epithelial tight junction barrier. Particular membrane pumps and channels highly control transcellular transport over the apical plasma membrane. On the other hand, paracellular transport is protected by the TJs that form an incessant, border between adjacent epithelial cells (Anderson & Van Itallie, 2009), regulating molecular transport and influencing trans-epithelial electrical resistance hence barrier integrity (Turner, 2009b).

1.2.4: Modulation of Barrier Function and tight junction by probiotic bacteria

Probiotics can augment barrier function by inhibiting the destruction of intestinal paracellular permeability. Moreover, It has been shown that *Lactobacillus rhamnosus* GG (LGG) up-regulates the expression of ZO-1, Claudin-1 and Occludin in the human colon-derived intestinal epithelial cell line, Caco-2 (Orlando *et al.*, 2014). Additionally, the mechanisms by which probiotics regulate intestinal barrier function are yet to be clarified. An analysis of the effects of *Lactobacillus plantarum* on Caco-2 intestinal cells prompted TLR2 signalling-mediated translocation of ZO-1 to the TJ region between epithelial cells (Karczewski *et al.*, 2010). Pre-treatment of Caco-2 monolayers with *L. plantarum* or the Toll-like receptor-2 (TLR2) agonist, Pam3- Cys-SK4 (P3CSK), significantly reduced the effects of phorbol ester-induced dislocation of ZO-1 and occludin and the linked increase in epithelial permeability. The phorbol ester, PMA, is an analogue of the protein kinase C (PKC)-activating diacyl glycerol (DAG); the understanding of this association will only be explained by investigating the relative utilisation of MAPK and PKC isoforms, these impacts, might be strain- and dose-specific. Furthermore it has been shown that the expression of TLR2 mRNA is up-regulated in IPEC-J2 cells (neonatal porcine mid-jejunum derived) when pre-treated with LGG, proposing that TLR2 recognition of Gram-positive probiotic bacteria plays a significant role in support barrier integrity (Zhang *et al.*, 2015). Additionally, pre-treatment with LGG inhibited the F4+ ETEC (Entero-toxigenic *Escherichia coli* K88)-induced increase in the pro-inflammatory cytokine, tumour necrosis factor-alpha (TNFα). The downstream effects of TLR activation contain two main signalling cascades, linking the MAPK and NFκB pathways. It has been demonstrated that probiotics can regulate intestinal epithelial permeability

up-regulation and stimulation of MAPK pathways (refer to Table 1.1) The MAPKs consist of numerous serine threonine protein kinases of which extracellular signal-related kinases (ERK), c-Jun amino-terminal kinases (JNK), and p38 are the primary members (Dent *et al.*, 2003). Preventing apoptosis means the capability to support efficient defences to pathogens and mucosal pathology by create a balance between cell proliferation and cell death that characterise a fine line between homeostasis. In addition to tight junction enhancement, probiotics have been shown to confer protection against many cellular stresses, which include oxidative stress-mediated apoptosis (Tao *et al.*, 2006). The LGG strain ATCC 53103 was shown to secrete p40 and p75 soluble proteins in fermented milk, where the p40 soluble protein has been shown to ameliorate cytokine-induced apoptosis in YAMC (young adult mouse colon) cells by the transactivation of EGFR and subsequent activation of the PI3K-downstream substrate, Akt /PKB (Tao *et al.*, 2006). Moreover, in a similar study, LGG also attenuated H_2O_2-induced disruption of the tight junction complex in IECs (Yoda *et al.*, 2014). These studies together reveal how LGG protein metabolism can enhance membrane barrier integrity and defensive responses by stimulation of the anti-apoptotic PKB/Akt in a PI3K-dependent manner to protect IECs from cytokine-induced apoptosis.

1.3: Gut-associated lymphoid tissue (GALT)

GALT is organised into isolated lymphoid follicles or peyer's patches, (PPs) located under the epithelial cell layer along the whole length of the gut. The epithelium covering the PPs is composed of cells that differ from the surrounding enterocytes; these cells are called micro-fold or (M) cells. M cells lack microvilli, have no glycocalyx covering, and intended to interrelate directly with antigens. The basolateral, cross section for GALT, shows forming a pocket that includes

T and B cells, dendritic cells (DCs), monocytes, and macrophages. GALT is regularly viewing, foreign materials (food and drinks), and commensal microorganisms about $1x10^{14}$ cfu/ml (Servin, 2004).

1.3.1: Innate immune recognition

Molecular mechanisms of host-microbial associations are difficult and complex to understand. Through infection, host immune cells identify evolutionarily preserved microbial components called Microbial associated molecular patterns (MAMPs) (Cinel and Opal, 2009; Diacovich and Gorvel, 2010; Strober et al., 2006). Over the last few years, some PRRs and MAMPs have been recognised (Strober et al., 2006; Yamamoto et al., 2004). Typical examples of MAMPs are lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acids (LTA) and lipoproteins (LP), microbial RNA and DNA (Mogensen, 2009). The recognition of MAMPs by PRRs triggers innate defence system. There are numerous functionally distinctive classes of PRRs that recognizes microbial MAMPs. The best characterised class of PRR is Toll-like receptors (TLRs) that identify several microbial products. 10 different types of TLRs are present in human (TLR-1–10) (Fitzner et al., 2008). Recognition of MAMPs by PRRs leads to the release of human innate defence molecules that include reactive oxygen and nitrogen species, bacteriolytic enzymes (lysozyme and phospholipase A2), antimicrobial peptides and the complement proteins (Bera et al., 2007; Fahlgren et al., 2003; Piris-Gimenez et al., 2005). Innate immune cells recognise bacterial and fungi to base an immune response. Peptidoglycan (PG) or murein is bacterial cell wall components, recognized by NODs , mannose binding lectin (MBL) and lysozyme cell wall modification of and pathogenic and commensal microorganisms employ to evade immune recognition during colonization of the host tissue.

1.3.2: Macrophage plasticity and polarization *in vivo*

Macrophages are an essential member of innate immunity and play a central role in inflammation and host defence (Gordon & Martinez, 2010). These cells have a defending roles and impact homeostatic functions, comprising tissue modifying in ontogenesis and transposition (Gordon & Martinez, 2010; Sica & Bronte, 2007). Cells of the monocyte-macrophage lineage have substantial diversity and plasticity. In tissues, mononuclear phagocytes respond to efficient signals, (e.g., microbial products, damaged cells, introduced lymphocytes) with distinct functional phenotypes. Macrophages may undertake classical M1 activation (stimulated by TLR ligand and IFN-γ) or alternative M2-activation (induced by IL-4/IL-13). These conditions mirror the Th1-Th2 polarisation of T cells (Biswas & Mantovani, 2010). While, Foey (2015) reviewed macrophage plasticity and polarisation relating to M2/homeostatic and M1 subsets regulates effector response, such as anti-inflammatory/regulatory and pro-tumoral contrasted with pro-inflammatory, immune activatory/CMI and anti-tumoral. M1 function is linked with iNOS/NO production, expression of pro-inflammatory cytokines (TNFα, IL-12 and IL-23). Pathogenic association of M1 function is linked with inflammatory diseases for example CD and CP. M2 function is related with Arg-1 activity, expression of suppressive cytokines, M2 polarization follows in response to downstream signals of cytokines such as (IL-4, IL-10 , IL-13, IL-10, IL-33, and TGF-β) (O'Shea & Paul, 2010; Wang, Liang & Zen, 2014). Pathogenic association of M2 function is associated with immunosuppressive diseases, and localised pro-tumoral environments of solid tumours. The M1 phenotype is characterised by the expression of high levels of pro-inflammatory cytokines, upregulation of reactive nitrogen and oxygen intermediates, promotion of Th1 response, and intense microbicidal and tumoricidal activity. After the stimulation

by lipopolysaccharide (LPS) and Th1 cytokines (such as IFN-γ and TNF-α), macrophages are polarized into M1 macrophages and characterized by TLR-2, TLR-4, CD80, CD86, iNOS, and MHC-II surface phenotypes. These cells induced different cytokines and chemokines (for example, TNF-α, IL-1α, IL-1β, IL-6, IL-12, CXCL9, and CXCL10), which exert positive feedback on unpolarized macrophages. It seems that NF-κB and STAT1 are the two major pathways involved in M1 macrophage polarization and result in microbicidal and tumouricidal functions (Martinez & Gordon, 2014; Murray, 2017). The connection between chronic inflammation and cancer, for example inflammatory bowel disease (IBD) and colorectal cancer (CRC), is indicative of the macrophage playing numerous roles in tumour development. Which particular macrophage function is vital during each phase of development is suggestive that the range of activities may be reproduced by plasticity in subset of TAMs. This inflammation-cancer association can be represented by the malignant transformation of oral phenotype (Biswas & Mantovani, 2010; Gordon,2010). Such a breakdown of regulation observed in CD would result in a dysfunctional innate immune response with downstream effects on the adaptive immune system and the commensal microbiota of the gut, which also plays an important role in barrier defences and mucosal tolerance. This total breakdown of barrier integrity and mucosal tolerance, coupled with the bias towards an inflammatory axis of Th1/ IL-12 and Th17/IL-23, results in a mucosal environment low in regulatory cytokines IL10 and TGFβ and high in IL-12p40. This inflammatory environment is conducive to M1-like macrophages activation/ differentiation with the corresponding up-regulation of proinflammatory cytokines and co-stimulatory molecule expression, (Martinez, 2010; Mantovani et al., 2002). Signals including IL-10, glucocorticoid hormones, molecules discharged from apoptotic cells and immune com-

plexes also profoundly impact monocyte-macrophage function. These signals induce expression of functional phenotypes that share nominated properties with M2 cells (e.g., high mannose and scavenger receptor expression), however, are distinct from them, for instance, in terms of the chemokine profile (Biswas & Mantovani, 2010). Plasticity and flexibility are the main structures of mononuclear phagocytes and their activation circumstances (Mosser, 2003). The phenotype of polarised M1-M2 macrophage can, to some extent, be reversed *in vitro* and *in vivo* (Guiducci et al., 2005; Sica & Bronte, 2007). Furthermore, pathology is frequently associated with dynamic changes in macrophage activation, with associated M1 cells concerned in originating and sustaining inflammation and M2 or M2-like cells connected with resolve or exhibiting chronic inflammation ,(**Figure 1.1**) (Martinez, Helming & Gordon, 2009). It remains unclear whether the mechanism of these switches includes the recruitment of circulating originators or the restoration of cells *in situ*. However, it is now apparent that specific or polarised T cells (Th1, Th2, Tregs) that are crucial orchestrators of polarised macrophage stimulation (Martinez, Helming & Gordon, 2009) display previously unanticipated flexibility and plasticity (O'Shea & Paul, 2010).

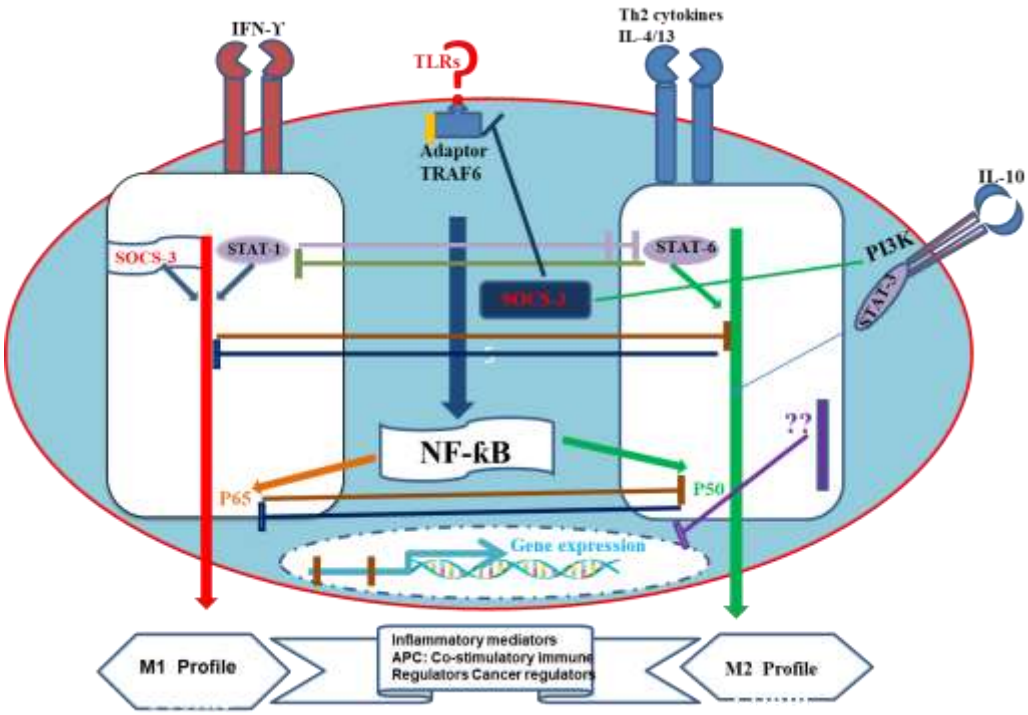

Figure1.1: Macrophages polarisation plasticity and effector phenotype can be determined by the manipulation of differentiation

Monocyte-derived macrophage, polarise towards the pro-inflammatory anti-tumour M1 subset(red arrows) by arrange of receptors, which include IFNγ-R and TLR activation (middle white box box) and signaling intermediates such as STAT-1, p65 NF-κB and SOCS3. Conversely, the polarisation towards the anti-inflammatory, M2 polarisation can be initiated by the receptors to the Th2-derived cytokines, IL-4/IL-13 The signaling intermediates associated with M2 macrophages include STAT3, and p50 NF-κB Cross-regulation is indicated by inhibitory (blunted) lines, which are colored purple to indicate M2 suppression of M1 polarisation and red to indicate M1 suppression of M2 polarisation (by STAT1), While the activation of its endogenous negative phosphatase regulator, PTEN, will indirectly bias polarisation towards M1 macrophages. Finally, activation of macrophage function by TLRs can be suppressed by inhibiting adaptor protein association and transduction of signals to TRAF6 and downstream effectors such as NF-κB and and endogenous-associated negative regulation through IL-10-induced SOCS3. Finally, activation of Mφ function by TLRs can be suppressed by inhibiting adaptor protein association and transduction of signals to TRAF6 and downstream effectors such as NF-κB and MAPKs via membrane-associated exogenous signaling and endogenous-associated negative regulation through IL-10-induced SOCS3. (Adapted from (Foey, 2015).

1.3.3: Molecular determinants of macrophage polarization

Macrophage polarisation and effector function is directed by a wealth of signals pathways and their component signalling molecules. For example, signals that regulate macrophage polarisation include NFκB, PI3K/PTEN, STAT3 and SOCS3. Members of the SOCS family control STAT-intermediated activation of macrophages. IL-4 and IFN-γ, the previous in conjunction with TLR stimulation, upregulate SOCS1 and SOCS3, which in turn inhibit the action of STAT1 and STAT3, respectively (Liu *et al.*, 2008; Whyte *et al.*, 2011). Macrophages are classified into two subgroups (M1 and M2 [M2a, M2b, M2c, M2d]) depending on their anti- or pro-inflammatory properties (Shapouri-Moghaddam *et al.*, 2018). Key transcription factors, for example NF-kB, STAT1, STAT5, IRF3, and IRF5 have been shown to regulate the expression of M1 genes. Many-faces of signalling molecules, transcription factors, and post-transcriptional regulators provoke the different forms of macrophage activation. Recognized IRF/STAT signalling pathways are stimulated by IFNs and TLR signalling to transform macrophage function toward the M1 phenotype (via STAT 1) or by IL-4 and IL-13 polarizing towards M2 subsets (via STAT6) (Murray, 2017; Sica & Bronte, 2007). M1 macrophages upregulate IRF5, which is essential for induction of cytokines (IL-12, IL-23, TNFα) intricate in provoking Th1 and Th17 responses (Krausgruber *et al.*, 2011). The IL-4 type I and type II receptors (Gordon & Martinez, 2010; Junttila *et al.*, 2008) activate STAT6, which in turn activates transcription of genes typical of M2 polarization, e.g.; mannose receptor (*Mrc1*), resist in-like α (*Retnla*, *Fizz1*), and chitinase 3–like 3 (*Chi3l3*, *Ym1*) (Pauleau *et al.*, 2004). IL-10 activates STAT3-mediated expression of genes (*Il10*, *Tgfb1*, *Mrc1*) associated with an M2-like phenotype. PTEN absolutely regulates TLR-induced IL-6 production; PTEN removal as well as constituent acti-

vation of PI3K was found to induce Arg-1 expression (Barrett, 2015). This is indicative that PTEN-ve macrophages expressed a functional phenotype similar to alternatively initiated or M2-like macrophages in a manner mediated by augmented activation of the transcription factors, C/EBPβ and STAT3. IL-10 signalling would perform to be basically associated with STAT3 and M2 polarisation, where STAT3 activation and IL-10 secretion are linked (Gunzl *et al.*, 2010) (**Figure 1.1**), and the STAT3-inducible cytokines IL-10 and IL-6, activate Arg-1 expression (Hasko *et al.*, 2000), a main marker of M2/ alternatively activated macrophage polarisation. If STAT3 plays a key role in M2 polarisation, it may represent a potential therapeutic target for the treatment of inflammatory pathology as evidenced by the conditioned STAT3 KO in mouse macrophages, which were refractory to IL-10 signalling and spontaneously established chronic entero-colitis (Riley *et al.*, 1999; Takeda *et al.*, 1999; Yao, et al., 2019). The polarisation of M1 macrophages is transduced by activation of the transcription factors NFκB and STAT-1 which induce the expression of M1-associated genes with further control of polarisation through the activity of SOCS3 (Liu *et al.*, 2008). In addition, the potential for differentiation towards an M2-like subset is prevented via STAT-1 inhibition of activation of the M2-polarising transcription factor, STAT-6, whereas the expressional knock-down (KO) of SOCS3 favours M2-polarisation (Liu *et al.*, 2008). Indeed Th2 cytokines induce Ym-1 expression (a poorly defined M2-associated molecule in mice) by a STAT6-dependent mechanism (Welch *et al.*, 2002). NFκB has been proposed to be integral to macrophage polarisation and effector function/disorder which enhanced the development of an anti-inflammatory M2-like macrophage phenotype (Weisser *et al.*, 2011). NFκB is also implicated in M2 polarisation, inversely, to p65 NFκB subunit involvement with M1 effector function, M2 polarisation processes are

driven by p50 NFκB subunits (Porta *et al.*, 2009). The targeting of NFκB would seem to play a role in macrophage polarisation and has been the subject of intense determinations in the re-education of tumour-associated macrophages (TAMs), originally defined as exhibiting a pro-tumoral M2-like phenotype (Hagemann *et al.*, 2008). Activation of the transcription factor, C/EBPβ is associated with the cAMP-dependent activation of CREB; cascades involving these transcription factors have been demonstrated to initiate M2 macrophage- specific gene expression and tissue reparative mechanisms (Ruffell *et al.*, 2009). The cAMP-activated factor CREB, is required for the full induction of C/EBPβ (Ruffell *et al.*, 2009), which trans-activates the Arg-1 gene promoter (Pauleau *et* al., 2004). As is the case with STAT 3, the expression and activity of IL-10 is associated with cAMP-mediated responses; whether this signalling pathway directly modulates polarisation or is an indirect consequence of IL-10 expression requires further investigation. What is clear, is that the profiles of pro-inflammatory and anti-inflammatory cytokines are differentially regulated by cAMP in a manner specific by original macrophage variation signals and stimulation signals in a PKC/cAMP/CREB axis (Foey et al., 2004; Pauleau et al., 2004). In addition to these signalling pathways, in influencing macrophage polarisation, it is likely that monocytes also display a level of polarisation. Fine control of macrophage polarisation and functionality is likely to be as a result of a complex cross-modulation between distinct signalling pathways rather than exclusive subset-specific pathway involvement.

1.3.4: Probiotic modulation of macrophage function

1.3.4.1: The Modulation of macrophage signalling and recognition

Probiotics regulate many macrophage-signalling pathways and have subsequent immunomodulatory functions on mucosal immunity. The vast majority of

the research has focused on the modulation of cytokine production by probiotic bacteria. Additional to the innate pro-inflammatory cytokines (TNFα, IL-1β, IL-6 and IL-8), specific consideration has been focussed on IL-10 and IL-12, as the production of these cytokines by macrophages and other immune cells in response to bacteria can control the type of immune response downstream of innate recognition. Also in intestinal epithelial cells, different signalling pathways are the target of probiotic regulation, including NFκB and ERK1/2, p38 and JNK MAPKs. The mechanisms by which the production of cytokine production is regulated by probiotics is being uncovered (Llewellyn & Foey, 2018).

1.3.4.2: Probiotic bacteria act as a modulator for pro-Inflammatory cytokines

Probiotics can inhibit inflammation by suppressing different signalling pathways at certain points. NFκB pathway is one of these signalling pathways, which may also be related to changes in MAPK pathways and PRRs. Different probiotics can downregulate IκBα phosphorylation or ubiquitination and the subsequent degradation of this NFκB inhibitor (Watanabe *et al.*, 2009). Selective strains of probiotics can inhibit LPS binding to the CD14 receptor, resulting in total reduction in NFκB activation and consequently, pro-inflammatory cytokine production (Menard *et al.*, 2004). Some of these probiotics can also inhibit MAPK pathway checkpoints, indicating that both NFκB and MAPKs play a role in pro-inflammatory cytokine production and that the use of probiotics, targeting these pathways may have a profound anti-inflammatory effect. For example, LTA (lipoteichoic acid, a TLR2 ligand) isolated from *Lactobacillus plantarum* (pLTA) inhibited the LPS-induced (TLR4-specific) TNFα production by decreasing the degradation of IκBα and IκBβ, resulting in the suppression of NFκB activation (Kim *etal.*,2008). Additionally, pLTA pre-treatment inhibited the phosphorylation

of ERK, JNK and p38 MAPKs in THP-1 monocytic cells, which is suggestive of signalling pathways modulated by endotoxin tolerisation. The levels of pro-inflammatory cytokines can also be regulated by the activation of suppressor of cytokine signalling (SOCS) family proteins. SOCS proteins are negative regulators of cytokine signalling pathways intermediate by JAK-dependent activation/phosphorylation of dimeric STAT transcription factors. The arrangement of JAK/STAT/SOCS isoforms establishing immune gene expression profiles, therefore macrophage functionality, as reviewed byO'Shea & Murray (2008). Additionally, STAT1 is linked with IFNγ and IL-12 signalling, although STAT3 is associated with anti-inflammatory signalling of IL-10 and IL-6. SOCS3 is inducible by IL-10 and IL-6 and is able to serve to both, inhibit pro-inflammatory cytokine gene expression as well as negatively feeding back to suppress IL-10 and IL-6 signalling. *Bifidobacterium* species decreased LPS-induced IL-1 and TNFα mRNA expression in murine RAW264.7 macrophage cells, which related with inhibition of IκB phosphorylation and augmented mRNA levels of SOCS1 and SOCS3 (Wald, 2003; O'Neill, 2008). A similar study also demonstrated that *B. breve*, LGG, and *L. helveticus* induced macrophage SOCS3 expression (Latvala *et al.*, 2011;Lee *et al.*, 2010). Together, these studies demonstrate that different inflammatory pathways can be modulated by different probiotics in order to induce anti-inflammatory effects. Such anti-inflammatory effects are not exclusively restricted to the direct effects of probiotic-dependent SOCS-suppression of inflammatory cytokines in macrophage cells. LAB inducing the expression of SOCS2 (*L. plantarum*) and SOCS3 (*L.acidophilus*), have been demonstrated to both activate/phosphorylate STAT-1 and STAT-3 whereas inactivating JAK2 and hence, downstream TNFα and IL-8 secretion (Lee *et al.*, 2010). Such effects on JAK2 would have intense and discriminatory effects on

macrophage polarization and subset-specific responses where JAK2 is essential for GM-CSF and IFNγ signalling but not for IL-6 and IFNα /β signalling. In addition to IL-10, G-CSF has been shown to have anti-inflammatory effects. *L. rhamnosus* strains GG and GR-1 have been shown to elicit the release of G-CSF from macrophages and that G-CSF has a paracrine effect on neighbouring macrophages and can suppress inflammatory responses (Kim *et al.*, 2006). G-CSF suppresses TNFα production, mediated by the activation of STAT3 and subsequently c-Jun inhibition. Furthermore, strain GR-1 treatment increased G-CSF production in normal human intestinal lamina propria cells. Reduced G-CSF production however, was observed in cells isolated from IBD patient tissue (Martins *et al.*, 2009). G-CSF-mediated mechanisms of action are being elucidated; using G-CSFR- deficient DCs, it was observed that GR-1-conditioned media induced significant IL-12/23 p40 production, which demonstrated that the G-CSF within the GR-1 conditioned media reduced IL-12/23 p40 production (Martins *et al.*, 2011).

1.3.4.3: Pro-inflammatory cytokine, TNFα

The production and release of cytokines from innate immune cells are acute responses to inflammation and infection in the body. Innate immune cells consist of populations of white blood cells such as circulating dendritic cells (DCs), neutrophils, natural killer (NK) cells, monocytes, eosinophils, and basophils, alongside with tissue-resident mast cells and macrophages (Iwasaki & Medzhitov, 2010). Inhabiting at the mainline of defence in immunity, these cells control attack by a wide range of viral, fungal, bacterial, and parasitic pathogens, partly by releasing a plethora of cytokines and chemokines to communicate with other cells and consequently orchestrate immune responses. This selection of soluble mediators secreted by different innate immune cells includes TNFα, IFN γ, inter-

leukins IL-1β, IL-4, IL-6, IL-10, IL-12, IL-18, CCL4/RANTES, and TGFβ. Cytokine release is directly evoked by immunoglobulin- or increase receptor-mediated signalling or by pathogens through a diverse selection of cellular receptors, including pattern recognition receptors such as TLRs (Iwasaki & Medzhitov, 2010; McGettrick & O'Neill, 2007). The Gram-negative bacterial coat component lipopolysaccharide (LPS), the main offender behind toxic shock syndrome and sepsis, is an extremely potent trigger of cytokine release through TLR4. For the immune system to function properly, the synthesis and release of cytokines has to be highly regulated and consecutively and temporally orchestrated. Accordingly, cascades of cytokines released by innate immune cells originally support inflammatory or allergic responses and then ensure that the responses are suppressed in an appropriate manner (Hu & Ivashkiv, 2009). Pro-inflammatory cytokines such as TNFα is generated by macrophages as a form of reaction to microbes, particularly in response to stimulation by LPS of Gram negative bacteria. TNFα is a significant mediator of acute inflammation and regulates the recruitment of macrophages to locations of infection via activating endothelial cells to secrete chemokines (Kigerl et al., 2009); TNFα additionally advocates the secretion of acute phase proteins. Furthermore, it has significant autocrine influences on major cellular origin, triggering them and improving their cytotoxic prospects (Beutler and Cerami, 1988). These conflicting influences of TNFα might partly be due to the presence of two distinct signalling pathways mediated through TNF receptor 1 (TNFR1; p55) and TNFR2 (p75).

1.3.5: Anti-inflammatory cytokines and their signal transducersIL-6 and IL-10

1.3.5.1: IL-10 /STAT-3

IL-10 cytokine is a main anti-inflammatory cytokine found within the human immune response. It is an effective inhibitor of Th1 cytokine, as well as IL-2 and IFN-γ. This activity leads to preliminary designation as cytokine synthesis inhibition factor (Howard et al., 1992; Lalani et al., 1997; Opalet al., 1998). In addition to its activity as a Th2 lymphocyte cytokine, (Opal, Wherry & Grint, 1998), IL-10 is also a potent deactivator of monocyte/macrophage pro-inflammatory cytokine synthesis (Clarke et al., 1998; Gerard et al., 1993). IL-10 is primarily synthesized by $CD4^+$ Th2 cells, monocytes, and B cells and circulates as a homodimer consisting of two tightly packed 160-amino-acid proteins, (Clarke *et al.,* 1998). IL-10 inhibits monocyte/macrophage-derived TNF-α, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, MIP-1α, and MIP-2α (Gerard et al., 1993; Marchant et al., 1994a). Furthermore, IL-10 inhibits cell surface expression of major histocompatibility complex class II molecules, B7 accessory molecules, and the LPS recognition and signalling molecule CD14, (Opalet al., 1998). Moreover, it inhibits cytokine production by neutrophils and natural killer cells. IL-10 inhibits nuclear factor κ B (NF-κB) nuclear translocation after LPS stimulation (Clarke et al., 1998) and promotes degradation of messenger RNA for the pro-inflammatory cytokines. In addition to these activities, IL-10 attenuates surface expression of TNF-α receptors (p75) and promotes the shedding of TNF-α receptors into the systemic circulation (Dickensheets et al., 1997; Joyce et al., 1994). IL-10 is present in sufficient concentrations to have a physiologic impact on host responses to systemic inflammation.

Physiologically inadequate IL-10 responses after systemic injury may have detrimental consequences too (Ware & Matthay, 2000). Human volunteers who were given IL-10 after endotoxin challenge suffer fewer systemic symptoms, neutrophil responses, and cytokine production than placebo-treated control subjects (Pajkrt et al., 1997). Moreover, mice who have genetic deletions of the IL-10 gene are more susceptible to endotoxin-induced shock than normal mice (Dai et al., 1997). IL-10 generally protects the host from systemic inflammation after toxin-induced injury, but renders the host susceptible to lethality from overwhelming infection in a variety of experimental studies (Greenberger et al., 1995; van der Poll et al., 1996). This observation should be kept in mind when administering anti-inflammatory cytokines in clinical medicine. This indicates that endogenous concentrations of IL-10 are important in limiting the inflammatory response to gut-associated bacteria. For this reason, IL-10 is tested and failed in early 2000's, in clinical trials as an anti-inflammatory therapy for inflammatory bowel disease amongst other potential indications. Initiation of STAT-3 is vital for the whole of the acknowledged outcomes of IL-10 (Lang et al., 2002, Takeda et al., 1999). IL-10 links to its cognate receptor (IL-10R), a tetramer comprised of two notably specific chains (IL-10R1 and IL-10R2). As such, the findings of IL-10 seem to be related to particular cells of the immune system. IL-10 binding to IL-10R initiates the IL-10/JAK1/STAT-3 cascade, where phosphorylated STAT-3 homodimers translocate to the nucleus in a matter of seconds to recruit the target genes being expressed (Hutchins et al., 2013).

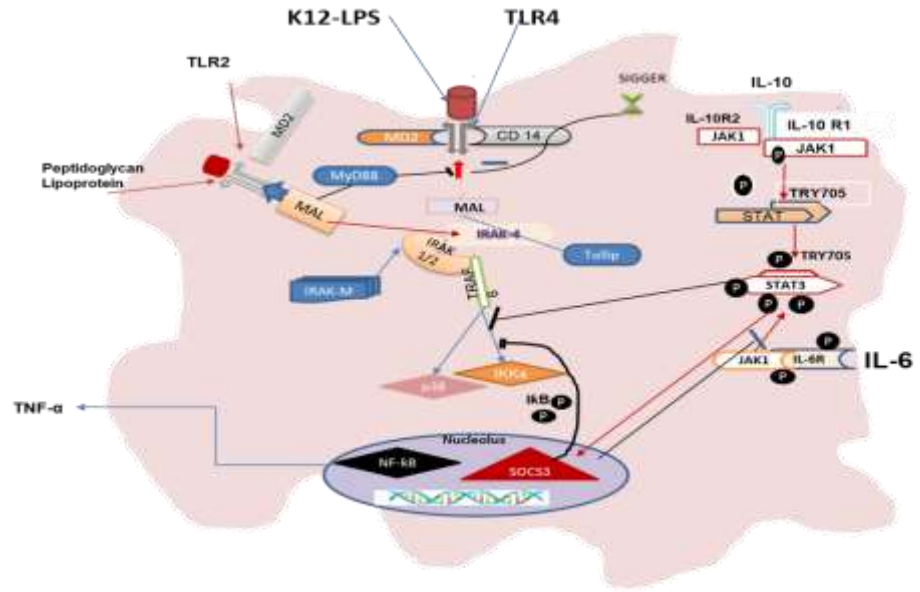

Figure 1.2: Collaboration between TLR2 and TLR4 signalling pathway and JAK/STAT signalling pathway

Stimulation by peptidoglycan or lipoprotein and *E.coli* Lipopolysaccharide LPS (K12-LPS); TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; CD14; MD2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; MyD88s, myeloid differentiation primary response 88 short version; IRAK-1,2,4 &M, IL-1 receptor-associated kinase; Tollip, Toll interacting protein; TRAF6, TNF receptor-associated factor-6; SIGIRR, Single Immunoglobulin IL-1 Receptor Related molecule; IKKs, IκB kinases; NF-κB, nuclear factor kappa B; P38, P38 mitogen-activated protein kinases; SOCS3, Suppressor of cytokine signalling proteins. As adapted from (Zhu et al., 2015, Cavaillon and AdibConquy, 2006a, Wang et al., 2011).

1.3.5.2: IL-6 and SOCS3

Studies have demonstrated that IL-6 has pleiotropic activity on several cells. Interleukin-6 (IL-6) is a four-helical protein of 184 amino acids. The cDNA of IL-6 was cloned in 1986 by Hirano et al., (1986). It was recognized that IL-6 belonged to a large family of cytokines, which all shared the four-helical protein topology (Bazan, 1990). The IL-6 receptor (IL-6R), a protein with an Ig-fold, binds IL-6 with nanomolar attraction (Yamasaki et al, 1988). Binding of IL-6 to the IL-6R, conversely, does not prime signalling. The compound of IL-6 and IL-6R links with the protein 130 gp thereby inducing its dimerization and starting intracellular signalling via the JAK/STAT pathway (Hibi et al., 1990). In addition to the activation of the canonical JAK/STAT pathway, it stimulates the phosphatase SHP-2 which is recruited to tyrosine phosphorylated gp130; SHP-2 is also

phosphorylated by JAK1 and thereupon mediates the activation of the Ras-Raf-MAPK signalling pathway(Scheller, Grötzinger & Rose-John, 2006). Rheumatoid arthritis (RA) is a chronic disease with joint and systemic inflammation resulting from immunological abnormalities and it has been found that IL-6 plays a key role in the development of this disease(Kishimoto, 2010;Yoshida, 2014). Research has shown (STAT3) as a downstream signalling effector of IL-6 reception (Alonzi et al., 2004). Progressive degrees of phosphorylated STAT-3 were connected with disease severity in people suffering from IBD, in addition to some animal models of colitis or enteritis (Suzuki et al., 2001). STAT-3 stimulation, especially in mucosal T cells, is a significant inflammatory event within the progress of IBD. SOCS genes are associated in negative regulation of the JAK/STAT pathway (Yoshimura et al., 2003). Current thought is that SOCS3 and STAT-3 are both expansively expressed inside human IBD and in animal IBD models (Suzuki et al., 2001). The stages of SOCS3 in the irritated intestine are probably insufficient to inhibit STAT-3 activation, thus encouraging chronicity of inflammation (Mitsuyama et al., 2006) (Figure **1.2**).

1.3.5.3: TOLL-Like Receptor

Toll-like receptors (TLRs) are the most studied of the pattern receptor (PRR) families and TLR4 is one of the most researched TLRs (**Table 1.1**). TLR4 specifically recognizes bacterial lipopolysaccharide (LPS) and its activation cheifly guides to the synthesis of pro-inflammatory cytokines and chemokines (Janssens & Beyaert, 2003). For example, the TLR4 agonist monophosphoryl A(MPL), a derivative of LPS has been used as an adjuvant for human vaccines in clinical trails. For example, GLA(glucopyranosyl lipid adjuvant), a synthetic lipid A, is presently under test in formulation of stable emulsion (SE) in candidate vaccines against tuberculosis (Meyer & McShane, 2013). A phase 1

trial of GLA/SE adjuvanted H5N1 vaccine is being studied as a potential prophylactic treatment against influenza infection (Carter *et al*., 2018; Treanor *et al.*, 2013).

The capability of pathogens to evolve quickly has applied strong evolutionary pressure on the mammalian immune system to adapt in parallel. Therefore, human and murine responses to TLR activation have some comparisons but also show differences, (Schroder *et al.*, 2012) (**Figure 1.2**). These differences may affect the prognostic value of mouse models for immunological studies, making extrapolation from mouse data to human difficult to achieve. However, although there are different TLR sequences among species, the basic biological function and down-stream signalling pathways appear to be significantly conserved (Ketloy *et al.*, 2008; Rehli, 2002).

TLRs, first identified in human in the 1990s, are members of type-1 transmembrane receptor family and are evolutionarily conserved proteins among vertebrates and invertebrates (Medzhitov, Preston-Hurlburt & Janeway, 1997; Werling & Jungi, 2003). TLRs are characterised by an extracellular leucine-rich repeat (LRR) domain for ligand binding and an intracellular toll/interleukin-1 (IL-1) receptor-like (TIR) domain. The latter is a highly preserved protein–protein interaction motif module crucial for signal transduction (Janssens & Beyaert, 2003;Kanzler *et al.*, 2007). The expression, ligand recognition, and signalling pathways of TLRs, as well as the immune penalties of their activation have been labelled at length elsewhere (Akira & Takeda, 2004; Kanzler *et al.*, 2007). In 1999, Qureshi et al. reported the TLR4 gene in the LPS chromosomal region as responsible for the defective LPS reaction in some mouse strains. They also recognised independent mutations in the TLR4 genes of two LPS-hyporesponsive mouse strains (C3H/HeJ and C57BL10/ScCr), suggesting that TLR4

is important for mediating responses to LPS *in vivo* (Poltorak et al., 1998;Qureshi *et al.*, 1999). Additional to the recognition of LPS, a main component of the outer membrane of Gram-negative bacteria, TLR4s from numerous species (e.g., humans and mice) recognize other components (Yang *et al.*, 2001) of pathogens such as mannuronic acid polymers from Gram-negative bacteria (Flo *et al.*, 2002), teichuronic acid from Gram-positive bacteria (Yang *et al.*, 2001), and viral components such as the F protein of respiratory syncytial virus (Haynes *et al.*, 2001; Kurt-Jones *et al.*, 2000; Ohashi *et al.*, 2000). In addition to exogenous PAMPs, TLR4 also binds endogenous molecules such as heat shock proteins in the mouse (Ohashi *et al.*, 2000; Vabulas *et al.*, 2001), in the rat (Kim *et al.*, 2009), and in humans (Roelofs *et al.*, 2006). Fibronectin type III extra domain A (Gondokaryono *et al.*, 2007) and saturated fatty acids (Kim *et al.*, 2007) are potentially recognized by human and mouse TLR-4, and heme by mouse TLR-4 (Figueiredo *et al.*, 2007). Recently, Choi et al.(2009) identified cholesteryl ester hydroperoxides, the active components of minimally modified low-density lipoprotein (mmLDL), as a new class of endogenous mouse TLR-4 agonists (Choi *et al.*, 2009; year Werling & Jungi, 2003). These findings indicated that molecules produced or circulating during abnormal situations, such as during tissue damage, are able to trigger TLR-4 dependent pathways (Werling & Jungi, 2003).

Structurally, TLR-4 forms a complex on the cell surface with several other proteins needed for ligand recognition (e.g., LPS) (Janssens & Beyaert, 2003; Werling & Jungi, 2003). In the serum, LPS is initially bound by LPS binding protein (LBP), which transfers LPS to CD14. CD14 is a glycosylphosphatidylinositol-anchored membrane protein that also exists in a soluble form and that binds LPS–LBP complexes with high affinity. While CD14 itself lacks an intracellular

domain for signalling, it associates with TLR-4 to form a functional LPS receptor complex. Binding of LPS also requires the MD-2 protein, which associates with the extracellular domain of TLR-4 (Park et al., 2009). Thus, the active LPS receptor complex includes CD14, TLR-4, and MD-2 although further study suggests that CD14 and LBP only enhance the TLR4-dependent LPS signalling and are not required for LPS binding and signalling (Lizundia et al., 2008).

Table 1.1: PRR ligands and subcellular localization

Receptor	Localization	Ligand	Origin of the ligand
TLR2	Cell surface	Lipopeptides	Bacteria
		Lipoproteins	G+ bacteria
		LTA	G+ bacteria
TLR2/1	Cell surface	Triacylated lipopeptide	G-bacteria, mycoplasma
TLR2/6	Cell surface	Diacylated lipopeptides	G+ bacteria, mycoplasma
TLR3	Intracellular compartment	dsRNA	Viruses, virus infected cells
TLR4/MD2 TLR4/CD-1	Cell surface, intracellular compartment	LPS	G- bacteria
TLR5	Cell surface	Flagellin protein	Bacteria
TLR7	Intracellular compartment	ssRNA	Viruses
TLR8	Intracellular compartment	ssRNA	Viruses
TLR9	Intracellular compartment, cell surface	DNA	DNA viruses, bacteria
TLR11	Cell surface	Uropathogenic bacterial components	Uropathogenic bacteria
NOD1	Cell cytoplasm	Meso-DAP	PG from G-, some G+, mycobacterium
NOD2	Cell cytoplasm	MDP	PG from G-, G+ bacteria, mycobacterium

PG, peptidoglycan; LTA, lipoteichoic acid; LPS, lipopolysaccharide; DAP, diaminopimelic acid; MDP, muramyl dipeptide; G+, Gram positive; G-, Gram negative; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA.This table adapted from (Wells, Loonen & Karczewski, 2010)**.**

1.3.5.4: The Inflammasomes

Inflammasomes are composed of multiprotein cytosolic complexes that fold to stimulate caspase-1 (Martinon, Burns & Tschopp, 2002). Inflammasomes multimeric stages are initiated in a range of cells including macrophages, dendritic cells, adipocytes, keratinocytes, and epithelial cells (Agostini *et al.*, 2004; Lissner & Siegmund, 2011). This activation of the pro-inflammatory cytokines (IL-1β and IL-18) is initiated either by NLR proteins NLRP1, NLRP3, NLRC4, NLRP6, and NAIP5 or by the DNA-sensing complex of AIM2, a member of the interferon-inducible HIN-200 protein family (Tschopp, 2010). Stimulation of these receptors by induced PAMPs leads to their oligomerization and consequent collaboration with the adaptor protein ASC and the CARD domain of caspase-1. ASC, (Figure 1.3), also presents a CARD domain that interacts with the CARD domain of procaspase-1 (Lissner & Siegmund, 2011). Inflammasome-activated caspase-1 is then used for the stimulation of the proinflammatory cytokines IL-1*β* and IL-18, both belonging to the IL-1 family. These inflammatory cytokines improve antimicrobial functions and support resistance to intracellular pathogens and support resistance, (Martinon, Burns & Tschopp, 2002), intracellular pathogens, (Martinon & Tschopp, 2007). The activation of NLRP3 required several mechanisms. Firstly, extracellular adenosine triphosphate (ATP) induces a P_2X_7R-reliant on pore creation in the cell membrane, consequently refereeing a shift from intra- to extracellular potassium (Kahlenberg & Dubyak, 2004). This, in turn, activates the formation of pannexin-1 channels into the membrane, allowing the higher molecular PAMPs and danger associated molecular patterns (DAMPs) penetration into the cell and thus direct activation of NLRP3 (Kahlenberg & Dubyak, 2004). A second suggested mechanism, results from the phagocytosis of crystalline or

particulate ligands, which enter the cell after the break of the phagolysosomes, where they can again directly bind and activate the inflammasome (Schroder & Tschopp, 2010). A third mechanism of activation involves reactive oxygen species (ROS), produced after exposure to PAMPs and DAMPs, which can activate the inflammasome via a ROS-sensitive ligand, thioredoxin-interacting protein (Cruz *et al.*, 2007; Zhou *et al.*, 2010). This is strongly highlighted by the finding that chemically activated ROS blockade suppresses inflammasome activation. The single essential function of the inflammasome is the consistent response to pathogen infections. Its main activators are whole microorganisms and PAMPs. Possible identifiable pathogens comprise fungi (*Candida albicans*, *Saccharomyces cerevisiae*), (Zhou *et al.*, 2010), bacteria, (*Staphylococcus aureus*, *Listeria*), and viruses, (influenza virus, adenovirus) (Kanneganti *et al.*, 2006; Mariathasan *et al.*, 2006). Apart from pathogens, exogenous mechanical factors, such as ultraviolet B irradiation, (Feldmeyer *et al.*, 2007), and particulate ligands, such as silica, (Muruve *et al.*, 2008) and asbestos, (Dostert *et al.*, 2008), can equally lead to inflammasome activation and IL-1β release. In recent years, the spectrum of activating factors has been broadened even more, since several host-derived molecules (ATP, hyaluronan) and metabolic stress, such as hyperglycemia (Maedler *et al.*, 2002) and free fatty acids (Joosten *et al.*, 2010), could been identified to potentially induce inflammasome activation. Consequently, although a representative of the innate immune system, the inflammasome serves not only as a sensor for infections, but also for metabolic danger.

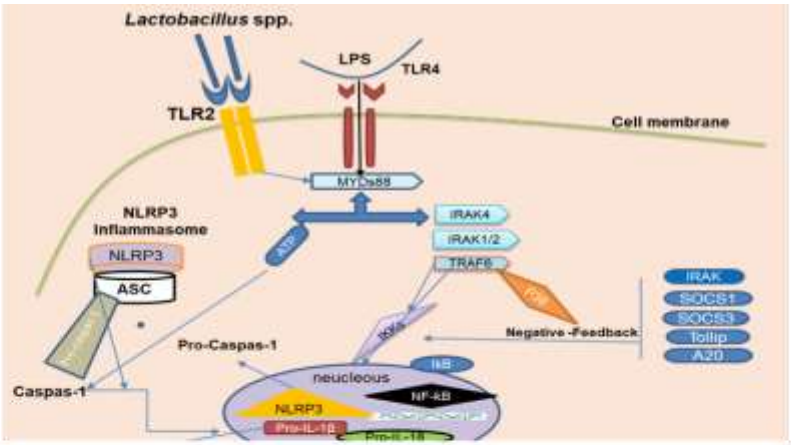

Figure1.3: Signalling proceedings associated with IL-1β and IL-18 production, inflammasome

TLR4/MD2 signal and TLR2 through either MyD88 to activate NF-κB. Activation of NF-κB results in increased transcription of nlrp3. Inflammasome activation follows in response to such a TLR4/MD2 signal and TLR2 through either MyD88 to activate NF-κB. Activation of NF-κB results in increased transcription of nlrp3. Inflammasome activation follows in response to such agonists as extracellular ATP. If TLR4 signalling increases NLRP3 protein levels such as with MyD88-competent LPS then the activation event permits the prior expressed NLRP3 pro-caspase-1, leading to caspase-1 processing to its active form and inflammasome assembly. Pro-IL-1β and pro-IL-18, which is associated with the inflammasome, is administered to mature IL-1β and IL-18 and secreted into the extracellularly, I presence of negative regulatory molecules IRAKM, SOCS1/3, Tollip and A20. Adapted from (Sun, 2017 ; Guo, 2015;Chilton et al., 2012).

1.3.5.5: TRAIL, Tumor necrosis factor-related apoptosis inducing ligand

TRAIL is a member of the tumor necrosis factor (TNF) family of cytokines. It is an effective inducer of apoptosis in numerous tumor cells but not in the majority of normal cells (Ganten et al., 2005; Koschny et al., 2007). However, systemically delivered TRAIL suffers from a rapid clearance from the body with an extremely short half-life (Kelley, 2001; Xiang, 2004). The receptor of TRAIL occurs on tumor cell called FasL. Some studies revealed that upregulation of FasL expression by tumor cells may enable the tumor cells to kill antitumor immune effector cells by activating lymphocytes express Fas (Houston, 2003). On the other hand, TRAIL exhibits much stronger apoptotic activity than other TNF family members, kills tumor cells more effectively than normal cells, and is unlikely to initiate inflammatory cascades following systemic administration. These unique features of TRAIL have attracted considerable attention on TRAIL as a potential

therapeutic to treat human cancers (Curnis et al., 2004; (Sheridan, 1997; Huang et al., 2005; Kim et al., 2004). TRAIL stimulated tissue regeneration by enhancing the resolution of pathological inflammation through the activation of the NLRP3 inflammasome pathway. In addition, TRAIL reduces the induction of colitis and inhibiting pro-inflammatory signalling, promoting tissue repair to maintain intestinal homeostasis through activation of the NLRP3 inflammasome (Kim et al., 2017).

Apoptosis is the physiological form of cell death, which is essential for the elimination of potentially dangerous cells, such as infected or transformed cells, and for the regulation of immune response (Schaefer et al. 2007). It also plays a vital role in tissue homeostasis, for instance, in the turn over of epithelial cells of the gastrointestinal mucosa. Dysregulated apoptosis contributes to carcinogenesis and is involved in inflammatory damage in chronic inflammatory diseases, such as chronic arthritis and inflammatory bowel disease. These new understandings into the immunological functions of TRAIL and TRAIL receptors led to the question of whether they are also involved in the development of inflammatory bowel diseases (IBD). The most important of which are Crohn's disease (CD) and ulcerative colitis (UC). Right now, there exist only limited data on the specific molecular mechanisms that lead to epithelial destruction during inflammation in CD and UC. A few years ago, some mechanisms that might play a role in the pathogenesis of IBD were discussed. Data showed that, for example, enhanced expression of chemokine receptors may be involved in the development of IBD (Autschbach *et.al*. 2005). Apoptosis is one of the key mechanisms that might influence immune functions as well as barrier functions of the gut. The notion is increasing that the dysregulation of apoptosis in intestinal epithelial cells and immune cells is involved in the pathogenesis of IBD. In epithelial

cells in IBD, increased apoptosis rates up to 5% in mild-to-moderately irritated colon specimens have been reported, resulting in a disordered barrier function (Strater *et al*.1997). Additionally, many studies propose that deficient apoptosis in activated lymphocytes and monocytes might take part in the development of IBD by an accumulation of inflammatory cells in IBD mucosa (Atreya, et al., 2000). Accordingly, the imbalance between pro- and anti-apoptotic factors of epithelial and immune cells might play a fundamental role in the pathogenesis of inflammatory bowel disease. In line with this assumption, Begue et al, (2006) showed TRAIL upregulation in epithelial cells in biopsy specimens from inflammatory colon and ileum. Interferon-γ and TNF-α potentially induced TRAIL in intestinal epithelial cells.

1.4: Endotoxin Tolerisation

Endotoxin tolerance is a phenomenon, which induces a cellular hypo-responsive state, whereby cells are unable to respond to further challenges, such as that with LPS**.** Endotoxin tolerance has been investigated *in vivo* and *in vitro* in both humans and animals (Biswas and Lopez-Collazo, 2009). The suppression of pro-inflammatory response, and inversion to anti-inflammatory response, are the most common pathways of endotoxin tolerance (Foster and Medzhitov, 2009). The physiological role of tolerance is to reduce the potential damage for the host tissue, due to prolonged production of pro-inflammatory cytokines. The suppression of pro-inflammatory responses occurs in some diseases, such as cancer. Tolerance stimulation and sensitivity to endotoxin tolerance is important to homeostatic function of the gut mucosa, effectively allowing the gastrointestinal tract to define immune response. Gut mucosal macrophages are important in endotoxin tolerance, through guiding the tolerisition process, by either suppression or induction of immunity. Macrophage tolerisation occurs

in many approaches, such as the stimulation of anti-inflammatory cytokines (e.g. IL-10 and TGFβ), down-regulation of PRRs (e.g.TLR4), detachment and secretion of cytokine receptors and PRRs and the induction of negative regulatory molecules, like Tollip, Myd88s, SARM, sTLRs, sCD14 and SIGIRR (O'Neill, 2008) also reviewed in (Foey and Crean, 2013). Endotoxin tolerisation of M2 subset macrophages has been shown to suppress pro-inflammatory cytokines with a concurrent up-regulation in anti-inflammatory cytokines such as IL-10 (Mantovani et al., 2005). However, it is extremely simplistic to allocate endotoxin tolerisation states to specific macrophage subsets owing to the diversities of macrophage phenotypes reflected by their activation and differentiation (Cavaillon and Adib-Conquy, 2006b). The preliminary *in vitro* research of endotoxin tolerance in human monocytes (Cavaillon and Adib-Conquy, 2006b, del Fresno et al., 2009) demonstrated re-stimulation with LPS to modulate expression of pro-inflammatory cytokines and chemokines like TNFα, IL-6, IL-1β, CCL3 and CCL4 (del Fresno et al., 2009, Draisma et al., 2009, Foster and Medzhitov, 2009, Mages et al., 2007). Genes of anti-inflammatory cytokines, like IL-10 and TGF-β and negative regulators, like IRAK-M were up regulated (Biswas and Lopez-Collazo, 2009). Tolerance mechanisms may start with inhibition of TLR expression (Nomura et al., 2000, Wang et al., 2002), whereas the down regulation of cascade of immune reactions is linked with increasing expression of TLR-4 surface protein.

Research has revealed that tolerance is associated with inhibition of TLR expression, down-regulation of cascade of immune reactions, linked with increasing expression of TLR4 surface protein and the lack of the necessary co-receptor subunits MD2 and CD14 (Abreu et al., 2001, Cario and Podolsky, 2000b, Otte et al., 2004). Moreover, suppression of cytokine signalling (SOCS)

regulates endotoxin tolerance through the negative regulation of signal transduction (Shin *et* al*.*, 2015). SOCS1 is an acute down-regulating factor for endotoxin signal pathways and its expression is promptly induced by endotoxin stimulation in macrophages (Duncan *et al.*, 2017). The studies revealed that SOCS1 induction could inhibit endotoxin-induced NF-κB and STAT1 activation in macrophages. Mice with SOCS1-deficiency were highly sensitive to endotoxin-induced shock, as well as secreting high levels of inflammatory cytokines (Duncan *et al.*, 2017). Whereas the heterodimeric p65/p50 NF-κB initiates TNFα and IL-12 expression in the primary stages of inflammation, homodimeric p50/p50 NF-κB in endotoxin tolerance suppresses the expression of these genes and boosts the expression of IL-10 and TGFβ. This agrees with the argument that the plasticity of NF-κB role inside adaptable stages of inflammation might acquire separate cell phenotypes from inflammatory to anti-inflammatory, reviewed in (Biswas and Lopez-Collazo, 2009). Besides TLRs, the cytoplasmic nucleotide-binding Oligomerization domain (NOD) proteins, NOD1 and NOD2, might play a role within the natural immune reaction (Cario and Podolsky, 2005; Watanabe, 2006). It has been proposed that NOD2 might control the TLR-signalling pathway in a detrimental manner.

The removal of macrophages and the restriction of pro-inflammatory cytokines in endotoxin tolerance are frequently connected with the expansion in anti-inflammatory cytokine generation, like IL-10 and TGFβ (Cavaillon and Adib-Conquy, 2006a, Schroder et al., 2003, Sfeir et al., 2001). Endogenous suppressors like IRAK-M, ST2, and short version of MyD88 (MyD88s) are connected with TLR4 downregulation and an absence of LPS reaction. IRAK-M expression has been suggested and verified within both, human and mice endotoxin tolerance models (Biswas and Lopez-Collazo, 2009). Extensive research has uti-

lised *in vitro* and *in vivo* concepts to develop our awareness of endotoxin tolerance. Foey and Crean (2013) indicate that macrophage subsets, resulting from the THP-1 cell line, demonstrated varying reactions to PG-LPS activation when they were pre-treated with *Porphyromonas gingivalis* LPS (PG-LPS). Pre-stimulation of M1-like macrophages with PG-LPS did not inhibit TNFα, IL-6 or IL-1β generation when treated with PG-LPS. In contrast, it did restrict the generation of TNFα and IL-6 but not IL-1β by M2-like macrophages when PG-LPS was applied. PG-LPS-stimulated M1 CD14hi macrophages expressed a greater degree of TNFα, IL-1β and IL-6, in contrast to M1 $CD14^{lo}$ macrophages. Sun *et al., (*2014), revealed that TLR4, TLR-2, IRAK-M, SOCS1 play an important role in tolerance. In addition, the level of IL-8 in tolerised THP-1 induced by (PG-LPS) upregulation significantly. While downregulation of TLR2 and TLR-4 in tolerised THP-1 expression by *P. gingivalis* LPS was observed. Moreover, re-stimulation of THP-1 with *P. gingivalis* LPS or *E. coli LPS* also enhanced IRAK-M and SOCS1, while maintaining the expression of IRAK4 and Tollip concurrently with the suppression of macrophage proinflammatory cytokines(Al-Shaghdali et al., 2019).

1.5: Pathological aspect of inflammatory bowel Disease, (IBD)

IBD is defined as a chronic inflammatory disease affecting the gastrointestinal tract, whose major forms are classified into ulcerative colitis (UC) and Crohn's disease, (**Figure 1.4 c / b**). Their causes are still unknown, even though several factors have been identified as major determining factors for induction or suppression. To describe IBD from genetic studies, it is essential to define the phenotype accurately, if gene association patterns whether Crohn's and UC are to be recognized. UC is characterised by widespread mucosal inflammation, restricted to the colon, with confluent inflammation covering proximally from the

rectum to varying extent, and of varying severity. Crohn's disease alternatively, is characterised by patchy transmural inflammation, which may affect any part of the gastrointestinal (GI) tract. Up to 10% of children with IBD affecting the colon, cannot be classified as having either UC or Crohn's disease despite full clinical, radiological (or other imaging), endoscopic and histological assessment, and are thus classified as having IBD unclassified (formerly termed indeterminate colitis) (Jess *et al.*, 2006; Sawczenko *et al.*, 2001). The role of the intestinal environment and enteric flora, in particular, appears to be of greater significance than previously thought. This complex relationship of genetic, microbial and environmental factors are related to the continuous activation of the mucosal immune and non-immune response, probably assisted by defects in the intestinal epithelial barrier and mucosal immune system, resulting in dynamic inflammation and tissue destruction (Chistiakov *et al.*, 2015). Under normal conditions, the intestinal mucosa is in a phase of ‘controlled’ inflammation regulated by a slight balance of pro-inflammatory (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, interleukin [IL]-1, IL-6, IL-12) and anti-inflammatory cytokines (IL-4, IL-10, IL-11). The mucosal immune system is the fundamental effector of intestinal inflammation and injury, with cytokines playing an essential role in modulating inflammation (O'Shea & Murray, 2008). It is suggested that the pathogenesis of IBD arises as a consequence of the irregular immune response to enteric bacteria, leading to overproduction of pro-inflammatory cytokines such as TNFα and IL-1β, promoting tissue injury of the gut mucosa. Management of people suffering from IBD include consideration of their lifestyle and suitable changes introduced, plus medical and surgical therapies. The principle that TNF-α plays a role in IBD pathophysiology, is emphasised by the successful use of anti-TNF treatments for patients who have CD (Targan et al., 1997).

Medical treatments include, corticosteroids, immunosuppressant agents and biologic treatments, containing varying anti-tumor necrosis factor elements (TNFα compounds) (Baumgart and Sandborn, 2007). The origins of IBD are still not clear although it supposes that genetically susceptible people are susceptible for suffering from it due to an unusual immune reaction towards the microorganisms of the intestinal flora, potentially resulting in dysbiosis of the microbiota. There is evidence to indicate that the dysregulated innate and adaptive immune pathways result in abnormal intestinal inflammatory reactions in individuals suffering from IBD. The majority of research undertaken within the last three decades has placed its emphasis on the function of aberrant adaptive immune reactions in the origins of IBD. Whilst CD has normally been perceived as being prompted by a Th1 reaction, UC has tended to be connected to an unconventional Th2 reaction (Fuss et al., 1996). Besides typical Th1 and Th2 reactions, a role for Th17 cells has also become evident (Geremia and Jewell, 2012). Evolution resulting from genome-wide association studies (GWAS) and immunological studies has most recently implicated the emphasis of IBD's origins towards mucosal innate immune reactions, including innate microbial sensing, epithelial barrier integrity, unfolded protein reaction, and autophagy (Geremia et al., 2014). Coqueiro *et al.* (2019), demonstrated, that lipopolysaccharides (LPS) of the gram-negative bacteria from the colon enter the circulation, stimulating circulating and local immune cells by binding to the Toll-like receptor 4, directing to the activation of the nuclear factor kappa-B (NF-κB). In response, there is an augmented synthesis of pro-inflammatory cytokines and increased inflammation of the intestinal epithelium.

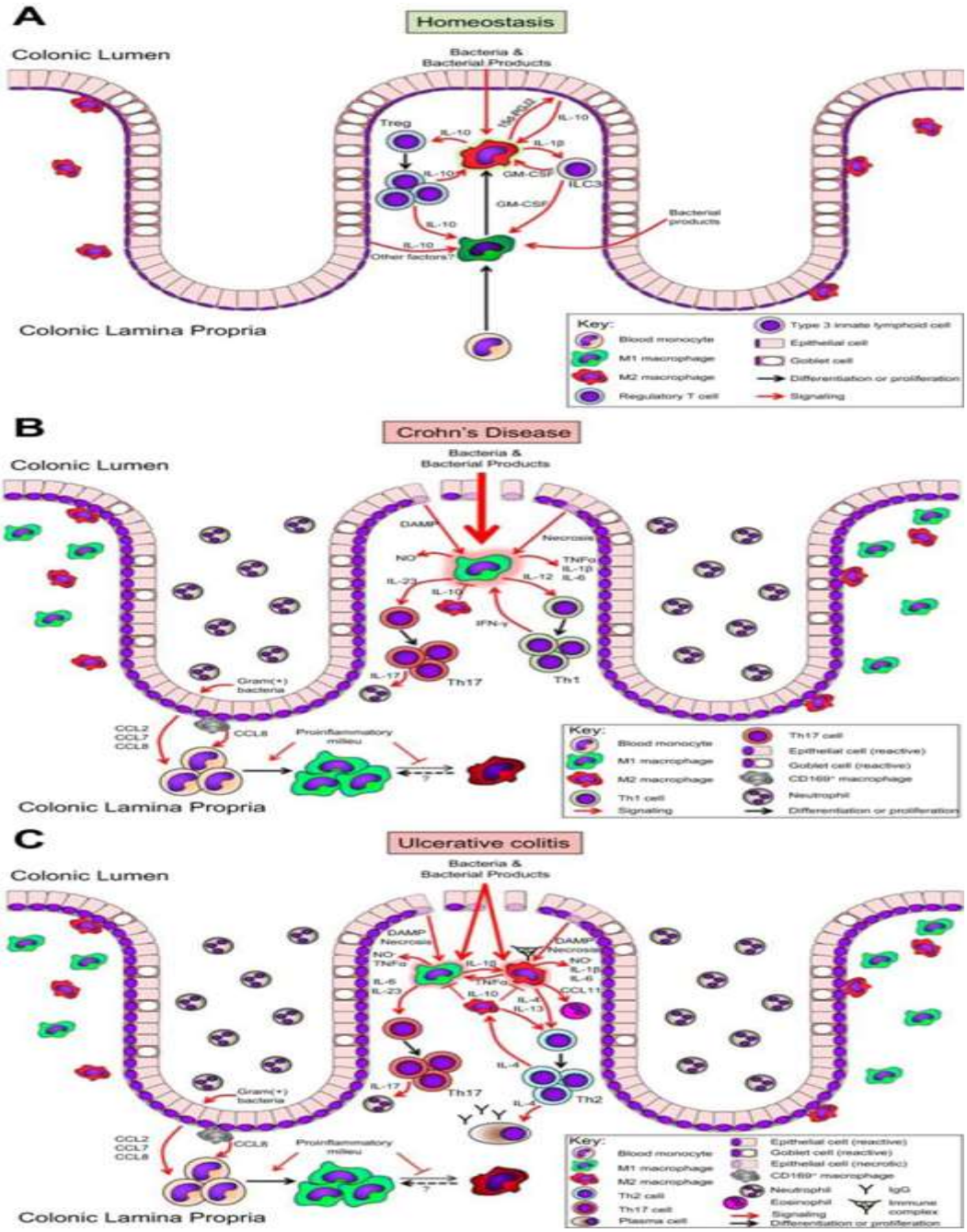

Figure1.4: Mechanisms of macrophage polarization. Colonic macrophage differentiation and phenotype in homeostasis and inflammatory

The colonic macrophage is a monocyte-derived tissue macrophage. A: in physiological conditions, monocytes extravasate into the colonic lamina propria and differentiate into macrophages, which initially develop a pro-inflammatory/M1 phenotype, but later acquire the characteristic anti-inflammatory/M2 phenotype. The signals that cause the colonic macrophage to replace the initial pro-inflammatory phenotype with an anti-inflammatory and protolerogenic profile are incompletely understood, but can include IL-10 secreted from intestinal epithelial cells (IECs) and regulatory T (Treg) cells, granulocyte-macrophage colony stimulating factor (GM-CSF) secreted from type 3 innate lymphoid cells (ILC3), and bacterial products from the colonic microflora. The colonic macrophage contributes to homeostasis by clearing bacteria and bacterial products that translocate into the lamina propria without mounting an immune response and by interacting with other mucosal cells. B: in Crohn's disease, monocytes recruited to sites of inflammation differentiate into macrophages that maintain their proinflammatory/M1 phenotype instead of transitioning to the characteristic anti-inflammatory/M2 phenotype

of colonic macrophages through mechanisms that remain poorly understood but that likely involve the proinflammatory milieu. Increased monocyte recruitment is thought to result from increased secretion of CCR2-ligating chemokines (i.e., CCL2, CCL7, CCL8), possibly resulting from IEC sensing of Gram-positive bacteria or activation of $CD169^{+}$ macrophages at the base of the colonic crypts. In response to the epithelial damage and bacterial translocation resulting from the disease, the M1 macrophages secrete several proinflammatory mediators, some of which can promote the expansion of type 1 helper T (Th1) cells and type 17 helper (Th17) cells. Th1 cells reinforce the proinflammatory phenotype of the M1 macrophages, whereas Th17 cells promote neutrophilic infiltration, possibly contributing to crypt abscess formation. C: in ulcerative colitis, as with Crohn's disease, monocytes differentiate into proinflammatory/M1 macrophages. However, atypical M2 macrophages [possibly M2b/M2(Ic)] produce inflammation-promoting factors, recruit eosinophils through CCL11 secretion, and signal type 2 helper T (Th2) cells to expand via IL-4 and IL-13 secretion. Th2 cells promote B-cell differentiation into plasma cells, and immunoglobulins (Ig) secreted by these plasma cells form immune complexes that can further reinforce the M2b/M2(Ic) macrophages. Expansion of Th17 cells induced by M1 macrophage-derived IL-23 leads to neutrophilic infiltration as seen in Crohn's disease (B). Adapted from(Isidro, 2016).

1.5.1: Activation of IL-1β and IL-18 in inflammatory bowel diseases IBD

IBD are chronic inflammatory conditions of the gut, for which the innate as well as the adaptive immune system perform a vital role. While the accurate mechanisms are not fully understood, the impression of an inadequate immune response against commensal microbiota in the gut lumen is generally recognised (Siegmund & Zeitz, 2009). For a long time, the inflammasome itself and its downstream cytokines IL-1β and IL-18, were thought to be fundamentally related to IBD development. The first indication for the association of IL-1β was described in 1989, when Mahida et al. demonstrated the enhanced production of this cytokine in mononuclear cells isolated from the actively inflamed mucosa of IBD patients (Mahida, Wu & Jewell, 1989). Similar results were later published for IL-18, reporting the increased expression of IL-18 mRNA and protein in the affected mucosa of IBD patients (Pizarro *et al.*, 1999). The increased production of cytokines were demonstrated in classical immune cells (macrophages and dendritic cells), as well as in intestinal epithelial cells (IEC).These results were reinforced by others who demonstrated the up-regulation of IL-18 in the mucosa

of CD patients, similarly describing a connection with the degree of inflammation. The perception of IL-1β and IL18 being the “hallmark” in mucosal inflammation was highlighted by *in vitro* studies, where the accumulation of IL-1β to epithelial cells altered tight junctions and accordingly intestinal permeability (Al-Sadi & Ma, 2007; Pizarro *et al.*, 1999). Conversely, the functional importance of such findings should be be thoroughly assessed in *in vitro and in vivo* studies.

1.5.2: Activation of the production of IL-12 and IL-23 in IBD

A-IL-12

Trinchieri (1995) reported that Interleukin-12 (IL-12) is a heterodimeric cytokine released regularly by phagocytic cells in response to bacteria, bacterial products, and intracellular parasites, and to some extent by B-lymphocytes. Regarding the structure of IL-12 being a heterodimer of p35 and p40, IL-12 influences cytokine production, mainly of IFN-γ, from NK and T cells. It also acts as a growth factor for stimulated NK and T cells, increases the cytotoxic activity of NK cells, and enhances cytotoxic T lymphocyte generation (Andrea *et al.*, 1992). *In vivo,* IL-12 acts in general at three stages throughout the innate resistance/adaptive immune response to infection. Early in the infection, IL-12 is generated and induces the production of IFN-γ by NK and T cells, which contributes to phagocytic cell activation and inflammation. The cell differentiation by priming $CD4^+$ T cells for high IFN-γ production; and IL-12 contributes to optimal IFN-γ production and to the proliferation of differentiated Th1 cells in response to antigens (Manetti *et al.*, 1994). The early preference expressed in the immune response depends on the balance between IL-12, which favours Th1 responses, and IL-4, which favours Th2 responses. Thus, IL-12 represents a functional bridge between the early nonspecific innate resistance and the subsequent antigen-specific adaptive immunity. *In vitro* studies of IL-12 secretion

from phagocytic cells showed that bacterial products and intracellular parasites were amongst the most powerful stimulators of IL-12 production (Andrea *et al.*, 1992). IL-12 is produced early in the infection (between a few hours -1 or 2 days), by phagocytic cells and antigen-presenting cells and stimulates NK cells and T cells to produce IFNγ and to an extent GM-CSF. The surface molecule, CD40 ligand on activated T cells also upregulates IL-12 production by interacting with CD40 receptor on IL12-producing cells. Downregulation of lL-12 production (negative feedback): several cytokines and other factors downregulate the ability of phagocytic cells to produce IL12 and the ability of NK and T cells to respond to lL-12. IL-10, a Th2 cell product, which can similarly be provoked by IL12, in both, T and NK cells, is a potent downregulator of IL12 production. Whereas the other Th2 cytokines, lL-4 and IL-13, can either downregulate, or upregulate IL12 production, conditional on the time of treatment. TGFβ and PGE-2 are other downregulators of IL12 production.

B-IL-23

A molecular study, that focused on the role of IL-23, found that a variant pathway of the IL-23R gene represents one of the most significant human genetic polymorphisms in multiple sclerosis (MS), and is linked to IBD susceptibility (Baldassano, 2007;Duerr, 2006; Huber, 2008; Illes, 2008). These results suggested that an IL-12 p40 monoclonal antibody that neutralizes IL-12 and IL-23, may perform as an effective treatment for Crohn's disease (Tang *et al.*, 2012b). Certainly, the best option in terms of controlling the inflammatory response, may be to block the activities of both IL12 and IL-23 (Jones, 2016). However, IL-12-dependent responses such as Th1 and cytotoxic T cell responses play a key role in host protective immunity, and the inhibition of these may leave patients susceptible to infection and cancer (Tang *et al.*, 2012a). A

study by Uhlig *et al.* (2006) directly addressed the roles of IL-12 and IL-23 in systemic and mucosal innate immunity and indicated that anti-CD40-induced systemic inflammation was dependent on IL-12 and not IL-23. Whereas, the converse was true for intestinal inflammation. IL-23 may permit a more discriminating block of the tissue inflammatory response while sparing systemic immunity. The merging of two widely different experimental influences on the IL-23 pathway, as a key regulator in the development of intestinal inflammation, is inspiring progress in IBD research. Obviously, many problems have to be addressed before these results can be translated into better therapies for patients with IBD. Upregulation of IL-23 should balance the anti-bacterial/fungal protection by the stimulation of local accumulation of innate immune cells. Unfortunately, in the absence of Th1 and Th2 effectors, these cells do not have the power to counterbalance adaptive immunity, but will attract more granulocytes/macrophages with high tissue destructive potential (Yannam, 2012 Guo, 2019). The suppression of IL-23 expression in advanced HIV/SIV infection however, raised the question of the mechanisms involved in the regulation of its production by cells of myeloid lineage (macrophages and dendritic cells) and their capability to support the protective antibacterial/antifungal function of Th17 cells as well as all other IL-23R expressing populations. All these features need attention and thoughtful investigation.

1.5.3: The pathology of colorectal cancer (CRC)

Colorectal cancer (CRC) is one of the the common cancers worldwide and causes leading cancer-associated deaths, (Tabung, et al., 2017). Many causes are related to the incidence of CRC such as junk food and unhealthy lifestyle, heredity, metabolic disorders, and genetic factors. Most of colorectal carcino-

mas originate from epithelial cells of the colorectal mucosa (Hamilton et al., 2000; Wang, 2010).

Yamaguchi et al., (2019), suggested that the production of cytokines is a good indicator of CRC. They measured the level of 13 cytokines, interleukin (IL)-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12p70, IL-13, IL-15, IL-17A, C-C motif chemokine ligand 11 (CCL11; Eotaxin), in the plasma of patients with CRC. They showed that IL-9 and IL-4 were associated with the presence of CRC potentially, in addition to TNF-α, IL-1β and IL-8. IL-9 is produced by CD4+ Th2 cells as well some of B lymphomas. Moreover, it has been shown to stimulate an increase in the proliferation of CRC cells and promotes tumorigenesis in CRC cells (Torres et al., 2013; Kantola e al., 2012). Generally, IL-4 is considered as an anti-inflammatory cytokine. IL-4 and IFN-γ are the most normally described cytokines in the inflammatory process. Sharp et al., 2017 reported that levels of the anti-inflammatory cytokine IL-4 were significantly elevated in advanced CRC, whereas IFN-γ levels did not change, (Sharp et al., 2017).TNF-α is a potent pro-inflammatory cytokine thought to be involved in the pathogenesis of inflammatory bowel disease (Kraus et al., 2009) and has been reported to promote inflammation and colitis-associated cancer (Ullman, 2011). In the blood of the patients with CRC, a significant elevation has been reported in the levels of TNF-α (Kamińska et al., 2000; Szkaradkiewicz et al., 2009). Dimberg et al. (2012) analysed 50 CRC patients and found significantly higher IL-8 (CXCL 8) levels in cancer tissue compared with paired normal tissue, and showed that CRC patients exhibited significantly higher plasma levels than healthy controls (Dimberg et al., 2012).

1.5.4: Inflammasome-associated human diseases

There is evidence for a number of inflammatory diseases being associated with inflammasome dysfunction. In a few of them, hyper-functional genetic mutations within the NLRP3 gene are detected and these are called, cryopyrin-associated periodic syndromes (CAPS). Amongst the so-called autoinflammatory diseases, CAPS comprise Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FACS), and neonatal-onset multisystem inflammatory disease (NO-MID). For each of them, a rapid and nearly complete response to the treatment with a soluble selective IL-1 receptor antagonist is characteristic (Goldbach-Mansky *et al.*, 2006;Hoffman *et al.*, 2004Justin & Leslie, 2011). Other autoinflammatory diseases display similar clinical manifestations, but these patients do not carry the described genetic mutation, such as familial Mediterranean fever, hyper-IgD-syndrome. These diseases are equally characterized by an immediate responsiveness to IL-1 blockade (Cascavilla, Bisceglia & D'Arena, 2010; Gattorno *et al.*, 2008). Furthermore, in these rare autoinflammatory diseases, there is increasing evidence for the involvement of the inflammasome pathway on other, more common, medical conditions for which hereditary as well as acquired components seem likely. One such disease is rheumatoid arthritis (RA), for which the combination of NLRP3 and CARD-8 gene pleotype represents a risk factor (Jiang *et al.*, 2000). IL-1 receptor blockade is an approved strategy to decrease disease severity and the destructive joint process in RA, and is comparable to other treatment options, such as tumor necrosis factor (TNF)-α blockade (Genant, 2001; Jiang *et al.*, 2000; Maedler *et al.*, 2002). In addition, there is emerging evidence for the crucial role of IL-1 on diseases associated with the metabolic syndrome, which, up until recently, have not been considered inflammatory diseases. Namely, type 2 diabetes mellitus has been

shown to develop in an inflammasome-dependent manner (Maedler *et al.*, 2002; Schroder *et al.*, 2012; Schroder & Tschopp, 2010; Stienstra *et al.*, 2011). The functional impact is strongly underlined by the clinical study where the blockade of IL-1 with anakinra, improved hyperglycemia and β-cell secretory function, and reduced markers of systemic inflammation (Larsen *et al.*, 2007; So *et al.*, 2007). Furthermore, IL-1 blockade has proven highly effective in gout, another inflammatory condition associated with the metabolic syndrome (Joosten *et al.*, 2010; Singh & Huston, 2009). In this case, free fatty acids have proven to be potent inducers of gout, attacking host tissue through inflammasome activation (Joosten *et al.*, 2010).

1.6: Reality beyond the choosing of a unique strain of Lactobacillus and consideration as probiotic bacteria

Lactobacilli are found in small numbers in the small intestine of adults, but some may be initiated from fermented foods or the oral cavity, which is home to few autochthonous species. In adult faeces only a small component of the microbiota ranging from 0.01 to 0.6% of total counts are detectable (Lebeer, et al., 2008a). While in new born, lactobacilli are found in the faeces in variable amounts ranging from 105 to 108 CFU/g with L. salivarius, *L. rhamnosus*, and *L. paracasei* being common species. Furthermore, lactobacilli are major members of the human vaginal microbiota where they play a defending role against urogenital infections (Falagas, Betsi & Athanasiou, 2007a; Falagas, Betsi & Athanasiou, 2007b). The lactobacilli have assumed significant role in considering acute infectious diarrhoea and in the prevention of antibiotic-associated diarrhoea in human clinical trials (Sazawal et al., 2006). The treatment with probiotic lactobacilli in the prevention of allergic diseases and in the treatment of allergic rhinitis/asthma has been reviewed (Kalliomaki et al., 2010; Vliagoftis et

al., 2008). Several medical studies on allergy have investigated Lactobacillus rhamnosus GG (LGG), which was revealed to inhibit atopic eczema and dermatitis (Kalliomaki et al., 2010; Kalliomaki et al., 2003). Following findings on the usage of LGG in the treatment of atopic eczema a therapeutic effect was suggested, (Isolauri et al., 2000; Majamaa & Isolauri, 1997; Viljanen et al., 2005). In general, there is promising evidence that specific lactobacilli probiotics are appreciated in the protection and treatment of diverse diseases but their successful application would benefit greatly from a better understanding of the mechanisms of probiotic action in both in vitro and in vivo clinical studies (Wells, Loonen & Karczewski, 2010a).

The rational behind choosing of the two strains of *Lactobacillus plantarium* strain (C28) and *Lactobacillus salivarius* strain (MS13), was based on previous investigations that took place in the University of Plymouth. Those studies revealed positive features of these bacteria that encourage their use as probiotic bacteria. Table 1.2 shows brief characteristics of the two lactobacilli strains.

Table 1.2: The validation of using the two strains of *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius* strain MS13

Accepted +, Good ++, very good+++, excellent ++++

Characterisation	*Lactobacillus plantarum* strain (C28) NCIMB 41605 (Demeckova, 2003)	*Lactobacillus salivarius* strain MS13 Salivarius NCIMB 41610 (Savvidou, 2009)
Mucus binding	+++	+
Adhesion to intestinal epithelial cells IPEC	+++ Pigs	++++ Pigs and Chickens
Adherence to CACO-2	+++	++++
TEER	Increased Caco-2cells	
Inhibition to pathogenic organisms	*Salmonella E.coli*	*Salmonella enterica Typhimurium* Sal 1344 duce Salm.*enteritidis (5188), Salm. enteritidis of chicken origin, Salm. Typhimurium, Esch. coli and Cl. perfringens*
Previous study	***In vitro* , *in vivo***	*In vivo*
Auto aggregation	**++**	**++++**
Resistant to pH <3.5 Tolerance to Bile	+++	++++

1.7: Aim and objectives

The rationale of this research was to shed light on the role of probiotic bacteria in the modulation of the immune response,and the interacion between epithelial cells and macrophages to activate or suppress immune responses of the gut.

The study was designed to investigate the immunomodulatory effect through two selective lactobacillus strains, *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) on macrophage immune response in homeostasis and inflammatatory enviroment. This was achieved by developing THP-1 cell line into macrophage–like phenotypes to design a model for the current study. Primarily, the creation of pro- and anti-inflammatory cells was investigated in resting and activated macrophages derived from THP-1 cells, differentiated with either PMA, to generate M1-like macrophages, or vitamin D3, to generate M2-like macrophages.

To further inverstigate macrophage subset management, the goal was, first, to establish cytokine profiles upon regulation of macrophage subsets by either *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) or stimulation by K12-LPS (homeostatic environment) or pro-inflammatory cytokines, E. coli LPS on inflammasome activation in macrophage subsets (reported in Chapter 3). Second, to examine the effect of endotoxin tolerisation on macrophage subsets, with respect to PRRs and negative regulatory molecules involved in endotoxin tolarance, and to study the cross-tolerance in response to probiotic bacteria and K12-LPS, and investigate the endotoxin tolerance of probiotic bacteria and the effect of probiotic bacteria homtolerisation on negative regulatory molecules (Chapter 4).

Finally, to explore the modulation induced by probiotic bacteria on (homeostatic environment), pro-inflammatory cytokines (TNFα, IL-1β), (inflammatory

environment) subsets in co-culture model considering their effect on epithelial cells relative to intestinal mucosal macrophage interacting with the epithelial cells (Chapter 5).

Chapter: 2

Materials and Methods

2.1: General materials

See the appendix

2.2: Methodology

2.2.1: Preparation of probiotic bacteria

Lactobacillus plantarum strain (C28) NCIMB 41605 obtained from National Collections of Industrial, Food and Marine Bacteria (NCIMB), Aberdeen, Scotland, *Lactobacillus salivarius* strain MS13 NCIMB 41610 from University of Plymouth collections collected by (Savvidou, 2009). These probiotic bacteria were cultured in de Man Rogosa sharp (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) at 37°C in 5% CO_2 for 24 hours (without shaking). For checking microorganism purity, they were streaked on MRS agar media and Gram–stained to check identity, where a single colony was used to inoculate broth media and the culture was incubated overnight in same optimal conditions.

2.2.2: Bacterial Growth curve in MRS media

Lactobacillus plantarum (C28) NCIMB 41605 and *Lactobacillus salivarius* strain (MS13) NCIMB 41610 were grown in MRS broth for 24 hours. 100 µl of broth was inoculated into three flasks (50 ml of MRS broth). The turbidity of the growth was checked by spectrophotometer at 440 nm every 32 hours at 37°C. The growth data was plotted on the growth curve presented as Turbidity OD against incubation time.

2.2.3: Growth in R10 tissue culture media

Probiotic bacteria *Lactobacillus salivarius* (MS13) and *Lactobacillus plantarum* (C28) were cultured aerobically at 37°C for 30 hours in R10 medium. To determine the bac-terial growth phases, each bacterial strain was monitored by optical density (OD 440 nm) to configure bacterial cell density.

These were then streaked on MRS agar to detemine the viable bacterial count at each time point in R10.

2.2.4: The biological density of probiotics (colony forming factors, cfu/ml)

The ratio of probiotic bacteria used this research was dependent on the ratio of macrophages in tissue cultured per ml. The density used was 10:1 (probiotic/macrophages), as referred in (Mercenier et al., 2000; Habil, 2013; Yoon et al., 2013).

2.2.5: Maintenance of THP-1 human cell line

The human pre-monocytic cell line, THP-1, was obtained from European Collection of Authenticated Cell Cultures (ECACC, UK), and routinely used for these studies between passages 7 and 31. THP-1 cells were maintained in R10 medium composed of Roswell Park Memorial Institute-1640 medium (RPMI-1640) medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine (100 U/ml penicillin, 100 µg/ml streptomycin) (R10). Cells were sub-cultured every 3-4 days at a ratio of 1:4 using R10 for cell dilution (Apostolou et al., 2001). Also used experimentally between passages 7 to 32 (Foey and Crean, 2013) and plated out at a final density of $1x10^{6}$ cells/ml in 12 or 24 flat-bottomed well, tissue culture plates (Sterllin, Newport, UK) in a humidified atmosphere of 5% CO_2incubator set at 37°C.

2.2.6: Caco-2 intestinal epithelial cell line

Human colon adenocarcinoma cell line (Caco-2 epithelial cells) were cultured in Dulbecco's Modified Eagles' Medium (DMEM) medium supplemented with 20% FCS, 2 mM L-glutamine.

A: Growth in trans-well plate

Cells were plated out at a density of $5x10^{5}$ cells/ml/well in 12-well pore size 0.4 µm transwell plates (Greiner bio-one, Stonehouse UK) and incubated in a hu-

midified atmosphere of 5% CO_2 at 37°C for 21 days for full cell differentiation (Habil, 2013, Hilgers et al., 1990). Caco-2 cells were sub-cultured every 3 days or once cells had achieved 80% confluence. This process was performed by removing cell media, followed by washing cells twice with Dulbecco's Phosphate-Buffered Saline (DPBS) and detaching cells by adding 2-3 ml of 0.25% v/v versene/trypsin (Ethylene-Diamine Tetra-Acetic Acid) EDTA (TE). Then, cells were incubated for 10-15 min at 37°C. The cells were checked during the time of incubation by light microscopy, and gently agitated until detached. To deactivate trypsin, D20 medium was added (Habil, 2013) and cells were re-suspended in a 0.5 ml of D20 medium before seeding according to the designed experiment.

B: Growth of Caco-2 in 48 and 96 wells plate

Cells were plated out at a density of 1.25 x 10^5 cells/ml/well in 48 wells 12 mm (Greiner bio-one, Stonehouse UK) plates(followed as in 2.2.6 a).To study the release cytokines from Caco-2, these cells were stimulated with either: TNF-α, IL-1β, IL-18 or K12- LPS.

2.2.7: Storage of human cell lines

2.2.7.1: Storage of THP-1 cell line

Pro-monocytic THP-1 cells were centrifuged at 400 g for 5 min, then the supernatant was discarded and the cell pellet was suspended in 1 ml of 15% FCS/ Dimethyl sulfoxide DMSO media (see Table1-1 appendix) where they were frozen at -80°C and consequently later stored in liquid nitrogen.

2.2.7.2: Storage of Caco-2 cell line

Caco-2 cells were grown until 80% confluence and harvested by centrifugation at 226 g for 5 min, the supernatant was removed, and the cell pellet was re-

suspended in 1 ml of storage medium (see 2.2.7.1)and then frozen at -80°C and consequently later stored in liquid nitrogen.

2.2.8: Determination of total cell number and cell viability

Evaluation of the number of cells and cell viability was checked after each passage. This was undertaken by staining 10 µl of cell suspension with 90 µl of 0.1% (v/v) Trypan blue (Sigma-Aldrich, UK). A Neubauer haemocytometer was used to determine total cell numbers. Cells were also stimulated with K12-LPS or by probiotic bacteria and viability was assessed later by 3-4-5-dimethyl-2.5 thiazol-2.5 diphenyltetrazolium bromide MTT or carrying out a sulforhodamine B (SRB) assay.

2.2.8.1: Measurement of Cell viability of macrophages by MTT assay

Viability of macrophages were checked by 3-4-5-dimethyl-2.5 thiazol-2.5 diphenyl tetrazolium bromide (MTT) according to (Mosmann, 1983). The principal of this method is carried out by measuring mitochondrial activity. The indicator of activity of the macrophages by metabolise the yellow colour in tetrazolium MTT pigment, to the purple formazan product by dehydrogenase enzymes. Monocytes, macrophages cells were seeded in 12 or 24 well plates. Treatment of cells was performed by using a suspension of cells that were treated with either lactic acid or with live bacteria at cell densities of $1x10^{6}$ cell/ml: 1x107 cfu/ml cells. The cells of each treatment were washed twice with (Phosphate buffer saline) PBS to discard the R10 media, 50 µg/ml of MTT was added to each well and incubated for 3 hours at 37°C, 5% CO_2. Live cells will degrade MTT through mitochondrial succinate dehydrogenase, resulting in the production of MTT formazan, then solubilized with Dimethyl sulfoxide (DMSO). Colorimetric development was measured spectrophotometrically by FLUOSTAR Omega 415-1244 (BMG-LABTEC plate reader, Germany) at 540 nm.

2.2.8.2: Determination cell viability by sulforhodamine B (SRB) assay

Based on the measurement of cellular protein concentration, SRB binds to protein basic amino acid residues of trichloroacetic acid (TCA)-fixed cell (Vichai & Kirtikara, 2006). The SRB assay represents an appropriate and sensitive assay to measure drug-induced cytotoxicity and is useful to quantify clonogenicity (Voigt, 2005). The SRB is anionic and bright pink; it can be quantitatively extracted from cells and solubilized for optical density (OD 570nm) measurement by weak bases such as Tris base. Colorimetric development was measured spectrophotometrically by FLUOSTAR Omega 415-1244 (BMG-LABTEC plate reader.

A: Determination of the viability of adherent cells by SRB assay

Proliferation and viability of adherent cells were confirmed according to Sekham, 1990. Treatment of the cells was performed using 50 µl of adherent cells ($5x10^3$ cells/well) of either Caco-2 or differentiated M1-like macrophage in 96-well plate. 50 µl of live C28 or MS13 bacteria, at a density of $1x10^7$ cfu/well, was added to each well with respect to number of cells not treated as control. After 24 h or overnight incubation, cells were fixed with 100 µl of 10% (w/v) trichloroacetic acid (TCA) at 4°C for at least 1 hour. The plates were washed with (deionised distilled water DDW) five times and cells were suspended in 0.4% SRB solution dissolved in 1% acetic acid at a volume of 100 µl/well for 30 min. Consequently, the cells were washed with 1% acetic acid for four times to remove unbounded stain. Finally, the plates were dried with tissue, the cells were suspended in 10 mM Tris base (non-buffered) solution, and ELISA reader (Molecular Devices, Manchester, UK) read the absorbance at 570 nm.

B: Determination of the viability of suspension cells by SRB assay

To investigate the viability of suspension cells (M2-like macrophage) after incubation with C28 and MS13, K12-LPS was used via a modified method of Sekham, (1990) cited from (Papazisis, 1997). (5000 / 50 cells/µl) of M2-like macrophage, cells were placed in 96 well plates. 50µl of live C28 or MS13 bacteria at $1x10^7$ cfu/well of C28 or MS13 were added to

every well for 24-48 hours. Cells were fixed immediately with 100 µl of 10% (w/v) trichloroacetic acid (TCA) at 4^oC overnight. Non-treated cells with C28 or MS13 suspension cells with supernatant were transferred into 15 ml tubes and centrifuged at 167 g for 1 min. The supernatant was discarded and the cell pellet was washed with DDw five times. On the other hand, treated suspension cells /well (cells were staked or adherent in bottom of the well). This procedure was used as the primary adherent method protocol.

2.2.9: Assessing the role of acidity on the viability of macrophages

Lactic acid is one of the main metabolic produces of the lactobacillus bacteria in the media due to glucose metabolism (Bintsis, 2018). The main challenge of using live pro-biotic bacteria was the accumulation of lactic acid on the surface of macrophages, which may influence macrophage effector function and viability. Different concentrations of lactic acid (Fluka, UK) were prepared (6.25, 12.5, 25, 50, 100 mM), and its viability was determined via the method in (2.2.8.1).

2.3: Macrophage subset polarisation

According to Schwende et al, (1996), the protocol of differentiation of pro-monocytic cells (THP-1) into pro-inflammatory M1-like differentiated by addition of 25 ng/ml of Phorbol-12-myristate acetate (PMA) for 3 days followed by minimum of 24 hours for PMA wash out. Anti-inflammatory M2-like macrophages

were differentiated by the addition of 10 nM 1, 25-(OH) $_2$- vitamin D_3 for 7 days. (**Table 2.1**).

2.3.1: Stimulation of macrophage cell subsets

Macrophage cells were stimulated with the bacterial pathogen associated molecular pattern (PAMP); serial dilution of *E. coli* strain K12 lipopolysaccharide (LPS) was expressed by the entire-pathogenic Gram-negative bacteria (Beutler and Poltorak, 2001). These cells were cultured in the presence of K12-LPS (10, 100,1000,10000 ng/ml) for 24 hours as the optimal time for cytokine release by macrophages in a humidified environment at 37°C, 5% CO_2. Cell supernatants were harvested and stored at -20°C until required for sandwich enzyme-linked immunosorbent assay (ELISA), such as TNF-α, IL-β and IL-10.

2.3.2: Time course kinetics of macrophage cytokine production

To confirm the best time-points for cytokine production from M1 and M2–like macro-phages induced by LPS, M1 and M2-like macrophages were stimulated with 100 ng/ml of K12 LPS *E. coli* and consequently cytokine production was measured at 4,8 and 24 hours. The supernatant was collected at every time point and stored in -20°C for assay by sandwich ELISA such as (TNF-α, IL-1β, and IL-10).

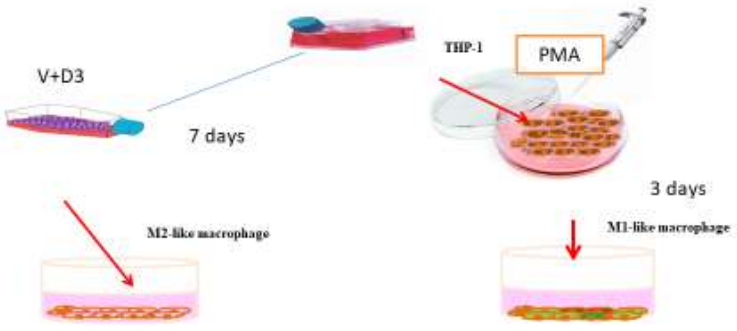

Figure 2.1: The steps of differentiation of THP-1 to M1 and M2 macrophage

Differentiate THP1-cell lines (1x10^6 cell/ml) with either 25 ng/ml of PMA for 3 days and washed out for 24 hr or 10 nM/ml of Vit. D3 for seven days replaced the overnight R10 medium.

2.3.3: Assessment of the role of live probiotics as immune-regulators of macrophage subsets

THP-1 derived macrophage subsets were used as a suitable model for immune cells (M1-like and M2-like macrophage subsets). Overnight growth of *Lactobacillus plantarum* and *Lactobacillus salivarius* in MRS media were centrifuged to discard media and then washed twice with PBS; the pellets were suspended in 10 ml of sterile PBS. The density *Lactobacillus* strains C28 and MS13 was used later in the experiments $1x10^7$ cfu/ml) in R10 media and its equivalent to 0.135, at 440 nm OD assessed by Spectrophotometer (SP) 20. The two strains of lactobacillus C28 and MS13 were incubated with $1x10^6$ cells/ml of either M1 or M2-like macrophages and incubated in optimum conditions for 24 hours. The supernatants were separated from the cells and stored at -20°C until required for sandwich ELISA such as TNF-α, IL-1β, IL-18, IL-23, IL-12, IL-10, or IL-6.

2.3.4: Time course of cytokine production is dependent on probiotic bacteria

To determine the best time-point for M1 and M2–like macrophage to produce the cytokines when incubated with $1x10^7$ cfu/ml of *Lactobacillus plantarum* and *Lactobacillus salivarius* for 4, 8, 12, 18 and 24 hours, the supernatant of macrophages were collected in Eppendorf tubes and centrifuged at 400 g for 15 min and the supernatants were stored at -20°C, until required for cytokine detection by ELISA. To demonstrate a physiological-relevant role for live probiotics bacteria, the colorimetric assay of MTT was carried out on both macrophages. Viability was routinely >90%.

2.3.5: Effect of live probiotics preparations on macrophage function

To investigate the ability of probiotics to modulate cytokine production of macrophages, differentiated M1 and M2–like macrophages were treated with 1x107

cfu/ml/well of *L.plantarum* and *L.salivarius*. The lactobacillus strain was prepared as described in sec-tion 2.3.3 in the presence or absence of *E. coli* K12 LPS. The negative control was un-treated M1 and M2 where 100 ng/ml LPS K12 was added to M1 and M2-like macrophages as a positive control and incubated for 24 hours in optimum condition. The supernatant was harvested after 24 hours. Cells were centrifuged and the supernatant stored in -20°C until required for assay by sandwich ELISA. The cells were also harvested for RNA extraction where cell lysis was carried out for detection of gene expression by RT-PCR or western blot (WB).2.3.6: The effect of NLRP3 inflammasome inhibition on probiotic-induced macrophage response.

2.3.5.1: The effect of NLRP3 inhibitor on macrophage subsets induced by C28 and MS13

THP1-cell lines ($1x10^6$ cell/ml) were differentiated with either 25 ng/ml of PMA for 3 days and washed for 24 hours with 10 nM/ml of Vit.D_3 for seven days after which was replaced by R10 medium overnight. M1 and M2-like macrophages were pre-treated with MCC950 10 μM (NLRP3 inhibitor) for 30 minutes, followed by stimulation with C28 or MS13 for 19 hours and 30 mins. The untreated cells with MCC950 were considered as a control for this experiment. The supernatants were stored in -20ºC until required for assay by sandwich ELISA using IL-1β.

2.3.5.2: The role of lactobacilli strain in modulation of IL-1β by macrophage subsets induce by K12-LPS in presence of the NLRP3 inhibitor

THP1-cell lines ($1x10^6$ cell/ml) were differentiated with either 25 ng/ml of PMA for 3 days and washed for 24 hours or incubated with 10 nM/ml of Vit.D_3 for seven days and refresh the cells with R10 medium overnight. Following pre-stimulation of M1 and M2 with 100 ng/ml of *E. coli* K12 LPS for four hours, me-

dia was removed and cells re-suspended in fresh media (**Figure 2.1**). Followed by adding 10nm of MCC950 (NLRP3 inhibitor) for 30 minutes , then adding the probiotic bacteria (C28 or MS13) $1x10^7$ cfu/ml for 19.5 hours, macrophages were centrifuged and stored in -20ºC until required for IL-1β cytokine assay by sandwich ELISA.

2.4: Macrophage subset tolerisation with LPS

M1 and M2-like macrophages were pre-treated with 100 ng/ml K12-LPS for 24 hours. The final density of cells was $1x10^6$ cells/ml plated out in 12 well flat-bottomed tissue culture plates. Before tolerisation with 100 ng/ml K12-LPS the medium was carefully removed and the cells were washed in fresh R10 media. The cells were then incubated with 100 ng/ml K12-LPS for 24 hours at 37°C, 5% CO_2. The cells were harvested for RNA extraction and the supernatant was separated and stored at -20 ^oC until required for a cytokine assay by sandwich ELISA, whilst the cells were prepared for detection of gene expression by RT-PCR.

2.4.1: Macrophage subset homo-tolerisation with probiotic bacteria

THP-1-derived M1 and M2-like macrophages were pre-stimulated with C28 and MS13 $1x10^7$ cfu/ml for 24 hours, washed and refreshed with R10 media, followed with post-stimulation with $1x10^7$ cfu/ml for another 24 hours. The cells were harvested for RNA extraction and the supernatant was separated and stored at -20 ^oC until required for a cytokine assay by sandwich ELISA, whilst the cells were prepared for detection of gene expression by RT-PCR.

2.4.2*:* Lactobacillus strains C28 and MS13 modulated endotoxin tolerance by macrophage subset

THP-1-derived M1 and M2-like macrophages were pre-stimulated with 100 ng/ml K12-LPS for 24 hours. This experiment followed as before with 2.4.2.

Then cells were incubated with 100 ng/ml K12-LPS and either C28 or MS13 for 24 hours at 37°C, 5% CO_2. Supernatant were separated and stored at -20°C until required for a cytokine assay by sandwich ELISA.

2.5: Epithelial cell stimulation

Caco-2 cells were treated with C28 and MS13 and pro-inflammatory cytokines (10 ng/ml TNF-α, 5 ng/ml IL-1β, 10ng/ml IL-1β and PAMP such as 100 ng/ml K12-LPS), were cultured for 24 hours. Cell supernatants were collected and kept at -20°C until required for evaluation by ELISA and the cell lysate for of gene expression analysis by RT-PCR.

2.5.1: Measurement of trans-epithelial electrical resistance (TEER) of Caco-2

To study the para-cellular transport properties (permeability) of epithelial cells grown on permeable filters and barrier integrity, the measurement of trans-epithelial electrical resistance (TEER) used. TEER measurement was done according to the modified protocol by (Teoh, Siow & Tan, 2000). Caco-2 cells were cultured in transwell plates for 21 days (Figure 2.2) and incubated either with C28 and MS13 or without bacteria. During the differentiation period and after bacteria treatment, cells were washed twice with 0.5 ml DPBS added to the inner insert and 1 ml to the outer compartment of the transwell. The electrical resistance was calculated using Epithelial Voltammeter (EVOM, Pharma, West Sussex UK), where each well reading was multiplied by the surface area of the transwell (0.33 cm^2) to calculate the final value in Ω cm^2.

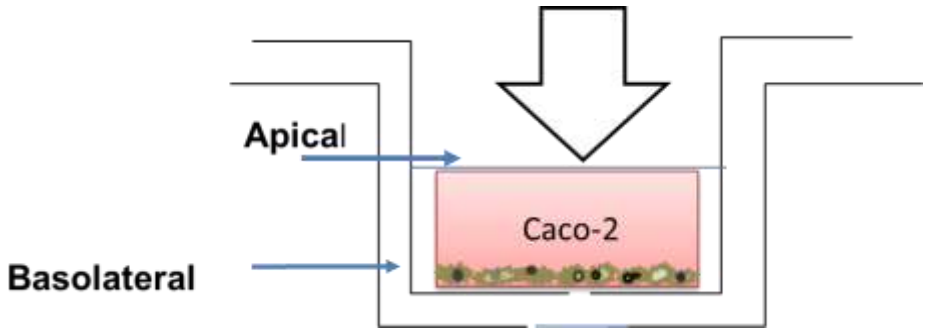

Figure 2.2: Epithelial cell grown up in transwell plate

Co-culture model of Caco-2 cell lines which were cultured in transwell to examine the Trans-epithelial electrical resistance (TEER).

2.5.2: Stimulation of the epithelial cells with different densities of lactobacillus strains

$5x10^5$ of Caco-2 cells were treated with different densities of C28 and MS13, $1x10^5$, $1x10^7$ and 1 $x10^8$ cfu/ml for 24 hours optimum conditions, where the ratio between a density of bacteria to a density of Caco-2 cells were 0.5:1, 20:1 , 200:1.

2.5.3: Co-culture system epithelial cell-macrophage

A co-culture model was created to mimic the role of *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) in modulation of the gut pathology. The epithelial cells cross-talked with macrophage subsets in the presence of LPS, TNF-α or IL-1β.The co-culture system was made using Caco-2 human epithelial cells and THP-1 monocyte-derived macrophage cell subsets, where M1-represented the inflammatory condition and M2-like macrophages represented the homeostasis of the gut (Watanabe et al., 2004 a). Briefly, six groups of Caco-2 cells were seeded on 12-well transwell plates ((0.4 µm pore size, Greiner bio-one, Stonehouse UK) at a cell density of $5x10^5$ cells/500 µl/well and cultured in D20 medium in a humidified 5% CO_2 incubator at 37°C for 21 days allowing for full cell differentiation. Three groups of Caco-2 cells

grown in transwell inserts were incubated with M1 or M2-like macrophages for 24 hours in the presence of C28 or MS13 added apically in the co-culture system, followed by the addition of K12-LPS basolateraly for 24 hours. The third group of Caco-2 cells were left without co-culture as a control as well as macrophages subsets controls (**Figure 2.3**). The apical and basolateral cell supernatant were collected and stored at -20°C for detection of cytokines by ELISA.

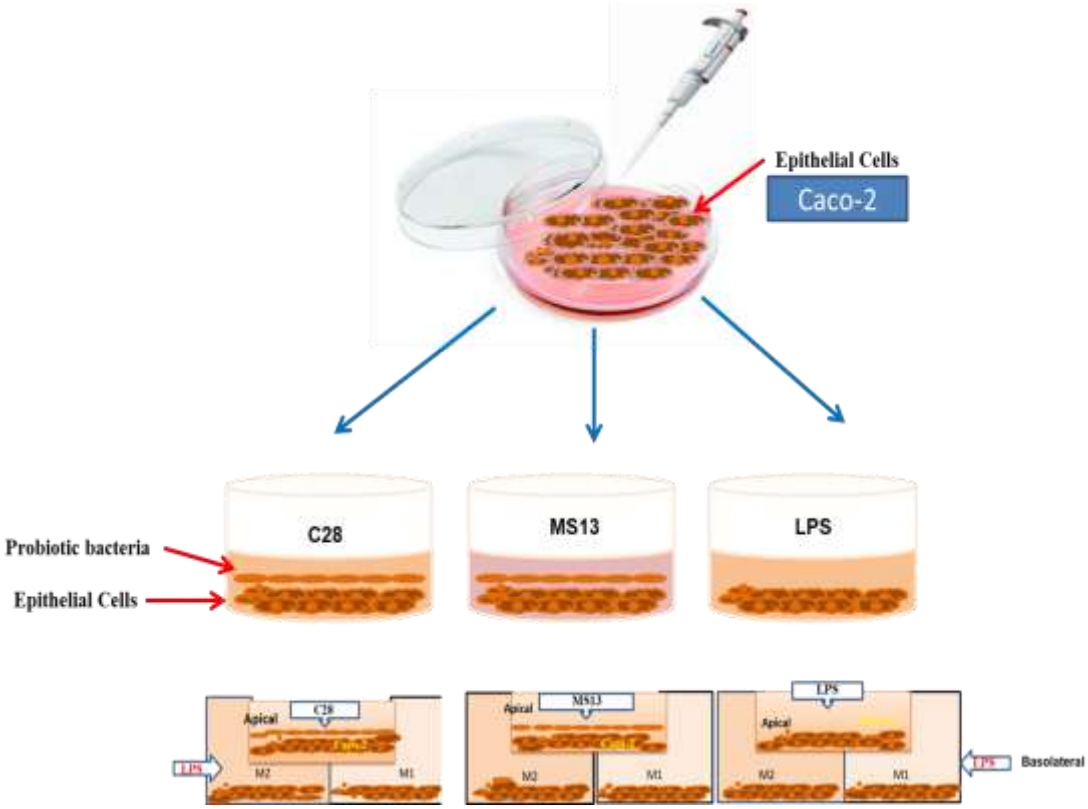

Figure 2.3: Experimental setup of the co-culture comprising of intestinal epithelial cell line ($CaCO_2$) and either macrophage subsets (M1-like or M2-like macrophages)

Co-culture model of Caco-2 cells which were cultured in transwell with either C28 or MS13 insert For mimicking gut mucosal tissue, with THP-1 derived M1-like macrophage and, LPS in basolateral side depending on designed experiment.

2.6: Cytokine measurements

Monocytes, macrophages, and epithelial cell production of the inflammatory cytokines: TNF-α, IL-1β, IL-18, IL-12, IL-23 and anti-inflammatory cytokine IL-10 and IL-6 were quantified by using sandwich ELISA. The 96-well immune-absorbance plates (Nunc, Fisher scientific, Loughborough,UK) were coated with

commercially available capture antibodies (R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK): anti-TNF-α (4 µg/ml), anti-IL-1β (1 µg/ml), anti-IL-18 (0.5 µg/ml), anti-IL-12 p35 (1 µg/ml), anti-IL-23 p19 (0.4 µg/ml), anti-IL-6 (1 µg/ml), anti-IL-10 (0.5 µg/ml), and incubated overnight at 4 ^{o}C. The plates were washed three times with PBS/Tween-20 (PBS, 0.05% v/v Tween-20), before being blocked with blocking solution (PBS, 2% w/v BSA) for 3 hours at room temperature, were placed in orbital shaker. The plates were then washed three times, incubated with serially diluted recombinant cytokines and test samples, and left overnight at 4°C. Plates were washed three times followed by incubation with biotinylated, anti-TNF-α (0.5 µg/ml), anti-IL-1β (0.5 µg/ml), anti-IL-18 (0.5 µg/ml), anti-1L-23 p40 (1 µg/ml), anti-IL-6 (1 µg/ml), anti-IL-10 (0.5 µg/ml), for 3 hours at room temperature. Plates were washed three times with PBS/Tween-20 and incubated with 50 µl/well of biotinylated-streptavidin horseradish peroxidase (HRP) at 1/250 dilution in 2% w/v BSA/PBS and incubated for 1 hour at room temperature. Finally, the plates were washed three times with PBS/Tween-20, followed by the addition of a colour reagent (Tetramethylbenzidine-TMB) 4µl/ml of PBS, Middlesex UK), and the reaction was stopped with 1.8 M sulphuric acid. Colorimetric development was measured spectrophotometrically by an OPTIMax tuneable microplate reader at 450 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA). Protocols were followed according to manufacturer's instruction. Standard curves, between ranges (7 to 5000 pg/ml), were applied to establish the cytokine concentration and compared with the recognized international standards available from National Institute for Biological Standards and Control (NIBSC) (Potter's Bar, UK). Reagents and antibodies used for ELISA are available in appendix 1,Table 2-1. The highest level of cytokine detec-

tion was 5 ng/ml and lower level of detection was 7 pg/ml. The inter-assay percentage CV values were not included as the high variability of cytokine amplitude between experiments depended on passage number, concentration of stimuli, and type of stimuli, therefore, all data are presented as a representative experiment of replicate experiments and the intra-assay percentage CV was 2.1- 5.2.

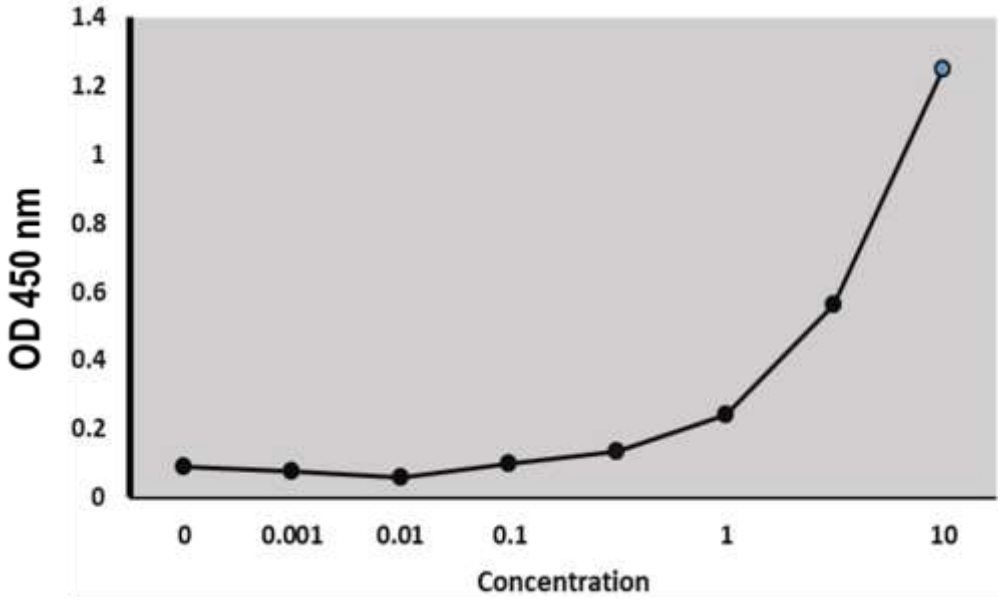

Figure 2.4: Standard calibration curve of ELISA

Standard curves, between ranges (7 to 5000 pg/ml), were used to determine the cytokine concentration and compared with the recognized international standards available from (NIBSC, Potter's Bar, UK). The absorbance (OD) was determined in a micro-plate reader at 450 nm.

2.6.1: Molecular biology methods

To find out the molecular basis of biological activity of the target gene of interest, polymerase chain reaction (PCR) was undertaken. Complementary deoxyribonucleic acid cDNA was synthesised followed by conventional PCR amplification using specific primers (see Figure 2.8). The accomplishment of qPCR was performed by using a normalisation of a housekeeping gene glyceralde-

hyde-3-phosphate dehydrogenase (GAPDH), which determined the threshold cycle (CT) value as a fold change.

2.6.1.1: Total RNA extraction

The extraction of total RNA was operated using a Sigma GenElute mammalian total RNA extraction kit, depending on the manufacturer's procedure for each treatment of the cells. Adherent M1-macrophages were washed twice with PBS, then adherent cells were detached by a sterile rubber cell scraper (Sterlin, UK), whereas M2 cells were harvested by centrifugation at 206 g for 5 min. Cells were re-suspended in 500 µl of lysis buffer provided by the kit, supplemented with 5 µl 0.02% v/v 2-mercaptoethanol for denaturation of RNase (reducing disulphide bonds and ruin the enzyme functionality of RNases released during the lysis step of RNA isolation). DNase digestion with RNase-Free DNase to remove potential DNA contamination was done by adding 10 µl of DNase and 70 µl of digestion buffer on the top of each filter of the column used for RNA isolation, the columns were incubated for 15 min at room temperature, and finally 50 µl of elution buffer was added.

2.6.1.2: RNA quantification and evaluation

Both the concentration of RNA and purity (contamination of proteins and DNA) were measured by Nanodrop (NanoDrop™ 2000/2000c Spectrophotometers, Thermo Fisher Scientific, UK). Quantification of RNA was determined using spectrophotometer. The ratio of OD260/OD280 had to be between 1.8 and 2.0 to exclude any contamination in the RNA sample extracted (Liu et al., 2003). Purified RNA was immediately stored at -20°C until required for gene expression analysis or experiment was continued to carry out reverse transcription of total RNA. The integrity of RNA sample was verified by running 1% formaldehyde agarose gel according to the protocol of Van Dessel et al.,2004. Briefly,

the formaldehyde agarose gel was prepared by adding 1.5 g agarose, 10 ml 10x MOPS into 87.5 ml distilled water and heated to dissolve all of the agarose. Consequently, 7 µl of Syber safe or red gel was added into a small gel set monitored by shaking to mix all the components of agarose liquid then cooled to 60°C prior to adding 26 µl formaldehyde whilst stirring in the fume hood. The mixture was transferred into the BIO-RAD gel set and left for casting. The gel was mounted in an electrophoresis tank and overlaid with 1x MOPS electrophoresis buffer. The loading buffer (2 µl 1X MOPS, 3.5 µl formaldehyde, 10 µl formamide, 0.4%w/v Bromophenol blue) was prepared where 15.5µl of this buffer and 4.5 µl of sample were mixed together and incubated for 10 min at 65°C to control RNase contamination and denaturation, and subsequently quenched on ice for 10 min, followed by brief centrifugation and then loaded into the gel well. The gels were run at 40-60 volts for 60 min, de-stained using deionised water for 45 min and reviewed under UV light using the documentary gel reviewer (Gel DocTM.XR, BIO-RAD, CA).

2.6.1.3 Reverse transcription of total RNA

Total RNA samples reversed transcribed to (cDNA) using high capacity RNA to cDNA reverse transcriptase. The manufacturer's protocol (Life Technologies Ltd, Dorset, UK) was followed and RNA samples diluted using RNase or DNase free water grade by taking 0.5 µg of each RNA sample diluted up to 9 µl in thin walled PCR tubes. All samples were reverse transcribed preparing the master mix accordingly to the amount of RNA samples that were needed to be reverse transcribed for each reaction by adding 10 µl/sample of the reverse transcriptase buffer. 1 µl/sample of the reverse transcriptase enzyme were added to the master mix and 11 µl/ sample of the master mix was added in equal amount into each diluted RNA sample. The reverse transcription conditions were 37°C for 1

hour stopped by heating at 95°C for 5 min, and then the run was held at 4ºC using 96 well (thermal cycler machine Vertti). All samples were stored at -20°C until needed.

2.6.1.4: Design of primers

The full-length sequences of the gene of interest was identified using the National Centre of Biotechnology Institute (NCBI). Primer express software provided with Quant Studio Real-Time PCR machine (Applied Biosystems, Lingly House, Warrington, UK), blast software at http://blast.ncbi.nlm.nih.gov/, the primers characteristics were assessed using Sigma DNA calculator website (http://www.sigmagenosys.com/calc/DNACalc.asp). The primers were synthesised by Eurofin MWG/Operon (Germany). The lyophilized primers were dissolved in appropriate volumes of nuclease free water to prepare 10 pmol of each primer depending on MWG instruction and then stored at -20°C. This study focused on TNF-α, IL-6, IL-1β, IL-10, IL-12 p35 ,IL-23 p19, TLR-2, TLR-4, Tollip, SOCS3, STAT3, NLRP3, TRAIL gene expression were quantified using RT-PCR (refer to appendix 1 Table 4-4-2 for primers details; see Appendix 1).

2.6.1.5: Real time –Polymerase chain reaction (PCR)

The samples that were transcribed to be cDNA from previous step (see section 2.2.6.3) was amplified using Power SYBR Green kit according to the supplier's instructions. Generally, the cDNA (0.5 µl) from RT reaction was used for each PCR reaction. The qPCR (Real time PCR) reaction conditions were 95°C for 10 min at the holding stage, followed by 40 cycles which included the denaturation at 95°C for 30 seconds, annealing at 53°C for 60 seconds, and then, the extension at 72°C for 120 seconds followed by a final 6 min extension at 72°C. All primers were designed and checked using primer express software. All primers that have been used are shown in Appendix Table. Fold increase values were

calculated as described by (Livak et al, 2001) (2^-(ΔΔCt)) i.e. RQ = 2 power – (ΔΔCt); the data of untreated cells represented as 1-fold increase (2^-(0) =1). All data were normally distributed (please see table 2 in Appendix, material and methods supplements).

The PCR calculations followed the equation below:

$$\Delta\Delta C\ t = \Delta Ct \text{ interest gene (resting cells)} - \Delta Ct \text{ endogenous control}$$

$$\text{Relative quantitation (RQ)} = 2^{-\{\Delta\Delta CT \text{ sample} - \Delta\Delta Ct \text{ control (resting cells)}\}}$$

Where CT is threshold cycle

2.6.2: Protein analysis methods

2.6.2.1 Cell lysis

M1 and M2-like macrophage cells, were washed gently after specific treatments with PBS followed by adding lysis buffer (RIPA buffer supplemented with a protease/phosphatase inhibitor cocktail). Cell lysate was spun down at 959 g at 4°C for 10 min to pellet cells. Supernatants were carefully transferred without disturbing the pellet and stored at -20°C to determine the protein concentration by Bradford method prior to WB (refer to appendix Table 3-1).

2.6.2.2: Protein quantification

This procedure followed the Bradford method (Bradford, 1976) that was modified for the micro-plate reader to quantify the protein of interest. This method is proposed for common use, especially in the detection of protein cell segments and secreted extrac-tions (Ernst and Zor, 2010). This assay was applied to measure the maximum absorb-ance for an acidic solution of Coomassie Brilliant Blue G-250 (See Appendix I) which shifts from 465 nm to 595 nm when associated with protein existence (Bradford, 1976). Standard of Bovine Serum Albumin (BSA) protein were used between 7 µg/ml and 2.5 mg/ml, which were pre-

pared by dissolving BSA in distilled water followed by adding an equal volume of dye reagent (Bradford) then incubating for 5 min at RT. The absorb-ance was measured in a microplate reader (Molecular Devices, Manchester, UK) at 595 nm where standard curve were plotted between absorbance value and protein concentrations, and the experiment samples were calculated according to ex-trapolation from the standard curve (**Figure 2.6-3.2**).

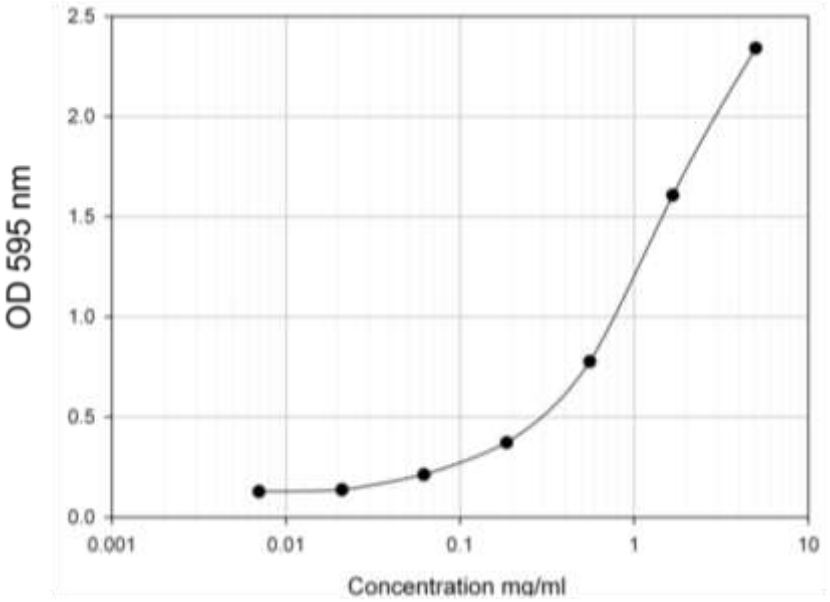

Figure 2.5: Standard calibration curve of BSA by Brafordford assay

BSA ‘s standard curve of Bovine serum albumin (BSA) protein [7 µg/ml: 2.5 mg/ml]. Theabsorbance (OD) was determined in a micro-plate reader at 595 nm. Data dis-played is a representative experiment with triplicate samples of n= 3 replicate experi-ments.

2.6.2.3: SDS-PAGE

A complex mixture of protein was identified using SDS-PAGE. This method was applied using a Laemmli discontinuous buffer system. To prepare SDS gel, glass plates (BIO-RAD, Hertfordshire , UK) were washed with detergent and splashed with deionised water, followed by 70% v/v ethanol then left to dry. The total concentration of the determining gel was 12.5% w/v. The essential gel so-lutions were mixed with fresh 1% w/v ammonium per sulphate (APS) and tetra-

methylethylene diamine (TEMED) (see appendix Table 3-1) for polymerisation and immediately loaded into the preformed space between the glass plates and filled to two-thirds the height of the largest glass plate. 2 ml of 10% v/v water saturated isopropanol was loaded immediately on top of the determining gel making a layer on top of the gel to discharge any air bubbles. The gel was kept for polymerisation for 30 min at room temperature. Then, the isopropanol layer was isolated, and the stacking gel 3% w/v was prepared, based on the recipe mentioned followed by adding APS and TEMED, and then directly loaded on top of the prepared gel. To make wells for sample loading, a comb was inserted into the loading gel, left in the gel for a few minutes for polymerisation, and then transferred to the electrophoresis container. The container was filled with running buffer covering the top of the gel. The comb was then carefully removed and the wells were washed with running buffer. 12 µl of each protein sample and 3 µl of loading buffer were boiled for 3 min to denature the protein, primed on ice for 2 min, briefly vortexed, centrifuged at 11269.44 g for 30 sec then added into each well of the gel.

The gel was run at 120 V for 2 h and the dye marker was observed (Hyper Page pre-stained protein Marker, Bio line, UK). The gel was removed from the electrophoresis system container, plates were removed, and the gel was ready for transfer to PVDF blotting membrane.

2.6.3.4: Western blotting (WB)

Western blotting is normally applied for recognising specific proteins stained with specific antibodies in a particular complex sample protein. Protein of interest in this investigation was NLRP3 (refer appendix 1). Proteins were detected by Criterion Xt precast gel (Bio-Rad Laboratories Hertfordshire Ltd, UK) where proteins were transferred to polyvinylidene fluoride membranes (PVDF, Sigma-

Aldrich Company Ltd., Dorset, UK) by using an electro-blotter system Criterion blotter (BIO-RAD, Hertfordshire ,UK), which were fixed to the same size as the gel, along with two pieces of filter papers. In order to prepare PVDF, it was saturated in methanol (100% v/v) for 15 seconds then distilled water for 2 min then in transfer buffer for 5 min. The blotting device system was organised as follows: black plastic cassette plate at the bottom, gauze, filter papers, gel, PVDF blotting paper, filter papers, gauze, and red plastic cassette at the top. The cassette was firmly closed before to take away the air bubbles with a roller, and then the cassette was placed in the tank where the red side towards the cathode (red electrode). Cold transfer buffer was added to the tank, an ice pack was placed at the bottom as well as a magnetic stirrer for cooling supply, and to avoid the overheating of the buffer, which might affect the blotting system.

The blotting system was connected to the power and the gel was electro-blotted at 30 V overnight at 4°C. When blotting is complete, the PVDF membrane was blocked with blocking solution (refer to appendix table 3-1) at room temperature for 60 min on the orbital roller and the membranes were primed overnight with the specific interest primary antibody in 1% BSA in TBST at 4°C on the roller. PVDF membrane was washed with TBST three times, 5 min each at RT on the roller and then incubated for 60 min at room temperature with suitable secondary antibodies together with horseradish peroxidase (HRP). The membranes were washed three times for 5 min with TBST at room temperature on the orbital roller and then washed once for 5 min with TBS on the roller to remove traces of Tween-20. To detect peroxidase activity from HRP-conjugated antibody, developing colour for protein detection was achieved by using, 2 ml Pierce™ ECL Western Blotting Substrate. After washing with TBS, the membrane was placed on a plastic sheet. Developing solution was carefully loaded

over the entire membrane for few minutes. The protein bands were pictured by a gel documentation system using EC3 imaging system (UVP ultra-violet product, Ca, UK). Band densities were quantitated by Image J 1.47 t software.

2.7 Statistical analysis

All values are presented as mean of 3 or more independent experiments and standard error of the mean (SEM). Data were analysed and presented using Sigma plot version 13 and Microsoft Excel 2016. Significant differences were determined using one-way ANOVA ($p^* < 0.05$, $p^{**} < 0.01$ and $p^{***} < 0.001$) when applicable. Followed by Tukey's Ad-Hoc. analysis.

Chapter 3:

Live probiotic strains modulate macrophage subsets cytokine response

3.1: Introduction:

Lactobacilli and other probiotics, together with commensal organisms, can interact with mucosal immune cells or epithelial cell (Bain & Cerovic, 2020; Holmgren & Czerkinsky, 2005; Wells, 2011). The interaction between epithelial cells and macrophages is integral to the environment of the gut; therefore, mucosal immune fate is crucial to deter-mine the balance between tolerance and immune activation or inflammation (Foey, 2011; Wells, 2011). The macrophages of the innate immune system represent the first line of defence and are present in most tissues of the human body (Alberts et al., 2002). Macrophages of the intestine produce inflammatory markers such as IL-10 to inhibit the release of pro-inflammatory cytokines, including IL-12 and TNF-α produced by innate inflammation (Murray & Wynn, 2011). Macrophages are a type of phagocytic cell that ingest and kill pathogens, exogenous antigens or endogenous matter, such as bacterial-al microorganisms or their intracellular components (Kayama & Takeda, 2015). Endogenous matter includes apoptotic bodies and cellular debris (Zhang et al., 2009). While, challenging macrophages with *Candida albicans* or *Escherichia coli* lipopolysaccharide (LPS) stimulated distinct cytokine profiles, these were modulated by probiotics such as *Lactobacillus rhamnosus* LR32, *Lactobacillus casei* L324m, or *Lactobacillus acidophilus* NCFM, which resulted in increased levels of IL-10 and IL-1β, and a corresponding reduction of IL-12 production (Matsubara et al., 2017).

The M1-macrophage is able to direct the induction of the pro-inflammatory cytokine re-sponse through the production of TNF-α, IL-1β, IL-18, IL-6, IL-8, IL-12, and IL-23 (Foey, 2015). While, challenging of macrophages with *Candida albicans* or *Escherichia coli* lipopolysaccharide (LPS) stimulated the cytokines profile, then were altered by the probiotics such as *Lactobacillus rhamnosus* LR32,

Lactobacillus casei L324m, or *Lactobacillus acidophilus* NCFM) or associated with *Escherichia coli* lipopolysaccharide (LPS), which finally led to increased levels of IL-10 and IL-1β, and reduction of IL-12 production by macrophages (Matsubara et al., 2017). On the other hand, M2–macrophages are associated with the production of anti-inflammatory cytokines such as IL-10, TGFβ and IL-1Ra. M2–macrophages control homeostasis of the intestinal mucosal system during the tolerance stage (in more detail in chapter 4) (Biswas & Lopez-Collazo, 2009). The activation of macrophages by LPS results in the activation of NF-ƙB transcription factor, orchestrating gene expression leading to the activation of inflammation (Liu et al., 2017). The timed release of cytokines such as IL-12 and IL-18 induce cell proliferation of T-helper 1 (Th1), whilst the production of IL-23, IL-6, and TGFβ activates the differentiation of T-helper 17 (Th17). Fujino et al. (2003) showed that there are an amplified number of IL-17-producing cells in the inflamed gut of patients with IBD in comparison to healthy controls (Sakaguchi et al., 2008).

The plasticity of macrophage polarisation between pro-inflammatory (M1-macrophage) and anti-inflammatory (M2-macrophage) determines tissue repair by increasing the re-flection of phenotypes, pathogenic factors and immune tolerance (Zhang et al., 2009). Mohammadi et al. (2019) reported the effect of an edible component on macrophage polarisation of curcumin. The authors showed that this edible component could induce polarisation by repolarising macrophages toward the M2-macrophage phenotype, by reducing macrophage inflammatory responses, inhibiting MAPK activation and trans-location of p65/p50 NF-ƙB. M2-macrophages are able to direct the immune response of the gut dominantly by using chemokines and other innate effectors. The pres-

ence of M2-macrophages in mucosal gut guides tolerance and drives the homeostatic state (Belkaid & Harrison, 2017).

Activated macrophage phenotypes produce different types of molecules such as chemokines and cytokines; their activation drives a variety of functions. This includes the activity of TNF-α, interleukin-1β (IL–1β), IL-10, IL-8, IL-12, and IL-23 (pro-inflammatory) and IL-10 (anti-inflammatory cytokine) (Verreck, 2004). IL-1β cytokine plays a crucial role as a pro-inflammatory mediator, driving inflammation of the gut and the immune system (Dinarello, 2010). The activation of IL-1β is strictly controlled by its expression, maturation, secretion and stimulation, by inflammasomes (Zhou et al., 2011). In such pathologies IBD; such as CD, the macrophage phenotype may exhibit a state of plasticity between a pro-inflammatory M1-like subset and a regulatory M2-like subset. Accordingly, M1 could emerge due to the classical pathway activation due to the interaction between pro-inflammatory signals, such as interferon-γ (IFN–γ) and mi-crobial products such as lipopolysaccharide (LPS) (Mosser, 2003). This change in mac-rophage effector phenotype is likely to result from modulatory signals derived from soluble and cell-contact molecules that are present in the mucosal environment (Foey, 2008). There are three types of M2-macrophage; M2a is induced by IL-4 and IL-13 (Haribhai et al., 2016; Mohamed Hamed et al., 2020), while M2b is induced by immune complexes and a TLR agonist. M2b performs immune regulatory functions by initiating Th2 lymphocyte anti-inflammatory cytokines through the secretion of IL-10 (Mosser & Edwards, 2008), IL-1ra, and IL-6. In addition, the M2c subset is induced by IL-10 and plays an important role in suppressing the inflammatory response by secreting TGF-β and IL-10 (Gordon & Martinez, 2010; Murray & Wynn, 2011). Macrophage dysfunction results

in both dysbiosis of commensal microbiota and the breakdown of mucosal tolerance associated with infectious inflammation or IBD.

Cheng et al. (2018) reported that airway inflammation could be ameliorated by the inhibition of NF-kappa B expression in asthmatic mice, accompanied by the inhibition of TLR2 and NLRP3 activation. The activation of TLR and NLR occur in response to nutrient imbalance, such as lipids and glucose (Torres et al., 2019). Furthermore, Wen et al. (2013) investigated cells of myeloid lineage, such as macrophages or dendritic cells, to study other features of inflammasomes. They reported that cytosolic LPS could differentially trigger the inflammasome. Moreover, NLRP3 is an intracellular signalling molecule that is sensitive to pathogens and other environmental host derived factors (Wen et al., 2013).

The inflammasome is effected by the interaction between ASC and the cysteine prote-ase, pro-caspase -1. Caspase-1 could activate the cleavage of the active form of IL-1β and IL-18, whereby they act as inflammatory mediators of cell death, known as pyro-ptosis (Schroder & Tschopp, 2010; Wen et al., 2013). Pathogens then stimulate the macrophage by activation of microbe-associated molecular patterns (MAMPs), activating the cascade of antigen-presenting cells (APCs) to modulate their function (Schaefer et al., 2018).

3.1.1: THP-1 a model for macrophage subsets

THP-1 could react as mimicking native monocyte-derived macrophages in several re-spects. This provides a valuable model to study mechanisms that are involved in mac-rophage differentiation and regulation. Several studies have characterised macrophage markers in subsets differentiated by PMA or Vit.D3. Cell lines can thus be differentiated into two distinct macrophage subsets; M1-like and M2-like (Schwende et al., 1996) (Table 3.1).

Table 3.1: Validation markers involved in THP-1 derived macrophage subsets

Marker	*M1(+PMA)*	*M2(+Vit.D_3)*	**Reference**
TNF-α generation expression And LPS-stimulated TNF-α (protein)	↑	↓	(Schwende et al.,1996) (Chanput et al., 2010) (Daigneault et al.,2010)
LPS –stimulated IL-10	↓	↑	(Matilainen et al.,2010)
LPS-stimulated IL-1β	↑	↓	(Chanput et al.,2010)
LPS-induced IL-12 and IL-12p40 (mRNA)	↓ ↑	↑ ↓	(Gynther et al.,2011) Chanput et al.,2010)
TLR expression	TLR2 (high) TLR4 (NS)	TLR10 (high)	(Serena Tedesco, 2018) (Verma et al.,2014)
iNOS (m RNA)	↑	ND	(Chanput et al.,2010)
IL-8 gene expression (mRNA)	↑	↓	Chanput et al.,2010)
Arginase(mRNA)	ND	↑	Chanput et al.,2010)
MRC-1,Dectin-1 (m RNA) and MRC-1gene expression	↓	↑	Chanput et al.,2010)
DC-SIGN	↓	↑	Chanput et al.,2010)
CD36,PPAR-γ ,CD204,PKC delta isoform PPAR-γ ,CD204	↑	ND ↑	(Baeili et al.,2011) (Tedesco, 2018)
CD68 mRNA) expression CD206 (mRNA)expression	↓ ↑	↑	(Daigneault et al.,2010) (Tedesco, 2018)
Morphological changes[1]	pseudopodia	Ruffled	**(**Schwende et al.,1996)

Bron et al., (2017) reported on the characterisation of probiotics as modulators of macrophage signalling pathways. The authors concluded that probiotic could

↑: increase, ↓: decrease, ND: not defined, NS: not significant

assist to decrease gastrointestinal disease symptoms. Specific probiotic bacterial strains are able to impact barrier integrity, as shown in *in vitro* cell lines, animal models and clinical trials. These were used to assess whether probiotics can response to the diseased, returning normal health. The predominant strain of promoted probiotic bacteria belong to the genera, Lactobacillus and *Bifidobacterium* (Jungersen *et al.*, 2014). Savvidu (2009) revealed probiotic bacteria, particularly *Lactobacillus salivarius*, as inhibition of *Salmonella enterica Typhimurium Sal* 1344 nal growth in poultry gut. The role of *Lactobacillus* strain was identified as a probiotic agent and their nutritional and antimicrobial effects were reported to be immunomodulatory in nature. It has been revealed that they can interact with the immune system at numerous stages, involving cytokine production, mononuclear cell proliferation, macrophage phagocytosis, modulation of autoimmunity and immunity with bacterial pathogens (Vitini et al., 2000). The *L.plantarum* strain C28 can resist bile or acid conditions and can adhere to intestinal sections such as mucus or epithelial cells. This strain can also express potent IL-12 induction (Savvidou, 2009). The efficacy of immunomodulation LAB could be enhanced through the use of live cultures rather than killed bacteria (Vesely et al., 1985; De Simone et al., 1986). Therefore, the role of both probiotic bacteria of *Lactobacillus plantarum* strain (C28) (NCIMB 41605) and *Lactobacillus salivarius* strain MS13 (NCIMB 41610) *in vivo* were reported as efficient by Savvidou (2009) and Demeckova (2003). Both studies showed the inhibition of pathogenic bacteria as the main findings.

This study focused on the long-term effectiveness of live probiotic treatments in modulation of LPS-induced cytokine expression by macrophage subsets *in vitro*. It has been recognised that pathogens can induce pro-inflammatory cytokines such as TNF-α, from both monocytes and macrophage directly. Here,

TNF-α plays an important role in innate immunity.: Given the important roles of macrophages in determining mucosal tolerance or activation, in direct response to pathogens and the fact that these responses may be directly affected by both endogenous commensal organisms and the addition of exogenous bacteria, this chapter focusses on the role of probiotic strains and their ability to modulate macrophage-mediated immune responses. The objectives being tested are:

Objective 1: The probiotic bacteria behaviour in growth depends on the abundance of nutrients in the growth media and the effects of metabolic materials on the viability of macrophages.

Objective 2: Cytokine production of macrophage subsets differentially respond to probiotic strains.

Objective 3: THP-1 derived M1 and M2-like macrophages differentially respond to *E.coli* K12-LPS.

Objective 4: Live *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius* MS13 differentially modulates K12-LPS inflammation driven by M1-like and M2-like macrophages.

Objective 5: Live probiotic bacteria strain (C28, MS13) differentially induces the expression of NLRP3 production in the presence or absence of K12-LPS.

3.2: Results

3.2.1: Optimisation of probiotic growth condition

3.2.1.1: Probiotic bacteria growth in MRS media

The probiotic bacteria *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) were grown in MRS broth for 32 hours (Figure 3.2.1). The growth curve showed that the bacterial lag phase was between 0-2 hours and the exponential phase was between 2-6 hours. As expected, the stationary growth phase for both bacteria was between 6-24 hours. Remarkably, the C28 and MS13 growth curve showed another log phase between 24-26 hours (bi-phasic) and the C28 showed another stationary phase between 26-32 hours. Whereas, the MS13 had a second stationary phase, between 26-28 hours, followed a decline of growth after 28 hours (Figure 3.2.1).The pH value at the start and end of the growth were **between 7.4 - 5.5.**

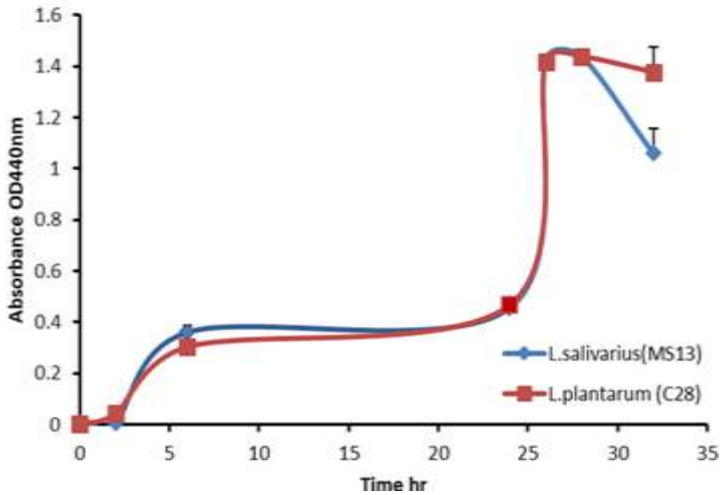

Figure 3.1: Growth curve of *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* (MS13) in MRS bro

Lactobacillus plantarum (C28) *and Lactobacillus salivarius* (MS13*)* were inoculated and cultured aerobically at 37^{o}C for 32 hrs in MRS broth. In order to determine bacterial growth phases, turbidity of bacteria was read at an optical density of 440 nm at the indicated time points in hours. All turbidity readings were controlled by zeroing optical densities of MRS media without bacteria at the indicated time points, prior readings the bacterial culture samples. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments.

3.2.1.2: Growth in R10 tissue culture medium

To examine the ability of lactic acid bacteria to grow in R10 media **(Figure 3.2.2)**, the phases of the growth curve were checked at 0, 2, 6, 24, 26, 30 hours. The lag phase of C28 and MS13 were between 0 - 2 hours, whilst the exponential phase was between 2 - 6 hours of growth. Continuously, exponential phase occurred in both strains at 6 – 24 hours in MS13 and C28. The stationary phase was differentiated between the two groups; MS13 was between 24 - 28 hours, whilst the stationary phase of C28 remained unclear and appears to be merged with the cell lysis phase.

To obtain the optimum condition of probiotic growth in R10 **(Figure 3.2.3)**, the growth pH value was checked at 0, 2, 6, 24, 26, 30 hour time points to preserve the viability of probiotic bacteria in R10 media. The results showed slight differences in pH readings between strains of R10 growth preserved at neutral point. At starting time, the pH was 7.9 and 7.4 of R10 + MS13 and R10 + C28, respectively. Whereas, the pH value of R10 media of both strain was ~ 7 at 24 hours.

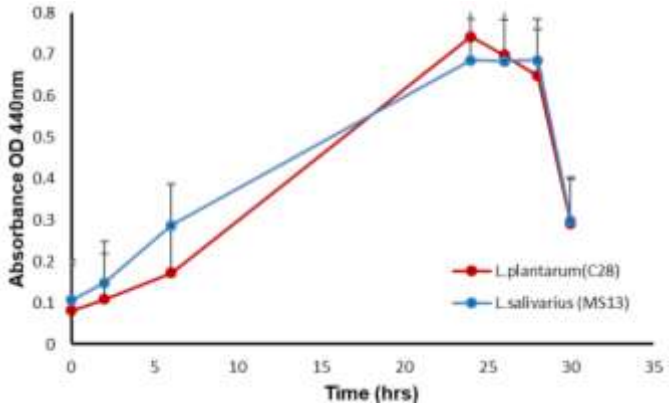

Figure 3.2: Growth curve of *Lactobacillus plantarum* (C28) and *Lactobacillus salivarius* (MS13) in R10 media

Lactobacillus plantarum (C28) and *Lactobacillus salivarius* (MS13) were cultured anaerobically at 37^{o}C 5% CO2 for 30 hrs in R10 tissue culture medium. In order to determine bacterial growth phases, turbidity of bacteria was measured at an optical density of 440nm at the indicated time points in hours. All turbidity readings were controlled by zeroing optical densities of R10 media without bacteria at the indicated time points, prior to reading the bacterial culture samples. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments.

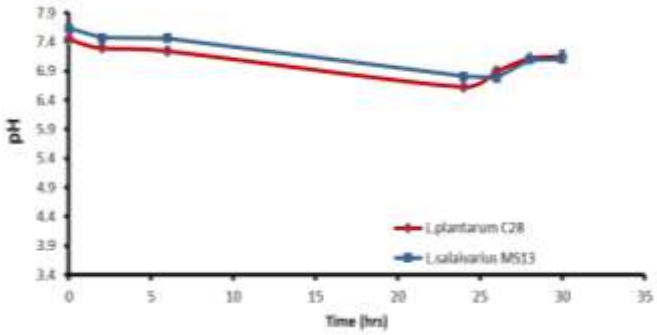

Figure 3.3 pH value of the growth media of *L.salivarius* and *L.plantarum* in R10 media

Lactobacillus plantarum (C28) and *Lactobacillus salivarius* (MS13) were cultured aerobically at 37°C for 30 hrs in R10 tissue culture medium. In order to determine the preserve the pH of the growth media in optimum condition, the pH of growth media was measured through growth period between 0-30 hours. Data displayed are experiments with triplicate samples of n=3 replicate experiments.

3.2.1.3: Optimisation of probiotic and M1 and M2-like macrophage culture condition

Based on previous studies, lactic acid is one of the metabolic products of Lactobacillus probiotic bacteria. This process involves the utilisation of D-glucose from the media before being conferred to lactic acid. A range of lactic acid concentrations 6.25, 12.5, 25, 50 and 100 mM were chosen, depending on concentration of D-glucose in the R10 media (11.01 mM). Two concentrations above and two below 25 mM were used to predict the amount of lactic acid metabolised in the media. Firstly, the pH value of different concentrations of lactic acid in R10 were checked and compared with R10 alone. The results showed the pH value of high concentration of lactic acid (100 mM) slightly decreased to 7.2, however, the pH of all concentrations was preserved at neutral 7.8 **(Figure 3.2.4)**. On the other hand, the effect of different lactic acid concentrations was examined for the viability of macrophages (M1 and M2-like macrophages) **(Figure 3.2.5**). Two groups for every M1 and M2-like macrophage were selected. The first group were grown in R10 for 24 hours without lactic acid and the second group consisted of different concentrations of lactic acid: 0, 6.25, 12.5, 25,

50, 100 mM (Fluka, UK). These were checked using a colorimetric MTTassay. Viability results reported 97-100% viability and no significant difference was observed between untreated M1 and M2, particularly those grown in 6.25 and 12.5 mM of lactic acid. Whereas, the viability of M1-like macrophages in 25 mM lactic acid slightly dropped to 97%, compared with untreated M1-like macrophages. Surprisingly, the viability of both M1 and M2 grown in high concentration of lactic acid is preserved at the 100% range (**Figure 3.2).**

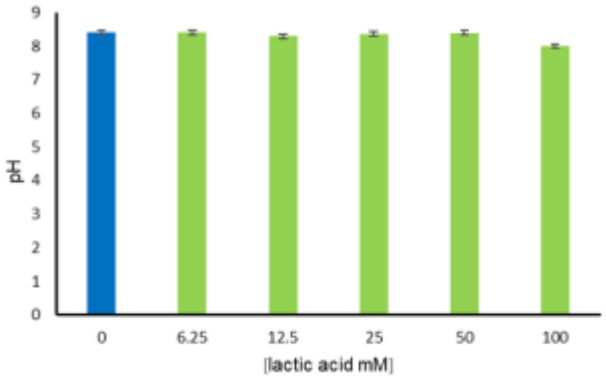

Figure 3.4: Exogenous addition of lactic acid partially reduces pH in R10 culture medium

Different concentrations of lactic acid (6.25, 12.5, 25, 50,100 mM) were added to R10 medium. The effect of lactic acid on R10 of tissue culture medium (green bar) compared with R10 without lactic (blue bar) for every concentration indicated. Data displayed is an illustration of triplicate samples of n= 1 experiment.

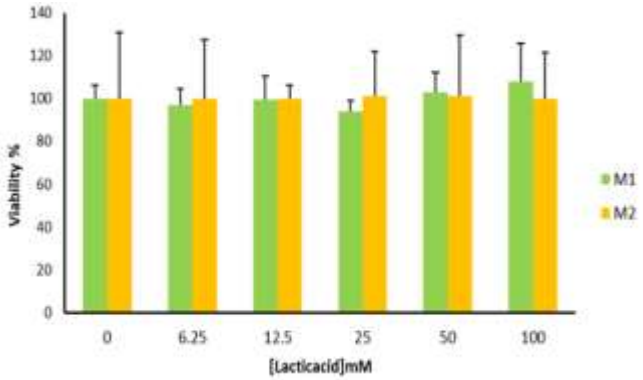

Figure 3.5: Lactic acid does not affect macrophage subset viability

were differentiated to M1-like (green bars) and M2-like (orange bars) macrophages, treated with serial dilutions of lactic acid (6.25,12.5,25,50,100 mM) and incubated at 37^{o}C 5% CO_2 in a humidified atmosphere for 24 hrs. Macrophage viability was measured by MTT assay 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide at 540 nm. Viability was calculated as Absorbance of (sample) /absorbance of control sample (without lactic acid) in percent. Data displayed is a representative experiment with triplicate samples of n= 1 replicate experiment.

3.2.2: Probiotic strains induce macrophage subsets cytokine kinetics

To study the ability of live *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius* strain MS13, cytokine production of M1 and M2-like macrophage were induced at specific time points **(Figure 3.2.2)**. Five time points were chosen (4, 8, 12, 18, and 24 hours) and three cytokines were examined (TNF-α and IL-1β) as pro-inflammatory cytokines. IL-10 was also studied as an anti-inflammatory cytokine. TNF-α production from M1-like macrophage induced by C28 $1x10^7$ cfu/ml was significant between 8-24 hours of incubation (600-1600 pg/ml) respectively ($p \leq 0.001$) (**Figure 3.2.6 a**). The induction of TNF-α by MS13 occurred gradually between 12 - 24 hours of incubation (400-1600 pg/ml), respectively ($p<0.01$). This is in comparison with untreated M1-like macrophages. Finally, there was no significant difference in TNF-α level of M1-like macrophages between induction by C28 or MS13, evident at 24 hours of incubation. On the other hand, there was no significant difference of TNF-α M2-macrophage induced by C28 or MS13 between 4-12 hrs of stimulation. The stimulation of TNF-α M2-like macrophage was not significant induced by C28 and MS13 between 4 - 11 hours. Both C28 and MS13 significantly induced the production of TNF-α M2-macrophage between 12 - 24 hours, whilst TNF-α level in M2-macrophages varied between the probiotic strains. Moreover, there were significant levels of TNF-α M2-macrophage induced by C28 between 18 - 24 hours using 600 and 950 pg. /ml. This was compared with untreated M2 ($P \leq 0.001$). There was a significant level of TNF-α M2-macrophage induced by MS13 at 300-500 pg/ml during 18 - 24 hours of stimulation ($P \leq 0.001$) (**Figure 3.2.6 b**).

IL-1β, a second pro-inflammatory cytokine, was examined in M1 and M2 macrophages. There was a significant production of IL-1β M1-like macrophage in-

duced by C28 and MS13 gradually increased between (12 to 24 hrs) compared with untreated M1 (P≤0.001) (**Figure 3.2.6 c**). Furthermore, IL-1β levels in M1-like macrophage induced by C28 gradually increased between 18 - 24 hours (103 - 203 pg/ml), while the induction of IL-1β of M1-like macrophage by MS13 was 110 pg/ml between 18 - 24 hours, in comparison with untreated M1 macrophages. The significant levels of IL-1β in M1 between 18 - 24 hours induced by C28 (103-303 pg/ml; p=0.004), compared with untreated M1-like macrophages. M2-like macrophages released IL-1β induced by C28 between 8 - 18 hours of incubation (50 - 230 pg/ml) (P<0.05, P≤ 0.001); while, MS13 induced IL-1β in M2 macrophages of two durations. The first duration was at 8 hours of incubation (49 pg/ml; P<0.05) and the second induction was at 24 hours (300 pg/ml; P=0.0001).

IL-10 is an anti-inflammatory cytokine that exhibits a delayed production following that of pro-inflammatory cytokines (Mosser & Zhang, 2008). The stimulation of M1-like macrophage IL-10 by probiotic C28 was significant between 4 - 12 hours (180 pg/ml) following suppression to 120 pg/ml at 18 - 24 hours, compared to untreated M1 (**Figure 3.2.6 e**). The suppression of IL-10 in M1 by C28 was at 24 hours, compared to untreated M1 (110 pg/ml; p≤ 0.001). Conversely, the significant production of IL-10 M1 induced by MS13 was evident at three time points, 8, 12, and 24 hours, compared to untreated M1 (P≤0.001 and P≤ 0.05). On the other hand, M2 IL-10 secretion was significantly augmented by the C28 probiotic at time points of 4, 8 and 24 hours with concentrations between 120 - 100pg/ml (p≤ 0.01, p≤ 0.001 and p≤ 0.05), compared with untreated M2-like macrophage (70 pg/ml). In contrast, probiotic MS13 not significantly induce IL-10 in M2-like macrophage, compared to untreated M2 at 4, 8, 12, 18 hours. MS13 significantly suppressed the production of IL-10 in M2-like macro-

phage at 24 hours (P≤ 0.01; 60 pg/ml) compared with untreated M2-like macrophages (100 pg/ml). Viability results for every time points reported between 80-100%.

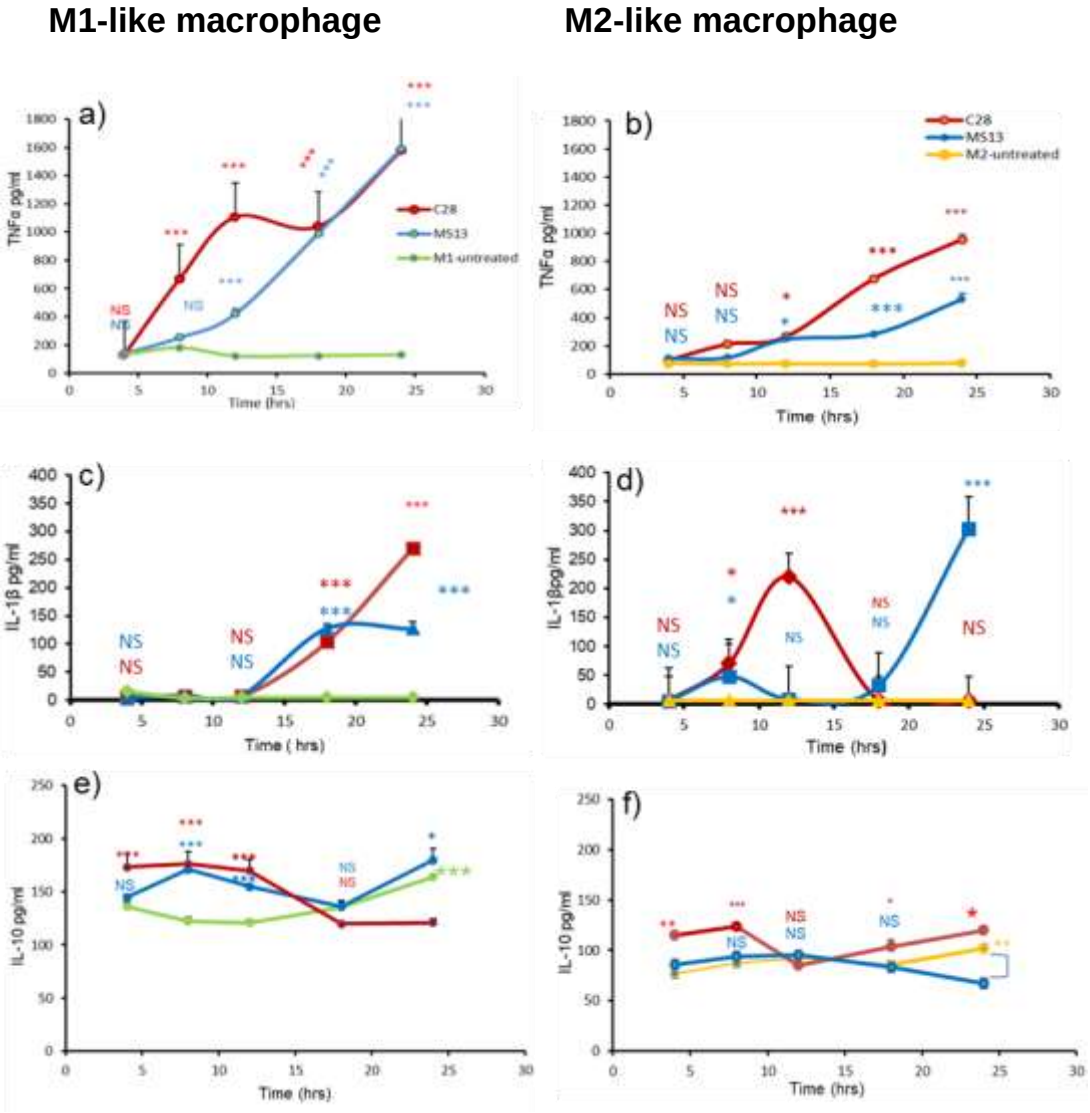

Figure 3.6: Probiotic strains differentially induce macrophage subset cytokine kinetics

M1-like macrophages (a,c,e) and M2-like macrophage (b,d,f) were incubated with live *Lactobacillus plantarum* strain (C28), and *Lactobacillus salivarius* strain (MS13), at a density of $1x10^7$ cfu/ml over a time period 24 hrs in 37 ^oC / 5% CO_2. TNF-α (a, b), IL-1β (c, d) and IL-10 (e, f) cytokine production is expressed as the mean ±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects of C28 or MS13 were compared to the control and are indicated as * ,* P≤ 0.05, ** ,**P≤ 0.01, *** ,***P≤ 0.001 and NS,NS = no significant difference. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

3.2.3: The stimulation of macrophage subsets by *E.coli* K12-LPS

Previous data revealed the ability of probiotic bacteria to stimulate TNFα, IL-1β and IL-10 cytokines from macrophage subsets. This experiment was designed to obtain cytokine production profiles by macrophage subsets and to identify the right concentration of K12-LPS macrophage subsets. The subsets respond differentially to different concentrations of *E.coli* of K12-LPS and the increase of cytokine production depends on the concentration of K12–LPS. K12-LPS induced high levels of pro-inflammatory cytokine TNF-α of the M1-like macrophage, while IL-1β significantly increased in M2-like macrophage compared to M1-like macrophages induced by K12-LPS. However, the dose causing 50% of the high level of TNF-α induced by K12-LPS was 650 pg/ml and 450 pg/ml for M1 and M2-like macrophage, respectively. The ED_{50} 30 ng/ml and 60 ng/ml K12-LPS of M1 and M2 like macrophages **(Figure 3.2.7a)**. IL-1β M2-like macrophage levels significantly increased with concentrations of K12-LPS at 1, 10, 100, 1000 and 10000 ng/ml. The ED_{50} of K12-LPS between 15ng/ml and 20 ng/ml resulted in 125 pg/ml and 175 pg/ml of IL-1β of M1 and M2, respectively **(Figure 3.2.7b).**

Interestingly, a high concentration of K12-LPS at 100, 1000 and 10000 ng/ml induced a significant production of IL-1β of M2-like macrophages ($P \leq 0.001$), compared with M1-like macrophage. The effective concentration of K12-LPS was 0.5 ng/ml and 1 ng/ml of M1 and M2-like macrophages, respectively, producing 100 pg/ml and 150 pg/ml of IL-1β. Concentrations of K12-LPS at 1000ng/ml induced high levels of IL-10 of M1 (550 pg/ml) and M2-like macrophage (680 pg/ml). M2-like macrophage released a high level of IL-10 compared to M1-like macrophage ($p \leq 0.001$). However, the ED_{50} of K12-LPS was 70 ng/ml and 35 ng/ml for M1 and M2-like macrophages, respectively. After the

administration of 100 ng/ml K12-LPS, M1-like macrophages induced cytokines TNFα /IL-10, and IL-1β /IL-10 were identified at a ratio of 7:3 and 1.2, compared to unstimulated controls. On the other hand, M2 macrophages induced TNFα/IL-10 and IL-1β/ IL-10 at a ratio of 5.2 and 1.2 when stimulated with 100 ng/ml K12-LPS. Cytokine expression between these two macrophage subsets were significant (P≤ 0.001, TNFα), (P≤ 0.001, IL-1β) and (P≤ 0.001, IL-10) **(Figure 3.2.7c)**.

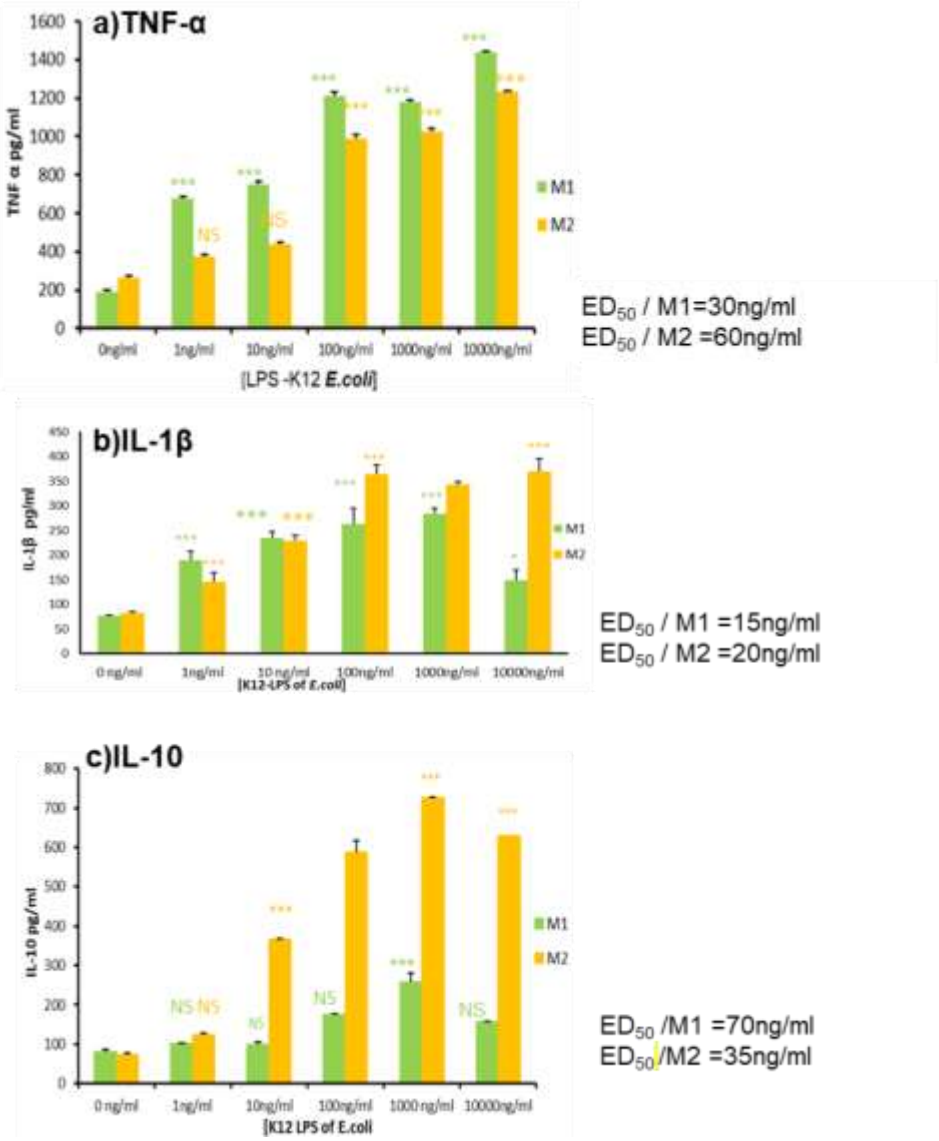

Figure 3.7: K12-LPS stimulated M1 and M2–like macrophage subsets exhibit a differential cytokine profile

M1 and M2 macrophages were generated by differentiating of THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 3 days and wash out for one day (M1) or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, (M2) respectively. M1 and M2 macrophage subsets were stimulated with *E.coli* K12-LPS at concentrations 1, 10,

100,1000,10000 ng/ml for 24 hrs. Cytokine production of M1 and M2 is expressed as the mean ±SE in pg/ml for TNF-α (a), IL-1β (b), and IL-10 (c). Data displayed represents triplicate samples for n= 6 replicate experiments. Significant differences in cytokine production of M1 and M2-macrophages which were stimulated by K12-LPS, compared to (0ng /ml) macrophage and are indicated as, * *p≤ 0.05, ***p≤ 0.005*** ***p≤ 0.001 and not significant = NS, NS, (M1 significance is indicated in green, whereas M2 significance is indicated in orange). ED50 values indicated in ng/ml for each of a-c.

3.2.4: Time course of cytokine production by K12-LPS stimulated M1 and M2-macrophage subsets

Previous experiment have identified concentration of K12-LPS of *E.coli* that induce the cytokine profile of M1 and M2-macrophage. K12-LPS concentrations of *E.coli* that resulted in the induction of TNF-α and IL-10 macrophage subsets was 10ng/ml. Only 1ng/ml can induce IL-1β of M1 and M2-like macrophage **(Figure 3.2.7)**. 100 ng/ml of K12-LPS was subsequently used to avoid any mistaken experiments resultant of small concentrations. Macrophage subsets differentially responded to *E. coli* LPS (100 ng/ml) at different time points of stimulation. The results **(Figure 3.2.8)** demonstrated that cytokine production in stimulated K12-LPS M1-macrophages and M2-macrophages are time dependent. Three cytokine profiles demonstrated a THP-1-derived macrophage phenotype in response to 100 ng/ml of K12-LPS, TNF-α, IL-1β and IL-10 **(Figure 3.2.8)**. M1-like macrophages released high levels of TNF-α and IL-1β cytokines than M2-macrophage (P≤0.001). Nevertheless, M2 macrophages produced significant levels of IL-1β after four hours of stimulation with K12 LPS, compared to M1-like macrophage (P ≤0.01).

The M1-like macrophage TNF-α production increased significantly at 4 hours and 8 hours of incubation. Levels of TNF-α were measured between 2000 and 3000 pg/ml (P ≤0.001); comparisons were made between groups (**Figure 3.2.8a)**. However, levels of TNF-α M1 and M2-like macrophages decreased to 100pg/ml at 24 hrs of incubation. Peak production of TNF-α of M1-like macro-

phage occurred at 8 hours, then dropped at 24 hours. Significant levels of IL-1β in M2-like macrophage were recorded at 4 hours with 40 pg/ml ($p \leq 0.05$). IL-1β M2-like macrophage levels decreased at 8 hours then rose at 24 hours. However, no significant difference was recorded between the production of IL-1β of M1-macrophages at 4 and 24 hours. Finally, the production of IL-10 at four hours in M1-like macrophage produced a high level compared with M2-like macrophages (**Figure 3.2.8 c**). Furthermore, IL-10 levels in M1-like macrophages gradually decreased after 8 hours of stimulation, in opposition to significant IL-10 M2-like macrophage levels. This then increased from 8 to 24 hours ($p \leq 0.01$).

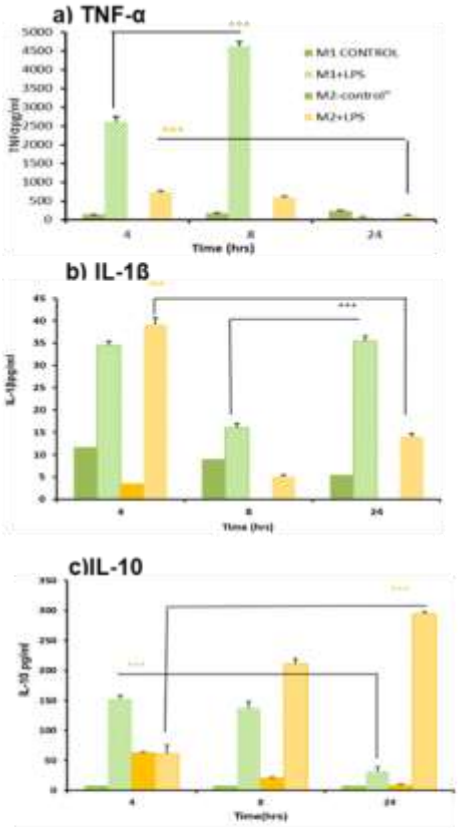

Figure 3.2.8: Kinetic of cytokine production of macrophage induced by K12-LPS

M1 and M2 macrophages were generated by differentiating of THP-1 monocytes with either 25 ng/ml phorbol 12-myristate 13-acetate(PMA) for 3 days and wash out for one day (M1) or 10 nM 1,25-(OH)2 vitamin D3 for days, (M2) respectively. M1 and M2 macro-phage subsets (were stimulated with 100 ng/ml LPS-K12 (4, 8, 24 hrs). Production of cytokine were expressed as the mean ±SE in pg/ml for TNF-α (a), IL-1β (b), and IL-10 (c). Da-ta displayed represents triplicate samples for n= 6 replicate experiments. Significant differences in cytokine production of M1 and M2-macrophage which were stimulated by K12-LPS and compared between stimulation time points are indicated as***, ***p≤ 0.001. M1 significance indication in green where M2 significance is indicated in orange).

3.2.5: Probiotic strains selectively modulate LPS-induced macrophage subset cytokine production

Previous results indicated that the optimal LPS concentration for cytokine secretion is 100 ng/ml **(Figure 3.2.7)** and the optimal time is 24 hours incubation (**Figure 3.2.8)**, whereas the optimal time for probiotic induction of macrophage cytokine secretion varies up to 24 hours, dependent on the cytokine investigated and probiotic strain **(Figure 3.2.6)**.

TNF-α

TNF-α is a pro-inflammatory cytokine; the most potent stimulus for eliciting TNF-α production by macrophages is LPS. Stimulations with probiotic bacteria in the presence of LPS modulate inflammation of macrophages subsets. This was investigated in the context of a homeostatic environment, whereby there was no LPS stimulation **(Figure 3.2.9a/b)** and in an inflammatory environment, with simultaneous LPS stimulation (**Figure 3.2.9 c/d**) over the duration of 24 hours. In a non-inflammatory, homeostatic environment, C28 and MS13 achieved induction of TNFα ($P<0.001$), compared to untreated M1 and M2-macrophages. On the other hand, during inflammation by K12-LPS, M1 macrophages released TNF-α induced by K12-LPS (black) at 500 pg/ml, compared to the *Lactobacillus plantarum* strain (C28) which upregulated the production of TNFα induced by K12-LPS (2800 pg/ml) (red) significantly, ($P=0.001$). As can be further seen in (Figure 3.2.9), MS13 failed to stimulate TNF-α M1-macrophage induced by K12-LPS (blue), while C28 modulated TNF-α compared to levels of TNF-α M1 induced by K12-LPS (black). C28 failed to stimulate a significant change in TNF-α of M2-like macrophage induced by K12-LPS, compared to the induction of TNF-α induced by K12-LPS (black). MS13 significantly stimulated M2-like TNF-α induced by K12-LPS (2000 pg/ml), compared with the induction of M2-macrophage by K12-LPS (270 pg/ml) ($p<0.001$).

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simultaneous LPS stimulation (**Figure 3.2.9 c/d**) over the duration of 24 hours. In a non-inflammatory, homeostatic environment, C28 and MS13 achieved induction of TNFα ($P<0.001$), compared to untreated M1 and M2-macrophages. On the other hand, during inflammation by K12-LPS, M1 macrophages released TNF-α induced by K12-LPS (black) at 500 pg/ml, compared to the *Lactobacillus plantarum* strain (C28) which upregulated the production of TNFα induced by K12-LPS (2800 pg/ml) (red) significantly, ($P=0.001$). As can be further seen in (Figure 3.2.9), MS13 failed to stimulate TNF-α M1-macrophage induced by K12-LPS (blue), while C28 modulated TNF-α compared to levels of TNF-α M1 induced by K12-LPS (black). C28 failed to stimulate a significant change in TNF-α of M2-like macrophage induced by K12-LPS, compared to the induction of TNF-α induced by K12-LPS (black). MS13 significantly stimulated M2-like TNF-α induced by K12-LPS (2000 pg/ml), compared with the induction of M2-macrophage by K12-LPS (270 pg/ml) ($p<0.001$). The comparison between homeostasis and inflammation revealed that levels of TNFα of M1 were increased by C28 during the inflammation stage compared to homeostasis by 6-fold **(Figure 3.2.9 c versus Figure 3.2.9 a)**. MS13 suppressed TNF-α level of M1-like macrophage in the inflammation stage by 50% compared with its level in homeostasis (**Figure 3.2.9a**). On the contrary, C28 suppressed TNF-α level of M2-like macrophages induced by K12-LPS by 50%.

The comparison between homeostasis and inflammation revealed that levels of TNFα of M1 were increased by C28 during the inflammation stage compared to homeostasis by 6-fold **(Figure 3.2.9 c versus Figure 3.2.9 a)**. MS13 suppressed TNF-α level of M1-like macrophage in the inflammation stage by 50% compared with its level in homeostasis (**Figure 3.2.9a**). On the contrary, C28 suppressed TNF-α level of M2-like macrophages induced by K12-LPS by 50%,

compared with levels of TNF-α of M2-like macrophages in homeostasis **(Figure 3.2.9 b/d)**. Furthermore, MS13 increased the production of TNF-α in M2-like macrophages during inflammation **(Figure 3.2.9 d)** 6-fold, compared to homeostasis **(Figure 3.2.9 b)**.

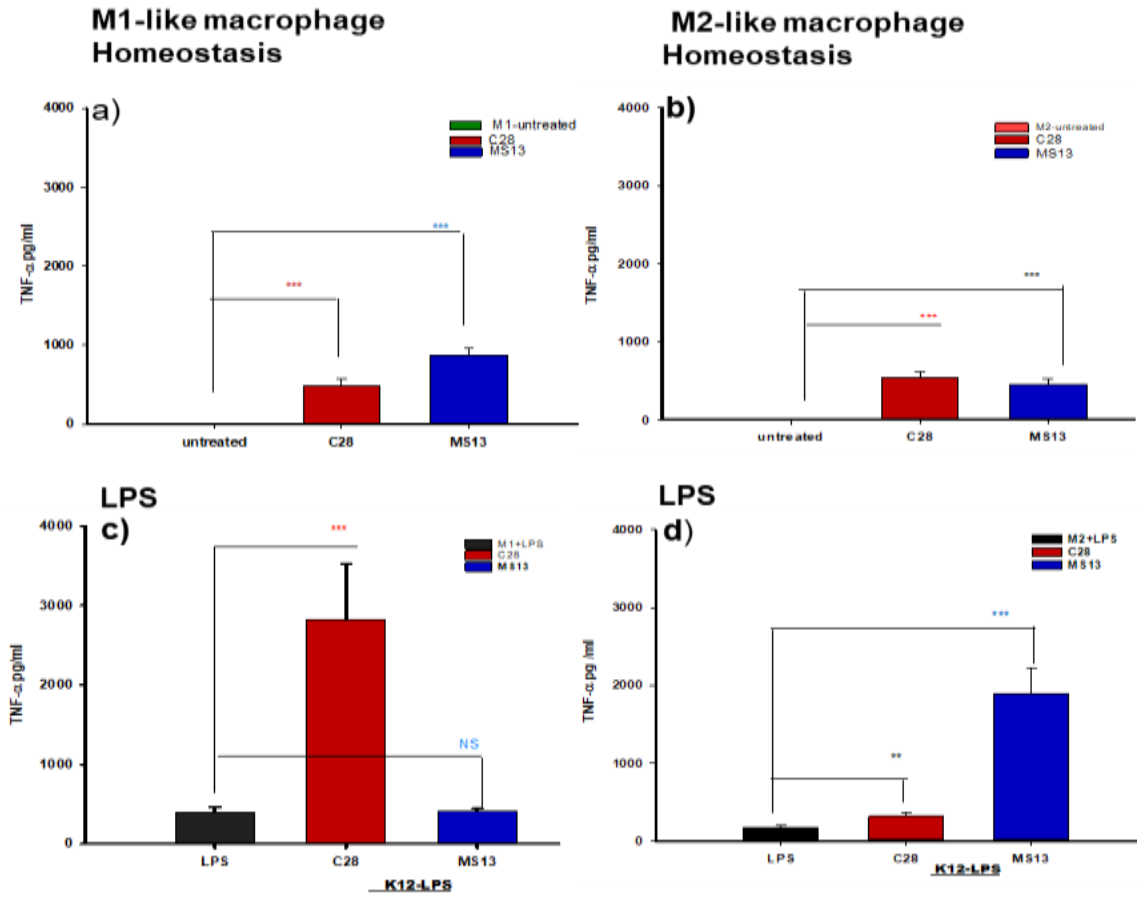

Figure 3.9: Live probiotic bacteria differentially modulate macrophage subset TNF-α which is stimulus dependent

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hrs wash out or 10 nM 1,25-(OH)2 vitamin D3 for 7 days. Then, M1 (a) and M2 (b) were directly treated with live Lactobacillus plantarum strain (C28) or Lactobacillus salivarius strain (MS13) for 24 hr. M1 (c) and M2 (d) were stimulated with 100 ng/ml E.coli K12 LPS alone or in the presence of 1x107 cfu /ml live (C28) or (MS13) for 24 hr. TNF-α production is expressed as the mean ±SE in pg/ml .Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects of C28 and MS13 on macrophage subsets stimulated with K12-LPS (c, d) compared with LPS macrophage, or (homeostasis a, b) compared with untreated macrophage subsets are indicated as, ***, *** P= 0.001 and not significant=, NS, NS. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

IL-1β

IL-1β mediates the inflammatory response of the host to infections, similar to that of TNF-α. The first investigation was in the context of a homeostatic environment with no LPS stimulation **(Figure 3.2.10a/b)** and in an inflammatory environment with simultaneous LPS stimulation (**Figure 3.2.10c/d**) over a 24 hour duration. In a non-inflammatory, homeostatic environment, C28 and MS13 activated the production of IL-1β in M1-like macrophage (20pg/ml). This was significant ($p< 0.001$) compared to untreated M1-like macrophage. Similarly, C28 and MS13 significantly induced the production of IL-1β in M2-like macrophage at 120 and 100pg/ml, respectively ($p<0.001$) compared to unstimulated control levels of 7pg/ml. In an inflammatory environment (LPS present), both C28 and MS13 induced significant increases in M1 derived IL-1β; C28 induced at 230pg/ml ($p< 0.001$) and MS13 induced at 100pg/ml ($p<0.05$) compared with the LPS control (20pg/ml) **(Figure 3.2.10c).** The response of M2 macrophages in an inflammatory environment increased significantly ($p<0.001$), where the LPS control levels of IL-1β (10pg/ml) were upregulated 19-fold to 1300 pg/ml ($p\leq0.001$) and 800 pg/ml ($p\leq 0.001$) in the presence of C28 and MS13, respectively.

Remarkably, in M1 macrophage homeostasis and inflammatory environment responses, LPS **(Figure 3.2.10 c, black)** alone induced significant levels of IL-1β secretion compared to unstimulated M1 (**Figure 3.2.10 a,** green). M1-macrophage IL-1β was augmented 10-fold by probiotic strains, whereby C28 induced at 200 pg/ml **(Figure 3.2.10 c, red)** compared to 20 pg/ml for C28, in the absence of LPS **(Figure 3.2.10 a, red)** ($p<0.001$). MS13 augmented IL-1β in the presence of LPS to 100 pg/ml (**Figure 3.2.10 c, blue**), compared to 20 pg/ml in the absence of LPS (**Figure 3.2.10 a, green**) ($p<0.001$). Secretion of

IL-1β M2-like macrophage induced by LPS (**Figure 3.2.10 d, black**) increased 10-fold compared to unstimulated LPS (**Figure 3.2.10 - b, yellow**). M2 IL-1β secretion was augmented by probiotic strains in the presence of LPS, whereby C28 induced at 1300 pg/ml (**Figure 3.2.10 d, red)** compared with 130 pg/ml for C28 in the absence of LPS (**Figure 3.2.10 b, red**). MS13 augmented IL-1β in the presence of LPS to 800 pg/ml (**Figure 3.2.10 d, blue**) compared to 100-pg/ml in the absence of LPS (**Figure 3.2.10 b, blue**) (P<0.001).

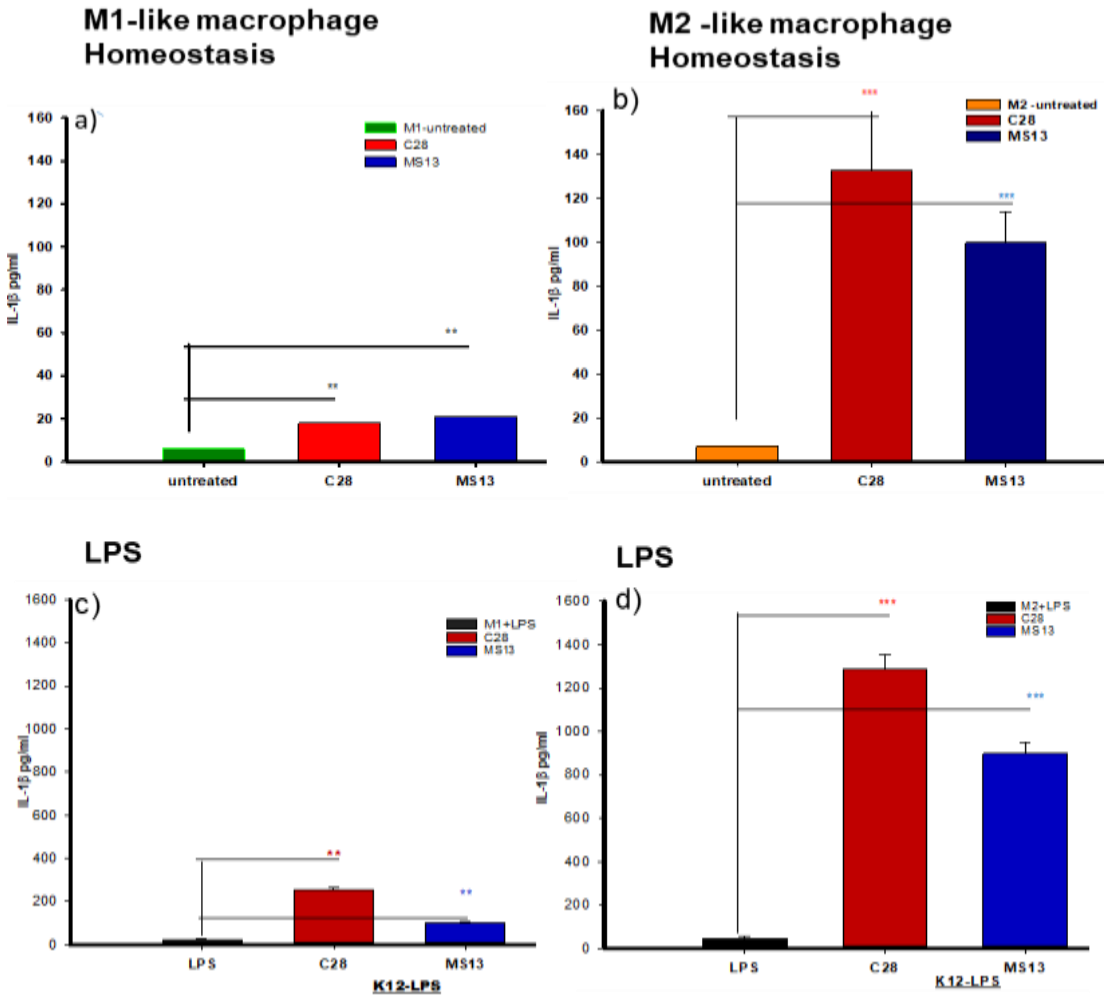

Figure 3.10: Live probiotic bacteria differentially modulate IL-1β of macrophage subset which is stimulus dependent

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hrs wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 (a) and M2 (b) were directly treated with live *Lactobacillus plantarum* strain (C28) or *Lactobacillus salivarius* strain (MS13) for 24 hrs. Whereas, M1 (c) and M2 (d) were stimulated with 100 ng/ml *E.coli* K12 LPS *alone or* in the presence of $1x10^7$ cfu /ml live (C28) or (MS13) for 24 hr. IL-1β production is expressed as the mean ±SE in pg/ml .Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments.

Significant effects of C28 and MS13 on macrophage subsets stimulated with K12-LPS (c, d compared with LPS macrophage or (homeostasis a, b) compared with untreated macrophage subsets are indicated as, ***, *** P= 0.001 and not significant=, NS, NS**.** (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

IL-10

IL-10 is an anti-inflammatory cytokine that displays delayed *de novo* synthesis following that of pro-inflammatory cytokine. The modulatory effects of the C28 and MS13 probiotic strains of IL-10 secretion were investigated in the context of a homeostatic environment with no LPS stimulation (Figure 3.2.11 a/b) and an inflammatory environment with simultaneous LPS stimulation (Figure 3.2.11c/d) over a duration of 24 hours. In a non-inflammatory homeostatic environment, C28 and MS13 failed to induce significant secretion of IL-10 by M1-like macrophage (Figure 3.2.11a). In contrast, both C28 and MS13 induced significant secretion of IL-10 by M2, where C28 induced 168.33 pg/ml ($p<0.01$) and MS13 induced 305.66 pg/ml ($P<0.001$), compared to unstimulated control at 102.4 pg/ml (Figure 3.2.11 b). In an inflammatory environment (LPS presence), the response to these probiotic strains varied to that of homeostatic environments. C28 and MS13 induced significant increases in M1-derived IL-10; C28 induced at 101.6 pg/ml ($p<0.05$) and MS13 induced at 131 pg/ml ($p<0.01$) compared with LPS control at 98 pg/ml (Figure 3.2.11 c). The M2 macrophage response in an inflammatory environment was different, where LPS control levels of IL-10 (485 pg/ml) were suppressed to 404 pg/ml ($P<0.001$) and 406 pg/ml ($p<0.001$) in the presence of C28 and MS13, respectively.

Interestingly, when comparing M1-macrophage responses between homeostatic and inflammatory environments, LPS alone (Figure 3.2.11c, black) failed to induce significant up-regulation in IL-10 secretion compared to unstimulated M1 (Figure 3.2.11a, green). M2 IL-10 secretion was augmented by probiotic strains, only in the presence of LPS, where C28 induced 400 pg/ml (Figure 3.2.11 d, red) compared to

100pg/ml for C28 in the absence of LPS (Figure 3.2.11b, red). MS13 augmented IL-10 in the absence of LPS 410 pg/ml (Figure 3.2.11d, blue) compared to 310 pg/ml in the absence of LPS. (Figure 3.2.11b, blue).

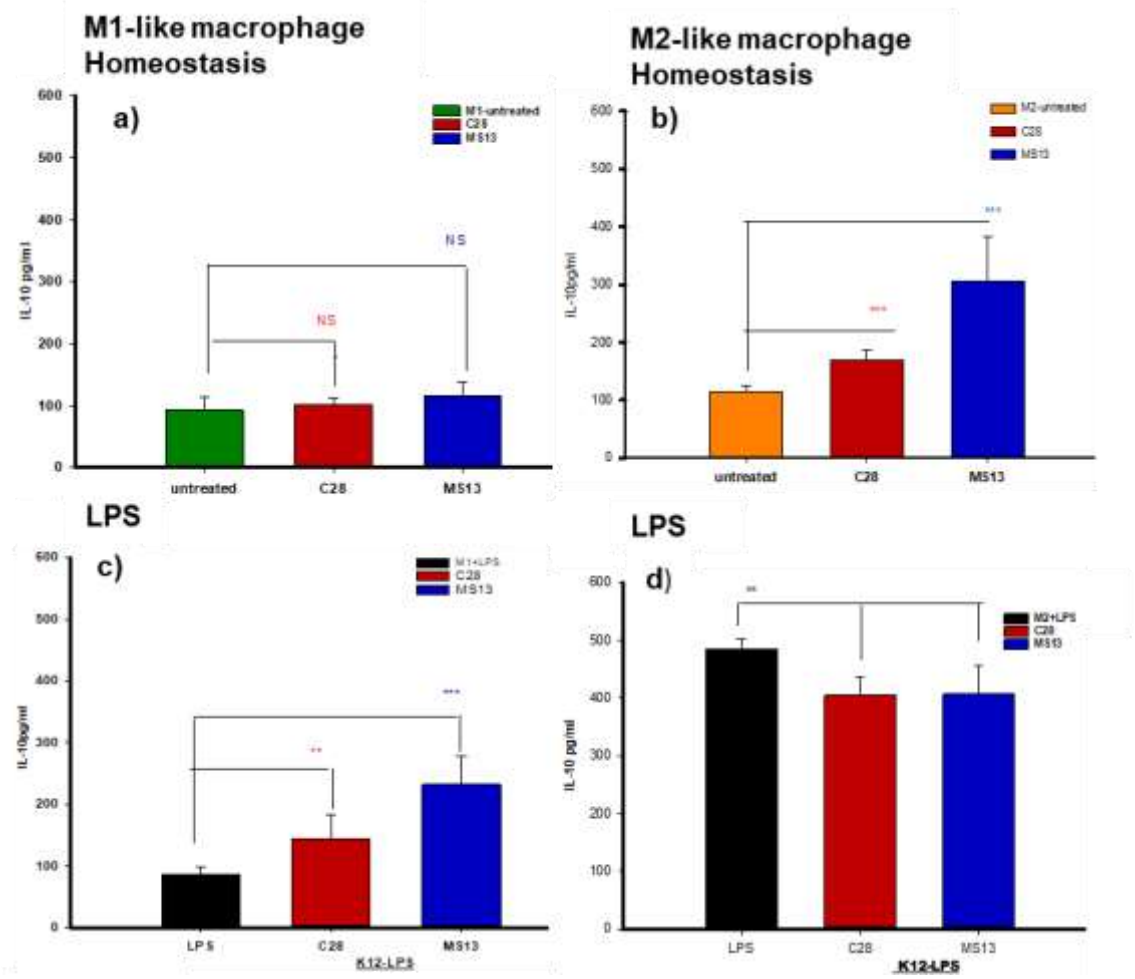

Figure 3.11: Live probiotic bacteria differentially modulate macrophage subset IL-10 which is stimulus dependent

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hrs wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 (a) and M2 (b) were directly treated with live *Lactobacillus plantarum* strain (C28) or *Lactobacillus salivarius* strain (MS13) up to 24 hrs. Whereas, M1 (c) and M2 (d) were stimulated with 100 ng/ml *E.coli K12 LPS alone or* in the presence of $1x10^7$ cfu /ml live (C28) or (MS13) for 24 hr. IL-10 production is expressed as the mean ±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects of C28 and MS13 on macrophage subsets treated with K12-LPS (c, d) compared with LPS macrophages or (homeostasis a, b) compared with untreated macrophage subsets control are indicated as, ***, *** P= 0.001 and not significant=, NS, NS**.** (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

3.3: Can probiotic bacteria induce activation of NLRP3?

3.3.1: MCC950 suppression of macrophage subset IL-1β induced by live probiotic bacteria: NLRP3-dependence

To investigate the role of probiotic bacteria for activation of NLRP3 molecules, our previous results (**Figure 3.2.6 c/d** and **Figure 3.2.10a/b)** that live C28 and MS13 stimulated the production of IL-1β of macrophage subsets, as well as K12-LPS. In this section, the aim was to investigate the concentration of MCC950 that suppressed IL-1β production. Varying concentrations of MCC950, a NLRP3 inhibitor, 0.01, 0.1, 1, 10,100 μM were applied. C28 and MS13 induced significant production of IL-1β of M1 macrophage at 1556 and 770 pg/ml, respectively. This is compared with unstimulated M1 that produce at 190 pg/ml ($p<0.001$) over a duration of 19 hours and 30 minutes. Pre-treatment with NLRP3 inhibitor for 30 minutes revealed that IL-1β of M1-like macrophage was significantly suppressed to 45 and 97 pg/ml induced by C28 and MS13, respectively ($p< 0.001$). However, these concentrations showed no significant difference between the concentrations. On the other hand, M2 released IL-1β induced by C28 and MS13 at 3340 pg/ml and 2300 pg/ml, respectively, in comparison to unstimulated M2 7pg/ml. IL-1β inhibition of M2-like macrophage was normalised with the induction by C28 (live bacteria) to produce 1556 pg/ml and 2300 pg/ml of IL-1β. Alternatively, MS13 results in the production of 770 pg/ml and 3340 pg/ml of IL-1β (**Figure 3.2.12 c/d**). The concentrations of MCC950 used here suppressed the production of IL-1β in M1 and M2-like macrophages between 56-77 pg/ml. The MCC950 concentration (10 μM) was similar to that used by Coll et al. (2015). This concentration was used in the following experiment in inhibition of NLRP3 whereby the stimulation of macrophage subsets was investigated by K12-LPS over a 4 hour. During this time, the production of

NLRP3 was activated and inhibited by MCC950 for 30 minutes, re-activating the production of NLRP3 for 19 hours and 30 minutes.

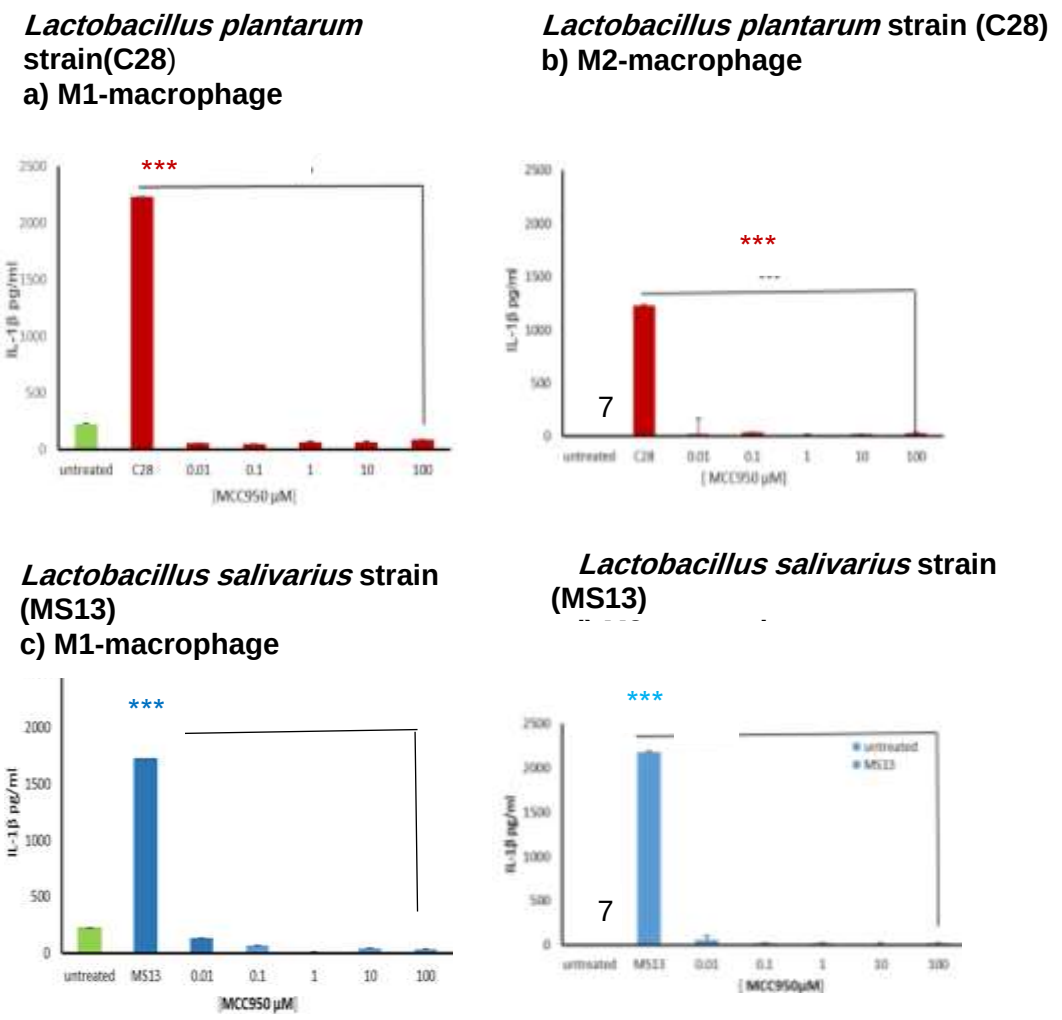

Figure 3.12: Probiotic induction of macrophage IL-1β is dependent on NLRP3 inflammasome

M1 and M2-like macrophage were generated by differentiating with either 25 ng/ml phorbol 12-myristate 13-actate (PMA) for 3 days + 24 hrs wash out, or by 10nM of Vit.D3 for 7 days. M1 and M2 like macrophages were treated with different concentrations of MCC950 0.01, 0.1,1, 10,100 µM for 30 mins prior to stimulation with either C28 or MS13 up to 20 hours. IL-1β production is expressed as the means ± SE with triplicate samples of n=3 replicate experiments. The significant effect of inhibition by MCC950 compared to untreated M1 and M2-macrophages are indicated as p***,***≤ 0.001. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

3.3.2: Probiotic bacteria stimulate gene expression of NLRP3 inflammasome of M1 and M2-like macrophages

Previous results demonstrate the role of probiotic bacteria C28 and MS13 as IL-1β producers from M1 and M2-like macrophage. This was identified through the

activation of inflammasome NLRP3 through an NLRP3 inhibitor. To confirm the role of probiotic bacteria C28 and MS13, the inflammasome NLRP3 was directly modulated. This section reveals the results of NLRP3 mRNA expression of M1 and M2-like macrophages in 24 hours. Results showed a significant decrease ($P \leq 0.001$) whereby C28 inhibited gene expression of NLRP3 of M1, in comparison to M1-untreated MS13 and K12-LPS. No significant difference was identified regarding the stimulation of NLRP3 between untreated MS13 and LPS. Gene expression of NLRP3 in M2-like macrophage induced by probiotics and K12-LPS showed that C28 and MS13 significantly upregulated expression of NLRP3 ($P \leq 0.001$), compared to M2-untreated macrophages and K12-LPS. M2-like macrophages expressed C28-induced NLRP3 up to 2.5-fold more than M2-untreated, whereas a 2-fold increase was recorded in MS13-induced M2-untreated and K12-LPS (**Figure 3.2.13**).

3.3.3: Detected protein production of NLRP3 inflammasome of macrophage subsets induced by C28 and MS13

The NLRP3 protein was also detected by western blotting after 24 hours of treatment by live C28 and MS13 and stimulation with 100ng/ml of K12-LPS (**Figure 3.2.14**). The NLRP3 protein in M1-like macrophage induced by MS13 was upregulated compared with C28 and K12-LPS or M1-untreated after 24 hours. Whereas, M2-like macrophages displayed upregulated NLRP3 protein induced by LPS, MS13 and C28, compared to M2-untreated.

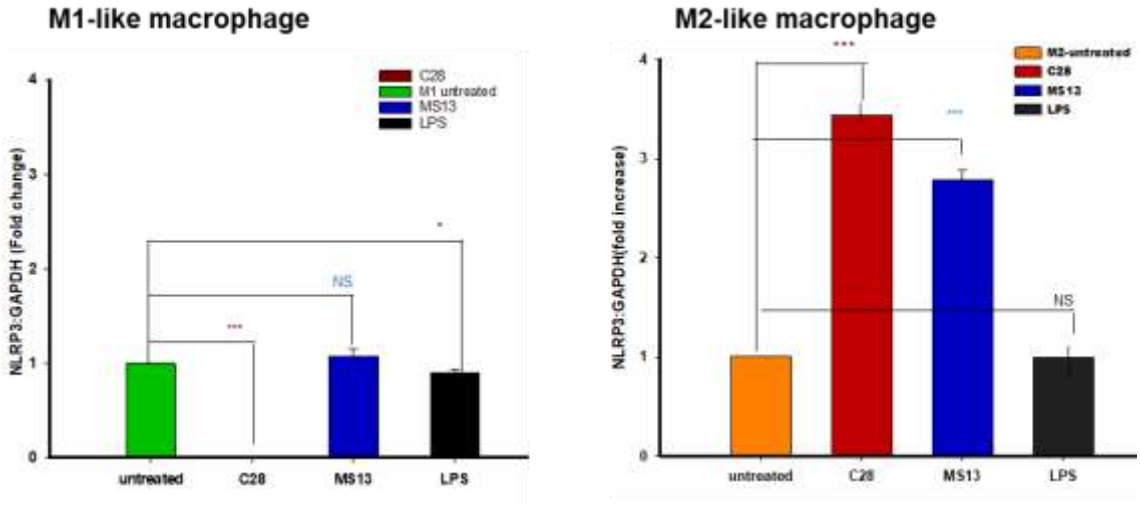

Figure 3.13: Lactobacillus plantarum strain C28 and Lactobacillus salivarius strain MS13 differentially modulate

NLRP3 gene expression in a macrophage subset-dependent manner. M1 and M2-macrophage subsets ($1x10^6$ cells/ml) were treated with ($1x10^7$ cfu/ml) of live (C28, MS13), or stimulated by 100ng/ml of *E.coli* K12-LPS for 24 hrs. Gene expression (mRNA level) is expressed as fold change using GAPDH as a reference gene and resting cells as a calibrator sample as described by (Livak *et al*., 2001) using $2^{-\Delta\Delta ct.}$ Data displayed is a representative experiment with duplicate samples of n=2 with replicate experiments. The significance effect of gene expression of NLRP3 of M1 and M2 macrophage treated by C28 and MS13 or K12 –LPS compared with untreated macrophage subsets are indicated as *** , ***P ≤0.001, NS=no significant. (C28 significance is indicated in red, whereas MS13 significance is indicated in blue).

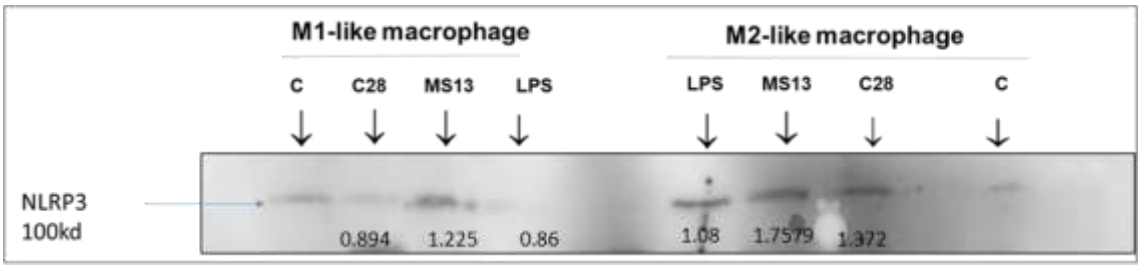

Figure 3.2.14: Probiotic bacteria differentially modulate macrophage subset NLRP3 protein

M1 and M2 macrophage subsets were treated with either C28 or MS13 for 24 hrs or stimulated with 100ng/ml K12-LPS for 24 hrs. NLRP3 protein was detected by western blot, this experiment was repeated 3 times and the ratios of optical density were compared with untreated M1 and M2-like macrophages (indicated numerically below NLRP3 band).

3.3.4: Probiotic bacteria stimulate gene expression of IL-1β mRNA in M1 and M2-like macrophage

Gene expression of IL-1β induced by live C28, MS13 and K12-LPS showed no significant difference of expression in M1-macrophage after 24 hours. Gene expression of IL-1β in M2-like macrophage induced by C28 was upregulated compared to other treatments, such as MS13, K12-LPS and M2-untreated after 24 hours of stimulation. However, gene expression of IL-1β was significantly downregulated by MS13

(P ≤0.001and 0.05) compared with other treatments (C28 and K12-LPS) (**Figure 3.2.15**).

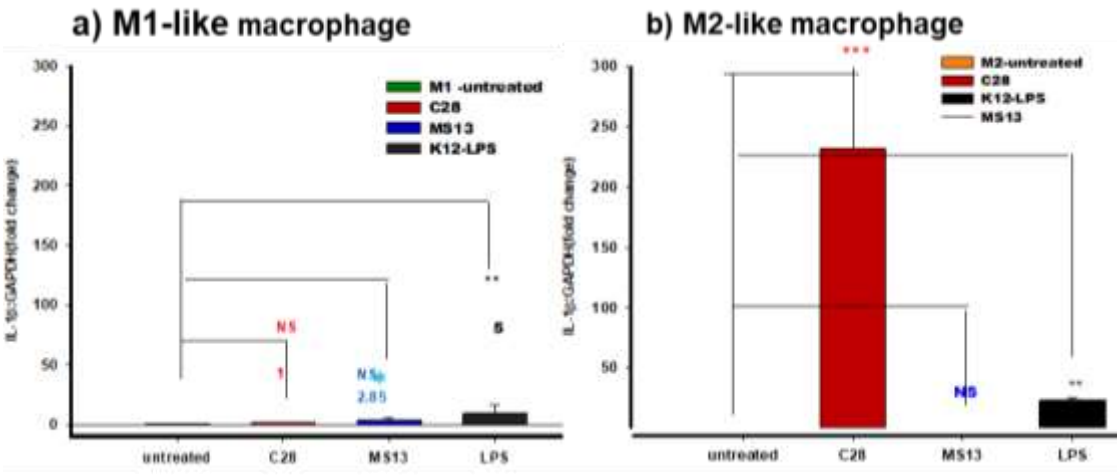

Figure 3.15: Modulation of IL-1β gene expression of macrophages by *Lactobacillus plantarum strain C28 and Lactobacillus salivarius strain MS13*

M1 and M2-macrophage subsets (1x10^6 cells/ml) were stimulated with (1x10^7 cfu/ml) **li**ve (C28, MS13) and 100 ng/ml K12-LPS for 24 hrs of stimulation. RNA was extracted from the cells to test mRNA expression. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample, described by (Livak et al., 2001) using $2^{-\Delta\Delta ct.}$ Data displayed are a representative experiment with duplicate samples of n=2 replicate experiments. Significant stimulation effects of C28 and MS13 or LPS on macrophage subsets were compared with untreated macrophage subsets are indicated as ***, *** P= 0.001, no significance=NS, NS. (C28 significance is indicated in red, whereas MS13 significance is indicated in blue).

3.3.5: MCC950 inhibited IL-1β production of M1 and M2-like macrophages, induced by LPS augments probiotic strains

LPS-primed M1 and M2-like macrophages were treated with MCC950, and then modulated with live C28 and MS13. MCC950 inhibited the release of IL-1β in response to both probiotic bacteria, C28 and MS13 (Figure 3.2.16 a/b). IL-1β production dropped upon treatment with NLRP3 inhibitor after stimulation with K12-LPS. MCC950+ treatment were compared to levels of IL-1β with MCC950- in both M1 and M2-like macrophages. IL-1β levels of M1-like macrophages induced by LPS and C28 were significantly inhibited ($p \leq 0.001$) after treatment with MCC950 to 75 pg/ml, in comparison with IL-1β MCC950-levels (2300 pg /ml) (Figure 3.2.12). IL-1β M2-like macrophage were induced by LPS for 4 hours, then regulated by C28 (2800 pg/ml), compared to IL-1β that was significantly suppressed by MCC950 ($P=0.001$) to 370 pg/ml (Figure 3.2.16b). IL-1β inhibition of M1 and M2-like macrophages that were significantly induced by LPS and MS13 by MCC950+ dropped to 100pg/ml and 200 pg/ml, respectively, in comparison with MCC950-, at 1200 pg/ml and 3800 pg/ml (Figure 3.2.16 c).

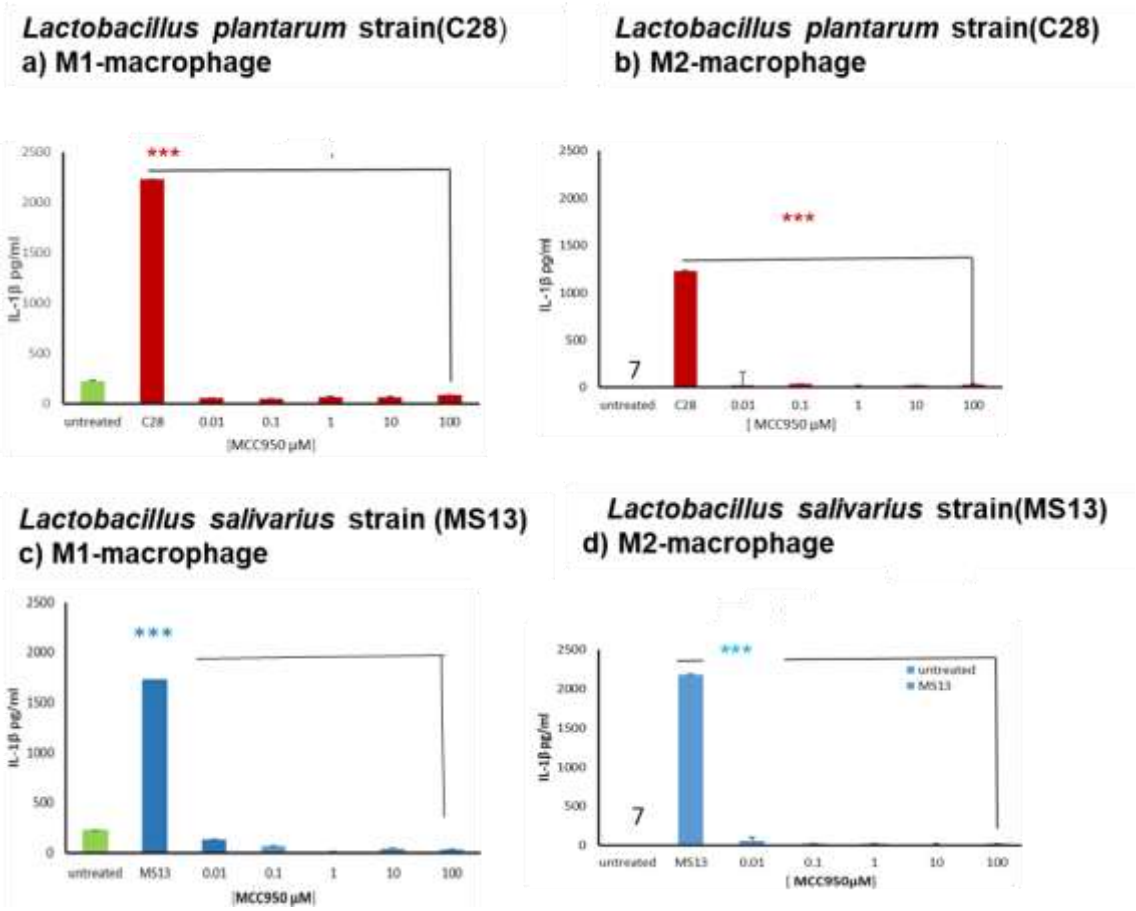

Figure 3.2.16: Probiotic strain augmentation of LPS-induced macrophage IL-1β is NLRP3-dependent

THP1-cell lines (1x10^6 cell/ml) with either 25 ng/ml of PMA for 3 days and washed out for 24 hr or 10 nM/ml of Vit.D_3 for 7days replaced with fresh R10 media for the over-night. Stimulated M1 and M2 with 100 ng/ml of *E.coli* K12 LPS for 4 hrs and washed out then re-suspended with fresh media and add MCC950 (10 µM) NLRP3 inhibitor 30 mins. Probiotic bacteria (C28 or MS13) 1x10^7 cfu/ml added for 19.5 hrs. Data displayed is a representative by 4 independent experiments. IL-1β production is expressed as the means ± SE. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. The significant effect on inhibition by (MCC950+) compared with the non-inhibition effect in M1 and M2-macrophages are indicated as p***, ***≤ 0.001. (C28 significance is indicated in red, whereas MS13 significance is indicated in blue.).

3.4 Summary of chapter 3 results:

Table 3.2: Cytokines induction by C28 and MS13 by macrophage subsets

Cytokine induction	M1-Like macrophage		M2-Like macrophage	
	Live C28	Live MS13	Live C28	Live MS13
TNF-α	↑	↑	↑	↑
IL-1β	↑	↑	↑	↔
IL-10	↓	↑	↑	↓

Table 3.3: The ratio between TNF-α production and IL-10 and IL-1β from M1 and M2 –like macrophages

	M1-like macrophage		M2-like macrophage	
	TNFα/IL-10	IL-1β/Il-10	TNF-α/IL-10	IL-1β/IL-10
K12-LPS	5.1	0.125	0.2	0.1
C28	3.77	0.25	2.62	0.8
MS13	16	3.53	1	0.3
C28 +LPS	18.66	2.5	0.625	3.25
MS13 +LPS	1.25	0.5	4.5	2.25

Table 3.4: Summary of C28, MS13 and K12-LPS induction, activation and modulation of NLRP3 inflammasome and IL-1β in M1 and M2 macrophage subest

	M1-like macrophage			M2-like macrophage		
Treatment	C28	MS13	K12-LPS	C28	MS13	K12-LPS
Secreted IL-1β ELISA	↑	↑	↑	↑	↔	↑
IL-1βmRNA	↔	↑	↔	↑	↑	↔
IL-1β MCC950	↓	↓	↓	↓	↓	↓
NLRP3 mRNA	↓	↔	↔	↑	↑	↔

Increased↑ Decreased↓ **No change**↔ WB=western blot

3.5: Discussion:

3.5.1: Validation of growth in different media

A. The growth of Lactobacillus in MRS media

Lactic acid bacteria ferment glucose to lactic acid. Glucose metabolism is subject to carbon catabolite repression, with pyruvate as the central metabolic branching point (Cocaign-Bousquet *et al*., 1996; Ganzle & Follador, 2012; Karin, 2003). *Lactobacillus* strains produce lactic acid in both aerobic or anaerobic conditions (El Attar *et al.*, 2000; Verluyten, Leroy & de Vuyst, 2004). The ability of *Lactobacillus plantarum* and *Lactobacillus salivarius* to grow biophysically in MRS media in aerobic conditions was surprising. The growth curves of both strains involved two log phases and two stationary phases. This result had not been previously described. The Lactobacillus bacteria in this investigation may have exhausted glucose from the MRS media by the end of the first stationary phase, as lactobacillus is capnophilic bacteria. It then approaches another carbon source in an aerobic environment. The distribution of final-products/ deriving from glucose catabolism was impacted by oxygen. Acetate increases and lactate decreases with the oxygen concentration; ethanol was only formed anaerobically. Moreover, oxygen promoted the energetically favourable conversion of lactate into acetate, particularly noticeable under fully oxygenated conditions (Chakravorty, 1964; Ferreira et al., 2013). As mentioned in the literature review, a high concentration of acetate (52-75 mM) in aerobic *Lactobacillus plantarum* ATCC14917, ATCC8014 and ATCC14431 cultures on prolonged incubation in MRS + 55.6 mM glucose (Murphy & Condon, 1984). The concentration of glucose in MRS broth was 110 mM and may accumulate more acetate in the growth culture. On the other hand, some studies found no significant differ-

ences in the metabolic products, such as lactic acid, acetic acid and ethanol under aerobic and anaerobic conditions (Gänzle, 2015; Karin, 2003; Smetanková et al., 2012). These studies mentioned acetate as the major fermentation end product, leading to homoeotic fermentation (Nosova et al., 2000). Finally, might be other carbon source or metabolic products that available in media has been used by C28 and MS13, to continue their growth aerobically.

B. The growth of Lactobacillus in R10 media

The low concentration of D-glucose in R10 in comparison with MRS media presented as the first challenge in this research of probiotic bacteria and macrophage subsets. However, both probiotic bacteria strains were grown in R10 media anaerobically, whilst they failed to grow aerobically (**Figure 3.2.2**). Surprisingly, long exponential phase was clear in growth curve, although to the scarcity of glucose. Maybe due to tendency of C28 and MS13 to exploit other carbon sources from the environment, such as L-glutamine. Mahajan and Sahoo (1998) mentioned the ability of probiotics to utilise nutrients, such as glucose and amino acids, in the gut. Similarly, Botta, (2017) studied the effect of using different concentrations of L-glutamine (2 and 6 mM in DMEM), on the growth of two strains of *Lactobacillus plantarum* and the production of butyric acid. (Li *et al.*, 2015) mentioned the importance of L-glutamine in enhancement of *Lactococcus lactis* growth. CO_2 as a source of carbon molecules was also mentioned as an important source of protein formation. Furthermore, lactic acid bacteria were shown to grow in low pH, possibly due to high levels of intracellular potassium concentrations. This affords the appropriate concentration for acids anions (Diez-Gonzalez and Russell, 1998). Thus, the bacteria become more

resistant to organic acids than other bacterial species, such as *salmonella* (Van Immerseel et al., 2006).

3.5.2: The effect of increasing lactic acid on the viability of a macrophage subset

Lactic acid as a product of *Lactobacillus* is an antibacterial growth inhibitor for pathogenic bacterial growth, such as salmonella growth. This was detected in the chicken intestine in the presence of *Lactobacillus salivarius* (Beal et al., 2002; Savvidou, 2009). On the other hand, Lactobacilli fermented glucose to lactic acid in the media, causing a decrease of pH in growth media.

The present study was designed to determine the effect of lactic acid on the viability of macrophages in the presence of live lactobacillus strains. We found that the viability of M1 and M2-like macrophages were 100% in all concentrations of lactic acid **(Figure 3.2.4).** Kapus et al. (1994) and Warburg (1956) reported that extracellular accumulation of lactate was the main reason for tumour acidity. The macrophage subset viability increased due to the lactic acid (25 - 50mM) concentration and this may be due to an increase of acidity.

In contrast to earlier findings by (Peluso *et al.*, 2007), the limitations of live bacteria in cell culture is the accumulation of lactic acid through bacterial cell growth. Habil (2013) mentioned these difficulties, in addition to (Peluso *et al.*, 2007) justified the use of heat to killed and secrete protein probiotic bacteria strains. This finding revealed no evidence of suppressed viability of M1 and M2-macrophage by live probiotic bacteria, especially with strains used in this study. Thus, live probiotic bacteria are an appropriate format, reflecting the environment of probiotic bacteria. Therefore, live probiotic bacteria will be used in this study.

3.5.3: Live lactobacillus strains differentially induce macrophage subsets cytokine

Several studies have focused on the ability of lactobacilli to modify macrophage cytokine production (Matsuguchi *et al.*, 2003;Kim *et al.*, 2008; Wang *et al.*, 2013;Kim *et al.*, 2014). However, the information on interactions between live lactobacilli and human macrophage cell line is uncommon. The present study was designed to shed more light on the ability of live probiotic bacteria strains (C28 and MS13) to modulate M1 and M2 macrophage responses. Furthermore, this study objective was to compare two different species of *Lactobacillus* for their differential ability to modulate macrophage subset responses. To this end, two species of Lactobacillus were used, *L.plantarum* strain (C28) and *L.salivarius* strain (MS13), and investigated for their ability to stimulate M1 and M2 macrophages to produce different cytokines.

Interestingly, *Lactobacillus* C28 and MS13 enhanced the production of TNF-α from both M1 and M2-like macrophage similarly, while differentially in IL-1β. M2s released IL-1β higher concentration than M1-like macrophage in both C28 and MS13. The growth phase was shown as important for the stimulatory role of C28 and MS13, to induce cytokine production of macrophage subsets. The macrophage supernatant of live bacteria were harvested at 24 hours and showed high cytokine levels compared with the level of cytokines in preliminary stimulation time-frames. Previous studies that examine the growth phase identified significant changes in gene and protein expression in bacteria during the growth phase and transition from exponential to stationary (Brisbin et al. 2015; Laakso et al., 2011). Similarly, Brisbin et al. (2015) mentioned that the high immunostimulatory effect of the late exponential growth phase in *L. acidophi-*

lus and *L. salivarius* impacts chicken macrophages. The results showed significant increases of nitric oxide (NO) production and phagocytosis in the macrophages.

Furthermore, the greatest number of detectable proteins was during the late-log and early-stationary phase (Cohen *et al.*, 2006). However, it is probable that growth phase-dependent changes in cell wall components, such as peptidoglycan, teichoic acid, or S-layer, play a role. These compounds have been shown to induce distinct cytokine profiles in macrophages (de Sa Peixoto et al., 2015; Kim et al., 2014; Kim et al., 2008; Naganuma et al., 2016; Ryu et al., 2008). Therefore, macrophage subset stimulation of the lactobacillus strain for 24 hours presents as sufficient time for bacterial growth and for the release of cytokine profiles.

3.5.4: THP-1 derived macrophage subsets and cytokine profiles in LPS stimulation

Macrophage in the gut appears divided into two phenotypes. M2-macrophages are anti-inflammatory and act to maintain the homeostatic environment of the gut. The other type, M1-macrophage, is classified as pro-inflammatory (Gordon and Mosser, 2003). The variation of functionality in macrophage subset depends on the environment. THP-1 was nominated as a model in this study, resulting in distinct macrophage cells. The cytokine levels and profile varied between monocytes differentiated by PMA (M1-like macrophages) and monocytes differentiated by vitamin D_3 (M2-like macrophages). Shojadoost et al. (2015) described the ability of vitamin D_3 to increase chicken macrophage production of nitric oxide in response to stimuli, such as LPS. Major inflammatory cytokines of the gut, which are inducible by LPS, include TNF-α, IL-1β, IL-8 and IL-6 (Fu-

nakoshi et al., 1998; Zuo et al., 2010). LPS is a heat-stable toxin linked with the outer membranes of gram-negative bacteria and belongs to the numerous studied pathogen-associated molecular patterns. It ubiquitously exists in the intestinal lumen and known for its association in intestinal inflammation (Guo et al., 2013).

Interestingly, these probiotic bacteria *Lactobacillus plantarum* (NCIMB 41605 C28) and *Lactobacillus salivarius* strain (NCIMB 41610 MS13) induced an up-regulated production of TNF-α from both M1 and M2-like macrophages. C28 and MS13 modulated M2-like macrophage towards a pro-inflammatory immune response. IL-1β of M2-like macrophage levels were higher than IL-1β of M1-like macrophage induced by C28 or MS13. This may be new information regarding probiotics and their impact on plasticity of macrophage-dependent subsets. On the other hand, these findings display live C28 and MS13 inducing the upregulation of pro-inflammatory cytokines. This compares with other studies whereby other lactobacillus strains, such as *Lactobacillus paracasei*, induce high levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and IL-12p70 in human dendritic cells (Bermudez-Brito, et al., 2013). In contrast, *L. salivarius* CECT5713 and *L. fermentum* CECT5716 strains significantly induced the production of a number of cytokines, including TNFα, IL-1β and IL-8. Upregulation of pro-inflammatory cytokines in M1 and M2 macrophages correlated with the downregulation of IL-10 induced by C28 and MS13. This may be due to the ability of probiotic bacteria to bind CD14 molecules and activate a TLR-mediated signal cascade in macrophages (Habil, 2013).

3.5.4: Live probiotic bacteria strains selectively modulate LPS-induced macrophage cytokine profiles

Generally, LPS induction established macrophage pro-inflammatory cytokines when activating NFκB (Silswal et al., 2005). C28 and MS13 upregulated pro-inflammatory and anti-inflammatory cytokines in the presence of K12-LPS. C28 modulates production of TNF-α induced by LPS, while MS13 supressed the production of TNF-α induced by LPS in 24 hrs of LPS treatment. Habil (2013) showed that heat-killed probiotics and their secreted proteins augmented LPS-induced TNF-α production in macrophage subsets.

TLRs are membrane bound and provide pathogen recognition that, upon ligand binding, activates signalling, leading to the production of pro-inflammatory cytokines. TLR4 recognises LPS from gram-negative bacteria, whereas probiotics may modulate these signalling pathways within macrophages, effectively regulating mucosal immunity. Research has focused on the probiotic modulation of cytokine production including pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8, with particular attention focussed on IL-10 and IL-12. The immune response can be determined using cytokine production by macrophage and other immune cells in response to microbes (Llewellyn and Foey, 2017). This study focused on the probiotic bacterial role in modulation of LPS-induced cytokine production by M1 and M2-macrophage. Interestingly, live probiotic bacteria differentially modulate pro-inflammatory cytokine production by macrophages. This result potentially identifies the role of probiotic bacteria in modulating endotoxin tolerance by K12-LPS in macrophage subsets: focus of the next chapter.

3.5.5: Probiotics modulate NLRP3 activation in M1 and M2-like macrophages

NLRP3 inflammasome is an important parameter checkpoint for innate immune defence as well as caspase-1. It modulates the monocytes through the cellular process of phagocytosis in the presence of gram-positive bacteria (Bertinaria et al., 2019; Sokolovska et al., 2013). The present study was designed to determine the role of probiotic bacteria in the induction of NLRP3 inflammasome *ex vitro*. Using MCC950 may be advantageous and has been shown in recent methods to investigate NLRP3 inflammasome activity (Coll et al., 2015; Lee et al., 2018; Perera et al., 2018; Xu et al., 2018). The cleavage of caspase-1 by this inflammasome results in its activation, subsequently cleaving pro-IL-1β (Phillips et al., 2016). This investigation details the role of probiotic bacteria strains C28 and MS13 in NLRP3 activation; moreover, MCC950 inhibited the production of IL-1β induced by C28 and MS13 in macrophage subsets **(Figure 3.2.12 and Figure 3.2.16).** Both untreated M1 and M2 macrophages expressed NLRP3, which may be a result of prior differentiation of THP-1 by PMA or by VD_3 **(Figure 3.2.13)**.

Our findings are consistent with other studies, where PMA-differentiated THP-1 cells produced high levels of NLRP3, compared to undifferentiated THP-1 (Kong *et al.*, 2016a; Kong *et al.*, 2016b). It was also reported that there was an additional role of vitamin D_3 in the regulation of the immune system, enhancing NLRP3-dependent release of mature IL-1β from macrophages (Tulk *et al.*, 2015).

While, Matsubara *et al.* (2017), described the role of probiotic bacteria treatment, such as *L. rhamnosus*, in macrophage inflammasome activation, resulting

in increased IL1-β production and increasing *Candida albicans* elimination (Miettinen et al., 2012). Meanwhile inflammasomes contribute to an anti-Candida response (Martinon et al., 2002). Macrophage stimulation of C28 and MS13 resulted IL-1β production and NLRP3 activation. Interestingly, gene expression of NLRP3 of M1 inhibited by C28 was supported by findings of NLRP3 protein production through the use of a 24-hour western blot. These findings demonstrated the regulatory role of probiotic bacteria C28 and MS13 in macrophage subsets. Further study on the activation of NLRP3 induced by C28, MS13 and macrophage subset IL-18 production is discussed in the following chapter.

Conclusions

Lactobacillus plantarium strain C28 and *Lactobacillus salivarius* strain MS13 probiotic bacteria grew aerobically and bi-phasically in MRS media, whereas grew mono-phasically in R10 tissue culture medium. Probiotic strain C28 and MS13 strains are immune-activatory, predisposing to a direct induction of pro-inflammatory mediators or manipulating macrophage plasticity to more inflammatory M1-like phenotype, whereas the induction of the anti –inflammatory cytokine, IL-10, was dependant on both the probiotic strain and macrophage subset. In this homeostatic setting (absence of pro-inflammatory signal such as LPS), MS13 induced a pro-inflammatory cytokine profile in M1 macrophages, whereas induced a more regulatory, homeostatic profile in the M2 subset; whereas C28 induced a moderate pro-inflammatory profile in both the M1 and M2 subsets. Conversely, in a pro-inflammatory setting (presence of LPS), MS13 induced a more regulatory, homeostatic profile in M1 macrophages and a pro-inflammatory profile in M2s. C28, on the other hand, induced a pro-inflammatory profile in M1s and a regulatory profile in M2s – exactly the opposite observed for MS13 in the inflammatory context. C28 and MS13 differentially modulate IL-1β expression and secretion of mature bioactive cytokine, which is dependent on the NLRP3 inflammasome, whereas amplitude of cytokine response between M1 and M2 macrophages may reflect a differential probiotic modulation of NLRP3 mRNA and protein levels. This chapters reavels a role for C28 and MS13 probiotic bacteria strains in activation of inflammasome molecules and macrophage cytokine profiles that uncovers more understanding of the potential role in controlling inflammatory responses to pathogenic microorganisms and complications of IBD in the gut.

Chapter: 4

The role of probiotic bacteria in the modulation of Endotoxin Tolerance induced by K12-LPS in macrophage subset

4.1: Introduction

Endotoxin tolerance describes a cellular hypo-responsive state, whereby cells are unable to respond to further challenges, such as that with LPS. Tolerance is impacted in inflammatory bowel disease and activated in cancer (Foster and Medzhitov, 2009). TLR4 predominantly recognises LPS and was the first PRR to be studied (Begue *et al.*, 2006; Beutler, 2003). Mucosal macrophages have a double functionality that determines tolerance to commensal organisms or immune response to entropathogens, such as *E. coli* K12-LPS. On the other hand, suppression of anti-inflammatory cytokines provide a positive outcome whereby the pathogen is capable of inducing protective immunity by the mucosal immune system towards harmful antigens (Chistiakov, 2015; Mantovani, 2005).

Endotoxin tolerance *in vivo* and *in vitro* in both animals and humans could be classified as a negative feedback response resultant of dysregulated inflammation, for example in conditions of sepsis (Biswas and Lopez-Collazo, 2009). On the other hand, endotoxin tolerance could be considered positive whereby pro-inflammatory damage is limited (Fu *et al.*, 2012). Generally, endotoxin tolerance occurs through repeated injection of animals and humans with small quantities of bacterial LPS or endotoxin. Following this, cells become rapidly refractory in response to the pyrexia metabolic lethal dose; thus, LPS hypo-responsiveness is observed (Biswas and Lopez-Collazo, 2009; West and Heagy, 2009). Endotoxin tolerance has been associated with suppression of pro-inflammatory cytokine production, including TNF-α, IL-6, IL-12 and IFN-β, due to alterations in signalling cascades that affect NF-ƙB, MAPKs and IRFs (Akira and Takeda, 2004; Perkins et al., 2010). Moreover, endotoxin tolerance has been associated with an upregulation in negative regulators of TLR signalling, such as IRAK-M,

ST2, SHIP-1, MyD88s and A20 (van 'T Veer *et al.* (2007) ; Xiong and Medvedev, 2011), and Tollip (Al-Shagdhali et al., 2019).

Macrophages are important in cellular homeostasis and defence, featuring high functional heterogeneity (Gordon and Taylor, 2005). The immune suppression roles of M2 macrophage resembles the features of endotoxin tolerance. M2 macrophages drive homeostasis and tolerance through the production of IL-10 and down-regulation of pro-inflammatory cytokines, such as TNFα. On the other hand, M1 macrophages are characterised by their strong capacity to present cytokine production including IL-12 and IL-23 (Mantovani et al, 2002). Early studies on tolerisation revealed differential suppression between M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages in response to LPS of an oral pathogen, *Porphyromonas gingivalis.* Studies reported that the pro-inflammatory M1-like subset was refractory to tolerance induced by *P. gingivalis*, whereas, the M2-like subset was sensitive to tolerance induced by *P. gingivalis* and suppresses inflammatory cytokines (Foey and Crean, 2013). Mucosal pathogen and *E. coli*-LPS are understood as able to induce endotoxin tolerance in macrophages (Sun et al., 2014; Biswas and Lopez-Collazo, 2009). Furthermore, the anti-inflammatory cytokine (IL-10) supresses the macrophage inflammatory responses (Moore et al., 2001), playing an important role in the modulation of endotoxin tolerance. Endotoxin tolerance is often linked with the over-expression of anti-inflammatory cytokines, such as IL-10 and TGFβ, contributing to the deactivation of monocyte/macrophages and suppression of pro-inflammatory cytokine expression by these cells (Schroder et al., 2003).

Tolerance to different stimulus, such as early exposure to TLR2 ligands, such as lipoteichoic acid, Pam3Cysk4 or MALP2, reduces macrophage tolerance to LPS (Wang *et al.*, 2014). With regards to intermediate signalling elements, het-

ero-tolerance appeared weaker than TLR4 or TLR2 homo-tolerance, with the exclusion of IKK kinase activity (Alshaghdali, 2018; Geisel *et al.*, 2007). On the other hand, IKK kinase activity appeared visible in hetero-tolerance. In contrast to Gram-negative, Gram-positive endotoxin, tolerance does not include down-regulation of TLR expression, defective MyD88 -TLR interaction or over-interaction (Alves-Rosa *et al.*, 2002). TNF-α secretion was also suppressed in *P. gingivalis* LPS-pre-treated, Eco LPS-challenged cells, but not vice versa (Dobrovolskaia, 2003; Alves-Rosa *et al.*, 2002). TLRs are acute components of the innate immune system that help defend the host from communicable disease through the recognition of pathogen-associated molecular patterns (del Fresno *et al.*, 2009).

This endotoxin tolerance phenotype could be used as a model to study tumour-associated macrophages in cancer, whereby an immunosuppressive phenotype is evident. Moreover, decreased production of inflammatory cytokines, such as IL-12 p40 subunit and TNFα, have been associated with an upregulation of anti-inflammatory cytokines, IL-10 and TGFβ(Biswas & Lopez-Collazo, 2009; Neurath, 2014). This association has been reported in *ex vivo* LPS stimulation (anti-inflammatory) macrophages in response to LPS of an oral pathogen, *Porphyromonas gingivalis* (Chen et al., 2008; Morimoto et al., 2009). Here, the pro-inflammatory M1-like subset appeared refractory to tolerance induced by *P.gingivalis*, whereas, the M2-like subset was sensitive to tolerance induced by *P. gingivalis,* whilst suppressing inflammatory cytokines (Foey and Crean, 2013). Endotoxin tolerance is often linked with the over-expression of anti-inflammatory cytokines, such as IL-10 and TGFβ, contributing to the deactivation of monocyte/macrophages and the suppression of pro-inflammatory cytokine expression by these cells (Schroder et al., 2003).

On the other hand, many members of the tumour necrosis factor (TNF) and the TNF receptor (TNF-R) superfamily have been shown to exert important functions in the immune system. One of these functions is the induction of apoptosis, which is mediated by certain members of the TNF and TNF-R families known as death ligands and receptors, respectively. Apoptosis is crucial for the proper function of the immune system. It assists different purposes, amongst them tasks as different as the removal of autoreactive T and B cells, the killing of infected cells by cytotoxic lymphocytes and the down-regulation of immune responses when an infection has been successfully dealt with (Opferman & Korsmeyer, 2003). One of the cell reprograming apoptotic molecules, such as TRAIL is upregulated by stimulation of monocytes and macrophages by LPS or IFN-β (Ehrlich *et al.*, 2003; Halaas *et al.*, 2000). Moreover, IFNγ can also induce the expression of TRAIL on the surface of monocyte, dendritic cells, and natural killer cells, NKs (Fanger *et al.*, 1999; Griffith *et al.,*1999).

Objectives

The main objectives for this chapter are to characterise probiotic strains *Lactobacillus plantarum* strain (C28) *and Lactobacillus salivarius* strain (MS13) for their differential modulation of endotoxin tolerisation in M1 and M2 macrophage cells.

Objective 1: Homo-tolerisation by probiotic bacteria C28 and MS13 differentially modulates M1 and M2 macrophage cytokine secretion**.**

Objective 2: Homo-tolerisation by C28 and MS13 induces the gene expression of cytokine production and negative regulatory role in macrophage subsets.

Objective 3: Endotoxin tolerisation *by E.coli* LPS can affect cytokine production in THP-1 derived macrophage subsets.

Objective 4: PRRs and negative regulatory roles in endotoxin tolerance are induced in THP-1-derived macrophage subsets.

Objective 5: Probiotic bacterial strains C28 and MS13 can modulate cytokine production of tolerised M1 and M2-macrophages induced by K12-LPS.

Results

4.2.1: Tolerisation impacts cytokine production of M1 and M2-like macrophages induced by probiotic bacteria

4.2.1.1: Tolerisation by strain C28 and MS13 differentially induce macrophage secreted Pro-inflammatory cytokines

The first aim of this chapter was to investigate whether incubating M1 and M2-like macrophage subsets with either of 2 lactobacilli, C28 and MS13, would induce tolerance to those lactobacilli. The production of cytokine by the macrophages was measured as an indicator of tolerisation. Tolerisation was induced by incubating the macrophages with either lactobacillus, (C28 and MS13), for 24 hours, then washing them. Macrophages were then stimulated for 24 hours with the lactobacillus it had been "tolerised" to. The immune responses of macrophages to each lactobacillus were evaluated following that incubation, by measuring the levels of cytokines produced by those macrophages. In order to determine whether tolerisation took place, the levels of those cytokines were compared in each macrophage subset, between those that had been "tolerised" before stimulation and those that were stimulated without prior tolerisation. Five pro-inflammatory cytokines were measured: TNF-α, IL-1β, IL-18, IL-12 and IL-23, as well as anti-inflammatory cytokine IL-10 and IL-6.

The results showed that M1 and M2-like macrophages, differentially responded to tolerisation by C28 and MS13 as revealed by their production of pro-inflammatory cytokines.

Upon stimulation, with C28, TNF-α production by M1-like macrophage tolerised by the same lactobacillus, (red bars) (Figure 4.1 a) significantly, increased by 2-fold (2800 pg/ml) versus (1200 pg/ml) ($P \leq 0.001$). A similar pattern was observed in M1-like macrophage, tolerised, then stimulated by MS13 (blue bars),

where the production of TNF-α, increased by 1.5-fold (2600 pg/ml) (P≤0.001), (1400 pg/ml) (**Figure 4.1 a**). In contrast, tolerisation of M2-like macrophages by C28 and MS13 (**Figure 4.1 b**) significantly suppressed the production of TNF-α upon stimulation by either lactobacillus. Tolerisation by C28 reduced TNF-α production by 34% (P≤0.001) (800 pg/ml), from (1200 pg/ml). Whereas, the level of TNF-α by M2-like macrophage tolerised by MS13 was suppressed by 50% (900 pg/ml), compared to that produced by macrophages stimulated with MS13 without tolerisation (1800 pg/ml), P≤ 0.001**).**

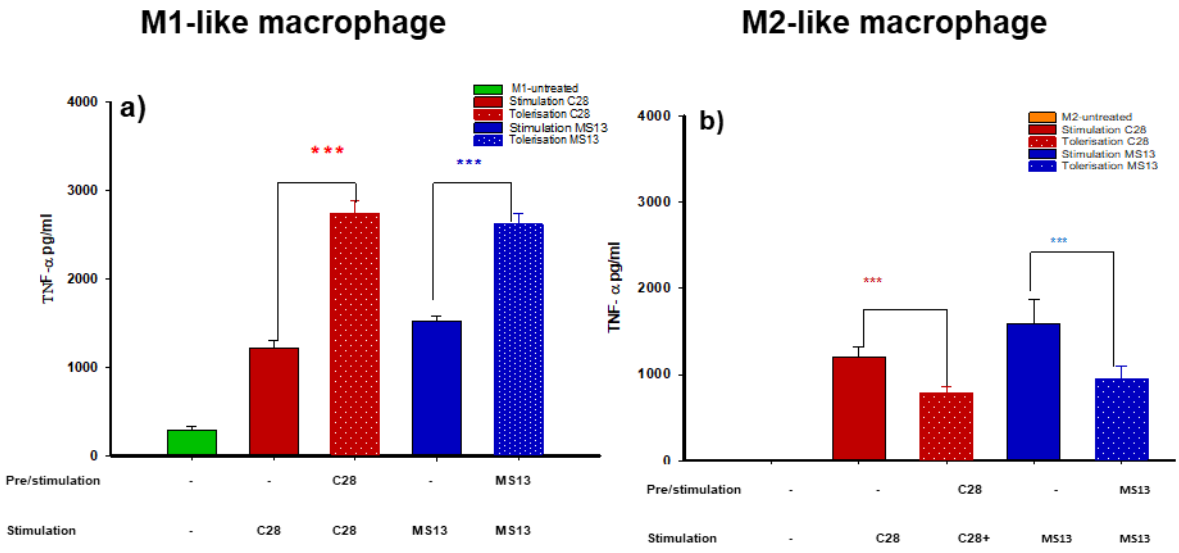

Figure 4.1: Tolerisation by C28 and MS13 differentially induced and suppress of TNF-α macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 green (a) and M2 orange (b) macrophage subsets/10^6 cells/ml for TNF-α, Data showed represents triplicate samples for n= 9 were pre-treatment with C28 and MS13 for 24 hours and post-treatment with same stimulus for another 24 hours ($1X10^7$ cfu /ml). TNF-α production is expressed as the mean ± SD in pg/ml of 6 replicate experiments. Significant effects on pre-treatment of modulation with C28 and MS13 compared to the post-treatment for the specified macrophage subset are indicated as, ***, *** p<0.001. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

The production of IL-1β by M1-like macrophages tolerised with C28 was significantly up regulated by 10-fold (5000 pg/ml), (P<0.001) (**Figure 4.2 a**). The

same pattern was observed in M1-like macrophage tolerised by MS13, where the production of IL-1β increased, (P≤0.01). Furthermore, the production of IL-1β by M2-like macrophage tolerised by C28 was significantly increased by 5-fold (P≤ 0.01), (100 pg/ml) (**Figure 4.2b**).

There were no significant differences in the levels of IL-1β produced by M2-like macrophages tolerised by MS13 as compared to those not tolerised before stimulation by the lactobacillus (**Figure 4.2b**). Interestingly, M1 and M2-like macrophage behaved differently in its production of IL-18 upon stimulation by C28 (**Figure 4.2 c/d**). The level of IL-18 appeared to be augmented in C28 tolerised M2-like macrophages (7-fold) (P=0.001). C28 significantly suppressed IL-18 production by tolerised M1-like macrophages (p=0.001). The levels of IL-18 produced by M1-like macrophages tolerised by MS13 was significantly up-regulated, (P≤0.001). Overall, no-significant differences were found between IL-18 produced by M2-like macrophage upon tolerisation by MS13.

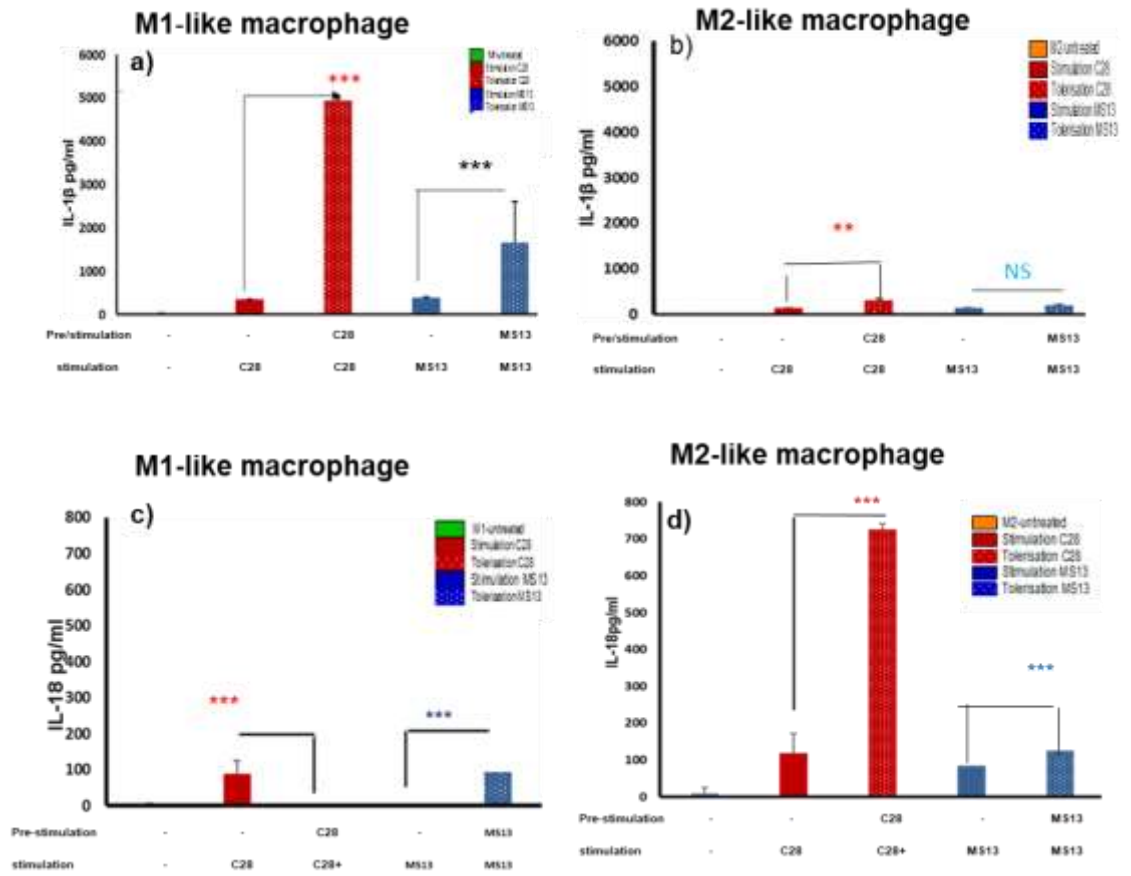

Figure 4.2: Endotoxin tolerance of C28 and MS13 differentially induced and suppress of IL-1β and IL-18 of macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-(OH)2 vitamin D_3 for 7 days. M1 green (a , c) and M2 orange (b ,d) macrophage subsets/10^6 cells/ml for IL-1β (a ,b) and IL-18 (c,d), Data showed represents triplicate samples for n= 9 were pre-treatment with C28 and MS13 for 24 hours and post-treatment with same stimulus for another 24 hours (1X10^7 cfu /ml). IL-1β and IL-18 production is expressed as the mean ± SD in pg/ml of 6 replicate experiments. Significant effects on pre-treatment with C28 and MS13 compared to the post-treatment for the specified macrophage subset are indicated as, ***, *** p<0.001 and not significant = NS, NS . (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

The production of IL-23 by tolerised M1-macrophage by C28 **(Figure 4.3 a)** was not different from macrophages that had not be tolerised prior to stimulation by the lactobacillus. Whilst the levels of IL-23 in M1–like macrophage tolerised by MS13 were up-regulated (7-fold) (350 pg/ml), compared with those not tolerised

(50 pg/ml) (P ≤0.001) (**Figure 4.3 b**). However, *Lactobacillus plantarum* (C28) were more effective in tolerising M2-like macrophage by releasing 100 pg/ml of IL-23, compared to stimulation without prior tolerisation, (1.5 fold) (p ≤ 0.01). The levels of IL-23 of MS13 tolerised M2-like macrophage did not change compared to stimulation of M2-like macrophages. There were no significant differences in the levels of IL-12 produced by M1-like macrophages tolerised by C28, compared to those not tolerised before stimulation by the lactobacillus **(Figure 4.3 c/d)**. The level of IL-12 of tolerised M1–macrophage by MS13 was suppressed by 57%, compared to IL-12 stimulation by M1 macrophage (P≤ 0.05) (**Figure 4.3 c and d**).

These results suggest the significant production of IL-23 by tolerised M1-like macrophage by MS13, while the significant tolerisation by C28 by M2-like macrophage to induce the cytokine production of IL-23. Whereas, no significant of the production of IL-12 by tolerised M1 and M2-like macrophage by the two lactobacilli strains.

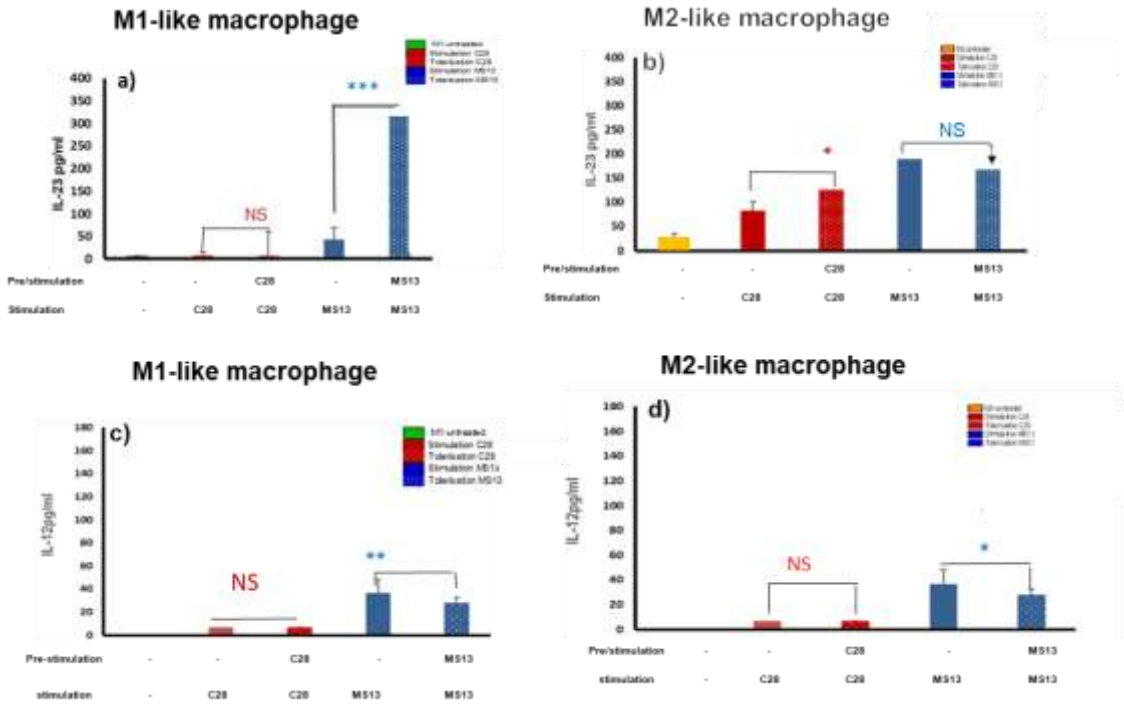

Figure 4.3: Endotoxin tolerance of C28 and MS13 differentially induced and suppress of IL-23 and IL-12 of macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 green (a , c) and M2 orange (b ,d) macrophage subsets/10^6 cells/ml for IL-23 (a ,b) and IL-12 (c,d), Data showed represents triplicate samples for n= 9 were pre-treatment with C28 and MS13 for 24 hours and post-treatment with same stimulus for another 24 hours ($1X10^7$ cfu /ml). IL-23 and IL-12 production is expressed as the mean ± SD in pg/ml of 6 replicate experiments. Significant effects on pre-treatment with C28 and MS13 compared to the post-treatment for the specified macrophage subset are indicated as, *, * p≤ 0.05 ,**, ** p≤ 0.001, *** p<0.001 and NS, NS not significant. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.1.2: Tolerisation by probiotic bacteria in endotoxin tolerance differentially mod-ulates anti-inflammatory cytokine of M1 and M2-like macrophages

In this section, the role of probiotic bacteria in tolerising macrophage subsets was invetigated.The results focus on the induction of anti-inflammatory cytokines by M1 and M2-like macrophages. Measurements of two anti-inflammatory

cytokines, IL-10 and IL-6, were taken. Data shows (**Figure 4.4 a/b**) that the level of IL-10 by tolerised M1-macrophage significantly increased when induced by C28 (1.5-fold) (1000 pg/ml), from (600 pg/ml) (P≤ 0.001). M1-macrophage tolerised by MS13 significantly suppressed production of IL-10 (75%) compared to the level of IL-10 by stimulation M1-like macrophages (980 pg/ml) (P≤ 0.001). On the other hand, IL-10 levels by tolerised M2-like macrophages significantly increased by induction with C28 and MS13 (2.5-fold/2-fold, respectively, compared to levels of IL-10 by stimulated M2-macrophage (400 pg/ml) and (600 pg/ml) respectively (P≤ 0.001).

The role of C28 and MS13 on toleraistion was further investigated for macrophages ability to produce IL-6 for up to 24 hours. Results showed that C28 significantly up-regulated production of IL-6 from tolerised M1 and M2–like macrophage by 3.8-fold (P≤0.001). MS13 differentially impacted the production of IL-6 by M1 and M2-like macrophages. Consequently, the tolerisation by MS13 of M1–macrophage suppressed the production of IL-6 (P=0.204) (**Figure 4.4 c**). In contrast, levels of IL-6 by tolerised M2–macrophage induced upon MS13 significantly increased (3-fold) (160 pg/ml) (P≤ 0.05) compared to levels of IL-6 by stimulation of M2–macrophage (50 pg/ml).

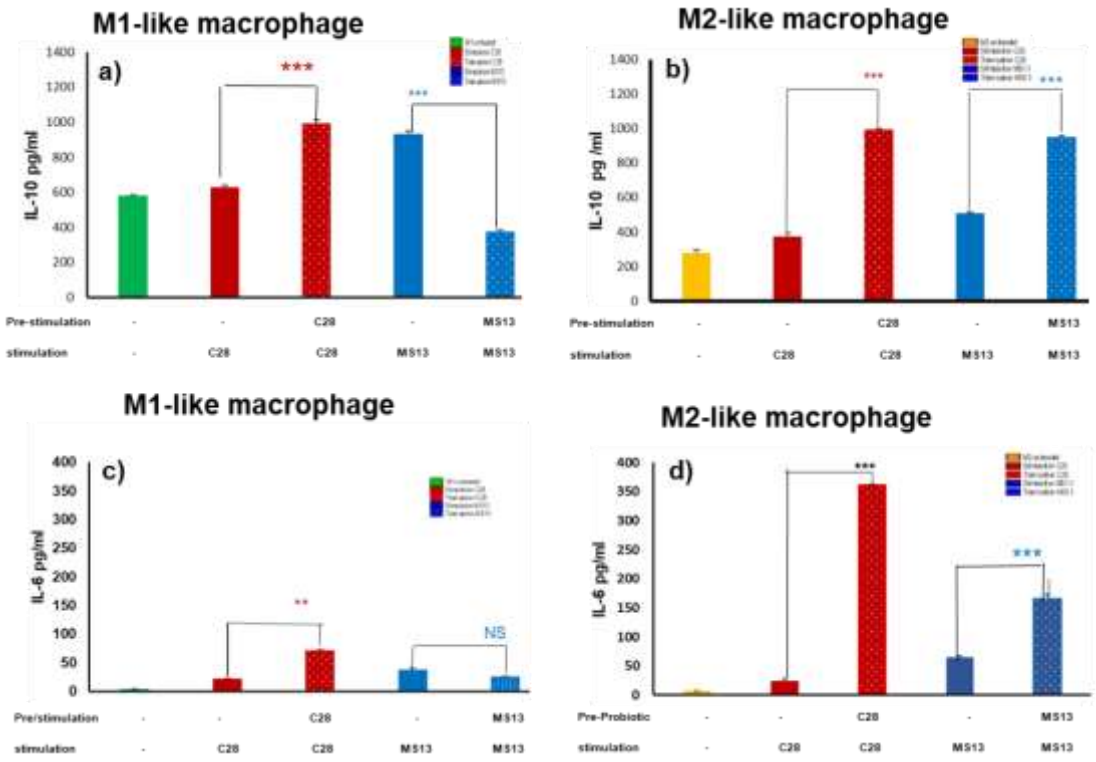

Figure 4.4: Endotoxin tolerance of C28 and MS13 differentially augmented of IL-10 and IL-6 of macrophages subsets

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 green (a , c) and M2 orange (b ,d) macrophage subsets/10^6 cells/ml for IL-10 (a ,b) and IL-6 (c,d), Data showed represents triplicate samples for n= 9 were pre-treatment with C28 and MS13 for 24 hours and post-treatment with same stimulus for another 24 hours (1X10^7 cfu /ml). IL-10 and IL-6 production is expressed as the mean ± SD in pg/ml of 6 replicate experiments. Significant effects on pre-treatment with C28 and MS13 compared to the post-treatment for the specified macrophage subset are indicated as, ***, *** p<0.001 (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.2: Effect of tolerisation on gene expression in macrophage subsets induced by probiotic bacteria

Earlier data (**Figure 4.1, 4.2, 4.3, and 4.4**) demonstrates that tolerisation by C28 and MS13 play a crucial role in modulating macrophage subset immune responses. Cell signalling controls immune response consequences. This im-

munomodulation may occur via modulation of macrophage PRR expression, including TLRs and NLRs. First, clarification of the role of probiotics in tolerisation of gene expression within cytokines TNF-α, IL-1β, IL-23 p19 and IL-12 p35 macrophage subsets were required. Gene expression of TNF-α in tolerised M1 and M2-like macrophage revealed down-regulation by C28 compared with 24 hr stimulation (4 and 2-fold) ($p \leq 0.001$). On the other hand, tolerised M1 by MS13 up-regulated the expression of TNF-α by (3-fold) compared with stimualted M1-macrophage ($p \leq 0.001$). There were no significant differences in the gene expression between tolerised M2 induced upon MS13 compared with stimulation stage (**Figure 4.5 a/b**). Gene expression of IL-1β in both tolerised M1 and M2–like macrophage induced by C28 were significantly upregulated (2.5-fold) (**Fgure 4.5 c/d)** ($p \leq .001$). MS13 differentialy downregulated gene expression of IL-1β in M2-like macrophage 10-fold, compared to stimulation ($P \leq 0.001$).

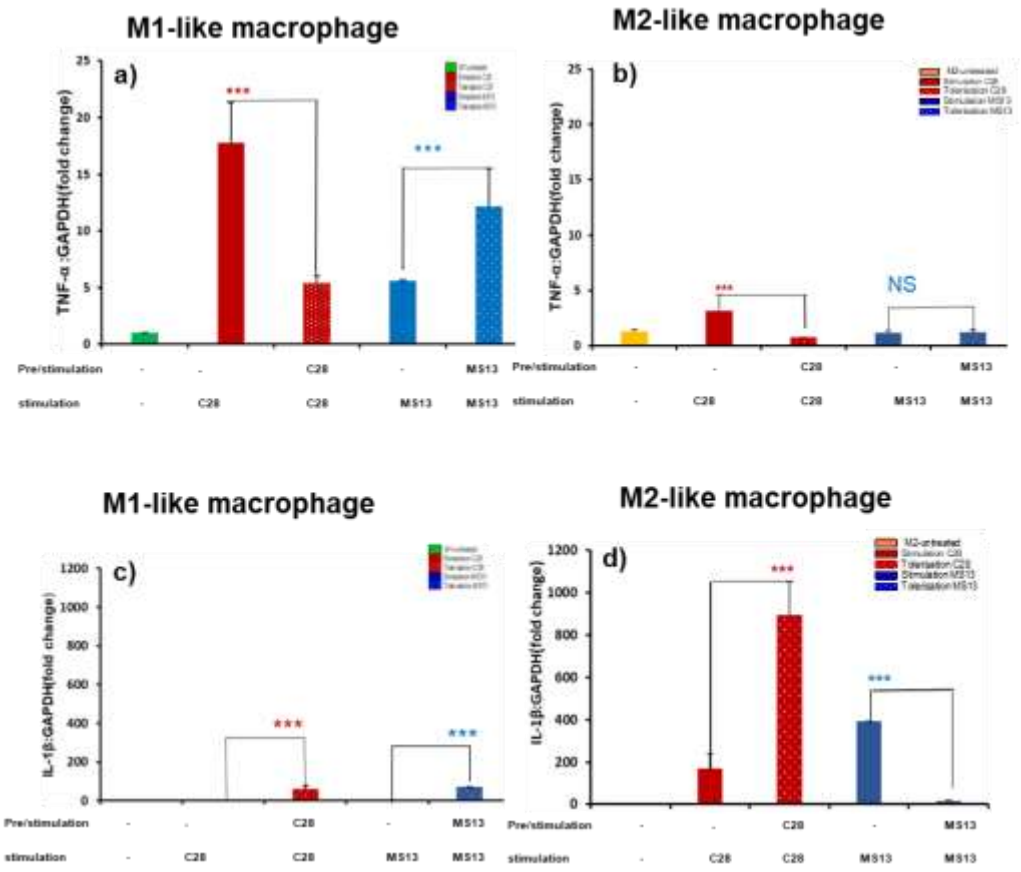

Figure 4.5: Probiotic bacteria induced differentially expression of TNF-α and IL-1β of tolerised M1 and M2-like macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13/MS13) for 24/24 hours. mRNA extracted from cells from each of these probiotic strain growth medium to test TNF-α and IL-1β expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as ***, *** P<0.001, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

The expression of IL-23 p19 in M1-like macrophage tolerised with MS13 was significantly downregulated 2-fold (P≤0.01) **(Figure 4.6 a/b).** The induction of exression of IL-23 in M1 and M2 -like macrophage with C28 did not show any changes compared with the stimulated macrophages subsets. On the other hand, the expression IL-12 p35 in C28-tolerised M1-like macrophages was up-regualted 10-fold, (p<0.001). MS13 tolerisation had no impact on the expression

in IL-12 p35 compared with the stimulation stage. Furthermore, no significant differences were detected in M1-like macrophages in gene expression of IL-12 p35 when compared between toleristion and stimualtion stages. However, IL-12 p35 gene expression M2-like macrophages, tolerised by C28 and MS13 significantly decreased. Here, the production was suppressed by 98 %, compared with 24 hours of stimulation (P<0.01).

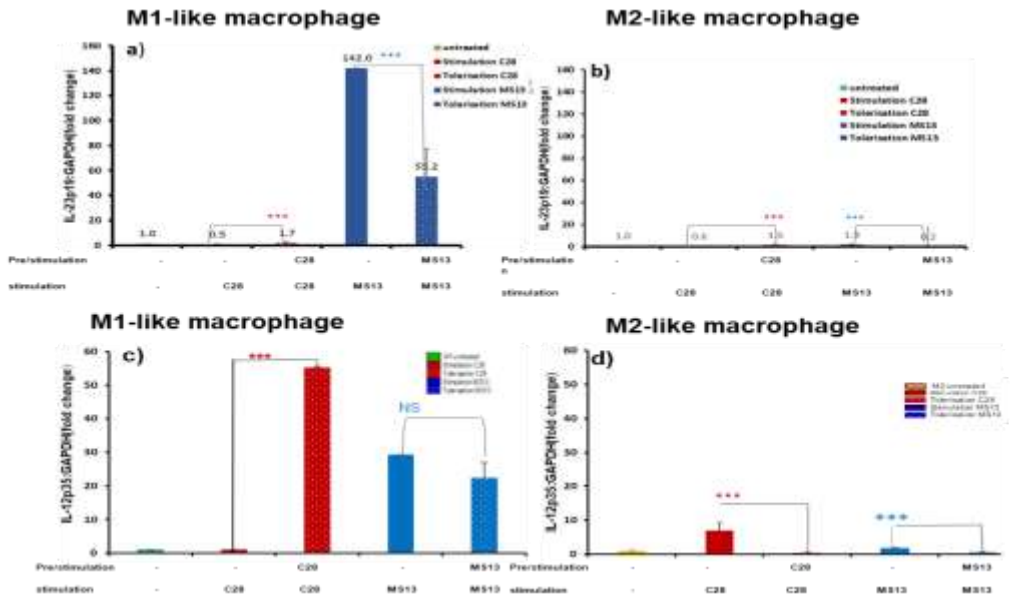

Figure 4.6: Probiotic bacteria induced mRNA of IL-23 p19 and IL-12 p35 of differentially in tolerised macrophage subsets

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13/MS13) for 24/24 hours. mRNA extracted from cells of each of these probiotic strain growth medium to test IL-23p19 and IL-12p35 expression by qPCR. Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as *,* P<0.05, **, ** P<0.01 and ***, *** P<0.005, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.3: Probiotic tolerisation differentially regulates gene expression of anti-inflammatory macrophage immune response

Previous data discussed the role of probiotic bacteria in the tolerisation of macrophages subsets, impacting the gene expression in pro-inflammatory cytokines. Continuously, this chapter presented data on the role of C28 and MS13 in the induction of the mRNA of anti-inflammatory cytokines in M1 and M2-like macrophage. Results revealed a significant downregulation of IL-10 gene expression by MS13 in tolerised M1 and M2-like macrophage by 80-60%, compared with stimulation ($P \leq 0.001$ and $p \leq 0.01$, respectively) (**Figure 4.7 a / b**). Gene expression of IL-10 in M1 and M2-like macrophage induced by C28 revealed no significant differences between tolerisation and stimulation. Gene expression of IL-6 was significantly upregulated in tolerised M1-like macrophages induced by C28 (*57*-fold), ($P \leq 0.001$). However, tolerisation of M1 and M2-like macrophage with MS13 showed and no significant difference in IL-6 mRNA, (**see Figure 4.7 c and d**).

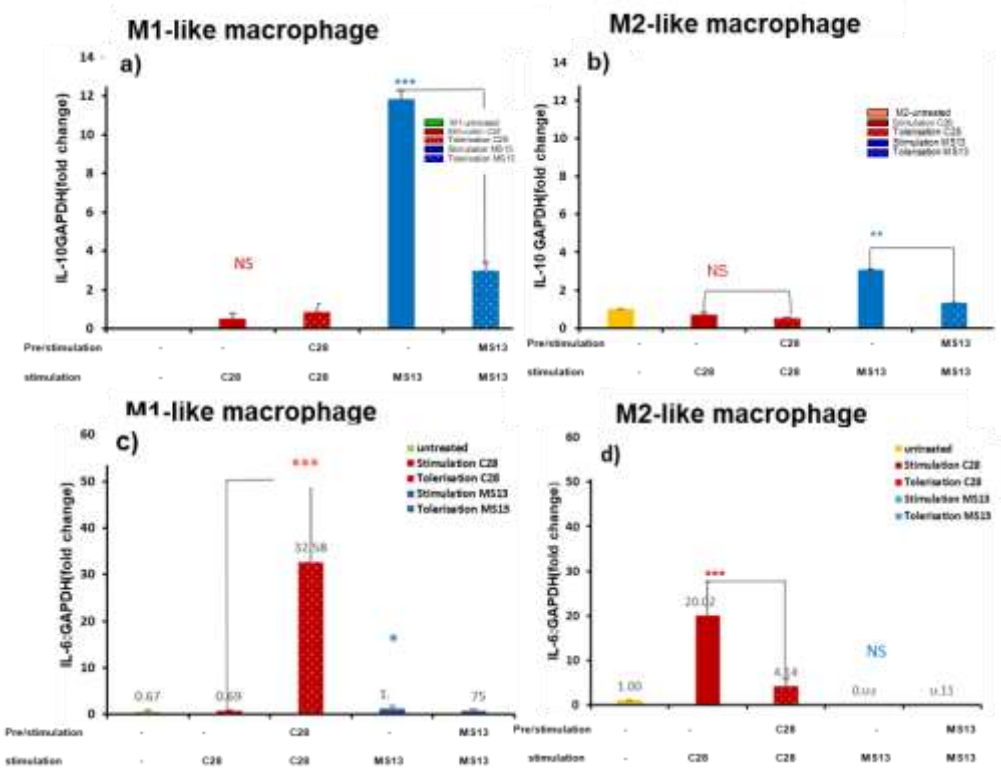

Figure 4.7: Probiotic bacteria differentially modulated IL-6 and IL-10 mRNA in tolerised macrophage subsets

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13/ MS13) for 24/24 hours. mRNA extracted from cells of each of these probiotic strain growth medium to test IL-10 and IL-6 expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as *,* P<0.05, **,** P<0.01 and ***,*** P<0.005, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.5: Tolerisation of probiotic bacteria on TNF-ligand family member (TRAIL) on macrophage subset

The importance of probiotic bacteria during apoptosis requires investigation, comparing the stimulation with tolerisation stages. Results revealed that TRAIL of tolerised M1-like macrophage significantly decreased with C28 by 100% ($p \leq 0.001$). Gene expression of TRAIL in tolerised M1 induced by MS13 appeared not to change, compared with the stimulation stage of M1-macrophage.

However, expression of TRAIL in tolerised M2-macrophage was up regulated by induction by C28 and MS13. Here, the increased expression was between 225 and 75-fold, respectively (P≤0.001) (**Figure 4.8 a/b**).

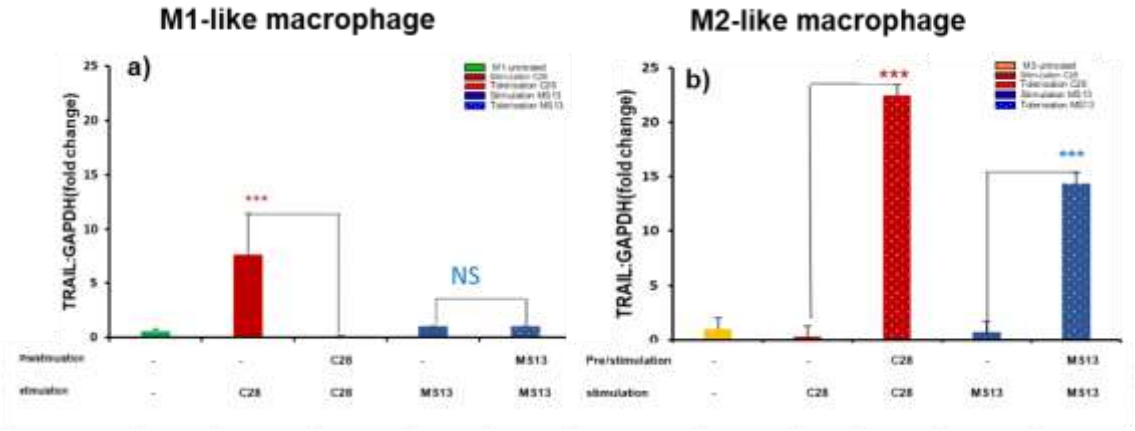

Figure 4.8: Gene expression of TRAIL suppressed by C28 and MS13 differentially in tolerised M1 and M2-like macrophages.

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (C28) (-/C28) or *Lactobacillus salivarius* strain (MS13) (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13 /MS13) for 24/24 hours from each of these probiotic strain growth medium to test TRAIL expression by qPCR gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as ***, *** P<0.005, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.6: Tolerisation by probiotic bacteria modulates macrophage subset TLRs expression

A: TLR4

To study tolerisation by C28 and MS13 on TLR4 and TLR2 receptor expression, gene expression was measured in macrophage cells. As shown in **Figure 4.9 a/b**, TLR4 gene expression was suppressed through recurrent induction by C28 in tolerised M1-like macrophage, compared with stimulation of C28 (99 %)

(P=0.001). There was no significant reduction in TLR4 induced with MS13 compared with stimulation of M1-like macrophage.

On the other hand, statistical tests revealed that tolerised M2-like macrophage upregulated expression of TLR4 induced by C28 (7000-fold), (P≤0.001). Moreover, expression of TLR4 in tolerised M2-like macrophage induced by MS13 upregulated (one-fold), (P ≤0.001).

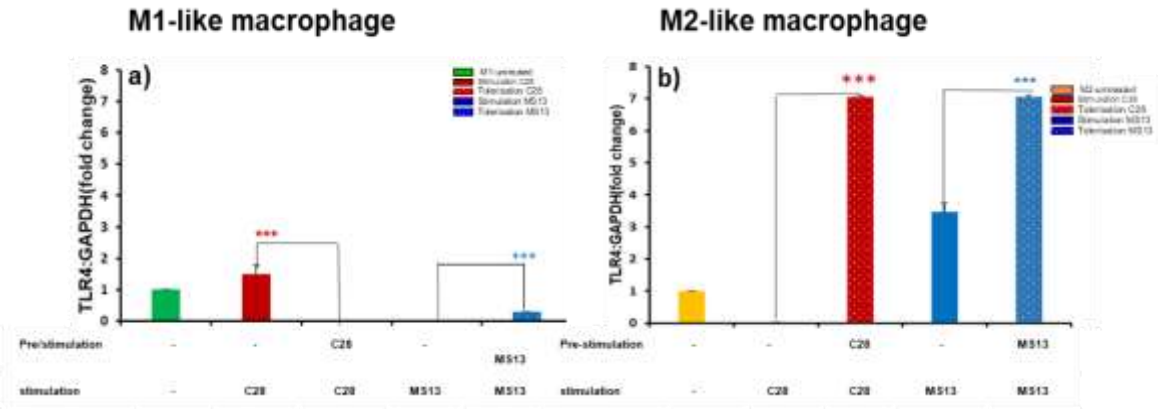

Figure 4.9: Gene expression of TLR4 differentially suppressed by C28 and MS13 in tolerised M1 and M2-like macrophage.

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/ MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13 / MS13) for 24/24 hours from each of these probiotic strain growth medium to test TLR4 expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as ***,*** P<0.005, NS ,NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

B: TLR2

Data shows strong evidence of suppression in TLR2 in recurrent treatment with C28 and MS13 of macrophage subsets. Interestingly, gene expression levels of TLR2 in C28 and MS13 stimulated M1-macrophage was not significantly increased compared with M1–untreated cells. Gene expression of TLR2 in tolerised M1-macrophages induced by C28 and MS13 were suppressed by 50%,

(P≤ 0.05 and P≤0.01). On the other hand, gene expression of TLR2 in tolerised M2-macrophage induced by C28 and MS13 were significantly suppressed by 80 and 90% respectively, (P≤0.001) (**Figure 4.10 b**). Further statistical tests revealed different levels of TLR2 gene expression between M1 and M2 macrophages. Stimulated M2-macrophages induced by C28 and MS13 expressed higher levels of TLR2 gene compared with TLR2 of stimulated M1-macrophage (P≤0.001), respectively (**Figure 4.10 a/ b**).

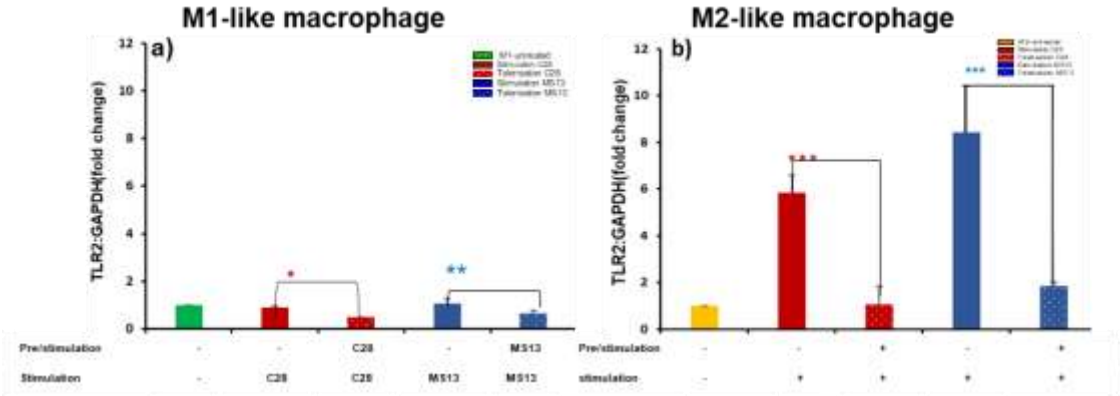

Figure 4.10: Gene expression of TLR2 suppressed by C28 and MS13 differentially in tolerised M1 and M2-like macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13 /MS13) for 24/24 hours from each of these probiotic strain growth medium to test TLR2 expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as *,* P<0.05, **,** P<0.01 and ***,*** P<0.005, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.3.7: Probiotics differentially modulate tolerisation of macrophage subset Inflammasome NLRP3 expression

Results from our preliminary findings investigating the tolerisation ability by C28 and MS13 regarding the secretion of IL-1β and IL-18 (**Figure 4.2**), can be compared to the tolerisation by *Lactobacillus plantrium* and *L.salivarius* in the induction or suppression of NLRP3 of M1 and M2-like macrophage expression. The results showed (**Figure 4.11a/b**) that macrophage subsets differentially supressed NLRP3 expression upon tolerisation with C28 and MS13. Gene expression of NLRP3 in M1-like macrophage tolerised by C28 was significantly upregulated (3500-fold), ($P \leq 0.001$). Induction by MS13 elevated NLRP3 expression in tolerised M1 by 0.5-fold, ($P \leq 0.01$) (**Figure 4.2 a**). Conversely, tolerised M2 by C28 exhibited down-regulation of NLRP3 expression (99%), ($P \leq 0.001$). Moreover, MS13 suppressed NLRP3 expression in tolerised M2-like macrophage (65%), ($P \leq 0.001$). Further statistical tests revealed levels of NLRP3 gene expression between tolerised M1 and M2 macrophage. Probiotic strains C28 and MS13 induced significant expression of NLRP3 in tolerised M1-macrophage, compared with gene expression of NLRP3 in tolerised M2-macrophage ($P \leq 0.001$) (**Figure 4.11 a/ b**).

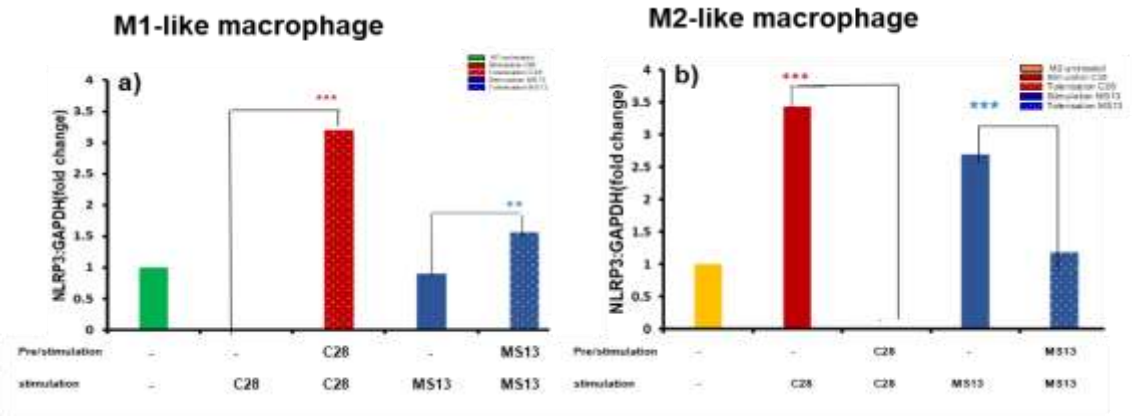

Figure 4.11: Probiotics bacteria induce the mRNA NLRP3 of tolerised macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13 /MS13) for 24/24 hours from each of these probiotic strain growth medium to test NLRP3 expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as**, ** P≤ 0.01, ***, *** P<0.005, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.7: Endotoxin tolerance with C28 and MS13 directed expression negative of regulator molecules in macrophage subsets

Tollip is a regulator molecule of TLR expression and plays an inhibitory role in TLR-mediated cell activation. **Figure 4.12 a/b** reveals that M1-like macrophages tolerised by C28, showed significant suppression of Tollip mRNA expression by 97% (P≤ 0.005). On the contrary, tolerisation of M1-like macrophages by MS13 augmented Tollip expression. It should be noted that neither difference was statistically significant. Furthermore, tolerisation by C28 of M2-like macrophages did not impact Tollip gene expression. There appears to be a clear effect of MS13 regarding the suppression of Tollip mRNA in M2-like macrophage by 59%. This was significant at the $p \leq 0.05$ level, (**Figure 4.12 b**).

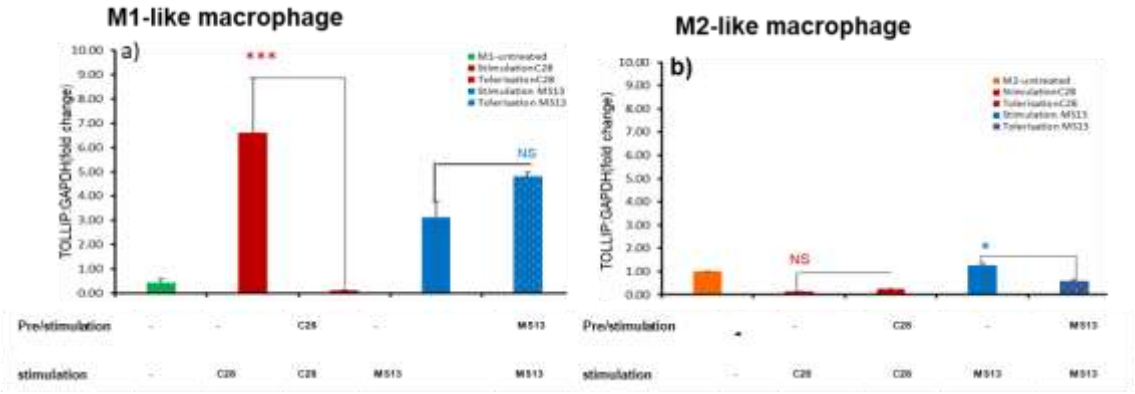

Figure 4.12: The tolerisation by C28 and MS13 differentially suppress the mRNA TOLLIP in macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13 /MS13) for 24/24 hours from each of these probiotic strain growth medium to test TOLLIP expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using 2– ΔΔCt. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as *,* P≤ 0.05, ***,*** P<0.005, NS, NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.8: The tolerisation of macrophage subsets by probiotic bacteria as measured by the induction of SOCS-3 mRNA and STAT-3 genes

To elucidate the benefecial role of C28 and MS13 in the modulation of an anti-inflammatory responses of macrophage subset, this part of the chapter investigates mechanistic signalling transduction controls of SOCS3 and STAT3 (**Figure 4.13**). The results revealed that tolerised macrophage subsets, by probiotic bacteria led to the suppression of SOCS3 mRNA expression. Moreover, the elevated expression of mRNA SOCS3 in stimulated M1–like macrophages after 24 hours with MS13, was suppressed in tolerised M1-like macrophages by 97.8% (P≤0.001). On the other hand, stimulation of SOCS3mRNA expression in M2-like macrophages over 24 hours was inhibited

by tolerisation by both C28 and MS13 (**Figure 4.13 c/d**). In addition, significant gene expression of STAT3 in tolerised M1 and M2-macrophage appeared suppressed with C28, (98%/M1) (P≤0.001). Finally, MS13 significantly up-regulated the expression of STAT3 in tolerised M1 and M2-like macrophages(54-63 fold), respectively) (P≤0.001) (**Figure 4.13 a and b**).

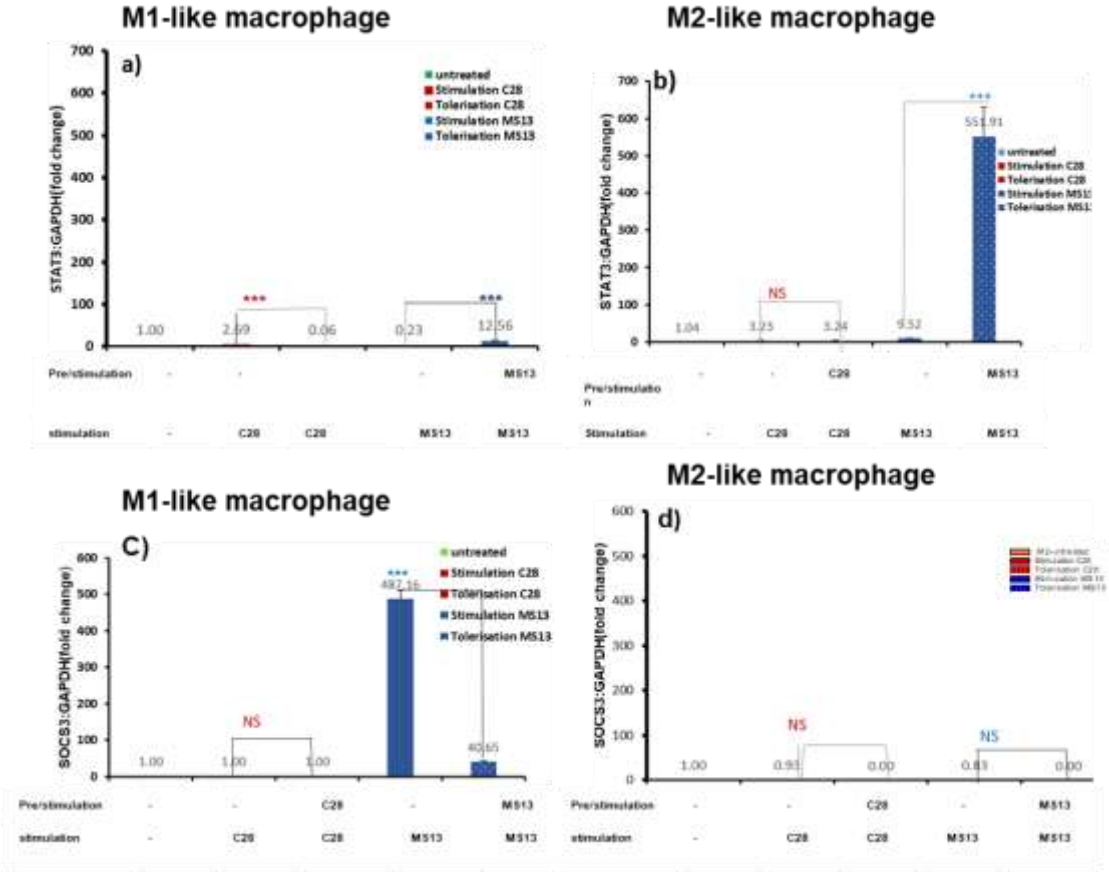

Figure 4.13: Probiotic bacteria modulate bacteria modulated the expression of SOCS3 and STAT3 in macrophage subset

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. Macrophage subsets ($1x10^7$ cfu/ml) stimulated with *Lactobacillus plantarum* strain (-/C28) or *Lactobacillus salivarius* strain (-/MS13) for 24 hours or tolerisation by (C28 /C28) or (MS13 /MS13) for 24/24 hours. mRNA was extracted cells from each of these probiotic strain growth medium to test SOCS3 and STAT3 expression by qPCR. gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as ***,*** P<0.005, NS ,NS (non-significant). (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

4.2.9: The viability of macrophages subsets tolerised with C28 and MS13

Consequent to the results in 3.2.1.1, the viability of macrophages were analysed for 24 hours. Here, we study the effects of tolerisation for 24 hours with live *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) on the viability of M1 and M2 macrophage. Viability was 97-100% compared to the untreated macrophages; no significant differences were noted between untreated M1 and M2-like macrophages, (**Figure 4.14**).

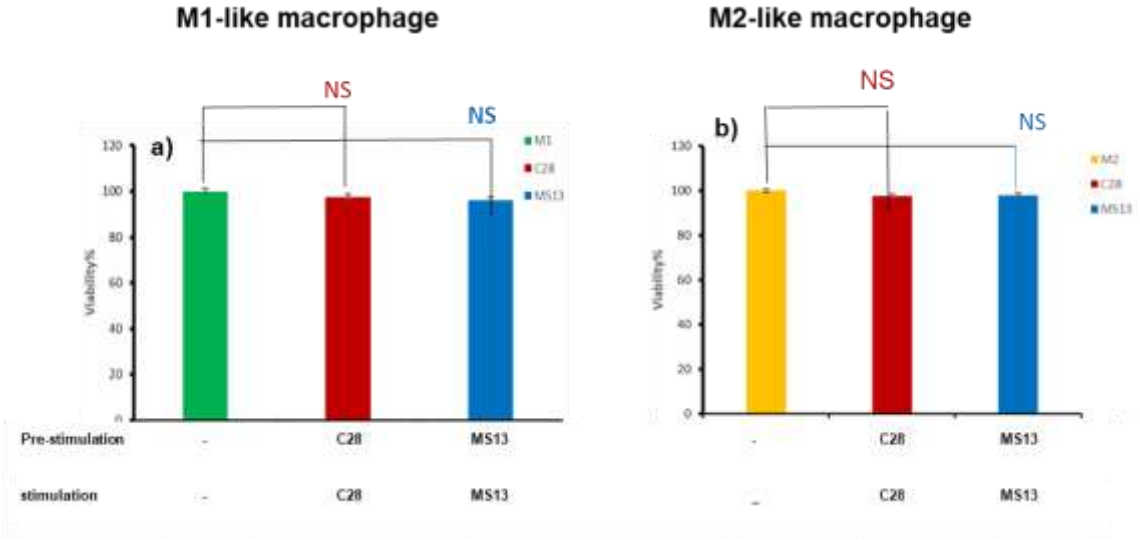

Figure 4.14: Determination the viability of M1 and M2-like macrophage by SRB

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days nd 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 – untreated (a) and M2-untreated (b). Viability was checked by SRB assay according to (Sekham,1990). Data displayed represents triplicate sample for n= 6 replicate experiments.

4.3: The role of recurrent exposure of macrophage subsets to *E.coli* LPS on the induction of endotoxin tolerance, as measured by cytokine secretion

4.3.1: The suppression of pro-inflammatory cytokines production by macrophages by K12-LPS

To study the effect of the induction of endotoxin tolerance by K12-LPS as measured by cytokine production, by M1 and M2-like macrophages subsets,

two stages of tolerisation were measured. The first stage was tolerisation with K12-LPS for 24 hours before washing out. Following this, the macrophages were stimulated with the same endotoxin for 24 hours. Five pro-inflammatory cytokines were measured in macrophages subsets: TNF-α, IL-1β, IL-18, IL-12, and IL-23. Anti-inflammatory cytokines, IL-10 and IL-6, were used as indicators of the immune response. Results were obtained to compare tolerisation and stimulation stages of M1 and M2 macrophages with K12-LPS. The level of TNF-α produced by tolerised M1-macrophage upon K12-LPS **(Figure 4.15 a) was** reduced to 60% (100 pg/ml), from (240 pg/ml) (P≤ 001). Whereas, the reduction of TNF-α levels, (40 pg /ml) by tolerised M2-like macrophage was 50% lower, compared to stimulation level by M2-like macrophage (80 pg/ml) (P≤ 0.01) **(Figure 4.15 b)**. Further statistical analysis revealed that the inhibition rate of TNF-α by tolerised M1-macrophage was significantly, different when compared to the rates of inhibition by M2-like macrophages (P≤0.05).

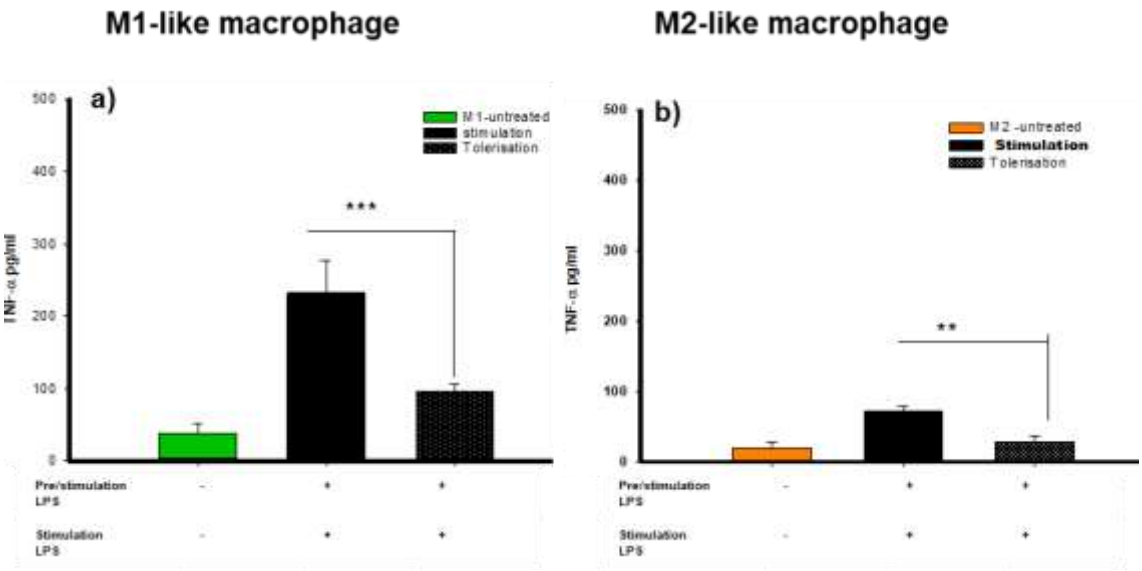

Figure 4.15: Suppression of TNF-α secretion by endotoxin tolerised macrophages

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days**.** M1–untreated (a) and M2-untreated (b). Macrophage subsets were further pre-stimulation with 100 ng/ml K12-LPS (black) for 24 hours, tolerisation prior to stimulation with 100 ng/ml K12-LPS incubated. The sign (-) = no LPS whereas (+) = LPS added

for both pre-stimulated and stimulated cells=tolerisation. Cytokine production is expressed as the mean secretion ± SD in pg/ml for TNF-α. Data showed represents triplicate samples for n= 9 replicate experiments. Significant effects on suppression were found compared to the stimulation LPS control for the specified macrophage subset which were indicated as ** P ≤ 0.01 and *** P ≤ 0.001.

The level of IL-1β produced by tolerised K12-LPS (+/+) M1-macrophage **(Figure 4.16 a)** decreased by (52%) (40 pg/ml) (P≤ 0.001) compared with IL-1β level by M1-macrophage induced by K12-LPS for 24 hours (100 pg/ml). Whereas, the production of IL-1β by M2-like macrophage induced by K12-LPS (-/+) (380 pg/ml) significantly reduced to (50 pg/ml) in tolerised M2-macrophages. However, there was a significant rate of IL-1β reduction by tolerised M2-like macrophage compared to the rate of IL-1 β by tolerised M1-macrophage **(Figure 4.16 a &b).** The rate of IL-18 production by tolerised M1–like macrophage induced by K12-LPS **(Figure 4.16 c)** was significantly suppressed (84%, 7 pg/ml) compared to stimulation levels (50 pg /ml), induced by K12-LPS after 24 hours (P ≤ 0.001). The tolerisation of M2-macrophages by K12-LPS led to a significant reduction of IL-18 production by 91% (7 pg/ml) compared to the stimulation level of IL-18 by M2 macrophage (120 pg/ml) (p≤0.001) (**Figure 4.16 d**). However, there were no significant differences in the reduction rate of IL-18 by tolerised M2-like macrophage compared to IL-18 produced by tolerised M1-macrophage.

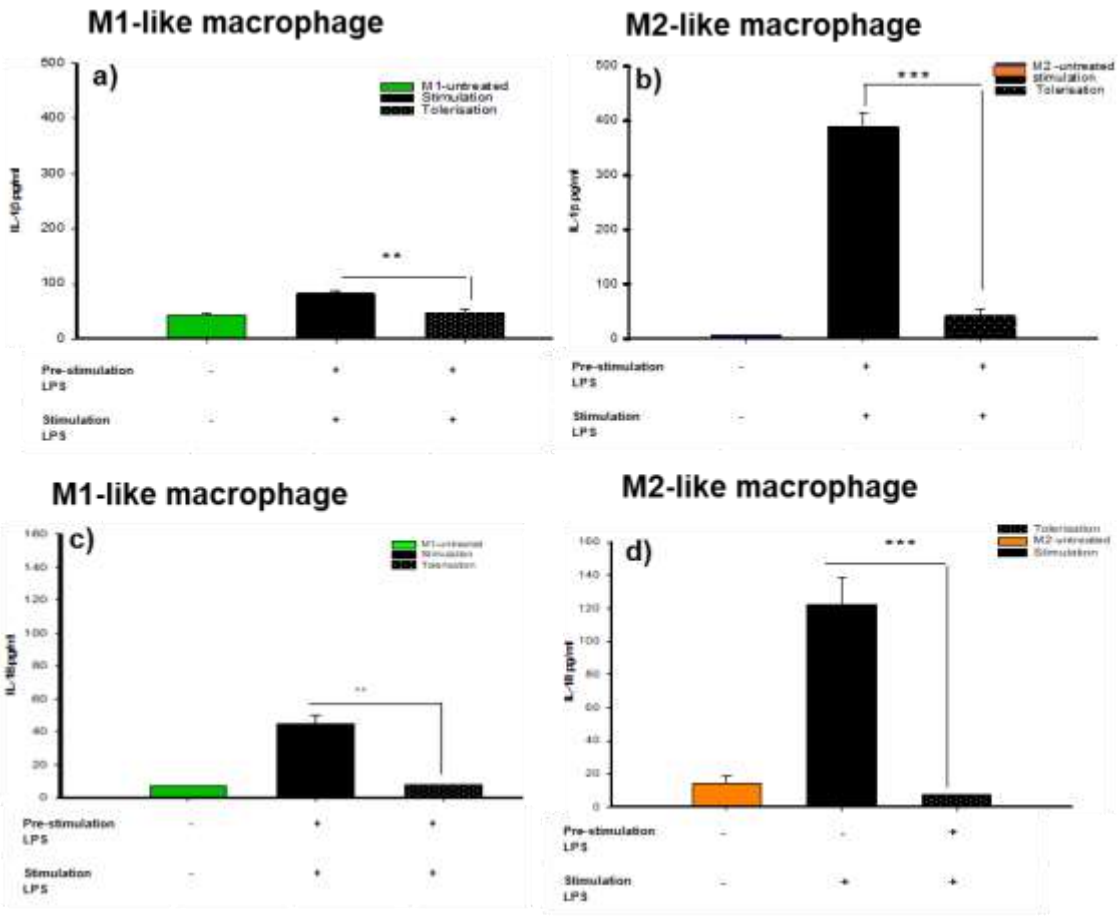

Figure 4.16: Suppression of pro-inflammatory cytokine IL-1β and IL-18 secretion by endotoxin tolerise macrophage

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-(OH)2 vitamin D3 for 7 days. M1 –untreated and M2-untreated. Macrophage subsets were further pre-stimulation with 100 ng/ml K12-LPS (black)for 24 hours, tolerisation prior to stimulation with 100 ng/ml K12-LPS incubated. The sign (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells=tolerisation. Cytokine production is expressed as the mean secretion ± SD in pg/ml for IL-1β (a &b) and IL-18 (c &d). Data showed represents triplicate samples for n= 9 replicate experiments. Significant effects on suppression were found compared to the stimulation LPS control for the specified macrophage subset which were indicated as ** P ≤ 0.01 and ***P ≤ 0.001.

Remarkably, the comparison of IL-23 production of macrophage subsets between stimulation and tolerisation stages induced by K12-LPS, revealed no significant differences **(Figure 4.17 a/b).** Similarly, IL-12 production of tolerised M1-macrophage did not show a significant difference when compared to stimulation by K12-LPS. Conversely, the level of IL-12 in tolerisation treatment of M2-like macrophage increased from 7 to 25 pg/ml, (P≤0.001) (**Figure 4.17**).

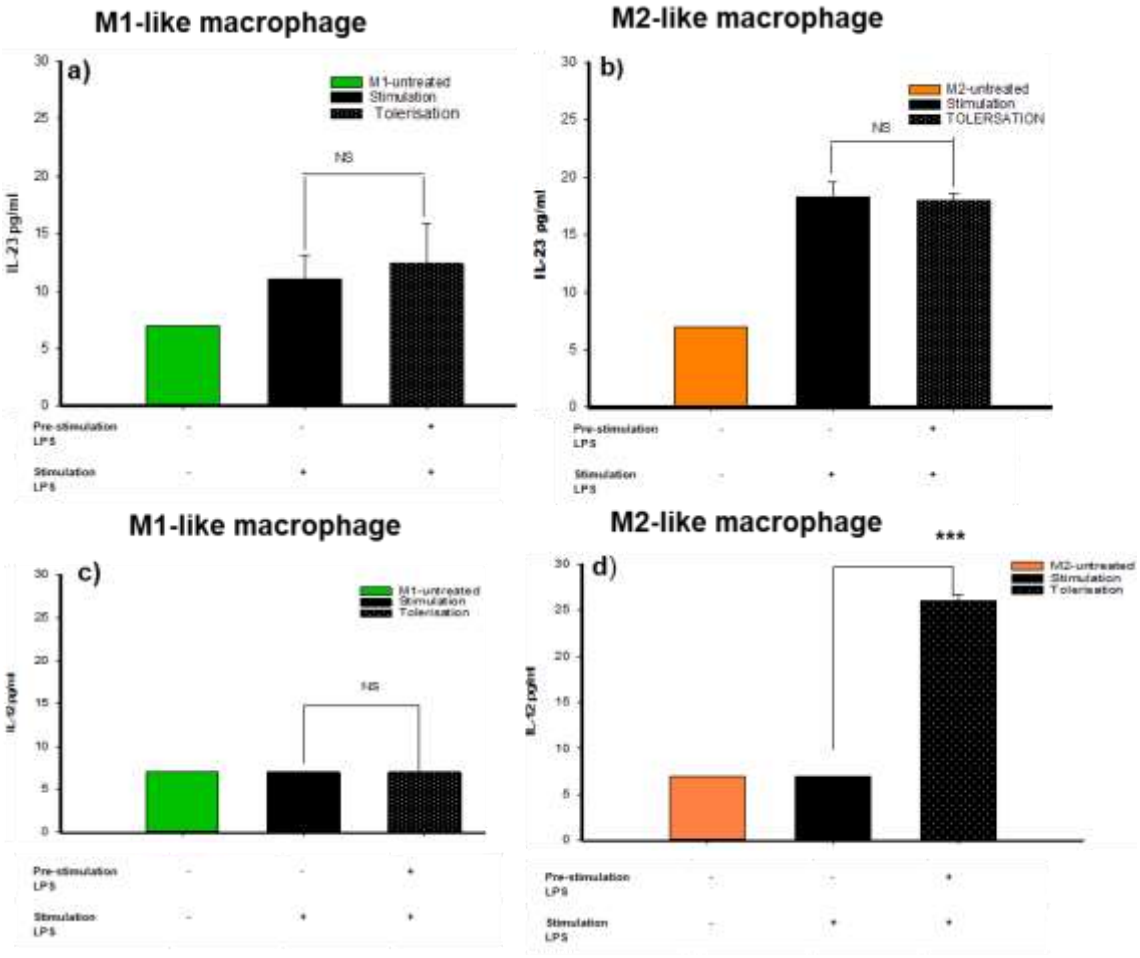

Figure 4.17: Suppression of IL-23 and IL-12 secretion by endotoxin tolerise macrophages

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-(OH)2 vitamin D3 for 7 days. M1 –untreated (a, c) and M2-untreated (b, d). Macrophage subsets were further stimulated with 100 ng/ml K12-LPS (black) for 24 hours (-/+) tolerisation (+/+) prior to stimulation with 100 ng/ml K12-LPS incubated the sign (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells=tolerisation. Cytokine production is expressed as the mean ± SD in pg/ml for IL-23 (a & b) and IL-12 (c &d). Data showed represents triplicate samples for n= 9 replicate experiments. Significant effects on suppression were found compared to the stimulation LPS control for the specified macrophage subset which were indicated as ***p<0.001 and NS=not significant.

4.3.2: Anti-inflammatory macrophage cytokines respond differentially to endotoxin tolerisation

To investigate the effects of endotoxin tolerance (K12-LPS) on the production of anti-inflammatory cytokines by tolerised M1-and M2-like macrophages, a comparison was made. Data was compared between the tolerisation (+/+) and stimulation (-/+) stage of macrophage subsets induced by K12-LPS. The produc-

tion of IL-10 by tolerised M1 macrophages was inhibited by 50% (50 pg/ml) **(Figure 4.18 a),** compared with the stimulation stage (100 pg/ml) (P≤ 0.005). On the other hands, IL-10 production by tolerised M2-like macrophages was inhibited by 80% by K12-LPS (50 pg/ml), compared to the stimulation stage (400 pg/ml) (P≤ 0.001) **(Figure 4.18 b)**. There was no difference greater than 50 pg/ml observed between levels of IL-10 in tolerised M1 and M2–like macrophage **(Figure 4.18 a/b).** Following this, the effect of endotoxin tolerance by K12-LPS on the production of IL-6 by tolerised M1–macrophage was studied, **(Figure 4.18 c/d**). Tolerance inhibited IL-6 production by 75% (10 pg/ml), compared to the stimulation stage with K12-LPS (40 pg/ml) (P≤0.001). Production of IL-6 by tolerised M2-macrophage was significantly suppressed by 92% (10 pg/ml), compared to the stimulation stage, (120 pg/ml) (P≤0.001) **(Figure 4.18 d).** Overall, the impact endotoxin tolerance by K12-LPS on IL-6 levels was not significantly different between tolerised M1 and M2-macrophages.

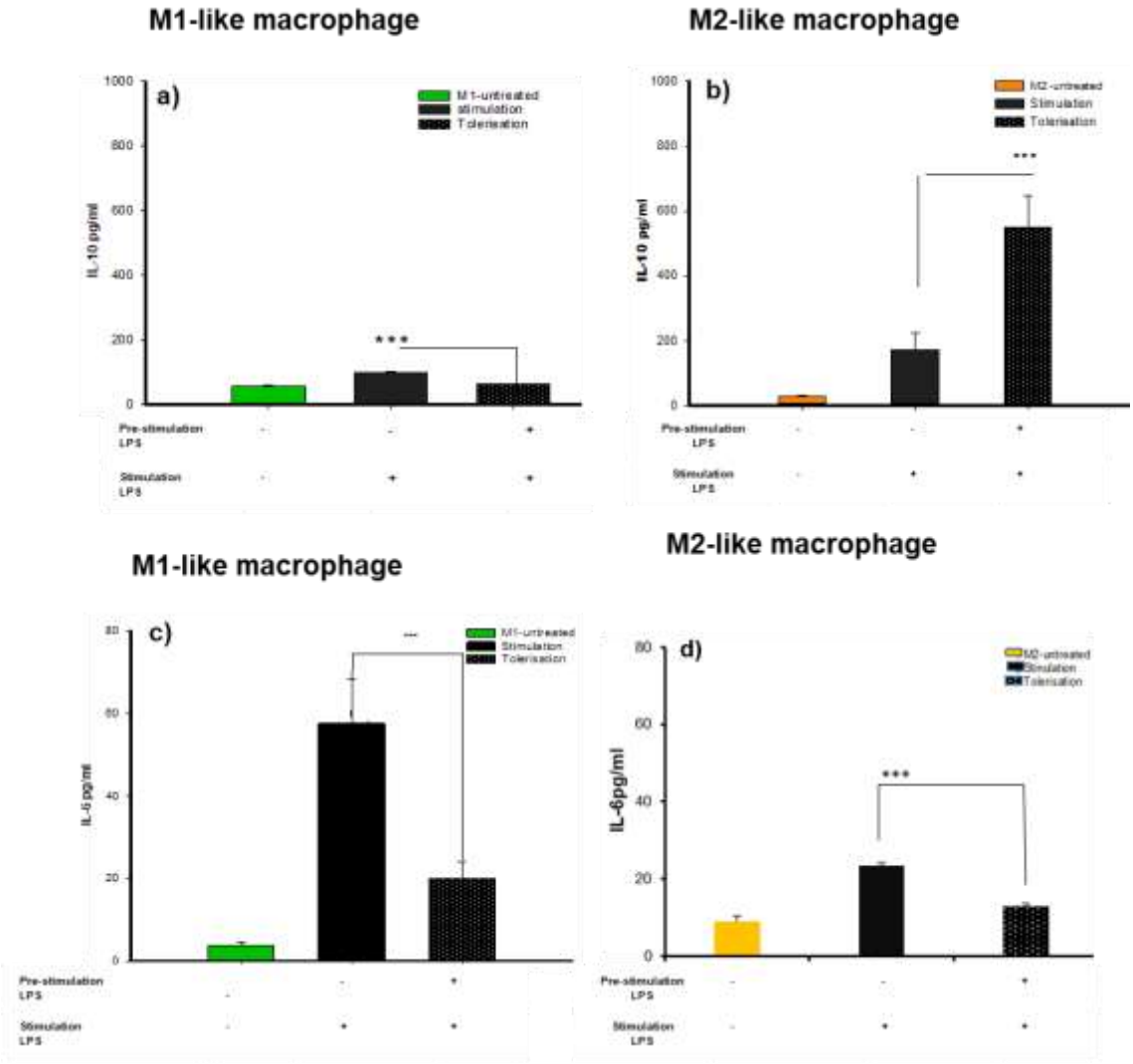

Figure 4.18: Suppression of IL-10 and and IL-6 secretion by endotoxin tolerise macrophages

Diffrentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 – untreated (a,c) and M2-untreated (b,d). Macrophage subsets were further stimulated with 100 ng/ml K12-LPS (black)for 24 hours (-/+) tolerisation (+/+) prior to stimulation with 100 ng/ml K12-LPS incubated the sign (-) = no LPS whereas (+) = LPS added for both pre-stimulated and stimulated cells=tolerisation. Cytokine production is expressed as the mean secretion ± SD in pg/ml cells for IL-10 (a &b) and IL-6 (c &d). Data showed represents triplicate samples for n= 3 replicate experiments. Significant effects on suppression were found compared to the stimulation LPS control for the specified macrophage subset which were indicated as **p≤ 0.01,***p<0.001.

4.3.3: Pro-inflammatory mRNA cytokines suppression in endotoxin tolerised macrophages

Previous results revealed the effects of endotoxin tolerance of *E.coli* K12-LPS (homo-tolerisation) on cytokine production by M1 and M2-like macrophages (**4.3.1.** and **4.3.2)**. In this section, gene expression of pro-inflammatory and anti-

inflammatory cytokines were studied the when macrophage subsets were challenged with *E.coli* K12-LPS. This was compared to tolerisation by C28 and MS13 and the effects on gene expression of TNF-α relative to GAPDH was measured to LPS-stimulated macrophages. Then, the tolerisation stage was compared to K12-LPS for 24 hours. Gene expression of TNF-α in stimulated M1-like macrophages increased 5-fold, compared to untreated M1-like macrophages, whilst gene expression of TNF-α in tolerised M1-like was significantly inhibited by 40%, (P≤0.05) **(Figure 4.19 a)**.

Gene expression of TNF-α in stimulated M2-macrophages increased 2-fold compared to gene expression of TNF-α in untreated M2-like macrophages **(Figure 4.19 b)**. Gene expression of TNF-α by tolerised M2-like macrophages was significantly suppressed, resulting in a 50% decrease, (P≤ 0.05). Moreover, IL-1β relative to GAPDH gene expression in stimulated M1-macrophages increased 20-fold, compared to untreated M1-like macrophage **(Figure 4.19 c).** IL-1β expression in tolerised M1-like macrophages was significantly suppressed by 25%, compared to stimulated M1-like macrophage by K12-LPS 24 hours (P ≤0.05). On the other hand, gene expression of IL-1β in tolerised M2-macrophage significantly increased 15-fold, compared to stimulated M2-like macrophages after 24 hours with K12-LPS (p≤0.005). Lastly, gene expression of IL-1β in stimulated M2-likemacrophages with K12-LPS increased 15-fold compared to untreated M2 (**Figure 4.19 d**).

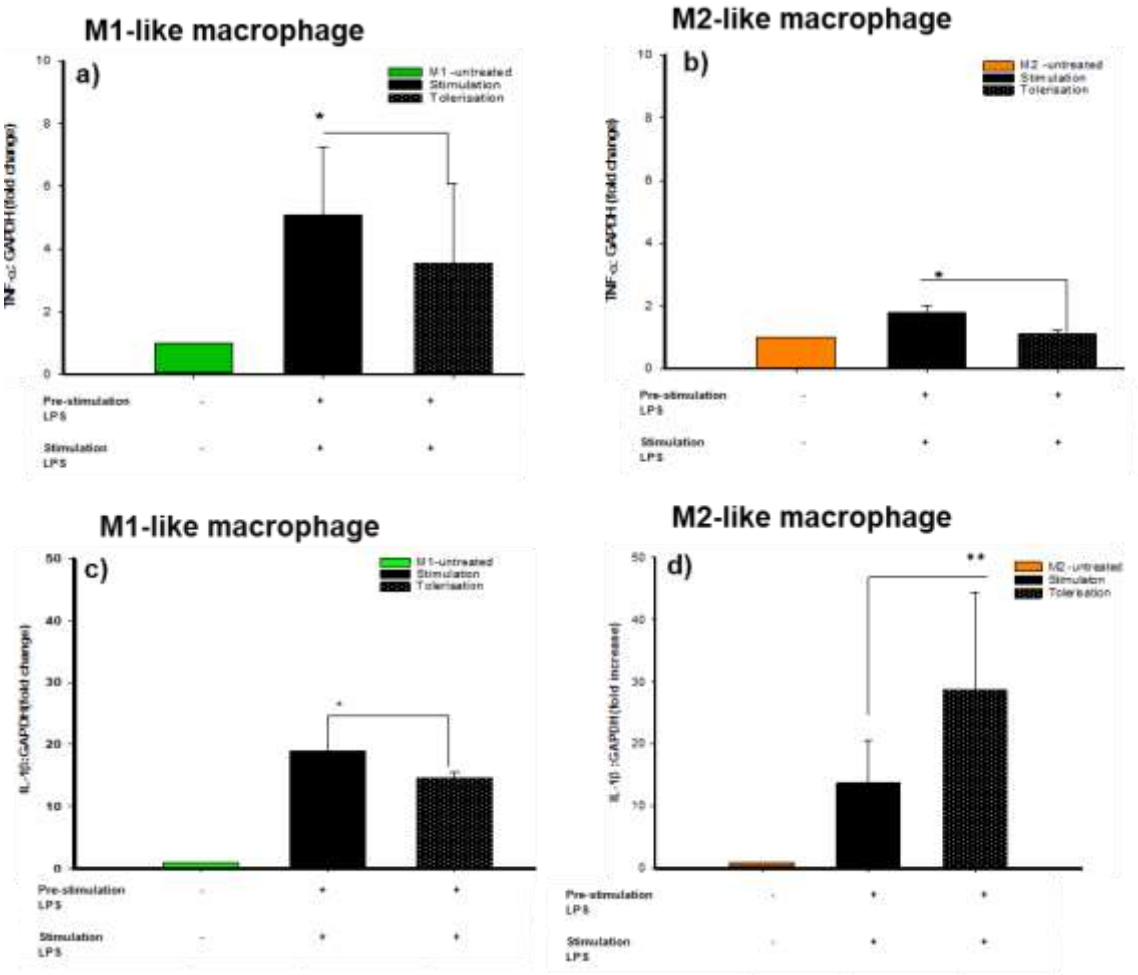

Figure 4.19: Differentially suppression of pro-inflammatory cytokine TNFα and IL-1β mRNA in endotoxin tolerised macrophage subsets

M1 (a) and M2 (b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS for 24 hours prior to stimulation with 100 ng/ml K12-LPS incubated for a further 24 hours (-) = no LPS, whereas (+) = LPS added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA gene expression of TNFα (a &b) and IL-1β (c&d). Gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n= 3 replicate experiments. Significant effects compared to the positive control (-/+) are indicated as * $P < 0.05$, ** $P < 0.01$.

mRNA expression of IL-23p19 (Figure 4.20 a) in stimulated M1-like macrophages with K12-LPS was elevated 1-fold, compared to untreated M1-like macrophages. Gene expression of IL-23 p19 in tolerised M1-like macrophages increased by 27-fold, compared to stimulated M1-like macrophages ($P \leq 0.001$). mRNA of IL-23 p19 in tolerised M2-like macrophages exhibited decreased ex-

pression compared to stimulated M2-like macrophages by 40% (P≤0.05). Gene expression of IL-23p19 in K12–LPS stimulted M2-like macrophages increased by 2-fold, (Figure 4.20 b). Furthermore, gene expression of IL-12p35 in K12-LPS tolerised M1-like macrophages significantly increased in 24 hours by 2-fold (Figure 4.20 c), (P≤ 0.001) (Figure 4.20 d). Whearas, the expression of IL-12p3 tolerised M2-like macrophages significantly decreased by 50% compared to stimulated M2-macrophage with K12-LPS after 24 hours (P≤0.01).

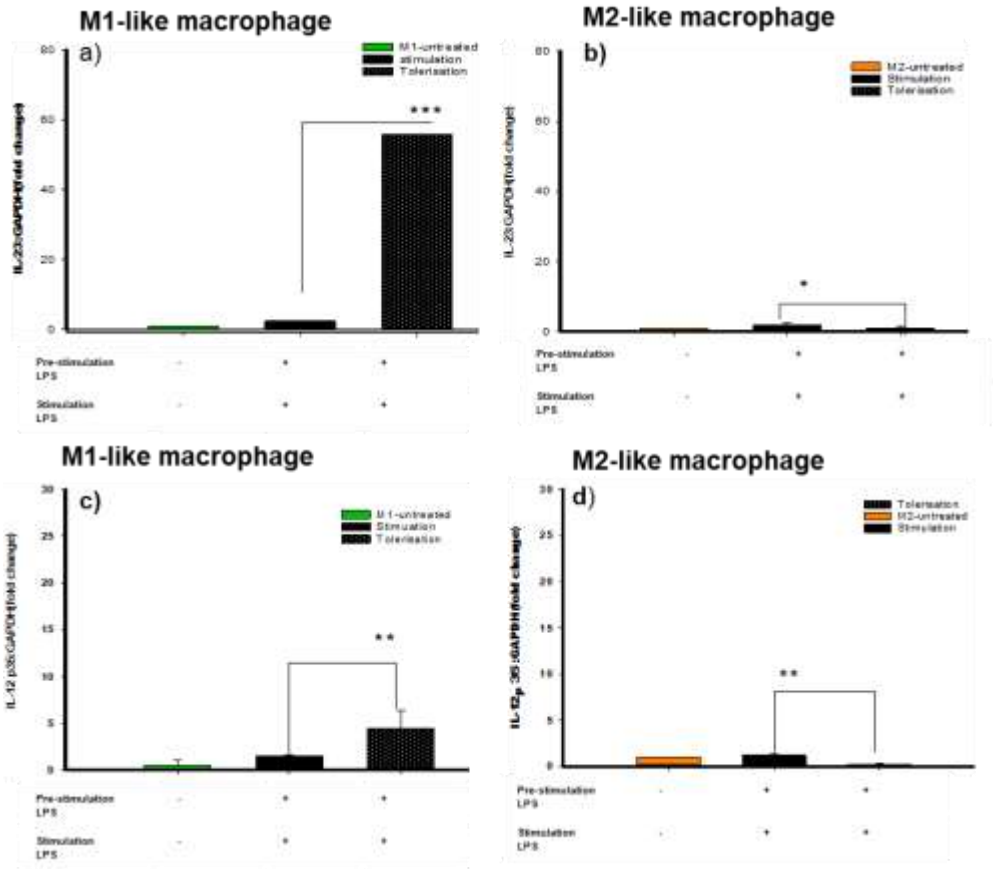

Figure 4.20: Differntially suppression of pro-inflammatory cytokine IL-12p35 and IL-23p19mRNA in endotoxin tolerised macrophage subsets

M1 (a,c) and M2 (b,d) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS for 24h prior to its stimulation with 100 ng/ml K12-LPS which was incubated for a further 24 hours. The sign (-) = no LPS, whereas (+) = LPS were added for both pre-stimulated and stimulated cells. mRNA was extracted from the cells to test the mRNA expression of IL-23 p19 (a,b) and IL12 p35 (c,d) gene. Gene expression (mRNA level) was expressed as fold change using GAPDH as a reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct.). Data showed represents triplicate samples for n= 3 replicate experiments. Significant effects on suppression were found compared to the stimulation. * P ≤ 0.05, ** P ≤ 0.01 and , *** P ≤ 0.001.

4.3.4: Expression of anti-inflammatory cytokines mRNA in endotoxin tolerised macrophages

Our previous results (**Figure 4.19 a/b**) investigated the effects of endotoxin tolerance by K12-LPS on the secretion of anti-infalmmatory cytokines such as IL-10 and IL-6 by M1 and M2-like macrophages. In this section, results compared the gene expression of IL-10 and IL-6 in tolerised macrophage subsets in comparssion with the stimualtion stage. The investegation revealed that the expression of IL-10 in tolerised M1-like macrophages was significantly upregulated 2-fold, compared with M1-like macrophages stimultaed K12-LPS for 24 hours ($P \leq 0.001$). mRNA expression of IL-10 in tolerised M2-like macrophages significantly increased 4-fold, ($P \leq 0.001$). A significant difference was noted between IL-10 expression between tolerised M1 and M2-like macrophages ($P \leq 0.001$). However, there was a significant change in gene expression of IL-6 in both M1 and M2-like macrophages. Gene expression of IL-6 was supressed by 89% in tolerised M1-like macrophages ($P<0.001$). M2–like macrophage tolerisation inhibited gene expression of IL-6 by 80%, ($P=0.001$) (**Figure 4.21 c / d**).

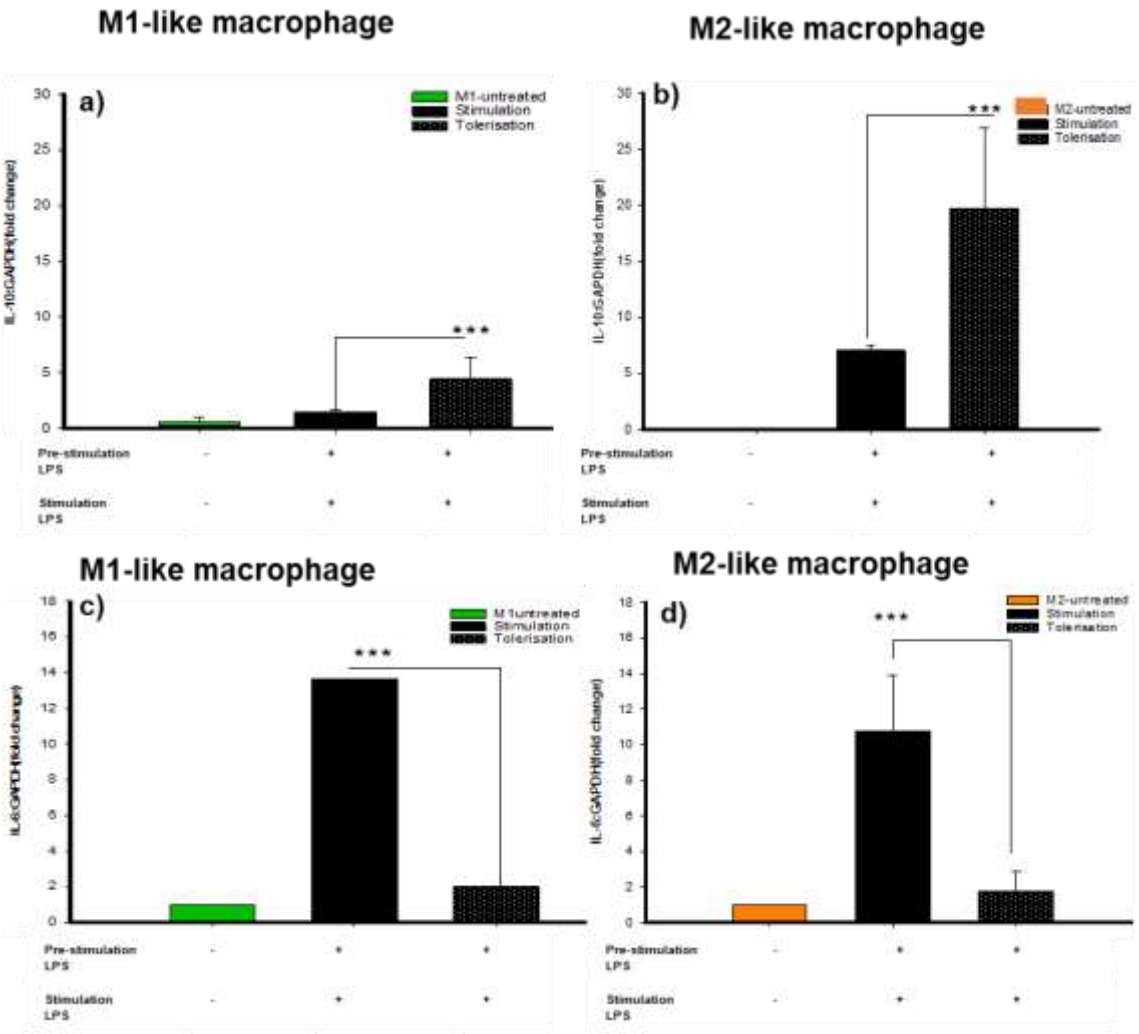

Figure 4.21: Expression of mRNA differentially anti-inflammatory cytokine in endotoxin tolerised macrophage

M1 (a,c) and M2 (b,d) Macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS(black) for 24 hours prior to its stimulation with 100 ng/ml, K12-LPS was further incubated for 24 hours. The sign (-) = no LPS , (+) = LPS were added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA expression of IL-10 (a, b) ,IL-6 (c,d) Gene expression (mRNA level) is expressed as fold change using GAPDH and has a reference gene as well as a resting cells as a calibrator sample that was described by (Livak et al,2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n= 3 replicate experiments. Significant effects compared to the positive control (-/+) are indicated as *** $P < 0.001$.

4.3.5: Endotoxin tolerance of K12-LPS on TRAIL gene expression of macrophage subsets

Tumour necrosis factor-related apoptosis inducing-ligand (TRAIL) is a member of the tumour necrosis factor (TNF) family of cytokines. It is a potent inducer of apoptosis in several tumour cells, but not in the majority of normal cells (Steven

et al.,1995). In addition to regulating the immune response through apoptosis, an investigation of the effect of TRAIL on endotoxin tolerance through cell death was conducted. Results showed that M1 and M2-like macrophages differentially stimulated and modulated TRAIL gene expression by K12-LPS. Observations revealed that TRAIL gene expression was up regulated in tolerised M1-like macrophages, (P≤0.001). Whereas, mRNA of TRAIL was downregulated in tolerised M2-like macrophages compared to K12-LPS stimulation (-/+) by 80% (P≤0.001) (**Figure 4.22 a/b**).

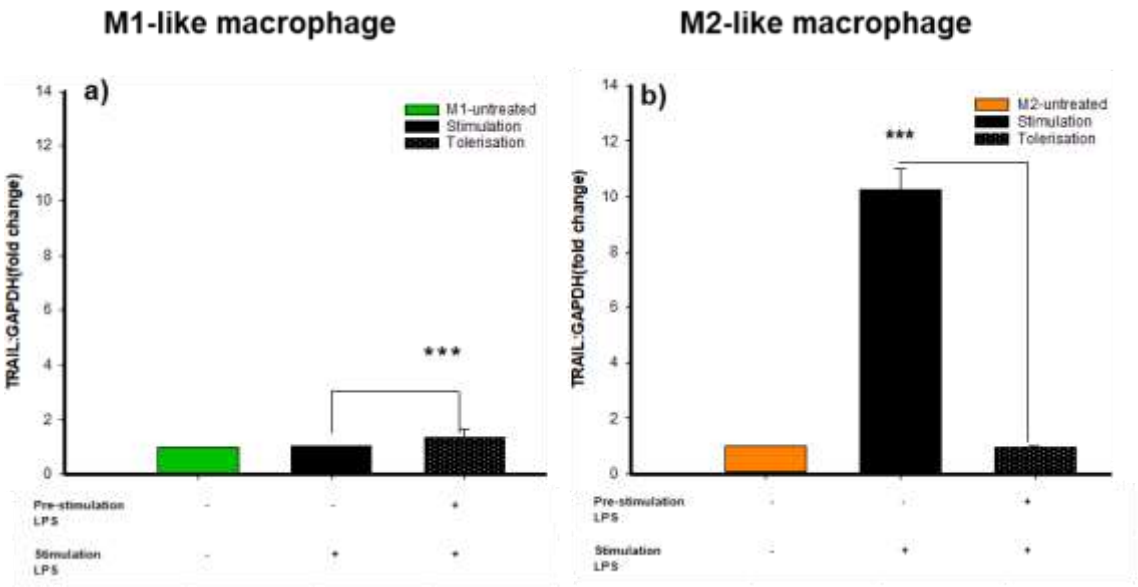

Figure 4.22: Endotoxin tolerisation effect on'macrophage TRAIL gene expression

M1 (a) and M2 (b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of TRAIL gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as ** P<0.009 and ns = no significant.

4.3.6: Endotoxin tolerance regulates macrophage subset's gene expression of pattern recognition receptors TLR4 & TLR2

Previous data showed the outcome of endotoxin tolerance on the regulation of pro-inflammatory and anti-inflammatory cytokines stimulated by macrophage subsets. Cell signalling controls the products of endotoxin-tolerance via Pathogen Recognition Receptors (PRRs), expression, such as TLR2 or TLR4.

A. TLR4

The the gene expression of various effector molecules, was studied in cells stimulated with K12-LPS through 24 hours of stimulation and 24 hours of tolerisation by K12-LPS. There were insignificant differences between stimulation and tolerisation between tolerised M1-like macrophages and stimulated M1-like macrophages induced by K12-LPS (**Figure 4.23 a**) (P=0.567). Gene expression of TLR4 in tolerised M2-like macrophages revealed significant upregulation compared to stimulation, by 4-fold (P≤0.001).

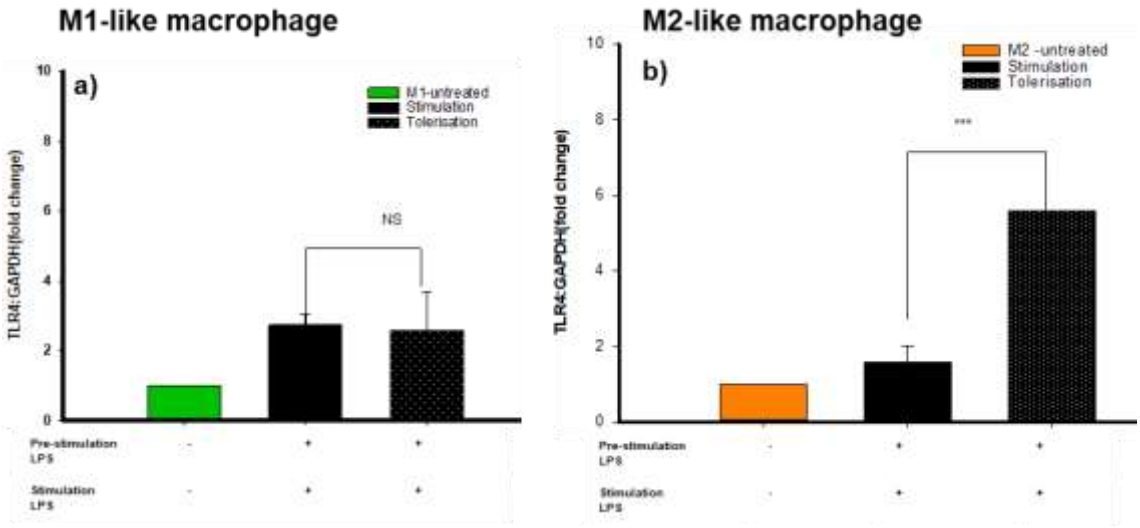

Figure 4.23: Endotoxin tolerisation effect on macrophage TLR4 gene expression

M1 (a) and M2 (b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of TLR4 gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as* P<0.05 , ** P<0.009.

B. TLR2

To investigate the role of TLR2 in modulating endotoxin tolerance by K12-LPS, the gene expression of TLR2 was examined in macrophages induced with K12-LPS. Twenty-four hours post-treatment, mRNA expression of TLR2 showed 80% upregulation by M1-like macrophages, compared with tolerisation treatment (P≤0.001). No changes were noted in TLR2 gene expression levels between stimulation in M2-like macrophages and tolerisation. (**Figure 4.24 a / b**).

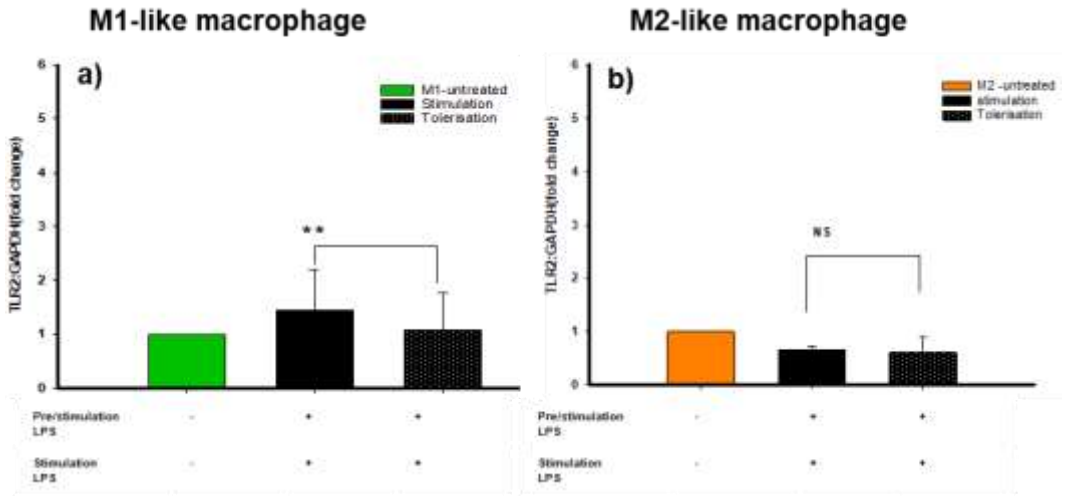

Figure 4.24: Endotoxin tolerisation effect on macrophage TLR2 gene expression

M1(a) and M2(b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of TLR2 gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as ** P<0.009 and ns = no significant.

4.3.7: Inflammasome NLRP3 is involved in the endotoxin tolerisation mechanisms in macrophages

This study investigates the role of endotoxin tolerance and considers the differences between M1 and M2-like macrophages. Expression of NLRP3 gene of in M1 and M2-like macrophages tolerised by K12-LPS, leads to the secretion of IL-18/IL-1β cytokines (**Figure 4.25**). Endotoxin-tolerised M1-like macrophages, showed significant up-regulation of the gene expression of NLRP3 by 99-fold (P≤0.01). Whereas, expression of NLRP3 in M2-like macrophages presented no significant differences in gene expression of NLRP3 between stimulation and tolerisation.

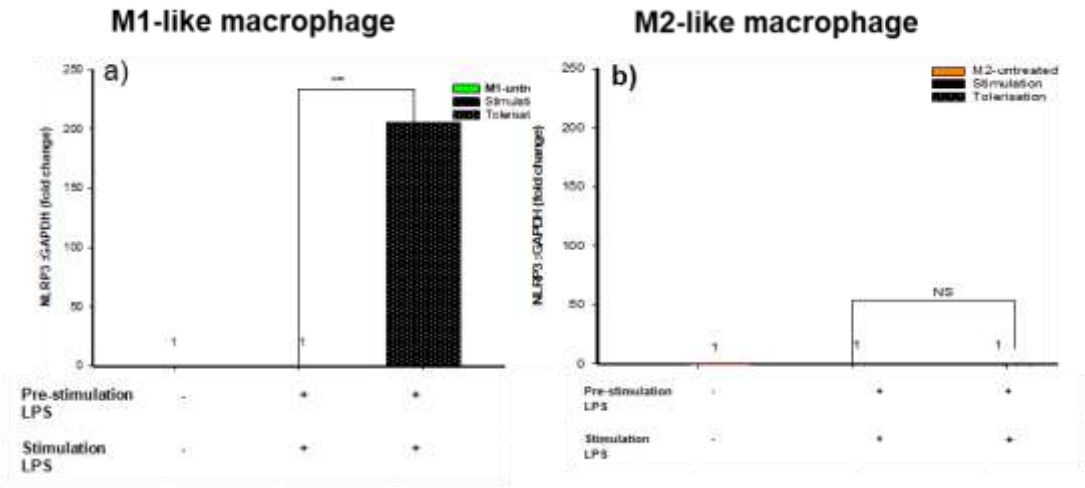

Figure 4.25: Endotoxin tolerisation effect on macrophage NLRP3 gene expression

M1 (a) and M2 (b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA expression of NLRP3 gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as ** P<0.009 and ns = no significant.

4.3.8: Negative regulatory molecules are associated with endotoxin tolerisation mechanisms

Further study of the role of tolerisation on selective regulatory molecules was carried out, to identify mechanisms of endotoxin tolerance and to understand differences in endotoxin tolerance between M1 and M2-like macrophages, particularly negative regulatory molecules, focused on Tollip, SOCS-3, and STAT3. Endotoxin tolerised macrophage subsets displayed different levels of negative regulatory gene expression.

A. Tollip

Endotoxin- tolerised M1-like macrophages, showed significant up-regulation of Tollip mRNA (80%, P=0.001) (**Figure 4.26**). Anti-inflammatory M2-like macrophages showed no significant difference in gene expression of Tollip between stimulation and tolerisation groups.

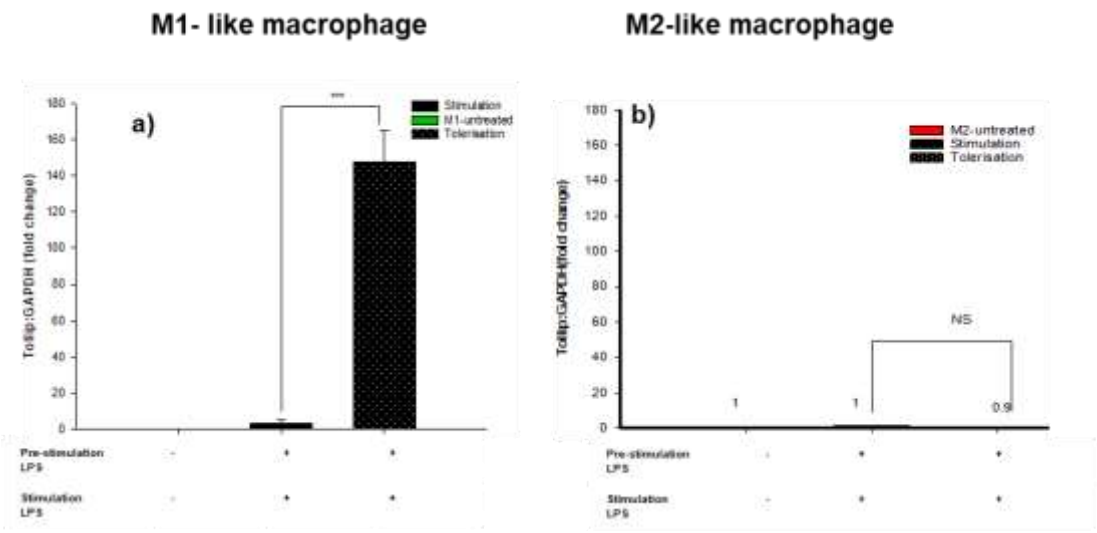

Figure 4.26: Endotoxin tolerisation effect on macrophage Tollip gene expression

M1 (a) and M2 (b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA expression of Tollp gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as ** P<0.009 and ns = no significant.

B. Expression of SOCS-3 and STAT-3 genes in endotoxin tolerisation macrophages

To identify JAK2-STAT-3 pathway functions in macrophage subsets, STAT-3 associated with both production and stimulation of IL-6 and IL-10 was investigated. The effect of endotoxin tolerance on SOCS-3 mRNA expression was studied, inducible by STAT-3 activation (Heinrich et al., 2003; Hutchins et al., 2013). RT-PCR analysis revealed that STAT3 in tolerised M1-macrophage was upregulated 19-fold, upon 24 hours stimulation, followed by 24 hours of challenge with K12-LPS (**Figure 4.27**). Endotoxin tolerisation showed up-regulation of mRNA of STAT-3 by (40-fold) (P≤0.001) in endotoxin-tolerisation of macrophages. Macrophages were upregulated after a long exposure to LPS (24/24) hours. However, M2-like macrophages showed significant downregulation by

75% (P≤0.001), of gene expression of STAT3 in tolerisation for 24 hours by K12-LPS.

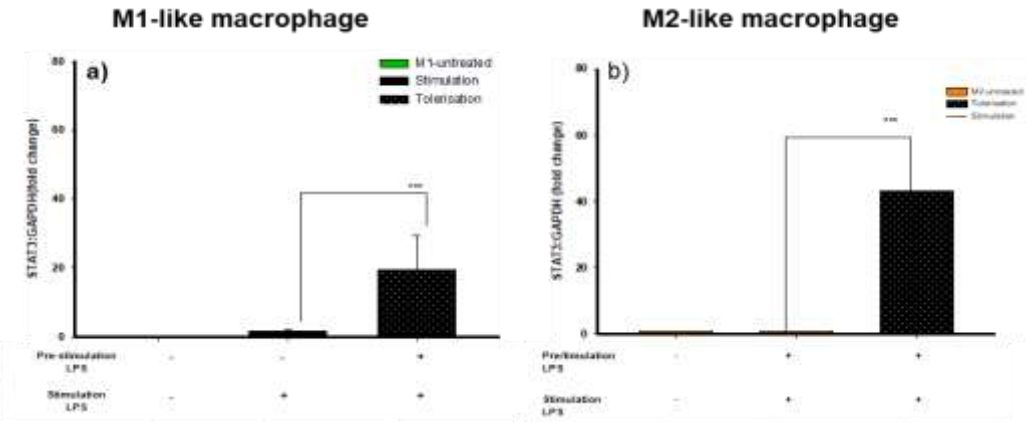

Figure 4.27: Endotoxin tolerisation effect on macrophage STAT3 gene expression

M1 (a) and M2 (b) macrophage subsets were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. mRNA was extracted from cells to test the mRNA expression of STAT3 gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as ** P<0.009 and ns = no significant.

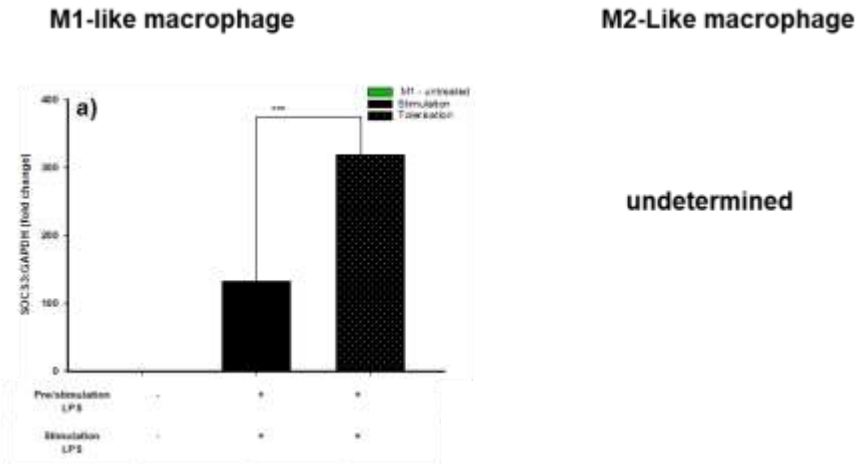

Figure 4.28: Endotoxin tolerisation effect on macrophage SOCS3 gene expression

M1 (a) and M2 (b) macrophage subsets(undetermined) were pre-stimulated with 100 ng/ml K12-LPS (black) 24 hours prior to stimulation with 100 ng/ml K12-LPS and was incubated for a further 24 hours, with the sign (-) = no LPS whereas (+) = LPS were added for both pre-stimulated and stimulated cells. RNA was extracted from cells to test the mRNA expression of SOCS3 gene. Expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by (Livak et al, 2001) using 2-ΔΔct. Data displayed is a representative experiment with duplicate samples of n=3 replicate experiments. Significant effects were noted compared to the LPS positive control (-/+) and are indicated as ** P<0.009 and ns = no significant.

4.4: Role of probiotic bacteria in modulating K12-LPS endotoxin tolerisation of macrophages

The role of *Lactobacillus bacteria* in the modulation of the endotoxin tolerance of M1 and M2–like macrophages, induced by K12-LPS, was studied (**Figure 4.15, 4.16, 4.17, 4.18).** Here, we focused on modulation of tolerisation of macrophage subsets by C28 and MS13. Samples were pre-treated with K12-LPS for 24 hours, washed out, and post-treated with K12-LPS + C28 or MS13 for a further 24 hours. Results showed that C28 and MS13 upregulated the production of TNF-α differentially from tolerised M1 and M2-like macrophages. Production of TNF-α modulated by C28 M1-like macrophage increased non significantly by 0.4 fold and significantly increased by MS13 by 26-fold (**Figure 4.28/a**) (P≤0.001). While C28 and MS13 upregulated TNF-α production of tolerised M2-like macrophage induced by K12-LPS 87-54 fold by LPS respectively (**Figure 4.28 /b**) (P≤0.001).

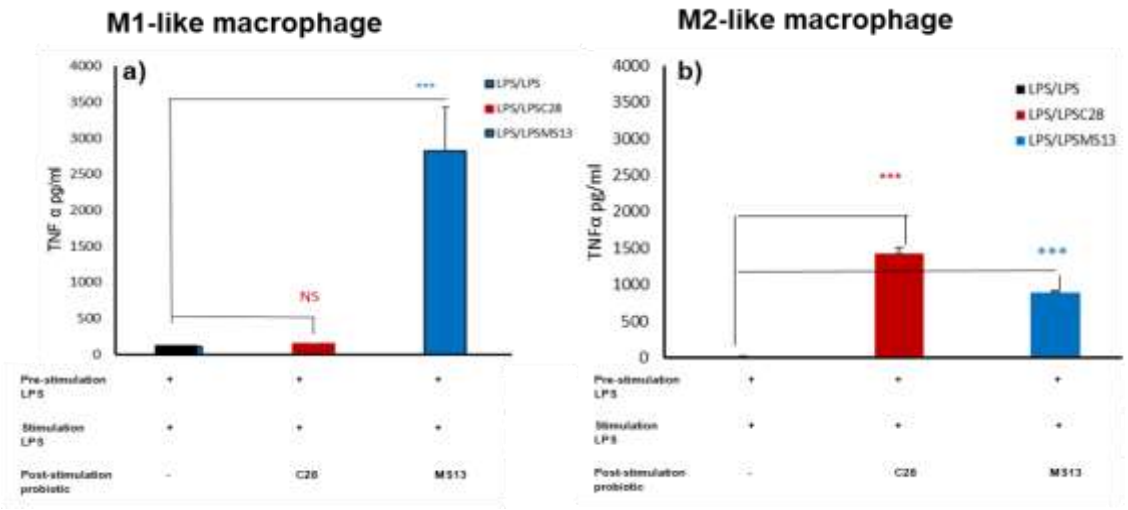

Figure 4.28: Probiotics modulate TNF-α tolerised macrophage by K12-LPS. Macrophages were generated by differentiating

THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 (a) and M2 (b) were pre-stimulate with 100 ng/ml K12-LPS for 24 hours and post-stimulated with K12-LPS (black bar) , then modulated with ($1X10^7$ cfu/ml) or C28 (red bar) or MS13 (blue bar) (LPS/LPS +C28 or MS13) for 24 hours. TNF-α production is expressed as the mean ±SE in pg/ml. Data displayed is a representative experiment in compared to the tolerisation of TNFα by K12-LPS/LPS and with triplicate samples of n=4 replicate experiments. Significant ef-

fects of C28 and MS13 on macrophage subsets tolerised with K12-LPS compared to the respective tolerisation LPS untreated control are indicated as,***,*** P= 0.001 and not significant=,NS, NS. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

Results revealed that C28 differentially modulated the production of IL-1β in tolerised M1 and M2-like macrophages. C28 induced IL-1β upregulation by tolerised M1 and M2-like macrophages, by K12-LPS 0.4-fold and 0.5-fold, respectively (**Figure 4.29 a/c**). In contrast with MS13 not impact the induction of IL-1β tolerised in M1 and M2-like macrophages compared to levels of IL-1β in tolerised M1 and M2-like macrophages by LPS/LPS (**Figure 4.16 a/b**). MS13 downregulated IL-1β of tolerised M1and M2-macrophages by K12-LPS by 10-80% respectively. C28 modulated IL-18 from tolerised M1-like macrophage and M2-like macrophage by 146-fold and 4-fold respectively (P=0.001 and 0.005), compared with tolerisation by M2-like macrophage induced by K12-LPS. MS13 modulated induction of IL-18 by M1 and M2-like macrophages 9-fold and 2.4-fold respectively (P=0.005 andP=0.001).

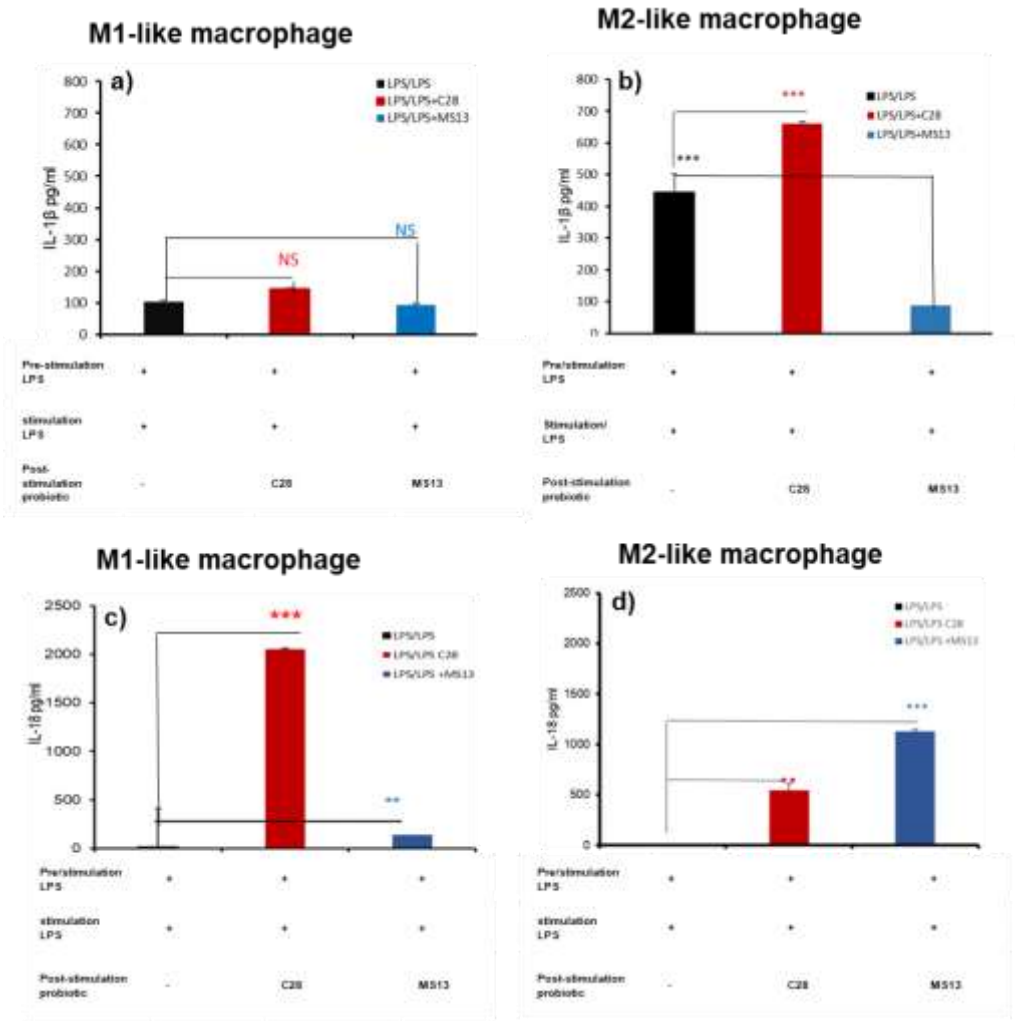

Figure 4.29: Probiotics modulate IL-1β and IL-18 tolerised macrophage by K12-LPS

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 (a) and M2 (b) were pre-stimulate with 100 ng/ml K12-LPS for 24 hours and post-stimulated with K12-LPS (black bar) then modulated with ($1X10^7$ cfu/ml) or C28 or MS13 (LPS/LPS +C28 or MS13) for 24 hours. IL-β and IL-18 production is expressed as the mean ±SE in pg/ml. Data displayed is a representative experiment in compared to the tolerisation of IL-1β and IL-18 by K12-LPS/LPS and with triplicate samples of n=4 replicate experiments. Significant effects of C28 and MS13 on macrophage subsets tolerised with K12-LPS compared to the respective tolerisation LPS untreated control are indicated as **,**P≤0.005,***,*** P= 0.001 and not significant=,NS, NS. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

C28 induced the production of IL-23 from tolerised M1 and M2-like macrophages by 24-fold and 9-fold, respectively ($p<0.001$). MS13 induced the production of IL-23 by M1 and M2–like macrophages, by 32-fold and 3.5-fold, respectively. Moreover, C28 and MS13 differentially regulated tolerisation of IL-23 production

by M1 and M2–like macrophages. No significant differences were found between endotoxin tolerance level of IL-12 and the regulation effect of both C28 and MS13 on M1-like macrophages. Whereas, the production of IL-12 by M2-like macrophage was up regulated by C28 and MS13 by 14-fold and 10-fold, respectively, compared to IL-12 induced by endotoxin tolerance experiments (**Figure 4.30**).

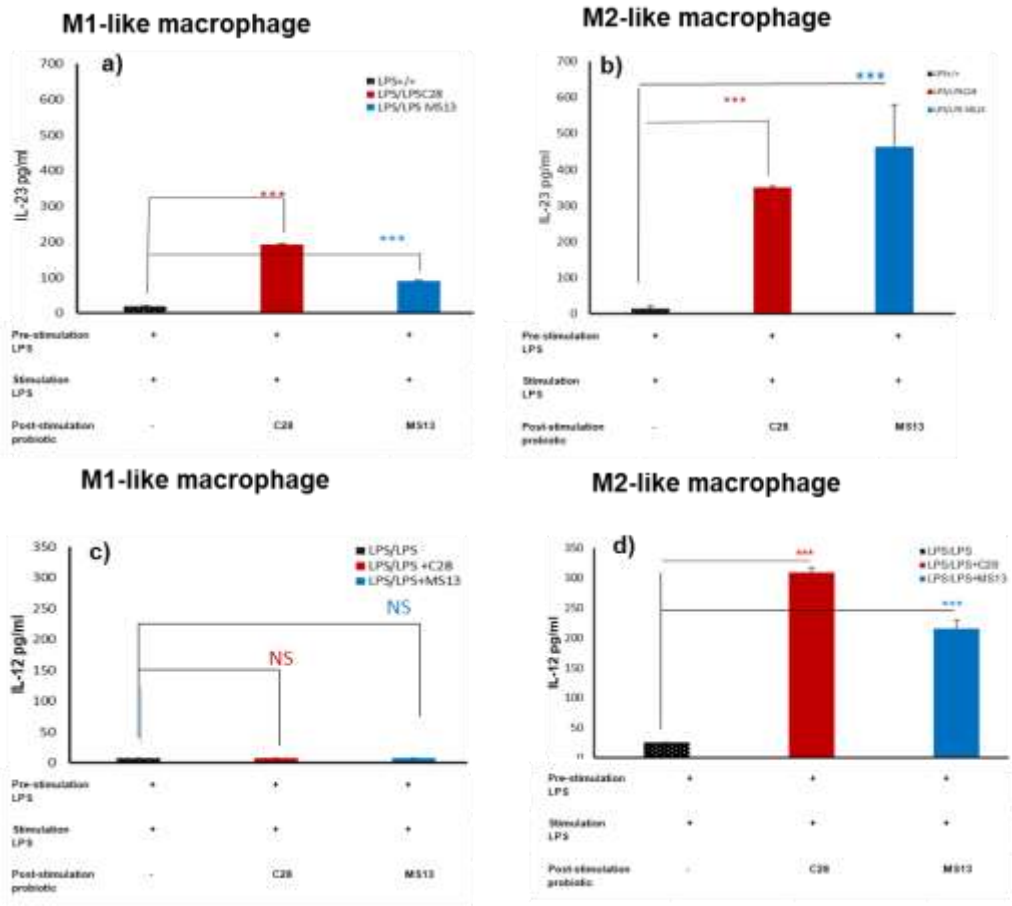

Figure 4.30: Probiotics modulate IL-23 and IL-12 tolerised macrophage by K12-LPS

Macrophages were generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days M1(a) and M2 (b) Macrophage subsets were pre-stimulate with 100 ng/ml K12-LPS for 24 hours and post-stimulated with K12-LPS 24 hours and modulated with ($1X10^7$ cfu /ml) either C28 or MS13. Cytokine production was expressed as the mean secretion ± SD in pg/ml/10^6 cells /ml for IL-23 (a, b) and IL-12 (c, d). Data showed represents triplicate samples for n= 3 replicate experiments. Significant effects on modulation with C28 and MS13 were compared to the tolerisation of IL-23 and IL-12 by K12-LPS (black bar), for the specified macrophage subset and are indicated as *** ,***p<0.001 and NS, NS not significant.

4.4.1: Modulation of anti-inflammatory endotoxin tolerance by C28 and MS13

To investigate probiotic bacteria in modulation of IL-10 and IL-6 production by M1 and M2-like macrophages, induction levels were measured. There is no efficacy of C28 on the production of IL-10 by tolerised M1-macrophage tolerised M1 by K12-LPS. While, MS13 downregulated the production of IL-10 by tolerised M1 by 63%. Probiotic bacteria C28 and MS13 upregulated the production of IL-10 by tolerised M2-like macrophages by K12-LPS by 2.46 fold and 0.46 fold significantly ($P \leq 0.001$). C28 downregulated the production of IL-6 10% of tolerised M1-like macrophage by K12-LPS compared with endotoxin tolerised M1-like macrophages. While MS13 upregulated IL-6 of tolerised M1-like macrophages by K12-LPS not significantly by 0.2-fold. Whereas, there was a significant upregulation of IL-6 by K12-LPS tolerised M2-like macrophage induced by C28 and MS13, (23-13-fold respectively ($p<0.001$).

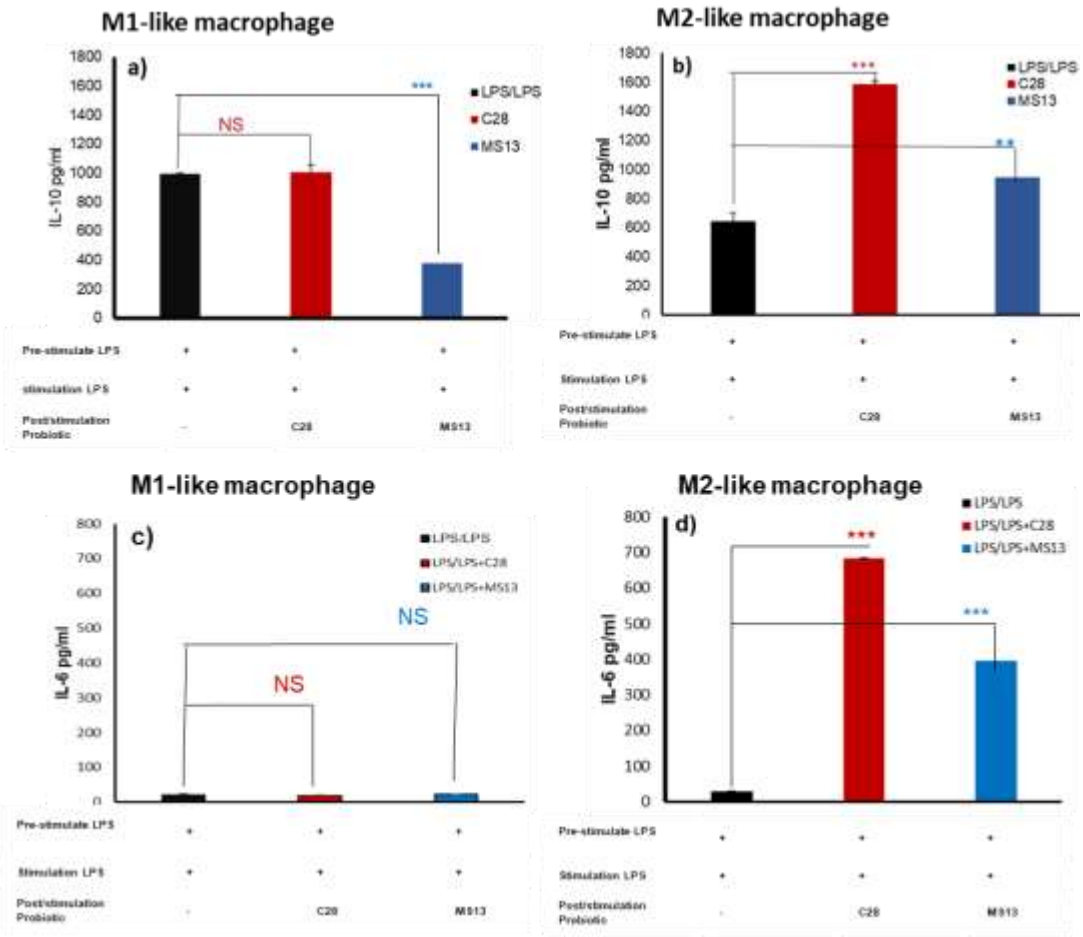

Figure 4.31: Probiotic bacteria modulate IL-10 and IL-6 tolerised macrophage by K12-LPS. Macrophages were generated by differentiating

THP-1 monocytes with either 25 ng/ml PMA for 3 days and 24 hours wash out or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days. M1 (a) and M2 (b) were pre-stimulate with 100 ng/ml K12-LPS for 24 hours and post-stimulated with K12-LPS (black bar) , then modulated with ($1X10^7$ cfu/ml) or C28 or MS13 (LPS/LPS +C28 or MS13) for 24 hours. IL-10 and IL-6 production is expressed as the mean ±SE in pg/ml. Data displayed is a representative experiment in compared to the tolerisation of IL-10 and IL-6 by K12-LPS/LPS and with triplicate samples of n=4 replicate experiments. Significant effects of C28 and MS13 on macrophage subsets tolerised with K12-LPS compared to the respective tolerisation LPS untreated control are indicated as,***,*** P= 0.001 and not significant=,NS, NS. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

Summary of the results of chapter 4

Table 4.1: Modulation of cytokines,PRRS and negative regulatory molecules by macrophage subsets upon endotoxin tolerisation

Macrophage Cytokine expression mRNA		LPS-toler-ised		Tolerisation by probiotics			
		M1	M2	M1-C28	M1-MS13	M2-C28	M2-MS13
Cytokine mRNA	TNFα	↓	↓	↓	↑	↓	↔
	IL-1β	↓	↑	↑	↑	↑	↓
	IL-23p19	↑	↓	↑	↓	↑	↓
	IL-12 p35	↑	↓	↑	↔	↓	↓
	IL-10	↑	↑	↔	↓	↔	↓
	IL-6	↓	↓	↑	↓	↓	↔
PRRs	TLR4	↔	↑	↓	↑	↑	↑
	TLR2	↓	↔	↓	↓	↓	↓
	NLRP3	↑	↔	↑	↑	↓	↓
Negative Regulator Molecules	Tollip	↑	↔	↓	↔	↔	↓
	SOCS3	↑	ND	↔	↓	↔	↔
	STAT3	↑	↑	↓	↑	↔	↑
TNF-LIGAND	TRAIL	↑	↓	↓	↔	↑	↑

↑ ***increased,*** ↓ ***decreased, not determined =ND***

Table 4.2: Comparison of cytokine production between endotoxin tolerance of macrophage subsets by K12-LPS and tolerised macrophage by C28 and MS13

Cytokine secretion ELISA	LPS-Tolerised		Tolerisation by Probiotics			
	M1	M2	M1-C28	M1-MS13	M2-C28	M2-MS13
TNFα	↓	↓	↑	↑	↓	↓
IL-1β	↓	↓	↑	↑	↑	↔
IL-18	↓	↓	↓	↑	↑	↑
IL-23 p19	↔	↔	↔	↑	↑	↔
IL-12 p35	↔	↑	↔	↓	↔	↓
IL-10	↓	↑	↑	↓	↑	↑
IL-6	↓	↓	↑	↔	↑	↑

↑ increased, ↓decreased ,not significant ↔,

Table 4.3: Modulation of K12-LPS endotoxin tolerisation by probiotic bacterial post-stimulation ↑ increased-fold change, ↓decreased - % suppression ,no significant change -

Cytokine production	M1+C28	M1+MS13	M2+C28	M2+MS13
TNF-α	↑0.4	↑26	↑87	↑54
IL-1β	↑0.4	↓10%	↑0.5	↓80%
IL-18	↑146	↑9	↑4	↑2.4
IL-23	↑24	↑32	↑9	↑3.5
IL-12	–	–	↑14	↑10
IL-10	–	↓37%	↑1.5	↑0.5
IL-6	↓10%	↑0.2	↑23	↑13

4.5: Discussion

This chapter reveals a number of interesting findings. First, 24/24-hour treatment durations by live probiotic (*Lactobacillus*) bacteria differentially tolerised macrophages and impacted the production of pro-inflammatory cytokines. C28 and MS13 repeat-stimulation/tolerised M1-macrophage continued releasing TNFα and IL-1β, whereas pro-inflammatory cytokine IL-18 and IL-23 production was differentially regulated by C28 and MS13. Both C28 and MS13 tolerised M2-macrophages downregulated TNFα whereas MS13 also suppressed IL-12. However, with respect to anti-inflammatory cytokine secretion, C28 induced M1-like macrophage upregulation of IL-10 and IL-6, conversely MS13 suppressed M1 IL-10 secretion. C28 and MS13 tolerised M2-like macrophages upregulated their production of IL-6 and IL-10 (**Table 4.2**).

Secondly, endotoxin tolerance induced by *E.coli*-K12-LPS failed to demonstrate an appreciable differential subset-specific response. K12-LPS tolerised M1 and M2-like macrophage subsets exhibited down-regulation of pro-inflammatory cytokines (TNF-α, IL-1β and IL-18) whilst no changes were recorded in IL-23 levels. Furthermore, IL-12 was differently released in M1 and M2–like macrophages. With respect to anti-inflammatory cytokine stimulation, there was a differential modulation; M1-like macrophages down-regulated IL-10 production, whereas, M2-like macrophages upregulated of IL-10 (**see Table 4.2**).

Moreover, this differential suppression of macrophage pro-inflammatory cytokines appeared to be linked with the differential regulation of TLR4 and the negative regulators, Tollip, SOCS3 and STAT3. Thirdly, homo-tolerance by probiotics up-regulated the production of pro-inflammatory and anti-inflammatory cytokines dependent on the macrophage phenotype response and probiotic bacteria strain. Up-regulation of M1 and M2–like macrophage cytokines ap-

peared linked with the down-regulation of negative regulators. Overall, results showed heterogeneity in cytokine responses, negative signalling molecules with limited heterogeneity in PRR expression between macrophage subsets and probiotic strain (**Table 4.1**).

4.5.1: Homo-tolerance by probiotic bacteria differentially modulates pro/anti-inflammatory macrophages cytokine production

The innate immune system responds rapidly through activation of pattern recognition receptors (PRRs), such as TLRs. These interact with highly conserved molecules present in microorganisms (Modlin et al.,1999), playing an important role in the pathogenesis. Both TLR2 and TLR4 are key PRRs involved in sepsis. Tolerisation of M1 and M2-like macrophages with C28 and MS13 enabled a comparison with endotoxin tolerance. TNF-α, IL-1β, IL-12, IL-18, IL-23, IL-16 and IL-10 play an important role in directing the healthy of the gut. As known the pathogenicity of IBD due the pathological activation of mucosal immune system in responsis to the pathogens. In patients with CD ,the pattern of cytokines expressed by mucosal tissue generally tend to be consistent with Th1 response (pro-inflammatory response) including an early increase in the expression of IFN, IL-2, and IL-12, followed by subsequent increase in TNF-α and IL-18 (Sartor, 2006;Targan, 1997). Additionally, there looks also to be an increase in IL-10 and TGF-β levels. In patients with UC, the patterns of cytokine expression differ from that seen in CD, with an increased the expression of IL-6, IL-10, IL-15, and IL-13 (Madsen, 2002). This study produced promising evidence for the role of probiotic bacteria, precisely (C28 and MS13), in potentially controlling UC. Tolerisation by C28 and MS13 probiotic bacteria, upregulated IL-10, IL-6 differentially in tolerised M1-like macrophage. Its seems there is synergy between a down-regulation of TNF-α and upregulation of IL-10 and IL-6 in

C28 and MS13-tolerised M2. Conversely, with K12-LPS endotoxin tolerised M1 and M2 macrophages, this study demonstrated the downregulation of pro- inflammatory cytokines (TNF-α, IL-1β, IL-18) (Figures 4.15 and 4.16). These observations agreed with a previous study of endotoxin tolerance induced by *E.coli* K12-LPS, where LPS-treated (24/24 hrs) endotoxin tolerance of M1 and M2-like macrophages suppressed TNF-α, IL-1β, IL-18 (Alshaghdali, 2018). C28 and MS13 upregulated IL-1β and IL-18 protein secretion by M1 and M2-like macrophage subsets. This is possibly as a consequence of the PGN cell wall component of probiotic bacteria, previously described to upregulate IL-1β by M2-like macrophages (Alshaghdali, 2018). Interestingly, cytokine secretion by tolerised M1 and M2–like macrophage was upregulated by both C28 and MS13. Importantly, in M1-macrophages, homo-tolerance by probiotic bacteria differentially modulated pro and anti-inflammatory cytokine production, where C28 and MS13homo-tolerisation differentially upregulated IL-10 and IL-6 in M1-like macrophages.

Probiotic bacteria have several PAMPs/MAMPs within the cell wall such as LTA, PGN and lipoprotein (Lebeer, Claes & Vanderleyden, 2012). In this study, tolerance utilising live probiotic bacteria as a source of PAMPs was investigated (Ginsburg, 2002; Claes et al., 2012a; Meng *et al.*, 2014; Serti *et al.*, 2014). Tolerisation induced by *Lactobacillus plantarum* (C28) and *Lactobacillus salivarius* (MS13) differentially regulated pro-inflammatory cytokines in both M1- and M2-like macrophages. Previously, Kim et al. (2008), described pre-treatment by pLTA might be effective in preventing LPS-induced inflammation in septic shock. Furthermore, purified pLTA inhibited *S. aureus* LTA (a LTA)-induced TNFα production in THP-1 cells, exhibiting TLR2-mediated homo-tolerisation. In addition, pLTA modulation of NOD2 signalling has been suggested to suppress

IL-1β or TNF-α (Kim *et al.*, 2011). C28 probiotic strain suppression of M1-like macrophage TNFα mRNA, focused on specific cytokine expression time points, may be an interesting avenue for future work.

Research in our laboratory, analysing effective gram-positive bacteria effects, revealed the inhibition of TNF-α, IL-1β, IL-6 and IL-10 from tolerised M1 and M2 by PGN and LTA, compared to a positive control of LPS. PAN pre-treatment (24 hours) / tolerisation of M2-like macrophages, up-regulated IL-1β (Alshaghdali, 2018). It is possible that probiotic-derived PGN activates NOD-2 receptors to upregulate IL-1β. Probiotic pre-stimulation - stimulation differentially modulated M1 and M2 macrophage IL-12 mRNA; in contrast to Mantovani (2005), IL-12 was highly identified in M1 macrophages, however M1 IL-12 mRNA was augmented by C28. Interestingly, C28 augmented IL-23 p19 mRNA with little effect observed in protein secretion for both M1 and M2–like macrophages, whereas MS13 suppressed both IL23 mRNA and protein secretion in both macrophage subsets. Previous findings (Gerber & Mosser, 2001) reported that *in vitro* macrophages stimulated with pro-inflammatory stimuli, such as LPS or lipoteichoic acid, resulted in the induction of high levels of IL-12 and modest levels of IL-10, whereas stimulation with anti-inflammatory stimuli induced high levels of IL-10 and low levels of IL-12 or might abrogate IL-12 synthesis.

This study's findings could indicate that live probiotic bacteria are effective as tolerisation (repeat-stimulation) treatment for the gut via the mucosal immune system. Moreover, *Lactobacillus* C28 and MS13 strains differentially regulated IL-23 and IL-18 in both subsets of macrophages; MS13 suppressed IL-23 and augmented IL-18, whereas C28 augmented IL-23 mRNA and M2 IL-18, yet suppressed M1 IL-18. These IL-23 data suggest a role for MS13 in suppressing Th17 activity, whereas C28 may encourage Th17 responses. Similarly, consid-

ering the linkage between IL-18 and the induction of IFNγ, and its role in the activation of NK and Th1-mediated responses, Llewellyn and Foey (2017) reviewed stimulated signalling by probiotics, where expression and secretion of IFNγ and NK signalling was modulated by probiotic bacteria at many levels. Our IL-18 data is suggestive that MS13 may be beneficial for initiating both NK and cell mediated responses, important for anti-tumour immunity, whereas C28 may be beneficial in controlling inflammation: suppressing CMI driven by M1s, yet augmenting M2-driven CMI. Such an effect may suggest efficacy in the control of chronic inflammation characteristic of CD.

4.5.1.1: The experssion of TLR-2 and TLR-4 and other regulatory molecules in tolersied macrophage subsets modulate by probiotic bacteria

As far as is known, tolerance induction by live probiotic bacteria, has not been extensively studied compared to endotoxin tolerance induced by LPS. Here, pre-treatment and post-treatment by *Lactobacillus plantarum* strain (C28) or *Lactobacillus salivarius* strain (MS13) tolerance induction was investigated.

Surprisingly, the macrophage subset TLR-2-mediated signal of was markedly inhibited by C28 and MS13; however, this suppression did not affect TNFα levels of tolerised M1-like macrophages. In addition, the TLR4-mediated response was downregulated in tolerised M1 by both C28 and MS13. Inhibition of TLR4 and TLR2 in tolerised M1s may be connected with the stimulation of NLRP3 expression. Llewellyn and Foey (2017) reviewed inflammatory cytokine regulation upon probiotic bacteria induction of a range of endogenous negative regulators involved in TLR2 signalling, such as Tollip, IRAK-M and NFkB homodimer (Finamore *et al.*, 2014). For example, TLR2–ligation may suppress TLR4-mediated inflammatory response through expression/activation of IRAK-M and Tollip. Interestingly, the current study showed a probiotic strain-selective modu-

lation of STAT3 in tolerised M1s, associated with the opposite effect on expression of IL-10. In turn, this encourages inhibition of SOCS3. Moreover, TLR2 downregulation may explain MS13-M1 SOCS3 inhibition, whereas the upregulation of TLR4 may be associated with a corresponding MS13-dependent upregulation of STAT3 in both tolerised M1 and M2 macrophages.

In contrast with this result, STAT3 is associated with anti-inflammatory signalling of IL-10 and IL-6, whilst SOCS3 is inducible by IL-10 and IL-6, serves to suppress pro-inflammatory cytokine gene expression, and uses negative feedback to inhibit IL-10 and IL-6 signalling. Furthermore, *Bifidobacterium* decreased LPS-induced IL-1 and TNFα mRNA levels in murine RAW264.7 macrophage cells (Okada *et al.*, 2009), correlating with inhibition of IκB phosphorylation and increased mRNA levels of SOCS1 and SOCS3.

This study revealed the role of C28 and MS13 in the down-regulation of Tollip following the tolerisation of M1– and M2-macrophages. In contrast, the expression of endogenous negative regulators, such as A20, Tollip, IRAK-M, TRIAD3A and p50/p50 NFκB, acted to tolerise TLR signalling (Biswas & Lopez-Collazo, 2009). Tolerising mechanisms for innate immune signalling are diverse. These mechanisms consist of antagonism for PAMP binding, down-regulation of PRR expression and induction of suppressive cytokines. Understanding the role played by probiotics in motivating endotoxin tolerisation will allow specific and selective consumption of probiotic bacteria for the treatment of pathological conditions.

4.5.2: K12-LPS differentially suppresses M1 and M2 macrophages pro- and anti-inflammatory cytokines

An investigation into tolerisation and stimulation of M1 and M2-like macrophages by K12-LPS allows for an understanding of TLR4 signals. Endotoxin toler-

ance of M1 and M2-like macrophages by K12-LPS, predominantly suppresses pro-inflammatory cytokines of the macrophage subsets. Our results corroborate findings of previous work regarding K12-LPS tolerisation (del Fresno et al., 2009; Al-Shagdali, 2018), in particular, suppression of pro-inflammatory cytokines of both macrophage subsets and enhanced anti-inflammatory cytokines such as IL-10, TGFβ and IL1RA. TNFα suppression in protein secretion was supported by similar levels of suppression shown in TNFα mRNA (Draisma et al., 2009).

In this study, tolerised M1 and M2–like macrophages did not have defined differences in IL-23 and IL-12 protein secretion between stimulation and tolerisation, whereas of IL-23 mRNA expression was upregulated in M1–like macrophages and down-regulated in M2-like macrophages. IL-12 mRNA was upregulated in tolerised M1-like macrophages and down regulated in tolerised M2-like macrophages. Mantovani, et al. (2005) reported that M1 cells produce high levels of IL-12 and IL-23 and low levels of IL-10. In contrast, various M2 subsets share the phenotype of low production of IL-12 and IL-23 and variable levels of IL-10 and other inflammatory cytokines depending on the signal received. There was an increase in IL-10 gene expression in M1 and M2-like macrophages and lower protein production in M1-like macrophage than in M2-like macrophages. It should be noted that this study contradicts previous research in our laboratory of *Porphyromonas gingivalis*-LPS tolerisation (Foey and Crean, 2013). Here, macrophages also displayed a differential sensitivity to tolerance induced by *E.coli*-derived bacterial PAMPs; K12-LPS tolerised M1 and M2-like macrophage subsets down-regulated pro-inflammatory cytokine secretion, with a differential subset-specific effect on the anti-inflammatory cytokine, IL-10. Endotoxin tolerance is usually related to excess secretion of anti-inflammatory cytokines, such

as IL-10 and TGF-β, contributing to macrophage deactivation and the suppression of pro-inflammatory cytokine production (Biswas and Lopez-Collazo, 2009). Expression of IL-6 by tolerised M1 and M2-macrophages was suppressed. Furthermore, protein secretion of IL-6 was inhibited in both M1- and in M2–like macrophages. Endotoxin tolerance may represent selective reprogramming designed to reduce inflammatory damage. To identify genuine reasons of variance in cytokine production, distinct signalling pathways can be investigated. Activation of negative regulator signalling may suppress pro-inflammatory cytokines, such as IL-1β, where TLR signal cascade activates NF-κB (Kwon et al., 1995).

4.5.2.1: Signalling mechanisms in endotoxin tolerance in M1 and M2-like macrophages

TLR4 is involved in the recognition of Gram-negative bacteria and their associated endotoxins such as lipopolysaccharide (LPS), (O'Neill and Bowie, 2007). These results demonstrated that in response of repeating exposure to K12-LPS, the expression of TLR4 was unaltered in tolerised M1s, whilst up regulated in M2-like macrophages. *E.coli* LPS revealed different abilities to modulate expression levels of members of TLR family. This might lead to the reduction of excessive signal transduction activated by PAMP stimulation. The pathway TLR4 employs, leads to diverse expression through two different adaptors. MyD88-dependent TLR agonists have been described to induce tolerance to LPS by inhibiting IL-1R-associated kinase-1 activation (Sato *et al.*, 2002). As a TLR-4 agonist, K12-LPS can activate both MyD88-dependent and MyD88-independent pathways. Endotoxin tolerance was first categorised by suppression of the MyD88-dependent pathway, decreasing pro-inflammatory cytokines and upregulating suppressive molecules (Akira & Hoshino, 2003). TLR-2 is also known to signal through MyD88-dependent pathways (Sun et al., 2014).

Further exploration of the expression status of TLR-2, TLR-4 and other down-stream receptor signalling cascades was to elucidate a potential differential role in endotoxin tolerisation between macrophage subsets. This showed that response to recurrent LPS stimulation, TLR-2 and TLR-4 gene expression was suppressed (see **Table 4.1**), which may prevent extreme signalling transduction activated by persistent bacteria stimulation. Different regulatory mechanisms of TLR-2 and TLR-4 gene expression could modify cytokine profiles secreted by K12-LPS tolerised by macrophages. These activation pathways may explain cytokine suppression in endotoxin–tolerised cells. TLR-4 triggers both MyD88-dependent and MyD88 independent pathways, whereas TLR-2 downstream only activates via MyD88-dependent pathway (Sun et al., 2014).

Although, LPS is a TLR-4 agonist, LPS endotoxin tolerance appeared to a play role in modulating expression of NOD-like receptors, including inflammasome member NLRP3. LPS-endotoxin tolerisation differentially induced NLRP3 gene expression in a macrophage subset-dependent manner. After 24 hours LPS pre-treatment followed by LPS stimulation, NLRP3 mRNA expression was increased significantly in M1-like macrophages, whereas no change was observed in M2 macrophages. According to Gurung (2015), chronic TLR-2 and TLR-4 stimulation inhibits NLRP3 by an upregulation of IL-10. This upregulation controls endotoxin tolerance by suppressing pro-inflammatory cytokines and inflammasomes. Al-Shagdali (2018) referred to the role of NOD-2 in controlling endotoxin tolerance; where NOD-2 expression was increased in tolerised M1-like macrophages, compared with a decrease in M2-like macrophages.

Further investigation compared gene expression modulation of endotoxin tolerised M1-and M2-like macrophages. Negative regulators that have been studied are Tollip, STAT-3, SOCS-3 and TRAIL. The upregulation of Tollip companied

with downregulation of TLR4 and TLR2. This results agreed with Jezierska,et al., (2011); Tollip is associated with TLR expression and plays an important role as an inhibitor for TLR-mediated cell activation, Tollip directly connected with TLR2 and TLR4, acting to inhibit TLR-mediated cell activation. Suppression by Tollip is mediated through its capability to suppress IL-1 receptor-associated kinase (IRAK) activity after TLR activation (Zhang and Ghosh, 2002; Al-Shaghdali *et al.*, 2019).

In addition, other regulatory molecules were evaluated for their role in endotoxin tolerance induced by macrophage. These include STAT-3 and SOCS-3. STAT-3 is thought to play an important role in IL-10 mediated inhibition. Macrophage stimulates SOCS3 in response to pathogens and may be a key inhibitor for JAK/STAT signalling. IL-6 and phospho-STAT-3 are crucial for the pathology associated with inflammation such as Crohn's Disease and upregulation of SOCS3 expression. This suggests that SOCS3 may have a modulatory role in these diseases (Croker et al., 2008). Induction of STAT-3 also encourages expression of SOCS3. STAT-3 is reported to play an important role in IL-10-mediated inhibition of LPS-induced TNFα production (Naganuma *et al.*, 2016). The pathology associated with gut inflammation, such as Crohn's disease, are crucial for STAT3 phosphorylation and IL-6 release. This increases SOCS3 expression, making SOCS3 crucial to such inflammatory pathology (Croker et al., 2008).

The relationship between increased expression of STAT-3 and SOCS-3 opposed down-regulation of IL-6 gene expression and secretion. The decrease in IL-10 of M1 and increased secretion of IL-10 M2 tolerisation may reflect the up-regulation of STAT-3 (**Figure 4.14).**

Further study is required to identify endogenous mechanisms of endotoxin tolerance induced in macrophages, and how they are selectively related to distinct macrophage subsets induced in the harmful inflammatory response. A further focus on endotoxin tolerisation in cell death induction, through expression of TRAIL, facilitates elucidating this response. Basically, tumour cells express DR4 and DR5, the receptors for TRAIL and as such, are sensitive to TRAIL-induced apoptosis,(Amarante-Mendes et al.,2015 andRossin, 2019) whereas, normal cells/tissues demonstrated to be resistant, as well as being able to synthesize and release TRAIL. The current study found the expression of TRAIL followed negative regulators such as STAT3, SOCS3 and Tollip in tolerised M1s; which were upregulated, and down regulated in M2-like macrophages. Thus, endotoxin tolerance regulation may add an effect in the regulation on macrophage subsets of Crohn's disease through polarisation of macrophage subsets between anti-inflammatory M2-like and pro-inflammatory M1-like phenotypes.

4.5.2.2: Differential modulation of probiotic bacteria on endotoxin tolerance in macrophage

As mentioned above, endotoxin tolerance discriminates through the suppression of pro-inflammatory cytokines such as TNFα, IL-1β, IL-12, IL-18, IL-23 by tolerised M1 and M2–like macrophages (See table 4.2). In the pathology of mucosal macrophage such as IBD or cancer, the gut undergoes inflammation or tolerance (Sato *et al.*, 2002), either by homeostasis or cancer disease (Faria, Reis & Mucida, 2017) . This investigation shed light on the role of probiotic bacteria in modulating the immune response state of tolerised macrophage subsets (see Table 4.3). Endotoxin tolerance is also initiated upon pre-stimulation by TLR4-ligands with post-treatment by live probiotic bacteria and LPS. Heterotolerance or cross-tolerance may utilise different mechanisms to the conven-

tional homo-tolerisation method of endotoxin tolerance. In this investigation, M1-like macrophages show suppression of pro-and anti-inflammatory cytokines produced in homo-tolerisation. However, C28 or MS13 (containing possible TLR2 ligands) post-exposure of M1 and M2–like macrophages, up-regulated pro-inflammatory cytokine, in both macrophage subsets.

There is contradictory regulation by probiotics of IL-6 and TNF-α, as observed in conditions whereby TNF-α is augmented or highly expressed and where IL-6 expression is low or suppressed (Ahmed and Ivashkiv, 2000). Where IL-6 acts as a negative mediator of anti-inflammation, SOCS-1 induces by IL-6 to inhibit Th1 cytokine expression, reflecting a partially negative feedback mechanism (Diehl et al., 2000). Th-1 driven pathologies such Crohn's disease may benefit from probiotics that suppress harmful immune reactions or induce cytokines. Previous studies using hetero–tolerance such as LPS/PGN and LPS/LTA identified the upregulation of IL-6 in M1-like macrophage and no significant changes in IL-10 upon stimulation with PGN/LTA. M2-like macrophages also showed similar suppression in the presence of LPS/LTA or LPS/PGN of cytokines TNF-α, IL-1β, IL-10 and IL-6 (Alshagdali, 2018). Studies found no cross-tolerisation in MDP and TLR2 in human monocytes or TLR4 signalling in the secretion of IL-6 (Kullberg et al., 2008). Modulation of macrophages by live probiotic bacteria stimulates TNFα through the induction of the TLR-2–ligand in M1 and M2–like macrophages.

Shida et al. (2009) reported that peptidoglycan is recognised by TLR2 and NOD2; whereby a product of peptidoglycan (PGN) digestion, muramyl dipeptide (MDP), is recognised. Accordingly, Habil (2013) revealed that probiotic treatments selectively modulate LPS induction of Tollip expression in macrophage cell subsets. Zhang and Ghosh (2002) reported that upregulation of Tollip di-

rectly associates with TLR2 and TLR4, playing an inhibitory role in TLR-mediated and IL-1 receptor cell activation. Modulation of LPS tolerance by probiotics requires further study to understand gene expression and its influence.

4.5.2.3: Regulator activator molecules in tolerisation by probiotics in M1 and M2-like macrophages

To the best of the author's knowledge, tolerance to live probiotic bacteria has not been extensively studied through LPS induction or pre and post-treatment by *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) induction of tolerance upon TLR2.

Surprisingly, the TLR-2 receptor signal, induced with C28 and MS13 in macrophage subsets, was markedly inhibited, without affecting TNFα production of tolerised M1-like macrophages. The TLR4-mediated response was downregulated in tolerised M1 by both C28 and MS13. The inhibition of TLR4 and TLR2 of tolerised M1 might be associated with the expression of NLRP3. Llewellyn & Foey (2018) reviewed inflammatory cytokine regulation upon probiotic bacteria, inducing endogenous negative regulators of TLR2 signalling, such as Tollip, IRAK-M and the NF-ƙB p50/p50 homodimer. For example, the TLR2 ligation may induce a suppressive effect on the TLR4-mediated inflammatory response via expression or activation of IRAK-M and Tollip. Probiotic bacteria regulate inflammatory responses via the induction of a range of endogenous negative regulators of TLR signalling, such as IRAK-M, Tollip, A20, Ubcl2, Bcl-3 and p50/p50 NF-ƙB homodimer. Here, we show the selective inhibition of STAT3 in tolerised M1s, dependent on probiotic strain and the downregulated expression of IL-10, encouraging the inhibition of SOCS3. Moreover, downregulation of TLR2 may explain SOCS3 inhibition and the upregulation of TLR4 associated with the upregulation of STAT3 in tolerised M2 through probiotic bacteria.

In contrast with these results, STAT3 is associated with anti-inflammatory signalling of IL-10 and IL-6, while SOCS3 is inducible by IL-10 and IL-6. This can suppress pro-inflammatory cytokine gene expression, and negatively feedback to inhibit IL-10 and IL-6 signalling. *Bifidobacterium* species decreased LPS-induced IL-1 and TNFα mRNA levels in murine RAW264.7 macrophage cells (Okada *et al.*, 2009), correlated with inhibition of IκB phosphorylation and increased mRNA levels of SOCS1 and SOCS3. This study revealed the role of C28 and MS13 in the upregulation of Tollip by tolerisation of M1–like macrophages. In contrast, expression of endotoxin tolerance suppressed TLR signalling, including A20, Tollip, IRAK-M, TRIAD3A and p50/p50 NFκB (Biswas & Lopez-Collazo, 2009).

Understanding the role played by probiotics in motivating endotoxin tolerisation will allow specific and selective consumption of probiotic bacteria to treat pathological conditions. The tolerisation mechanisms by which innate immune signalling occur is diverse. These mechanisms have been partially defined, consisting of PAMP binding antagonism, down-regulation of PRR expression and the Induction of suppressive cytokines.

Conclusions

In conclusion, this study suggests that the tolerisation of macrophage subsets differentially respond to probiotic bacteria. Tolerised M1-like macrophages failed to suppress the pro-inflammatory cytokines, except IL-18. While both strains downregulated the gene expression of TLR2 in macrophage subsets. However, M1-like macrophage tolerisation by C28 resulted in the upregulation of the (TNF-α, IL-1β, IL-12) and anti-inflammatory (IL-10 and IL-6).

On the other hand, MS13 upregulated (TNF-α, IL-1β, IL-18, IL-23, IL-10) and downregulated (IL-12 and IL-6). *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius* strain MS13 drove the upregulation of TRAIL and NLRP3, accompanied with upregulation of cytokine production of TNF-α and IL-1β by M1-like macrophages.

While they downregulated TRAIL and NLRP3 as well as leading to the down regulation of TNF-α and IL-1β by M2-like macrophages. Generally, the tolerance induced by C28 and MS13 inhibited the negative regulatory molecules (Tollip and SOCS3) in macrophage subsets. MS13 upregulated STAT3 in M1 and M2-like macrophages. Probiotic bacteria upregulated anti-inflammatory cytokines (IL-10 and IL-6). Conversely, the effect of endotoxin tolerance by K12-LPS compared to the tolerisation by C28 and MS13, showed downregulation of pro-inflammatory cytokine production (TNFα, IL-1 β, IL-18) by tolerised M1 and M2-like macrophage subsets by K12-LPS.

Whereas, IL-10 and IL-6 were upregulated in M2-like macrophage, induced by endotoxin tolerance. Moreover, negative regulatory molecules Tollip, STAT3 and SOCS3 were upregulated in M1 and M2 –like macrophages. These findings can lead to better understanding of the role of probiotic bacteria, especially in

endotoxin tolerance states, to be selectively utilised in the control of gut mucosal inflammatory pathology such as CD or suppressive pathology, such as colorectal cancer (CRC).

Chapter 5:

Probiotic bacteria modulate inflammatory response of gut epithelial cells and epithelial cell-macrophage co-cultures

5.1 Introduction

The intestine is the central location of digestion, nutrient, and water uptake regulation (Kvietys and Granger, 2010). Its main functions include the uptake of nutrients across the intestinal epithelial barrier and to prevent the effects of harmful pathogens and their their antigens (Clayburgh,et al.,2004). For this ambivalent role, intestinal epithelial cells (IECs) and the gut-associated lymphoid tissue (GALT) collaborate through finely tuned cohabitation (Spahn & Kucharzik, 2004). In the gut, epithelial cells display a variety of functions. They act as a physical barrier, separating the external environment of microbiota and food antigens from the internal environment. Through tight junction (TJ) connections, the IECs form a semi-permeable barrier that restricts the translocation of luminal material. In the underlying lamina propria, non-inflammatory intestinal macrophages contribute to the protection from invading pathogens by active phagocytosis (Smythies et al., 2005). Epithelial cells and other cell types react to environmental factors such as cytokines and inflammatory molecules, a requirement when building up a gut model (epithelial cells /macrophages). Provoked macrophage responses are dependent on the tissue environment and the resulting cell subsets. During inflammation *milieu*, the macrophage resembles the M1 subset, whilst homeostatic macrophages resemble M2 macrophage subset (Mantovani et al., 2007). The uncontrolled immune responses of macrophages induced by stimuli are implicated in gut diseases, particularly Inflammatory Bowel Disease, IBD, associated with loss of gut tolerance.

The precise aetiology of IBD is a matter of debate. However, the understanding of the pathophysiology of IBD has advanced in recent years through studies applying *in vivo* and *in vitro* models (Ni *et al.*, 2020). In recent decades, re-

searchers have accelerated the investigation of AMP expression, especially, hBD-2. It is thought these could act as a bactericidal for other pathogenic microorganisms whilst modulating the mucosal immune response (Cheng *et al.*, 2019; Jäger, et al., 2013; Mahlapuu *et al.*, 2016). Thus, the determinative aspects directing the hBD-2 expression are very important for sustaining gut mucosa associated with barrier function. It is known that there is a clinical association between hBD-2 expression and the disease, especially between IBD and deficiency in hBD-2 production (Baumgart & Carding, 2007; McGuckin *et al.*, 2008; Wehkamp *et al.*, 2005). Within probiotic research, there is renewed interest in the role of probiotics in the treatment of gut diseases. In the host, probiotics modulate gut microbial content and the local immune response, whilst maintaining the integrity of the gut barrier (Klaenhammer et al., 2012; Courcoulas *et al.*, 2017).

Kotzampassi *et al.* (2012) reported that evidence from randomised clinical trials (RCTs) supports the therapeutic use of probiotics for reducing the incidence of antibiotic-associated diarrhoea (AAD), Clostridium difficile infection (CDI) and acute gastroenteritis. Probiotics reduce symptoms when administered in paediatric populations with acute gastroenteritis (El Feghaly *et al.*, 2013). In general, probiotics are beneficial to treat and protect from GIT diseases, such as pouchitis, infectious diarrhoea, irritable bowel syndrome (IBS), *Helicobacter pylori*, CDI, and ADD (Allen *et al.*, 2013; Ritchie & Romanuk, 2012; Sánchez *et al.*, 2017). Hence, the choice of probiotics is key for the treatment or prevention of GIT disease. Two factors need to be considered; (1) the type of probiotic stain and (2) the type of disease.

Caco-2 cells were used as a model for intestinal epithelial cells in this study. After differentiation, they become enterocyte/M-like cell. Micro fold cells are found in gut-associated lymphoid tissue (GALT) of the M-cells which lack microvilli (Mabbott *et al.*, 2013). However, like other epithelial cells, they are characterised by strong cell junctions and express markers such as Ulex europaeus agglutinin (UEA)-1 mediated endocytosis (Kimura, 2018; Rochereau *et al.*, 2013). The human receptor for poliovirus CD155 mediated *Poliovirus transcytosis* and *Salmonella* pathogenicity island 1 (SP-1) mediated *Salmonella* translocation, they express an advanced response to inflammatory stimuli (Garai et al., 2012; Martinez-Argudo & Jepson, 2008). Pathological cases of the gut may release inflammatory cytokines such as TNF-α and IL-1β, leading to the destruction of epithelial tissue in IBD conditions (Cesaro *et al.*, 2009; Neuman, 2007). Several studies showed that treating Caco-2 cells with TNF-α enhanced the induction of mediators including IL-8, ICAM-1, IP-10, MCP-1, TNF-α and MMP-1 (Sonnier *et al.*, 2010b; Treede *et al.*, 2008), whereas IL-1β treatment induced IL-2 and RANTES CCR1 receptors (RodrÍGuez *et al.*, 2001; Rodríguez *et al.*, 2012). A number of studies have shown that probiotics have a significant role in inducing cytokine expression in Caco-2 cells (Bahrami et al., 2011; Dickinson *et al.*, 2012; Hosoi *et al.*, 2003). It appears that probiotics modulate cytokine production in Caco-2 stimulated by inflammatory cytokine, particularly probiotics *L. paracasei or L. plantarum* directed IL-6 production in Caco-2 cells treated with IL-1β (Reilly, 2007). Furthermore, probiotics induce hBD-2, which is important for killing pathogenic microorganisms, and at the same time modulate the mucosal immune response (Huttner and Bevins, 1999, Klüver et al., 2006). Hence, the determining factors controlling the hBD-2 expression are very important in terms of maintaining gut mucosa associated with barrier function. Important to

the entire discipline of hBD-2 expression is the concept of a clinical association between disease, especially between IBD and deficiency in hBD-2 production (Baumgart & Carding, 2007; Wehkamp *et al*., 2005). Probiotic bacteria also modulate exogenous, membrane bound and intracellular cytokine-induced hBD-2. Whether this is through human intestinal epithelial cells or Caco-2 is unknown at present. Similarly, *in vivo* gut physiology prototypes use amalgamation of different cell types to investigate the immunomodulation of probiotics on the barrier cell function. Expressions of tight junction proteins are required for the formation of an epithelial barrier, maintenance of integrity and for polarity. Differentiation of epithelial cells occurs through a series of changes to form a monolayer with a tightly packed selectively permeable membrane with measurable trans-epithelial resistance. (Kim *et al.*, 2016; Parlesak *et al.*, 2004), have reported that co-culturing of Caco-2/PBMCs modulate cytokine kinetics during bacterial challenge (non-pathogenic *Escherichia coli*) in a grouped co-culture model. Bacteria were added apically to the Caco-2 cell layer and it was reported that the production of TNF-α, IL-12, IL-1β, IL-8, IL-6, IL-10, and TGF-β was significantly lower compared to bacterial stimulation of leucocytes beneath the Caco-2 cells (Parlesak *et al.*, 2004). Caco-2 modulates cytokine expression of the induction of leucocyte-epithelial cells in parallel with the modulation of the cytokine response in the gut mucosa. Similarly, (Haller *et al.*, 2000; Servin, 2004) reported that when co-cultured with human blood leucocytes, Caco-2 profiles of TNF-α, IL-1β, IL-8 monocyte chemoattractant protein 1 (MCP-1) and IL-10 were modulated in response to stimulation of Caco-2 cells with non-pathogenic (*Lactobacillus sakei*) and entero-pathogenic bacteria. The barrier function of epithelial cells was addressed by this co-culture model. According to Tanoue *et al.* (2008), this apical co-culture system of Caco-2 / RAW264.7 cells macrophage

(basolateral side) allows the assessing of the anti-inflammatory effect of food factors (Xu *et al.*, 2013). Furthermore the stimulation of RAW264.7 cells with LPS was followed by a reduction in trans-epithelial electrical resistance (TEER), an increase in TNF-α production and IL-8 mRNA expression in Caco-2 cells. Furthermore, treatment with anti-TNF-α antibodies inhibited TNF-α production and IL-8 mRNA expression. At the start of this chapter, an established trans-well co-culture system was developed, enabling the comparison of two statuses of gut physiology. The first was inflammation (Caco-2/M1) and the second was homeostatic (Caco-2/M2). This enabled the examination of immunomodulatory effects of probiotics on barrier function influenced by LPS-stimulated macrophages (Tominaga *et al.*, 2013). Fully differentiated Caco-2 at apical compartment and THP-1 cells were cultured and differentiated by PMA (M1-like macrophages) (Ingoglia, 2017). Alternatively, cells were differentiated with vitD_3 (M2-like macrophages at lower section, apically treated with probiotics (Kawano *et al.*, 2016) and basolaterally stimulated with LPS to resemble gut pathology (inflammatory) and normal homeostasis, respectively (Shah *et al.*, 2016). The investigation focused on probiotic regulation of the epithelial cytokine, hBD-2, TEER (as an index of intestinal epithelial barrier function) and ZO-1 protein (associated with barrier function expression). These were tracked with gene expression and immunohistochemistry at homeostatic and chronic inflammation model.

To summarise, epithelial Caco-2 cells are the most appropriate model to replicate the intestinal environment, allowing for the evaluation of chemical movement through the intestinal barrier and their transport mechanisms. Permeability values assessed with this model correlate well with human *in vivo* absorption data (Press & Di Grandi, 2008). Severe adherence of experimental conditions,

coupled with careful control of the cell-culture procedures is crucial for obtaining a meaningful correlation with *in vivo* data (Angelis & Turco, 2011). The THP-1 human monocytic cell line enable monocyte derived M1-like and M2-like macrophage cell subsets to be analysed. Consequently, these two cell lines built a strong gut physiology model, whereby Caco-2 epithelial cells reacted with M1-like or M2-like macrophages to model inflammatory gut physiology and normal homeostasis. When confluent, Caco-2 cells express characteristics of enterocytic distinction and functionality, with features that consist of microvilli, large vacuolated mitochondria, and smooth and rough endoplasmic reticulum (Lee et al., 2009). Lo et al. (2004) mentioned the transwell-based Caco-2/Raji B cell culture model as leading to differentiating enterocytes into M-cell-like cells. The benefits of Caco-2 were also co-cultured with other cell types such as PBMCs and macrophages.

The aims of this chapter is to address the role of probiotic bacteria for modulation of gut inflammation by using Caco-2 as epithelial cells

Objective 1: Live probiotic bacterial strains regulate the induction of cytokine production or expression through intestinal Caco-2 epithelial cells.

Objective 2: Optimising the growth of Caco-2/monoculture by TEER to study the effect of probiotic C28 and MS13, which modulate epithelial barrier function.

Objective 3: Live C28 and MS13 regulate the the secretion of IL-8 by Caco-2 as indicator of inflammation.

Objective 4: Probiotic lactobacilli strains C28 and MS13 modulate the expression of hBD-2 and the epithelial barrier ZO-1. Monoculture of epithelial cells ex-

hibit cell barrier modulation and different cytokine expression profiles in response to the stimulus of TNF-α and IL-1β.

Objective 5: Probiotics modulate epithelial cells in co-culture model; Caco-2/M1 versus Caco-2/M2: inflammatory vs homeostatic environmental modelling.

5.2: Results

5.2.1: Epithelial cells grow and form the barrier and tight junction

The growth of epithelial cell integrity was determined using TEER, to measure the efficacy of the barrier in epithelial cells. Data showed that the barrier and tight junction of epithelial cells progressed through incubation time, starting with the first day and continuing on to days 7, 14 and 21. The growth of the tight junction was significant on the 21st day of incubation (400-702 Ohms-cm^2), P=0.001 (**Figure 5.2.1**). Most obviously, the growth of barrier significantly progression in the epithelial cell barrier between 7 and 21 days, compared to day 1, P≤ 0.001.

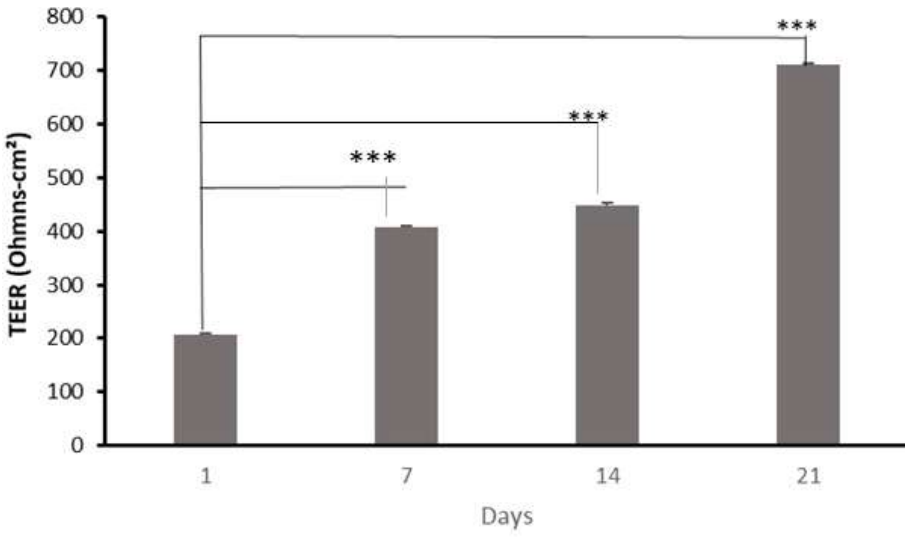

Figure 5.1: Time dependant development of epithelial cells barrier integrity

Caco-2 epithelial cells at density 5 x 10^5 cell/ml were differentiated in trans-well inserts for 21 days, the growth were checked regularly between the day one ,7 ,14 ,and 21 days TEER measurements were performed using EVOM epithelial voltmeter for Caco-2 epithelial cells. Data displayed represents duplicate samples for n=3 replicate exper-

iment epithelial cells. Significant effects compared to the control are indicated as *** P< 0.001.

5.2.2: IL-8 production induced by different serial dilution of *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* (MS13)

Probiotic treatments in cytokine production requires a specific bacterial density. Three densities of live probiotic bacteria were chosen from 1 $x10^5$, $1x10^7$ and $1x10^8$ cfu/ml, with the density of Caco-2 cell culture at $5X10^5$ cells/ml. Probiotic bacteria were live for 24 hours. Data in (**Figure 5.2.2**) show untreated Caco-2 cells (in grey) produced more IL-8 spontaneously. While, the effect of probiotic treatment on modulation epithelial cytokine production by IL-8. In particular, IL-8 production levels were inversely correlated with the density of C28. IL-8 was significantly suppressed by C28 and MS13 at density $1x10^5$ cfu/ml (49 pg/ml and 70 pg/ml, respectively), compared with untreated Caco-2 (grey bars) P≤0.001. Also, IL-8 decreased by augmenting the density of C28 to $1x10^8$ cfu/ml, whilst the concentration of MS13 at $1x10^7$ cfu/ml inhibited the production of IL-8 in Caco-2. All the densities of lactobacilli used in this experiment were decreased the level of cytokine production of IL-8 of Caco-2. Free to use of any concentration depends on the rate between bacteria cfu/ml / Caco-2 cell/ml that will be 2: 1 for next experiments.

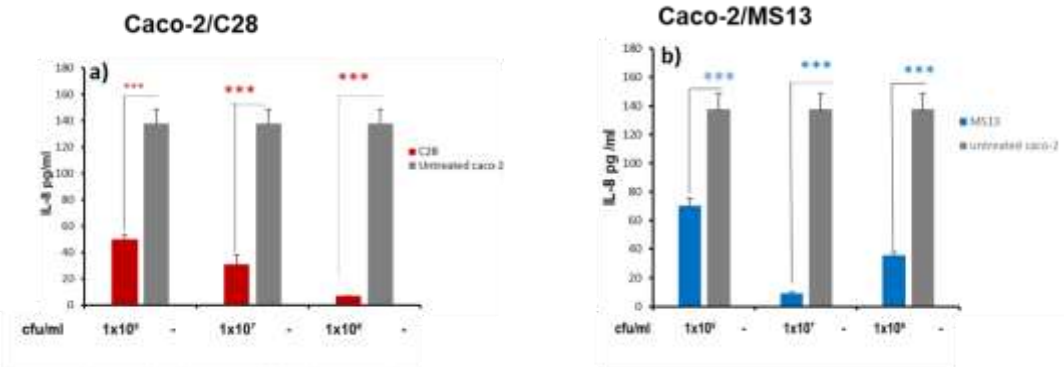

Figure 5.2: The level of the production of IL-8 by Caco-2 was varied depending on probiotic densities

Caco-2 epithelial cells were grown 21 days ($5x10^5$ cells/ml), then treated with different density of live *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) at density 1 $x10^5$, $1x10^7$ and 1 x 10^8 cfu/ml for 24 hrs. IL-8 cytokine production

is expressed as mean ±SE in pg/ml for Caco-2. Data displayed represents duplicate samples for n= 3 replicate experiments. Significant differences of the level of IL-8 induced by probiotic bacte-ria and untreated Caco-2 indicated as ***, *** P< 0.001.

5.2.3: *Lactobacillus plantarum* strain (C28) and *Lactobacillus salivarius* strain (MS13) induce epithelial cells to grow as well as the formation the barrier and tight junction

The effect of the two strains, C28 and MS13, of probiotic bacteria on the growth of epithelial cell integrity was determined using TEER, to measure the efficacy of the barrier function of epithelial cells. Caco-2 was fully differentiated ($5x10^5$ cell/ml) then incubated with C28 ($1x10^6$ cfu /ml) for 24 hours. The data (**Figure 5.2.3**) shows measuring TEER significantly progressed in the epithelial cell barrier in the presence of *Lactobacillus planetarium* (C28) compared with untreated Caco-2, P≤ 0.001. In addition, growth did not significantly progress in the epithelial cell barrier with MS13, compared with untreated Caco-2.

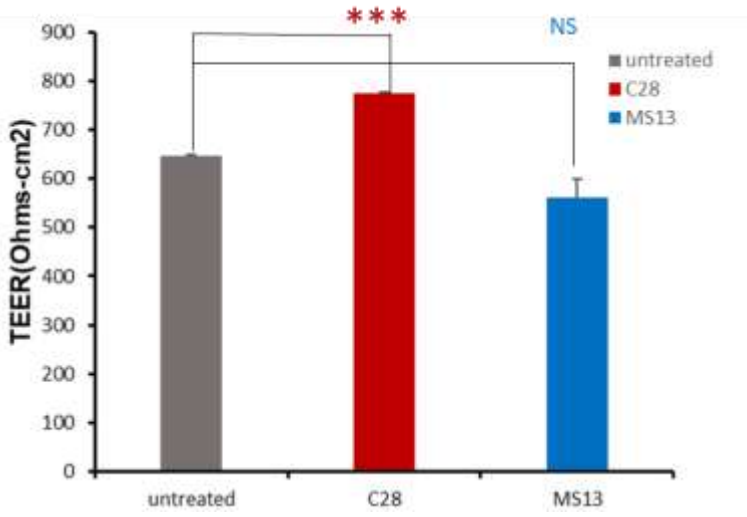

Figure 5.3: Probiotic bacteria *Lactobacillus plantarium* strain C28 and *Lactobacillus salivarius* (MS13) modulated epithelial cell barrier, using TEER

Caco-2 epithelial cells at density 5 x 10^5 cell/ml were differentiated in trans-well inserts for 21 days, followed by treatment with C28 and MS13 ($1x10^6$ cfu/ml) for 24 hours. TEER measurements were performed using EVOM epithelial voltmeter for Caco-2 epithelial cells. Data displayed represents duplicate samples for n=3 replicate experiment epithelial cells. Significant effects compared to the control are indicated as *** P< 0.001 and NS=not significant differences.

5.2.4: Kinetics of TNF and IL-1β induced epithelial cell IL-8 secretion

Next, we examined the time points of IL-8 production through the inflammation milieu of gut epithelial cells; points were at 2, 4 and 24 hrs of incubation. Results showed a significant stimulation of Caco-2 induced by TNF-α within 2 and 4 hours, compared with the level of IL-8 after 24 hours (350 and 200 pg/ml), respectively (p≤ 0.001) (**Figure 5.2.4 a**). In contrast, there was a significant increase of IL-8 stimulation in Caco-2 induced by IL-1β at 4 and 24 hours (10000 and 6000 pg/ml; p ≤0.0010, compared with 2 hours of IL-8 stimulation (**Figure 5.2.4 b**).

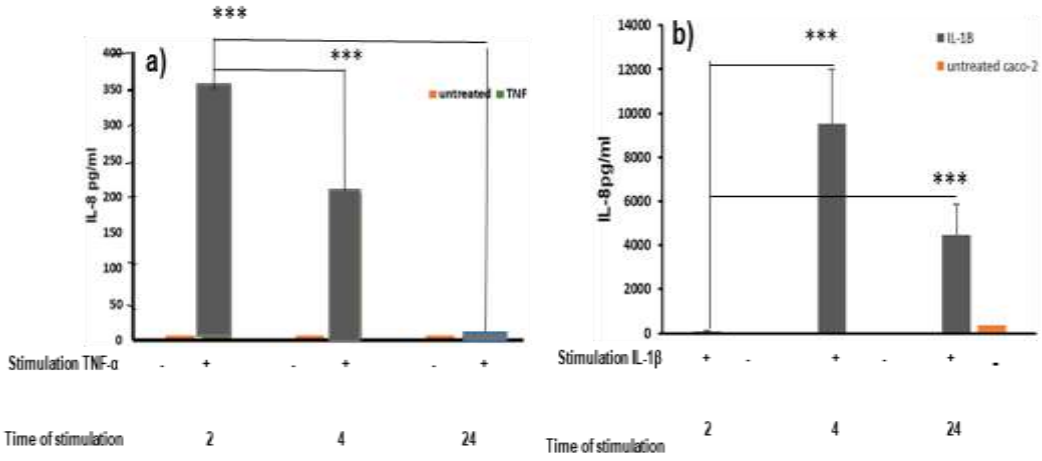

Figure 5.4: TNFα and IL-1β induction of IL-8 production of Epithelial cells time point dependant stimulation

5x10^5 cells/ml of Caco-2 epithelial cells were differentiated in trans-well inserts for 21 days, followed by addition of (a) [10 ng/ml] TNFα, or (b) [5 ng/ml] IL-1β for (2,4, 24 hrs) (b) and untreated cells (as a control). Cytokine production is expressed as the mean ± SE in pg/ml for IL-8, secreted into apical compartment. Data displayed represents duplicate samples for n= 3 replicate experiments. Significant differences between IL-8 production through time of stimulation of Caco2 cells are indicated as, *** P < 0.001.

5.2.5: Probiotic bacteria modulate the production of IL-8 in Caco-2 induced by TNF-α or IL-1β

To acquire baseline data about the role of live *Lactobacillus plantarum* (C28) and *Lactobacillus salivarius* (MS13*)* in modulating IL-8 production of Caco-2 epithelial cell behaviour. The levels of TNF and IL-1β -induced IL-8 was measured in homeostasis or chronic inflammation status (**Figure 5.2.5**). Diagram a-1 demonstrates the effect of probiotic bacteria on epithelial cells, whilst diagram b-1 and c-1 represent TNFα and IL-1β stimulation on epithelial cell monoculture and the response to these stimuli (a, b, c). The results showed that IL-8 production induced by C28 or MS13 decreased significantly by 75% and 57% (50 and 60 pg/ml, respectively), compared with the level of IL-8 of untreated Caco-2 (130 pg/ml; P≤0.001) (**Figure 5.2.5 a**) also showed earlier in (**Figure 5.2.2**). Stimulation of IL-8 production in Caco-2 was induced by TNF-α (350 pg/ml) in 2 hours (**Figure 5.2.5 b**) and was significantly suppressed by the treatment of C28 and MS13 by 49% (180 pg/ml), P≤0.001. On the other hand, the comparison between IL-8 production of Caco-2 induced by TNFα for 2 hours and untreated Caco-2 were significantly upregulated by 2.5-fold (p ≤0.006) (**Figure 5.2.5 a /b**). **Figure 5.2.5** c details the effects of treatment with C28 or MS13 on the production of IL-8 in Caco-2 induced by IL-1β. Here, IL-8 increased significantly by ½ fold, compared to the level of IL-8 in Caco-2 induced by IL-1β. Further analysis showed that the changes to IL-8 production by Caco-2 induced by IL-1β were not significant, compared to the level of IL-8 from untreated Caco-2 (**Figure 5.2.5 a/c**).

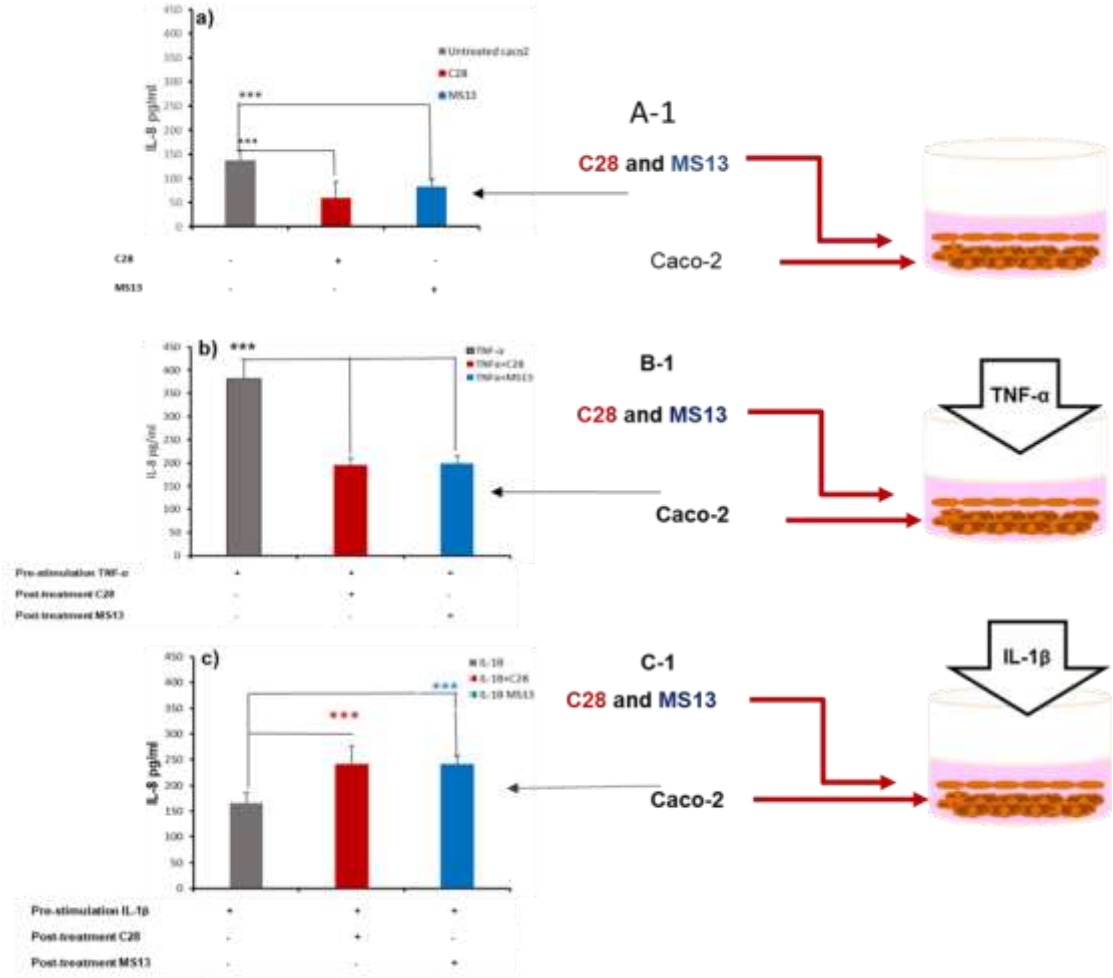

Figure 5.5: Probiotic bacteria differential modulated of IL-8 by Caco-2 /epithelial cells induced by TNF-α and IL-1β

5x10^5 cells/ml of Caco-2 epithelial cells were differenti-ate in trans-well inserts for 21 days, followed by addition of Caco-2 cells pre-treated 5 X 10^5 cell/ml with TNF-α or IL-1β for 2 hrs, followed by modulation with Lactobacillus plantarum (C28) and Lactobacillus salivarius (MS13) at a density of 1 x 10^6 cfu/ml for 22 hrs, Cytokine production of IL-8 induced by C28 and MS13 (a), IL-8 production induced by TNF-α (b) and the induction by IL-1β (c) are expressed as the mean ±SE in pg/ml. Data displayed is a representative experi-ment with triplicate samples of n=3 replicate experiments. Significant effects compared to the controls (untreated Caco-2 +TNF-α and IL-1β) (grey bar) are indicated as *** ***, *** P<0.001. (C28 significance is indicated in red whereas MS13 significance is indicated in blue**).**

5.2.6: Probiotic bacteria selectively regulate TNF-α or IL-1β induced hBD-2 in epithelial cells

This part of the study focused on the role of probiotic bacteria in the induction of hBD-2 of epithelial cells, the gene expression of hBD-2 immunity, the homeostatic stage (untreated Caco-2 and treatment with C28 and MS13) and the inflammation stage (in the presence of TNF-α or IL-1β). Results showed that no changes in gene expression of hBD-2 in Caco-2 were induced by C28, compared with untreated Caco-2. Gene expression of hBD-2 of Caco-2 was induced by MS13 and significantly upregulated by 2-fold, compared with untreated Caco-2 ($P \leq 0.01$) (**Figure 5.2.6 a**). On the other hand, stimulation of Caco-2 by TNF-α for two prior hours significantly induced hBD-2 gene expression of Caco-2 2-fold, compared with untreated Caco-2 ($P \leq 0.05$) (**Figure 5.2.6 a/b**). Whereas, treatment with C28 did not change gene expression levels of hBD-2 in Caco-2 induced by TNF-α, compared with the induction by TNF-α. Treatment with MS13 significantly supressed gene expression of hBD-2 in Caco-2 when induced by TNF-α (80%), compared with stimulation of Caco-2 with TNF-α. This value was 86% with induction of Caco-2 by MS13 ($P \leq 0.001$) (**Figure 5.2.6 a/b)**. On the other hand, stimulation of Caco-2 by IL-1β significantly upregulated gene expression of hBD-2 6-fold, compared with untreated Caco-2 ($p \leq 0.01$). Treatment by C28 and MS13 significantly suppressed gene expression of hBD-2 in Caco-2 induced by IL-1β (99%), compared with gene expression of hBD-2 in Caco-2 induced by IL-1β (**Figure 5.2.6 c**). Further analysis revealed that C28 and MS13 suppressed gene expression of hBD-2 in Caco-2 induced by IL-1β compared with gene expression by C28 and MS13 alone (**Figure 5.2.6 a/c**).

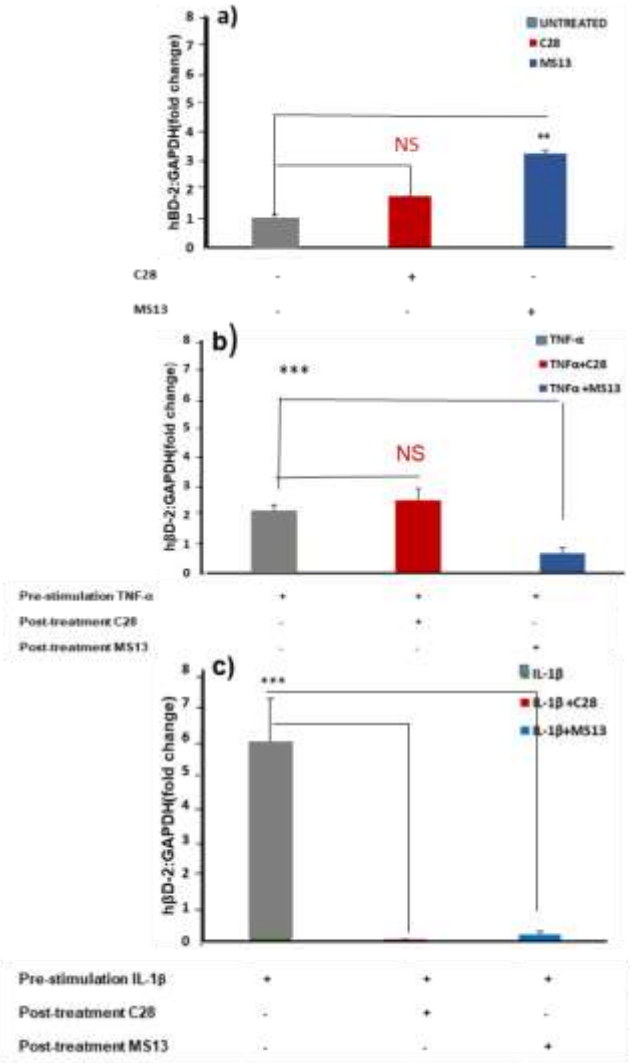

Figure 5.6: Probiotic bacteria differential modulated of the hBD-2 in Caco-2 /epithelial cellsinduced by TNF-α or IL-1β

Caco-2 cells pre-treated 5X10^5 cell/ml with TNF-α or IL-1β for 2 hours, followed by modulation with *Lactobacillus plantarum* (C28) *and Lactobacillus salivarius* (MS13) at a density of 1x10^5 cfu/ml for 22 hrs, a) Caco-2 epithelial cells (grey) pre-treated with 10 µg/ml of TNF-α (b) or 5µg/ml of IL-1β (c) for 2 hrs ,followed by modulation with C28(red) and MS13 (blue)1x10^5 cfu/ml for 22 hrs. , followed by stimulated by (c) then modulated by of 1x10^5 CFU/ml. hBD-2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al (2001) using 2– ΔΔCt **.** Data displayed is a representative experiment with triplicate samples of n=2 independent experiments. Significant effects compared to the (untreated Caco-2, C28 and MS13) are indicated as *** P<0.005 and NS=no significant difference. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

5.2.7: Probiotic bacteria modulated gene expression of ZO-1 in Caco-2 induced by TNF-α or IL-1β

To focus on the role of live C28 and MS13 in the induction of the epithelial cell barrier, gene expression of Zona Occludin-1 or ZO-1 was used. ZO-1 is a protein associated with the epithelial cell barrier function, linking trans-membrane proteins occludin, claudine and the actin cytoskeleton. an mRNA expression level of the ZO-1 gene was compared to GAPDH in Caco-2. Results showed that C28 and MS13 significantly augmented gene expression of ZO-1 in Caco-2 (2 and 2.82-fold respectively), compared with untreated Caco-2 ($P \leq 0.01$) (**Figure 5.2.7 a**). The induction ZO-1 expression in Caco-2 by TNF-α was unchanged, compared with untreated Caco-2 (**Figure 5.2.7 b/a**). Whereas, C28 suppressed ZO-1 levels in Caco-2 induced by TNF-α compared with Caco-2+TNF-α; this suppression was not significant. Interestingly, gene expression of ZO-1 in Caco-2 by MS13 was significantly induced by TNF-α 31-fold, compared with Caco-2/TNF-α and Caco-2/MS13 ($P \leq 0.001$) (**Figure 5.2.7 b/a**). On the other hand, induction of ZO-1 in Caco-2 by IL-1β was not significant compared with ZO-1 mRNA of untreated/Caco-2 cells (**Figure 5.2.7 c/a**). There was a significant induction of ZO-1 expression of ZO-1 of Caco-2 by C28 induced by IL-1β (200-fold), compared with Caco-2/ IL-1β ($P \leq 0.001$). In addition, MS13 did not change the levels of ZO-1 expression in Caco-2 cells induced by IL-1β, compared with Caco2/IL-1β. Further statistics demonstrated that MS13 significantly suppressed ZO-1 expression in Caco-2 (84%), compared with Caco-2 induced by MS13 ($P \leq 0.001$). Data is labelled, as bars are not visible due to low expression levels of ZO-1.

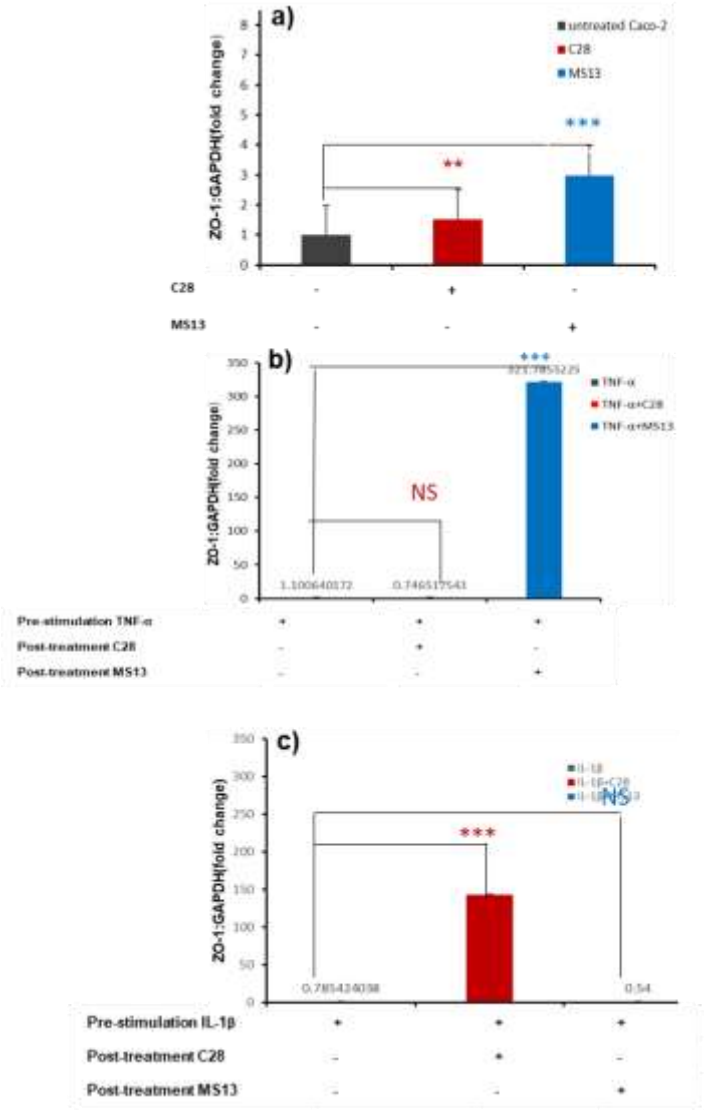

Figure 5.7:Probiotic bacteria modulated of ZO-1 in Caco-2 /epithelial cells induced by TNF-α and IL-1β

Caco-2 cells pre-treated 5X10^5 cell/ml with TNF-α or IL-1β for 2 hrs, followed by modulation with *Lactobacillus plantarum* (C28) and *Lactobacillus salivarius* (MS13) at a density of 1x10^5 cfu/ml for 22 hrs, a)Caco-2 epithelial cells (grey) pre-treated with 10 µg/ml of TNF-α (b) or 5µg/ml of IL-1β (c) for 2hrs, followed by modulation with C28(red) and MS13 (blue)1x10^{5}cfu/ml for 22 hrs. ,followed by stimulated by (c) then modulated by of 1x10^5 cfu/ml. ZO-1 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al.(2001) using 2– ΔΔCt . Data displayed is a representative experiment with triplicate samples of n=2 independent experiments. Significant effects compared to the (untreated Caco-2, C28 and MS13) are indicated as ** P<0.01 and ***,*** P<0.005 and NS, NS=significance. (C28 significance is indicated in red whereas MS13 significance is indicated in blue).

5.2.8: Probiotic strains differentially modulate homeostatic and inflammatory cytokine induction of TNF-α and IL-10 expression in Caco-2 epithelial cells

C28 and MS13 have both, selectively modulate epithelial cell barrier integrity (ZO-1), antimicrobial defence (hDB-2) and neutrophil responses (IL-8) in both homeostatic and pro-inflammatory environments. In a homeostatic environment, it is desirable that probiotics maintain barrier integrity and the ability to respond to pathogenic invasion. Exactly what happens to probiotic modulation in a pro-inflammatory pathological environment has not been documented. As a consequence of IBD manifestations predominated by TNFα or IL-1β, we investigated the modulatory capacity of these probiotic strains in the presence of these inflammatory cytokines and how this impacts the inflammatory environment. To this aim, gene expression of the pro-inflammatory cytokine TNFα and the anti-inflammatory cytokine IL-10 were investigated.

With respect to the induction of TNFα mRNA, untreated Caco-2 cells (homeostatic) resulted in low control levels, whereas in the presence of pro-inflammatory environments, TNFα mRNA was upregulated to a relative ratio of 100 ($p<0.001$, TNFα) and 9 ($p<0.001$, IL-1β). Both C28 and MS13 up-regulated TNFα mRNA by 8-fold in the homeostatic environment (**Figure**. **5.2.8 a)** ($p<0.001$). Probiotic modulation of TNFα mRNA induced in Caco-2 epithelial cells in the pro-inflammatory environment contained TNFα or IL-1β. Differential modulation was dictated by the inflammatory cytokine environment. In the case of an inflammatory environment predominated by TNF-α (**Figure 5.2.8 b**), C28 suppressed TNF-α by 83% ($p<0.01$), whereas MS13 suppressed gene expression of TNF-α in Caco-2 induced TNF-α by 60% ($p<0.05$). Probiotic modulation of TNF-α mRNA expression in an inflammatory environment was predominated

by IL-1β, however this resulted in a different response. C28 significantly upregulated the expression of TNF-α in Caco-2 induced by IL-1β by 74-fold ($p<0.001$) (**Figure 5.2.8 c**). Conversely, MS13 downregulated the expression of TNF-α in Caco-2 induced by IL-1β. This result was not significant (**Figure 5.2.8 c**).

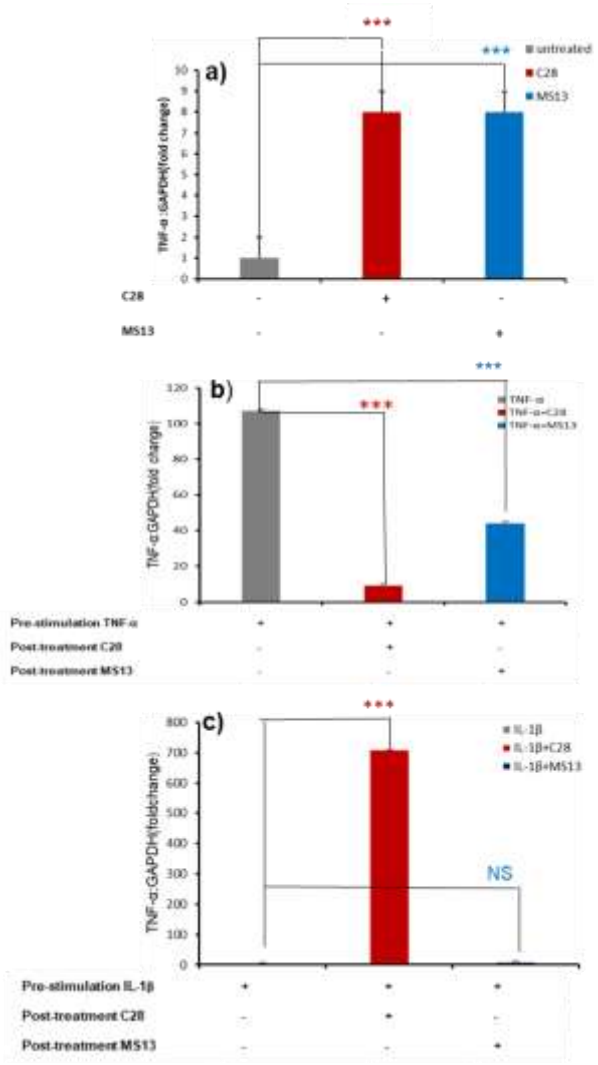

Figure 5.8: Probiotic bacteria differential modulated of TNF-α in Caco-2 /epithelial cells induced by TNF-α and IL-1β

Caco-2 cells were differentiated for 21 days, $5X10^5$ cell/ml after which time were either ; unstimulated (grey bar) or treated with C28 (red bar)and MS13 (blue bar) (a). Pre-treated with 10 ng/ml TNF-α (b) or 5 ng/ml IL-1β (c) for 2 hrs, followed by modulation with *Lactobacillus plantarum* (C28) and *Lactobacillus salivarius* (MS13) at a density of $1x10^5$ cfu/ml for 22 hrs. TNFα cytokine gene expression is expressed as the mean ±SE fold change, using resting unstimulated cells as a calibrator sample and GAPDH as a reference gene as described for the $2^{-\Delta\Delta}$Ct method by Livak et al., 2001. Data displayed is a representative experiment of triplicate samples for n=3 independent experiments. Significant effects of the probiotic strains in homeostasis (a)and inflammation (b/c) compared to unstimulated and in TNFα/IL-1β-stimulated controls are indicated as *** ,***$p<0.001$ and NS (non-significant).

With the induction of IL-10 mRNA (**Figure 5.2.9 a**), expression in treated Caco-2 cells (homeostatic) with C28 showed no change in IL-10 mRNA expression compared with untreated Caco-2. The induction of Caco-2 with MS13 resulted in significant up-regulation of IL-10 mRNA expression by 2.2-folds ($p<0.001$). Whereas, in the pro-inflammatory environment (**Figure 5.2.9**), IL-10 mRNA was significantly upregulated at a relative ratio of 3 ($p<0.001$) with TNFα, compared with untreated Caco-2. In contrast, in the presence of IL-1β (**Figure 5.2.9 a/c**), there was no significant difference compared with untreated Caco-2 epithelial cells.

Probiotic modulation of IL-10 mRNA induced Caco-2 epithelial cells in the pro-inflammatory environments containing TNFα or IL-1β. These exhibited differential modulation dictated by the inflammatory cytokine environment. In the inflammatory environment predominated by TNFα (**Figure 5.2.9 b**), C28 significantly suppressed the expression of IL-10 mRNA by 50% ($p<0.001$), compared with Caco-2/TNFα. On the other hand, MS13 suppressed IL-10 expression in Caco-2 induced by TNFα by 65% ($p<0.001$), compared with Caco-2/TNF-α. The probiotic modulation of IL-10 mRNA in the inflammatory environment was predominated by IL-1β, resulting in a different response. C28 downregulated the gene expression of IL-10 in Caco-2 induced by IL-1β upon 50% ($p<0.01$), compared with Caco-2/IL-1β. MS13 significantly upregulated IL-10 expression in Caco-2 induced by IL-1β by 128-folds ($p<0.001$) (**Figure 5.2.9c**).

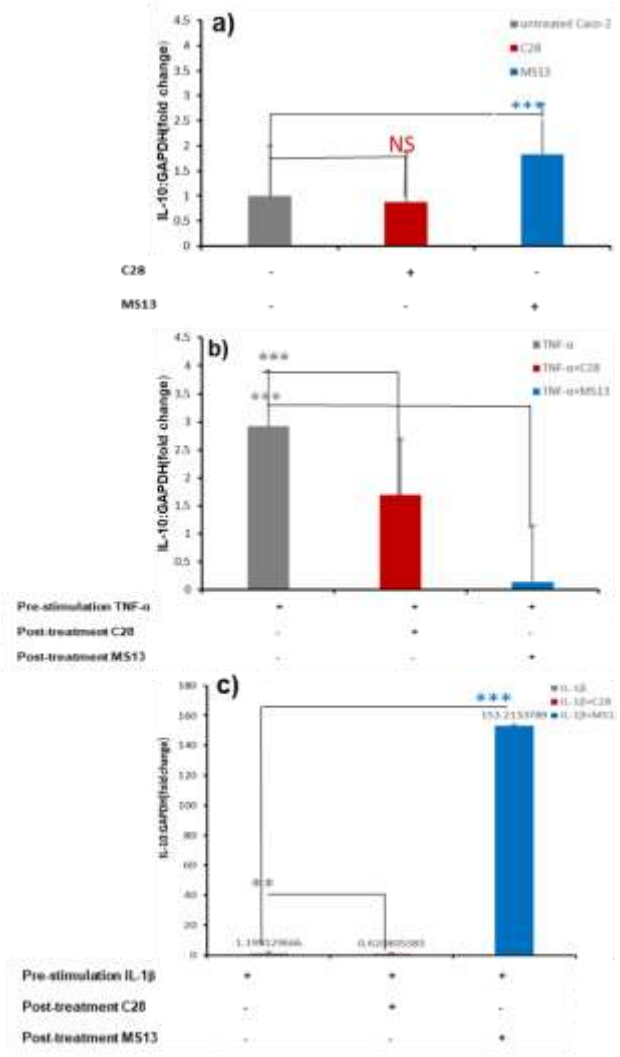

Figure 5.9: Probiotic bacteria differential modulated the expression IL-10 mRNA in Caco-2 /epithelial cells induced by TNF-α and IL-1β

Caco-2 cells were differentiated for 21 days, 5X10^5 cell/ml after which time were either ; unstimulated (grey bar) or treated with C28 (red bar)and MS13 (blue bar) (a). Pre-treated with 10 ng/ml TNF-α (b) or 5 ng/ml IL-1β (c) for 2 hrs, followed by modulation with *Lactobacillus plantarum* (C28) and *Lactobacillus salivarius* (MS13) at a density of 1x10^5 cfu/ml for 22 hrs. IL-10 cytokine gene expression is expressed as the mean ±SE fold change, using resting unstimulated cells as a calibrator sample and GAPDH as a reference gene as described for the $2^{-\Delta\Delta}$Ct method by Livak et al., 2001. Data displayed is a representative experiment of triplicate samples for n=3 independent experiments.Significant effects of the probiotic strains in homeostasis(a) and inflammation (b,c) compared to unstimulated , TNFα/IL-1β-stimulated controls are indicated as *** ,***p<0.001.5 and NS (non-significant).

5.2.9: Modulation of IL-8 production by induction with C28 and MS13 in co-culture models Caco-2 /M1 and Caco-2/M2

Previous results in Chapter 3 and 4 report the role of probiotic bacteria in the modulation of macrophages. In this chapter, we investigated the modulation of

the inflammation by inflammatory cytokines TNFα and IL-1β of Caco-2 epithelial cells. To mimic the pathogenic bacterial effect that invading the broken site of epithelial barrier such as LPS, probiotic administration as a supplement to confer human health, in presence K12-LPS. **Figure 5.2.10 f** details the experiment design. To obtain the effect of probiotic bacteria in co-culture of Caco-2/M1-macrophage induced by K12-LPS, C28 and MS13 were apically added to Caco-2 and basolaterally on M1-like macrophage (**Figure 5.2-10 a/b**). The grey bar (**Figure 5.2.10 a**) demonstrates the Caco-2/apical and the grey bar shows the M1-untreated (**Figure 5.2.10 b**) homeostatic condition. Surprisingly, un-treated Caco-2 produced high secretion of IL-8 with the concentration of IL-8 of un-treated M1-macrophage at 200 pg/ml. The second pair Caco-2+C28 /M1+LPS bars (red apical/ black basolateral) show how C28 probiotic significantly down-regulates IL-8 production in Caco-2 induced by LPS and IL-8 macrophages of M1 (basolateral) increased, compared with untreated M1 ($p \leq 0.001$).

Interestingly, in the third pair of bars Caco-2+MS13 /M1+LPS (blue apical/black basolateral), Caco-2 had significantly reduced IL-8 with the probiotic strain MS13 ($p \leq 0.001$). The last pairs of CaCO-2+LPS/M1+LPS (black apical/black basolateral) had significant reduction in IL-8 Caco-2/LPS ($p \leq 0.001$), compared with untreated Caco-2. However, IL-8 of M1-LPS was not significantly changed compared with untreated M1-macrophage. The same process was repeated with co-culture (Caco-2/M2-macrophage **(Figure 5.2.10 c/d**). For the control group, untreated Caco-2 (grey bar) released 850 pg/ml of IL-8. Untreated M2-macrophage located basolateral (yellow bar) (**Figure 5.2.10 d**) released low levels of IL-8 (7 pg/ml). In the second bar (Caco-2+C28 apical/ M2+LPS baso-lateral) (red/black), C28 significantly inhibited IL-8 production in the presence of probiotics C28 and MS13, respectively ($p \leq 0.001$). However, the production of

IL-8 M2-macrophage induced by LPS increased significantly, compared with IL-8 of untreated M2 (p≤0.001). The last bar of apical Caco-2 induced by LPS/ M2 induced by basolateral LPS showed that Caco-2 significantly inhibited the production of IL8 induced LPS in M2 (p≤0.001).

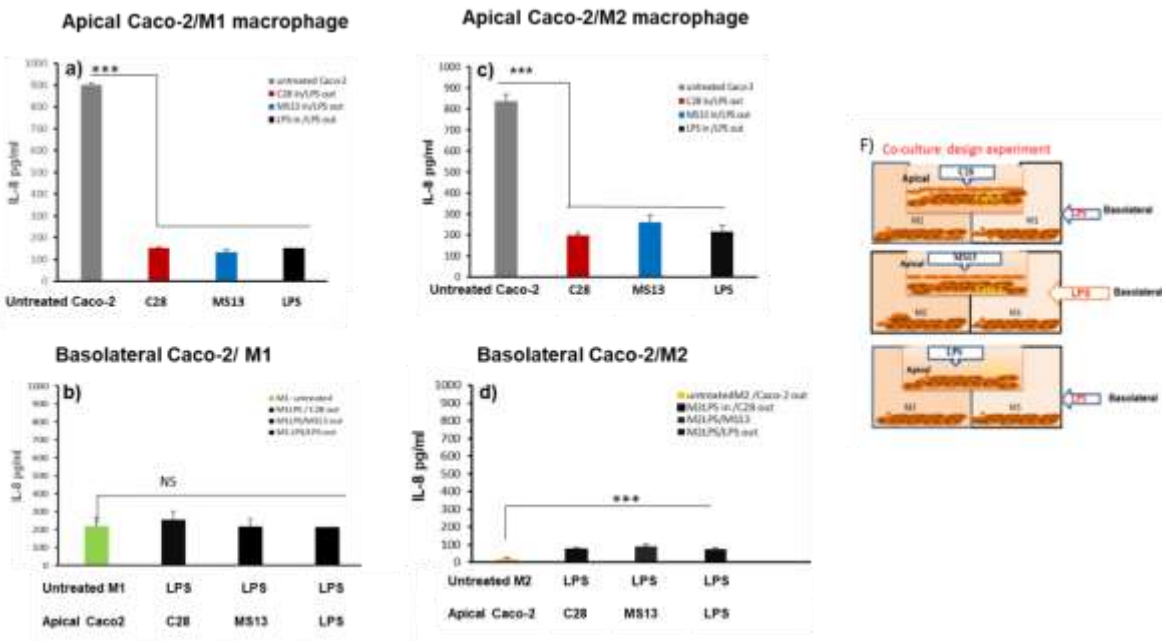

Figure 5.10: The impact of probiotic bacteria on the production of IL-8 by Caco-2 and macrophage subset when co-culture

The co-culture was a model for inflammatory environment**.** Co-culture models of Caco2/M1(a) and Caco2/M2 cells were stimulated *Lactobacillus plantarum* (C28) (red)and *Lactobacillus salivarius* (MS13)(dark blue) at a density of $1x10^5$ cfu/ml for 24 hours added to the apical side, with 100 ng/ml of K12-LPS (black bars)added apically as control.(homeostasis) or basolateral side (inflammatory) in the co-culture models. Caco2/M1 apical/basolateral (a/b) supernatant ,Caco2/M2 (c/d) apical/ basolateral supernatant (orange). IL-8 production is expressed as the mean ± SD in pg/ml n=2 replicate experiments. Significant effects of the probiotic strains apical co-culture Caco-2/macrophage subsets compared with untreated Caco-2, and untreated M1 and M2 macrophage in the presence of LPS and inflammation (b,c) compared to unstimulated , TNFα/IL-1β-stimulated controls are indicated as *** p.

Summary of Chapter Five

Table 5.1: Summary of the chapter Five, Cytokine production (IL-8) and gene expression of TNF-αmRNA, IL-10 mRNA, ZO-1 mRNA, and hBD-2 mRNA.

		IL-8	hBD-2 mRNA	ZO-1 mRNA	TNF-α mRNA	IL-10 mRNA
Homeostasis	**Caco-2** untreated	↑	*	*	*	*
	Caco-2/C28	↓	↔	↑	↑	↔
	Caco-2/MS13	↓	↑↑	↑	↑	↑
Inflammation	**Caco-2/TNFα**	↑	↑	↔	↑↑↑	↔
	Caco-2/IL-1β	↑	↑↑↑	↔	↔	↔
Modulation of the inflammation by probiotic	**Caco-2/TNF-α C28**	↓↓↓	↔	↔	↓↓↓	↓
	Caco-2/TNF-α MS13	↓↓	↓↓↓	↑↑↑	↓↓	↓↓↓
	Caco-2/IL-1β +C28	↑	↓↓↓	↑↑↑	↑↑↑	↔
	Caco-2/IL-1β +MS13	↑	↓↓↓	↓↓↓	↔	↑↑↑

↑ increased compared to the untreated caco-2 *, TNFα or IL-1β

↑↑↑↑ Upregulation compared to the control (untreated Caco-2 or TNFα or IL-1β)

↓↓↓ suppression compared to the control (untreated Caco-2 or TNFα or IL-1β)

↔ = no significance differences compared to the untreated Caco-2, or TNFα or IL-1β)

*Release and expression by untreated Caco-2

5.3: Discussion

The gut is a complex system, containing numerous collaborating cell types and microbiota. Models to study this system should take into account as many of these factors as possible. Indeed*, in vitro* cell models of the gut should functionally be similar to the *in vivo* models. There are numerous cell line gut models, utilising Caco-2, HT-29 (human epithelial cells originated from colon adenocarcinoma) and HIEC-6 (normal human epithelial cells originated from small intestine) (Cencic and Langerholc, 2010). Amongst these lines, Caco-2 cells are commonly used as a model of human intestinal epithelial cells, as they offer easy handling and maintenance. In culture, these cells undergo a process of impulsive differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005). They are also known for spontaneous differentiation from a colonic to a small intestinal-like phenotype (Pinto et al., 1983). Caco-2 cells offer transport and permeability characteristics, similar to human intestinal tissue (Lennernas et al., 1996; Rubas et al., 1993). The application of Caco-2 cells has generated valuable data regarding pharmacokinetics and toxicological impacts of compounds (Awortwe, Fasinu & Rosenkranz, 2014). However, cell monocultures are not capable of mimicking the complex structure defining the intestine.

Macrophages can recognise and respond to microbes or diseased/cancerous cells (Guerriero, 2018; Hao *et al.*, 2012). Through processing, they present antigen to the helper T-cell, motivating the adaptive immune responses (Santos *et al.*, 2019). Many studies have utilised a combination of cell types (co-culture model), to study the immunomodulatory effects of probiotic bacteria in a range of gut mucosa, such as DCs and epithelial cells. Hence, the objective of this

study was to study further the cell characteristics and find a model of intestinal mucosa in both homeostatic and inflammatory states. To achieve this, intestinal epithelial cells were stimulated with pro-inflammatory compounds, such as LPS, from intestinal microflora and cytokines such as TNFα and IL-1β. The present study was designed to regulate the immunomodulation of different stimuli (TNFα and IL-1β) by probiotic bacteria on epithelial cell barrier function (cytokine production and gene expression). To study stimulation of inflammation, TNFα or IL-1β were in two status; normal/homeostatic and chronic inflammation. Experiments for the induction of macrophages were directed by addition of LPS to the basolateral surface.

5.3.1: Probiotic strains selectively induce IL-8 in the Caco-2 intestinal epithelial cell model

The features of Caco-2 cell line represent a good *in vitro* model of the human small intestinal mucosa to predict the absorption of orally administered drugs (Linnankoski *et al.*, 2006), therefore, it is used, in this study, as a model to investigate the immuno-modulatory effects of probiotics. The gut epithelial cells are also actively involved in the defence of the gut by releasing cytokines, which orchestrate the recruitment (IL-8) of immune cells into the gut mucosa. Furthermore, other epithelial cell-derived cytokines perform important roles in immune-regulation (e.g. IL-10 and TGF-β), and tissue repair (e.g. TGF-β) (Wells et al., 2011 a). Thus, the present study, focused on the effect of probiotics on epithelial cell-derived cytokines. Though epithelial cells discharge an array of cytokines in response to different stimuli, there are differing reports about the spectrum of cytokine release either during normal homeostasis or in inflammation (Ohkusa et al., 2009, Bahrami et al. 2011b). It is well known that epithelial cells constitutively expressed IL-8; Becker, Koren and Henke (1993) reported

high levels of interleukin-8 (IL-8) mRNA but invisible levels (< 1 mRNA copy/cell) of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-6, IL-1, or tumour necrosis factor (TNF) mRNA. However, the expression of IL-8 by epithelial cells is increased after bacterial attack (Eckmann et al., 1993b). This is appropriate to recruit immune cells, such as neutrophils to the site of infection. The Neutrophil perform direct killing of pathogens by phagocytosis, or indirect killing by cytokines and antibodies. Conversely, accumulative infiltrate of immune cells will lead to chronic inflammation and tissue destruction.

In this part, two strains of probiotic bacteria were used to investigate the potential of probiotics in inducing IL-8 by Caco-2 cells. The results showed that live bacteria successfully inhibited IL-8, dependent on bacterial cell density cfu/ml and strain (**Figure 5.2.3 a**). These represent the role of C28 and MS13 in reduction of infiltrating cells to site of inflammation in the gut. These findings however, are contradictory (Malago *et al.*, 2010; Malago, Tooten & Koninkx, 2010); who reported elevation of IL-8 production by Caco-2, due to increasing cell density of *Lactobacillus salivarius*. They used between 200-500 live bacteria /Cell. While, Candela et al. (2008), reported that live *Lactobacillus acidophilus*, *L. plantaum*, *Bifidobacterium longum* and *B. lactis* induced IL-8 in monolayer HT-29 cells, also converse with (Roselli *et al.*, 2006). They studied the stimulation of Caco-2 by the treatment of uninfected cells with *Bifidobacterium animalis* or LGG, inducing small levels of IL-8, associated with migration of low neutrophil numbers. Messaoudi *et al.* (2012) described the role of *L. salivarius SMXD51* in the induction of immune defence cytokines such as, (IL-6, IL-8, IL-10 and β-defensin 2), and augmentation of barrier integrity of Caco-2 cells. Bahrami et al. (2011a) reported that treating Caco-2 and HT-29 epithelial cell lines with Lactobacilli and Bifidobacteria obtained from healthy people, results in different profiles of pro-

inflammatory (IL-1β, IL-6, IL-18 and TNFα) and anti-inflammatory (TGF-β, IL-4 and IL-10) cytokines, the differences are probably reflective of probiotic strain variation.

5.3.2: Probiotics selectively modulate TNF-α or IL-1β induced Caco-2 IL-8

This study focused on investigating the role of probiotic bacteria in the modulation of the inflammation, by TNF-α or IL-1β in epithelial cells. The results of this study showed the stimulation of epithelial cells with TNF-α or IL-1β resulted in the release of IL-8 from Caco-2 cells. IL-8 is known to play a dominant role in the beginning and maintenance of intestinal inflammatory and immune responses. It is well established that TNF-α and IL-1β induce and up-regulate IL-8 mRNA expression and protein secretion in Caco-2 intestinal epithelial cells (Bai *et al*., 2005; Savidge *et al*., 2006; Sonnier *et al*.,2010). Additionally, Moon *et al.* (2000), demonstrated stimulation of the Caco-2 cells with IL-1 β to also increase IL-6 mRNA and protein levels. Thus, pro-inflammatory cytokines can perpetuate inflammatory responses of intestinal epithelial cells. Additionally, this study data demonstrated that the probiotic bacteria, C28 and MS13, inhibited the production of IL-8 by Caco-2 induced by TNF-α. These results agreed with (Ren *et al.*, 2013), who suggested that the role of live lactobacilli strains and their conditioned media efficiently inhibited IL-8 production in TNF-α-induced Caco-2 cells. These results suggested that both, the structural components and the soluble cellular content of lactobacilli have anti-inflammatory effects. In contrast, both C28 and MS13 up-regulated IL-1β-induced IL-8 production by Caco-2 cells. Taken together, these data suggest that C28 and MS13 can both suppress and augment inflammatory responses, dependent on the inflammatory pathology being predominated by either TNF-α or IL-1β.

5.3.3: Probiotics selectively modulate TNF-α or IL-1β induced hBD-2 and ZO-1

Results showed that Caco-2 constitutively expressed hBD-2, whereas TNF-α and IL-1β inducted an increased expression of hBD-2 in Caco-2 epithelial cells. The ability of probiotic bacteria, especially the strains used in this study, to modulate the expression of hBD-2 by Caco-2 induced by TNF-α or IL-1β, was investigated (see **figure 5.2.6**). Schlee *et al.* (2008) reported that three stains of lactobacilli (heat-inactivated) induced Caco-2 expression of hBD-2 mRNA, dependant on time and the probiotic density. A high density of heat inactivated probiotic bacteria incubated with Caco-2 for 24 hours, resulted in high levels of hBD-2 expression after 6 hours compared with low densities of bacteria (optimal at 12 to 24 hours), while the lowest density of lactobacillus did not lead to changes of hBD-2 expression compared with untreated Caco-2. The results of our study showed that the live lactobacilli (MS13) $1x10^6$ cfu/ml upregulated Caco-2 hBD-2 expression in 24 hours. This ability of stimulation might be seen with live bacteria, even at low densities. While C28 induction of Caco-2 hBD-2 expression was not significant compared with untreated Caco-2 that might be due to the collection data time. Conversely, Paolillo *et al.* (2009), found that *L.plantarum* significantly induced Caco-2 cell hBD-2 mRNA expression and hBD-2 secretion in a time-dependent manner. While, Ghadimi *et al.* (2011), testing 11 probiotic bacteria strains, found two strains of bacteria, K11-Lb3 and K2-Lb6, to significantly induce the production of hBD-2 by Caco-2 cells. The induction of Caco-2 by TNF-α or IL-1β increased the expression of hBD-2 in epithelial cells, while the probiotic bacteria reduced the level of gene expression of hBD-2 induced by these pro-inflammatory cytokines. On the other hand (Habil *et al.*, 2014) studied the role of probiotic bacteria *Lactobacillus casei* strain Shirota

(LcS) and *Lactobacillus fermentum* strain MS15 (LF) in the modulation of Caco-2 hBD-2 in inflammatory environments predominated by IL-1β or TNF-α. LcS augmented IL-1β-induced hBD-2, while, MS15 enhanced TNF-α- and suppressed IL-1β-induced hBD-2, which were differentially regulated by endogenous IL-10, dependent on inflammatory environment.

It is well understood that tight junctions (TJs) control TEER: TJs are composed of diverse types of proteins, including ZO-1, ZO-2 and Claudin. The results of this study demonstrated that C28 augmented the tight junction integrity (TEER) and the expression of ZO-1 mRNA in Caco-2s (**Figure 5.2.3 and 5.2.7**). While, MS13 up regulated the expression of Caco-2 ZO-1, with no detectable change in TEER, compared by untreated Caco-2. While (Putaala *et al.*, 2014) highlighted the potential role of probiotic bacteria for use in the treatment of a disorder associated with Tight Junction function, where the probiotic bacteria, soluble metabolites of probiotic bacteria and/or a cell lysate is formulated for topical administration. Interestingly, C28 and MS13 exhibited a differential effect on ZO-1 expression, determined by inflammatory environment: in the presence of TNFα, MS13 augmented ZO-1 expression where C28 had little effect. Conversely, in an IL-1β-predominated inflammatory environment, C28 augmented ZO-1 and MS13 had no effect. In cells regulated by C28 and MS13, or inflammation by (TNF-α or IL-1β), ZO-1 showed less intensity and discontinuous labelling around the cell periphery indicative of a disorganization process of the tight junction proteins (Pyrgos et al., 2010), and this is similar to that observed with IBD, where tight junctional intercellular changes result in reorganization of TJ protein structure with subsequent changes in barrier properties (Capaldo and Nusrat, 2009). These observations, and that of others, indicate that inflammatory context of gastrointestinal pathologies will have a profound effect on deter-

mining whether probiotic strains are beneficial or detrimental to inflammatory pathology.

In addition, probiotic strain (BGZLS10-17) supernatant increased IL-1β-induced Caco-2 cells monolayer inflammation (production of IL-8) and significantly stimulated the expression of tight junction proteins (zonulin, occludin, and claudin 4), leading to conclude that inflammatory context can exert a differential effect on immune responsiveness and maintenance of intestinal epithelial barrier integrity (Sokovic et al., 2019).

5.3.4: Probiotics selectively modulate TNF-α or IL-1β induced cytokine TNF-α and IL-10

This study focused on considering the importance of probiotic bacteria in the modulation of Caco-2 IL-10 and TNF-α gene expression in homeostatic and inflammatory environments. This study showed that live C28 and MS13 induce Caco-2 gene expression of TNF-α in homeostasis (**Figure 5.2.8 a**). A strong relationship between TNF- α expression and the expression of TLR-2 ,TLR4, MD-2, CD14 has been reported, where stimulation of epithelial cells with specific TLR ligands induced a range of pro-inflammatory cytokines (TNFα, IL-1α, and IL-6) and anti-inflammatory cytokines such as IL-10 (Goto, 2008). In addition, Habil,(2013) studied the ability of heat killed *Lactobacillus fermentum* and *Lactobacillus casei* to stimulate Caco-2 TLR-2 ,TLR4, MD-2 and CD14 gene expression of, where these probiotics significantly up-regulated Caco-2 TLR2 ,TLR4, and CD14 while MD-2 non-significantly decreased. Habil,(2013) worked on the heat killed lactobacillus strains on Caco-2 whereas this study used live bacteria on Caco-2, however is more relative to present study. Conversely, the effect of probiotic bacterial modulation of Caco-2 gene expression of TNF-α in an inflammatory environment predominated by TNF-α and IL-1β was investi-

gated (**Figure 5.2.8 b/c**). In this study, Caco-2 epithelial cells expressed cytokines including TNF-α and IL-10, in agreement with other studies (Habil, 2013; Jarry, 2008). *Lactobacillus plantarum* strain (C28), and *Lactobacillus salivarius* strain (MS13), clearly suppressed the expression of TNF-α by Caco-2 in the presence of TNF-α (**Figure 5.2.8 b**). Conversely, heat killed *Lactobacillus plantarum* (C28) and *Lactobacillus casei*, upregulated TNF-α gene expression of Caco-2 in the presence of TNF-α (Habil, 2013). In addition, C28 upregulated TNF-α expression of Caco-2 induced by IL-1β (**Figure 5.2.8 c**), agreeing with findings of (Habil, 2013). In reviewing the literature, no data showed that the stimulation of epithelial cells with TNF-α or IL-1β differentially augmented gene expression of TNF-α and IL-10 and that these cytokines are regulatable by tolerisation, which in turn is selectively modulated in the presence of probiotics. Cytokine networking controls the fates and the outcomes of the epithelial cell immune response. Cytokine are always produced by specific cells in a cascade, (Brennan and Feldmann, 1992).

The main functions of IL-10 as an anti-inflammatory cytokine is to inhibit inflammation of the Bowel disease through down-regulation of MHC class II ,B7-1, B7-2 co-stimulatory molecule expression and decrease the production of IL-1β, TNF-α, and IL-8 (Herfarth & Schölmerich, 2002). Interestingly, TNF-α expression, was suppressed by adding C28 and MS13, (**Figure 5.2.8 b**). It appears that epithelial cells respond to the TNF-induction indirectly, via regulation and activation of other cytokines (Janes et al., 2006). This study showed TNF-α to induce IL-10 expression, compared with Foey et al. (1998), who reported TNF-α to have a vital role in inducing macrophage IL-10. Surprisingly, it was found, that MS13 performed similarly to TNF-α; inducing the expression of both IL-10 and TNF- α, in epithelial cells (**Figure 5.2.9**).

Interestingly, live MS13 might work as a modulator to reduce the inflammation of Caco-2 by IL-1β, where IL-10 was up-regulated. On one hand, both C28 and MS13 suppressed TNFα-induced Caco-2 IL-10 (**Figure 5.2.9 b/c**). These findings both compare and contrast with (Jarry *et al.*, 2008), whereby upregulation of mucosal TNF-α , IFN-γ, and IL-17 is linked with the depletion IL-10 in mucosal explants. In addition, high levels of IFN-γ were produced upon IL-10 depletion and were responsible for surface epithelium damage and crypt loss. Jarry *et al.* (2008) found that the addition of commensal bacteria to mucosa explant cultures depleted IL-10, mimicking the ability of endogenous LPS to induce IFN-γ. These findings demonstrate that IL-10 ablation leads to an endogenous IFN-γ mediated inflammatory response via LPS from commensal bacteria in the human colonic mucosa (Strober & Fuss, 2011; Wells *et al.*, 2011). Thus, reinforcing the careful consideration required for probiotic strain utilisation in the management of chronic inflammatory bowel diseases; probiotics can be both immune activatory and suppressive.

5.3.5: Live probiotic bacteria modulated LPS-induced cytokine production in different co-culture models

This part of the study concentrated on mimicking healthy intestinal mucosa, to investigate the immunomodulatory effects of probiotics using different cell types in a co-culture model (Mileti *et al.*, 2009). Several studies were performed, concerning THP-1 derived macrophages using an *in vitro* co-culture model to display and predict the bioactivities of a variety of food components (Chanput, Mes & Wichers, 2014; Kämpfer *et al.*, 2017; Watanabe *et al.*, 2004a; Watanabe *et al.*, 2004b). The great majority of macrophages found in the homeostatic intestine do not express several immune response receptors, such as the LPS-receptor CD14/TLR4, and are characterised by a remarkable inflammato-

ry energy, which justifies the lack of pro-inflammatory reactions towards non-self-antigens (Smythies et al., 2005). To establish the model of the inflamed intestine, the macrophages had to be able to respond to stressors, here LPS. Interestingly, lactobacilli strains C28 and MS13 that were used in this study, inhibited the production of IL-8 of apical of Caco-2/M1 and Caco-2/M2-macrophages, and no differences were noticed in the level of IL-8 of M1/Caco-2 induced by LPS-K12, compared with untreated M1-co-culture. This agreed with Habil (2013), who reported that heat killed *Lactobacillus caesi* and *Lactobacillus fermentum* significantly inhibited IL-8 secretion by K12-LPS-induced Caco-2/M1 and Caco-2/M2 (by 20-60%). This is in contrast with results from a co-culture model of Caco-2 and peripheral blood mononuclear cells (PBMCs), which resulted in a significant increase in production of pro-inflammatory cytokines after apical exposure to a non-pathogenic *E. coli* strain (Parlesak *et al.*, 2004a).

This study focused on probiotic bacteria in the immunomodulation of Caco-2/macrophage co-cultures, where *E. coli*- K12 LPS was used as a stimulus to directly expose the M1 and M2 macrophages to provoke an inflammatory response, equitable with barrier breach and inflammation associated with chronic inflammatory pathology. LPS is ubiquitously present in the intestinal lumen and known for its association in intestinal inflammation (Guo et al., 2013). Caco-2 cells express the LPS binding receptor CD14 as well as TLR-2 (Good, et al., 2012). The model of this study, apical Caco-2 was exposed to the probiotic bacteria C28 and MS13. C28 and MS13 play an important role in barrier integrity where they induced TJ ZO-1 expression in Caco-2 epithelial cells as shown in monoculture (**see figure 5.2.7**), and inhibited IL-8 production in Caco-2 monoculture (**Figure 5.2.5 a**) and co-cultures (**Figure 5.2.10**), suggesting that no translocation of LPS/bacterial products through the epithelial cell barrier oc-

curred, as a consequence of probiotic C28 and MS13 controlling Caco-2 permeability via ZO-1 expression (**Figure 5.2.7**). Unfortunately, the limitation of time did not allow going further to study TEER in co-culture of Caco-2. On the other hand, Kämpfer *et al.*, (2017), described a reduction in barrier integrity with Caco-2 cells co-cultured with PMA /macrophages stimulated by LPS / IFN-γ and associated with large concentrations of pro-inflammatory cytokines such as IL-8, IL-1β and TNF-α. Alshaghdali (2018) discussed the role of K12-LPS in barrier disruption in Caco-2/M1 and Caco-2/M2 co-cultures where macrophages were basolaterally stimulated by LPS. However, it is important to note that *E. coli* bacteria can interact with intestinal epithelial cells and translocate across the epithelium to the basolateral compartment, whereas *E. coli*-derived LPS remains on the luminal side of the IEC barrier (Neal & Webb, 2006). The results of this study showed in (**Figure 5.2.10)**, high level of production of IL-8 of Caco-2/macrophage co-cultures, compared to untreated Caco-2 monoculture (F**igure 5.2.5**). This might be due to the differential macrophage activity of M1 and M2-macrophages. (Satsu, et al. 2006) showed a significantly increased release of pro-inflammatory cytokines after PMA-treatment without additional stimulation of THP-1 cells.

Conclusions:

To conclude, this study, reports that the *L. salivarius* strain (MS13) and *L. plantarum* (C28) live probiotic bacteria effectively modulate Caco-2 barrier integrity, pro- and anti-inflammatory capability and anti-pathogen responsiveness, with a range of differential response profiles exhibited being dependent on probiotic strain and environment (homeostatic or inflammatory).

Homeostasis: In the context of a homeostatic environment (unstimulated Caco-2 cells), C28 resulted in the phenotype IL-8^{Down} ZO-1^{Up} TNFα^{Up} IL-10^{NC} hBD-2^{NC} whereas MS13 induced a phenotype, IL-8^{Down} ZO-1^{Up} TNFα^{Up} IL-10^{Up} hBD-2^{Up}. Both probiotic strains maintain barrier integrity (ZO-1^{Up}), maintaining or partially augmenting TEER, and exhibit a differential effect on inflammatory response and anti-microbial defence, where C28 may suppress neutrophil recruitment and effector responses (IL-8^{Down}), yet up-regulates TNFα; the level of which may support homeostatic defences but not pathological inflammation, with no detectable modulation of anti-inflammatory response or anti-pathogen response. Conversely, MS13 also may suppress neutrophil-mediated responses, yet exhibits a balanced regulation of inflammation (TNFα^{Up} and IL-10^{Up}) and maintains anti-pathogen defences (hBD-2^{Up}). Thus, when considering probiotic supplementation for maintaining homeostatic epithelial barrier function, MS13 would appear to be the most appropriate choice.

Inflammatory: In an inflammatory environment, (Caco-2 cells in the presence of either TNFα or IL-1β); for ***TNFα-predominated inflammation***, C28 resulted in the phenotype IL-8^{Down} Zo-1^{NC} TNFα^{Down} IL-10^{Down} hBD-2^{NC} whereas MS13 induced a phenotype, IL-8^{Down} ZO-1^{Up} TNFα^{Down} IL-10^{Down} hBD-2^{Down}. This is suggestive that C28 is generally anti-inflammatory, without compromising barri-

er integrity or anti-microbial defences, when in a TNFα-predominated inflammatory environment. MS13 is similar, but anti-microbial defences may be suppressed or changed in favour of other anti-microbial peptides. In the case of an ***IL-1β-predominated inflammation***, C28 induced an IL-8^{Up} ZO-1^{Up} TNFα^{Up} IL-10^{NC} hBD-2^{Down} phenotype whereas MS13 induced IL-8^{Up} ZO-1^{Down} TNFα^{NC} IL-10^{Up} hBD-2^{Down}. In this environment, C28 induced a pro-inflammatory profile (TNFα^{Up}, $IL8^{Up}$, IL-10^{NC}, suppressed anti-pathogen responsiveness (hBD-2^{Down}), yet augmented barrier integrity (ZO-1^{Up}). This is suggestive that C28 is generally pro-inflammatory with MS13 being more anti-inflammatory and regulatory; almost the opposite of the observations for the same strains in a different inflammatory environment.

These observations made for epithelial cell monoculture may or may not be representative of the 3-dimensional mucosal tissue. In recognition of this, a human cell line-based co-culture model of the human intestine was established, mimicking the intestine in homeostatic or controlled inflammation statuses. The lactobacillus strains downregulated IL-8 in the co-culture of Caco-2/M1 and Caco-2/M2 induced by K12-LPS. These results indicate that *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius* strain MS13 protect intestinal cells from the inflammation-associated response caused by *E.coli* LPS-K12 by partly reducing pathogen damage and by counteracting neutrophil migration, probably through the regulation of chemokine and cytokine expression.

Chapter: 6

Final Discussion

6.1: Probiotic bacteria maintain immune responsiveness and anti-pathogen responses

Maintaining health is a great challenge that faces a human body in a changing environment and immune responsiveness to pathogens. The most accurate and acceptable definition of health maintenance is a dynamic mechanism assessed in relation of ability to adapt to stress and maintain physiological homeostasis (Malone, 2017; Ockhuizen, 2012; WHO, 1948). Interactions between environmental factors, the intestinal epithelium, intestinal microbiota, the immune system, and health were explained by studying the effects of antibiotics, the Western diet and non-digestible carbohydrates on the microbiota (Zhao & Shen, 2010). Probiotic bacteria can produce excellent support to different states of health and disease. Mucosal homeostasis involves a persistent balance between pro- and anti-inflammatory elements. For decades, many studies have investigated the potential correlation between dysbiosis and extra-intestinal and intestinal diseases such as inflammatory bowel disease (IBD) (Ley et al., 2006; Stark et al., 2005).

In human IBD, inflammatory lesions exhibit an increase in build-up of macrophages that display enhanced pro-inflammatory cytokines such as, (IL-12, IL-6, IL-8, TNF-α), compared with healthy controls, (Hart, 2005; Lindsay, 2006; Hill, 2009). Modulation of macrophage mucosal immune responses is one of the possible methods for the prevention and cure of IBD, using substances of ecological origin; effectively restoring immune homeostasis, by redressing the gut microbiota, considered a notable therapeutic approach to treat IBD patients. To this end, faecal microbiota transplantation is used to support recent therapies in IBD management (Amoroso, 2020; Yousef, Babak & Ahmad Yari, 2018).

6.2: Do C28 and MS13 exhibit probiotic effects?

Lactic acid bacteria (LAB) are microorganisms that have a particularly important function in, maintaining health, especially the gastrointestinal tract and in defending it from infection by other pathogenic organisms. These characteristics qualify LAB as probiotic bacteria. Babak & Ahmad Yari, 2018, reported the ability of 45 strains out of 88 of these bacteria, to tolerate bile (0.3%) and acid at pH 2.5. They also have antibacterial effect on microorganisms. Based on the molecular identification and clustering, the 45 isolated LAB, were classified into three major groups: Enterococcus, Lactobacillus and Lactococcus. The main features of good probiotic bacteria are, survival in an acidic environment, such as in the stomach and adhesion to the epithelium. Previous research by Demeckova, (2003) and Savvidou, (2009), on the two strains of *Lactobacillus plantarum* strain, C28 and *Lactobacillus salivarius* strain, MS13, revealed both acid tolerance (pH ≤ 3.5) and bile resistance, facilitating transit through the stomach, and proliferation in the colon. Moreover, these strains are able to inhibit pathogenic bacteria such as *Salmonella enteric* and *Salmonella typhimurium* (Savvidou, 2009). In addition, these bacteria were able to auto-aggregate and have high affinity to adhere to epithelium cells. The auto-aggregation enhances the clearance of these pathogens from the gut. In addition to their nutritional and antimicrobial effects, many of them have immunomodulatory activity. It has been shown that lactobacilli can interact with the immune system at many levels, including cytokine production, macrophage phagocytosis and killing, as well as enhancing immunity to bacterial pathogens (Vitini et al., 2000). This study also revealed the ability of bi-phasic growth. The growth curve (see **Figure 3.1**) showed bi-exponential and bi-stationary phases. As mentioned in the methodology, shaking was avoided during the incubation period. In order to

avoid the release of hydrogen peroxide from the media that might have decreased the rate of growth. These findings disagreed with (Murphy and Codon 1984), who found that the growth rate of *Lactobacillus plantarum* was enhanced in a complex medium (55.6 mM glucose) and decreased under aerobic conditions. In addition, they found that growth rate was reduced much earlier due to an increase in the rate of oxygen utilization by the culture, which led to the accumulation of hydrogen peroxide H_2O_2.

6.3: Live probiotics homeostatically regulate macrophage subset production of pro-inflammatory cytokines

This study was designed to determine role of live *Lactobacillus* bacteria in the modulation of M1 and M2–like macrophages incubated for 24 hours. The aim was to determine whether this effect led to either upregulation or downregulation of immune responsiveness, effectively determining the impact of live *Lactobacillus plantarum* (NCIMB 41605) (C28) and *Lactobacillus salivarius* (NCIMB 41610) (MS13) on modulating a differentiated monocytic cell line, THP-1. Generally, the THP-1 cell line or pro-monocytes differentiate to either M1 or M2-macrophages by treatment with PMA and Vit.D_3, respectively (Auwerx, 1991; Chanput *et al.*, 2010; Schwende *et al.*, 1996). Some publications found similarities between THP-1 derived macrophages and human PBMCs derived macrophages, in some features like, polarising ability (plasticity), macrophage morphology and cytokine production (Tsuchiya *et al.*, 1982) (**see table 3.1**). Further research is required to investigate the similarities between these in vitro derived macrophage subsets and mucosal macrophages and their role in homeostasis, chronic inflammation, and endotoxin tolerisation.

Induction of macrophage subset by lactobacilli showed further comparable features related to their regulatory function. No data was found in the literature on

the question of how do live probiotic bacteria modulate macrophages subsets *in vitro* through 24 hours. Habil *et al.* (2011) however, studied the effects of heat killed probiotic bacteria and their secreted proteins on cytokine production by M1 and M2-macrophages induced by K12-LPS. M1 and M2-like macrophage TNF-α was upregulated, while IL-6 was suppressed. It is important to note that Habil et al. (2011) used heat killed probiotic bacteria, whereas this current study focussed on the immunomodulatory effects of live probiotic bacteria.

Surprisingly, the probiotic strains C28 and MS13 may have polarised M2-macrophages towards M1s, as evidenced by the production of pro-inflammatory cytokines TNF-α, IL-1β, IL-18, IL-23, and IL-12 (**see chapter 3 and 4**). Together with modulation of anti-inflammatory cytokines, IL-10 and IL-6 (**see Figure 6.1**); these results suggest a role of these probiotic strains in re-polarising and plasticity modulation of macrophage subsets. It is of note that the original function of M2 is predominantly to induce tolerance by the secretion IL-10. Whilst these probiotic strains differentially induced M1-like macrophage cytokine production, for example, C28-induced IL-18 and MS13-induced IL-23 in M1-like macrophages, both strains induced the production of TNF-α and IL-6 but failed to induce IL-10 in a homeostatic environment (probiotic only, no addition of LPS or TNFα/IL-1β). This study's findings, based on macrophage cytokine profiles induced, suggest an indirect role of C28 and MS13 in T cell stimulation and M1 positive feedback resulting in the activation of Th1 cell-mediated immunity, secretion of IFNγ and the resulting activation of cytotoxic T cells. This was also reflected in the possibility of activating other T cell mediated immune responses, such as the potential role of IL-23 in polarising Th17s in the presence of IL-1β (Atreya et al., 2000; Yamamoto et al., 2000). Moreover, IL-23 upregulates IL-17 production which in turn promotes neutrophil-mediated inflammatory responses;

this justifies the adoption of neutralising antibodies targeting the shared IL-12/IL-23 subunit (p40) for clinical trials in multiple inflammatory diseases including CD (Kanda and Watanabe, 2008). IL-23 and IL-17 in both in mice and human are important mediators of neutrophil recruitment, migration through the induction of granulopoiesis and production of G-CSF, IL-6, IL-1β, TNF-α, and neutrophil chemoattractant chemokines, including CXCL1-2, and -5 (Starket al., 2005;von Vietinghoff et al., 2008). Neutrophils are an important innate defence; however, they are also involved in the pathophysiological mechanisms driving CD.

To make a comparison between the previous studies and this study results might be difficult. This is because most previous studies used heat inactivated probiotics or cell wall components and secreted proteins (Kim et al., 2011; Habil, 2013; Wang et al., 2013), in order to avoid the potential reduction in macrophage viability by metabolic products of lactic acid bacteria. Previous research by Rocha-Ramírez et al. (2017) revealed that the activation of healthy peripheral blood-derived monocytes by heat inactivated (*Lactobacillus rhamnosus* GG, *L. rhamnosus KLSD, L. helveticus IMAU70129*, and *L. casei* IMAU60214) strains, resulted in the pro-inflammatory cytokine gene expression of IL-8, TNF-α, IL-12 p70, and IL-6, whereas IL-1β expression was stimulated only by *L. helveticus* and *L. casei* strains (after 24 hrs stimulation). The bacterial density (cfu/ml) of heat-inactivated bacteria (MOI 1: 500 of heat-inactivated lactobacillus), was 50 times higher than the density of live bacteria used in the current study (MOI 1:10, macrophage to live lactobacillus).

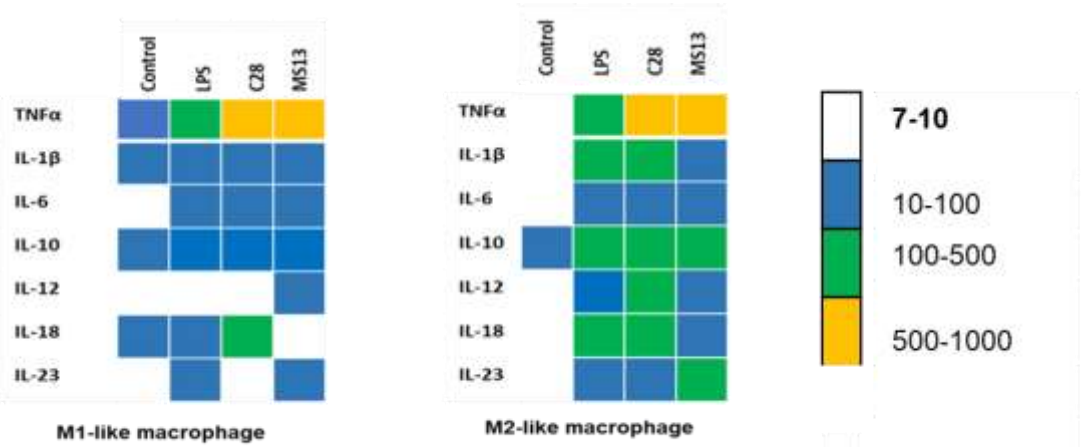

Figure 6.1: Probiotic strains differentially modulate macrophage subset cytokine protein secretion

6.4: The benefit of probiotic bacteria in the activation of the inflammasome

The study confirmed that NLRP3 inhibitor (MCC950) suppressed M1 and M2-like macrophage production of IL-1β, induced by *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius* strain MS13. These findings further support the principle that probiotic bacteria play a vital role in the maintenance health. The stimulation of the NLRP3 inflammasome by pattern-associated molecular patterns (such as LPS or MDP) results in caspase-1-dependent processing of the inflammatory cytokines IL-1β and IL-18 (Pétrilli *et al.*, 2007). Interestingly, this study revealed that the stimulation of macrophage subsets by LPS prior to that by probiotic bacteria upregulated the production of IL-1β, suggestive of either a pre-formed reservoir of IL-1β or a constitutive basal inflammasome activity. In contrast, (Wu *et al.*, 2018), found that *Lactobacillus rhamnosus* GR-1 decreased *E. coli*-induced caspase-1 activation and the production of IL-1β and IL-18. However, in parallel to increases in the expression of NLRP3 and caspase-1, expression of the adaptor protein ASC is reduced in PBMCs infected with *E. coli*, even in cells pre-treated with *L. rhamnosus* GR-1 (Wu et al., 2016).

It is important to note that while hyper-functional mutations of NLRP3 have been linked with several inflammatory disorders such as IBD, UC and CD in humans,

it is the hypo-functional mutations that are associated with an improved risk of CD (Xavier & Podolsky, 2007).

The activation of the NLRP3 inflammasome in M1 and M2-like macrophages by MS13, suggested a critical role for probiotic bacteria in the protection of the human body from many pathogenic bacteria. On the other hand, this study produced results which corroborate the findings of a great deal of previous work; Hirota et al. (2011) found that NLRP3$^{-/-}$ mutation mice to be more susceptible to experimental colitis and the induction of spontaneous colitis. This observation was linked to the suppression of IL-1β and downstream suppression of IL-10 and TGF-β. This increased inflammation associated with suppressed IL-10 and TGF-β, was not surprising, as earlier studies showed this to be the consequence of targeted deletion of this protective mediator. Additionally, *ex vivo* macrophages from NLRP3$^{-/-}$ mice failed to respond to bacterial muramyl dipeptide (Takada and Kotani, 1995).

6.5: Do probiotic bacteria inhibit macrophage subset immune responses in an inflammatory environment?

The altered behaviour of Lactobacillus bacteria in the modulation of the macrophage subsets in the case of inflammation (presence the K12- LPS), is a more acceptable model of an inflammatory condition *in vitro*. Generally, *E.coli* LPS-K12 induces the production of TNF-α, IL-1β, IL-18, IL-23 by M1 and M2-like macrophages, while no significant differences in production of IL-12 by stimulation of M1 and M2-like macrophages were observed (**Figure 6.1**). Typically, mucosal macrophage immune responses mimic the M2 subset and fail to express CD14, a co-receptor for LPS signalling (Fenton & Golenbock, 1998). The increase in the production of pro-inflammatory cytokines such as TNF-α, IL-8, IL-6, and IL-1β can be used as markers to measure the inflammatory response

(Bäckhed et al., 2005). Moreover, M1-like macrophages were characterised as TNFα ^{hi,} IL-1β^{lo}, IL- 18 ^{hi}, IL-12 ^{hi}, IL-23 ^{hi} and IL-6^{hi}, whereas M2 macrophages were TNFα^{lo}, IL-1β^{hi}, IL-18^{lo}, IL-12 ^{lo}, IL-23^{lo} and IL-6^{lo} (**Figure 6.1**). Except for the IL-12 and IL-23 profile, the TNFα, IL-1β, IL-6 and IL-18 data is supported by previously published data from our laboratory (Foey and Crean, 2013; Alshaghdali, 2018). This is in addition to the validation markers for THP-1 differentiation by PMA and Vitamin D_3 (**Table 3.1**).

6.6: Tolerisation by C28 and MS13 induced differential macrophage subset gene expression of cytokines and negative regulatory molecules

Endotoxin tolerance has been widely investigated in peripheral macrophages (Biswas & Lopez-Collazo, 2009; Medvedev *et al.*, 2002). In this study, the effect of two lactobacilli and K12-LPS-induced endotoxin tolerisation of macrophage subset cytokine production and expression of PPRs and negative regulatory molecules was investigated. The effects of probiotic bacteria on tolerisation has not been previously published, so results in this study are compared to conventional endotoxin tolerance induced by the enteropathogenic *E.coli* K12-LPS. Firstly, the results indicate an upregulation of cytokine production (TNF-α, IL-18, IL-23, and IL-10) by C28 and MS13 in M1-like macrophages. The monocolonisation influence is strain-dependent, the best result having been gained with *Lactobacillus paracasei* (NCC 2461). The tolerisation findings of this study showed that the suppression of TLR2 expression in M1 and M2-like macrophages by C28 and MS13 (**Figure 4.10**), was accompanied with the downregulation of SOCS3 and Tollip **(Figure 4.12 & 4.13 c/d)**. These findings contradict the explanation of Sun *et al.* (2017), who studied the effects of the inhibition of negative regulatory molecules such as Tollip and SOCS1 and SOCS3, due to the blocking antibody to TLR2 and low dose pre-treatment of LPS, resulting in

the further elevation of pro-inflammatory cytokines. Basically, Lactobacillus is a gram-positive bacterium with an LPS-deficient cell wall (Sun *et al.*, 2017). This study revealed that TLR4 expression was upregulated in tolerised M2-like macrophages by C28 and MS13, while MS13 alone augmented TLR4 expression in tolerised M1-like macrophages. Moreover, the expression of TLR4 was accompanied with the upregulation of TRAIL gene expression **(Figure 4.8 & 4.9)** (**Figure 6.3);** *Lactobacillus plantarum* strain C28 and *Lactobacillus salivarius strain* MS13 drove the upregulation of TRAIL. Furthermore, *Lactobacillus reuteri* was shown to inhibit colorectal cancer by downregulating NF-ķB-dependent gene products regulating epithelial cell proliferation (Iyer et al., 2008). These results suggested a role for these probiotic strains in both regulation of tolerance and pathogenesis, thus may play either a beneficial or a detrimental role in both inflammatory disease (CD) or suppressive disease (CRC) that is strain-specific.

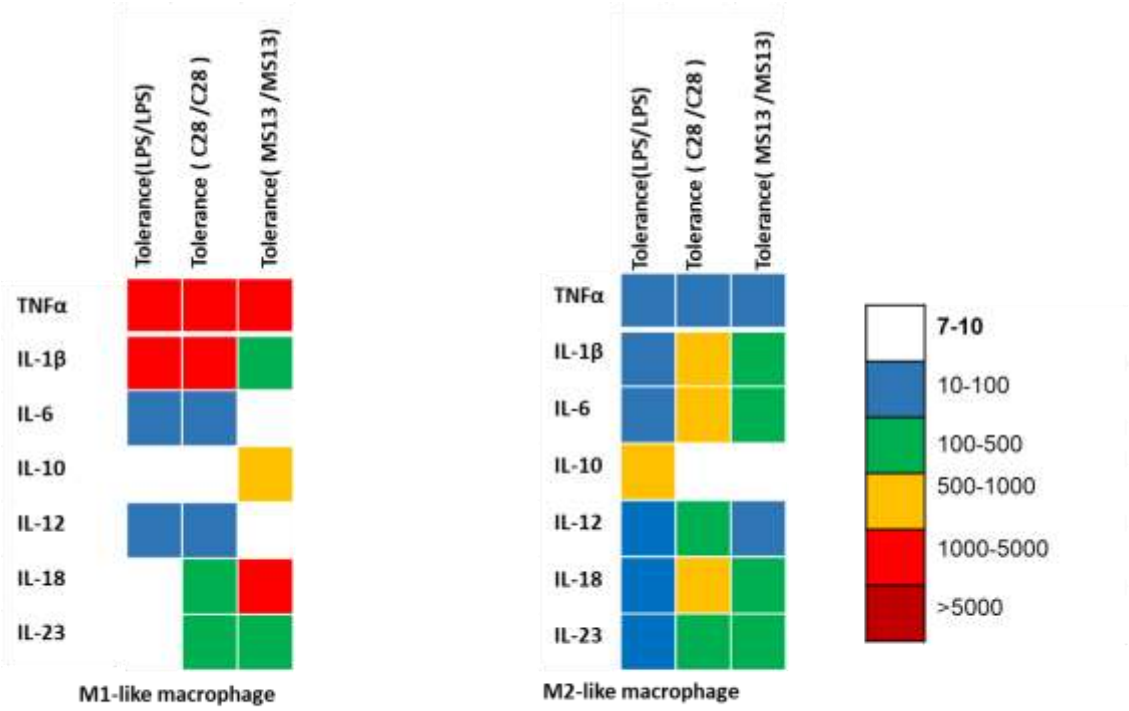

Figure 6.2: A Comparison of tolerisation macrophages subsets between (Endotoxin tolerance and tolerised by probiotic strains

6.7: *E. coli* K12-LPS Endotoxin tolerance induced differential macrophage subset gene expression of cytokines and negative regulatory molecules

The main characteristics of endotoxin tolerance are downregulation of pro-inflammatory mediators such as TNF-α, IL-1β, and IL-18 by M1 and M2 macrophages **(Figure 6.2**.) As well as the upregulation of anti-inflammatory cytokines such as IL-10 and IL-6 by M2-macrophages, these findings agreed with (Liu *et al.*, 2019; McClure *et al.*, 2015), who found that endotoxin tolerance upregulated the anti-inflammatory cytokines, IL-10 and TGF-β, as well as the C-X-C motif chemokine 10 (CXCL10) and suppression of TNF-α and IL-1β. While endotoxin tolerance upregulated the expression of IL-12 and IL-23 in M1- and M2-macrophages, investigations of LPS tolerised mice showed a decreased ability to produce TNF-α, IL-12, and other pro-inflammatory cytokines in response to subsequent LPS exposure (Wysocka *et al.* (2001); Balkhy & Heinzel, 1999). Interestingly, priming with different concentration of LPS (1, 5, and 20 μg) resulted in a consistent suppression of serum TNF-α, whereas low dose LPS, failed to suppress IL-12 production upon secondary LPS challenge. This study's findings of the upregulation of endogenous suppressors Tollip, SOCS3 and STAT3 (**Table 6.3**), is consistent with those of Al-Shaghdali et al. (2019) who induced endotoxin tolerance by 100 ng/ml K12-LPS in these same macrophage subsets. They found that Tollip was more associated with tolerised M1 macrophages; moreover, there could be other additional regulatory mechanisms to control LPS response in other subsets. Endotoxin tolerance is considered to be the regulatory mechanism of excessive inflammation (Liu *et al.*, 2019), however, the precise mechanisms are yet to be fully determined in distinct macrophage subsets. Further studies need to be conducted on the effects of endotoxin tolerance on cellular signalling, cytokine production, PRRs, and transcription factors.

Therapeutic adoption will be dependent on the specific effects on macrophage polarisation, plasticity, macrophage subset-specific endotoxin tolerisation mechanisms, and resulting downstream effects on the polarisation of T cell reactions (Foey and Crean, 2013).

Foey (2015) reviewed the interaction of M2-like macrophages with solid tumours. This was related to the role of M2- in driving the suppression and regulation mechanisms required for tumorigenesis and progression of solid tumours. High tumour associated macrophage (TAM) numbers have been shown as a poor prognostic marker in cancers, specifically in squamous cell carcinoma (Mills et al., 1992). Regarding the comparison of tolerance by K12-LPS and lactobacillus bacteria; K12-LPS macrophage endotoxin tolerisation suppressed pro-inflammatory cytokines such as TNFα and IL-1β, and TLR-4 mediated signals (Biswas and Lopez-Collazo, 2009). Macrophage functional inhibition might benefit infectious microbes but at the same time, prevent damaging inflammatory responses, hence allowing for a suitable environment for the pathogen to improve its numbers through growth. Overall, macrophage subsets were differentially sensitive to K12-LPS-induced endotoxin tolerance; M1- and M2-macrophages exhibited a suppression of TNF-α, IL-1β, IL-18, and IL-6, whereas M2 production of IL-12 and IL-10 were increased, while no significant change was observed in the level of IL-23 cytokine production in both tolerised macrophages.

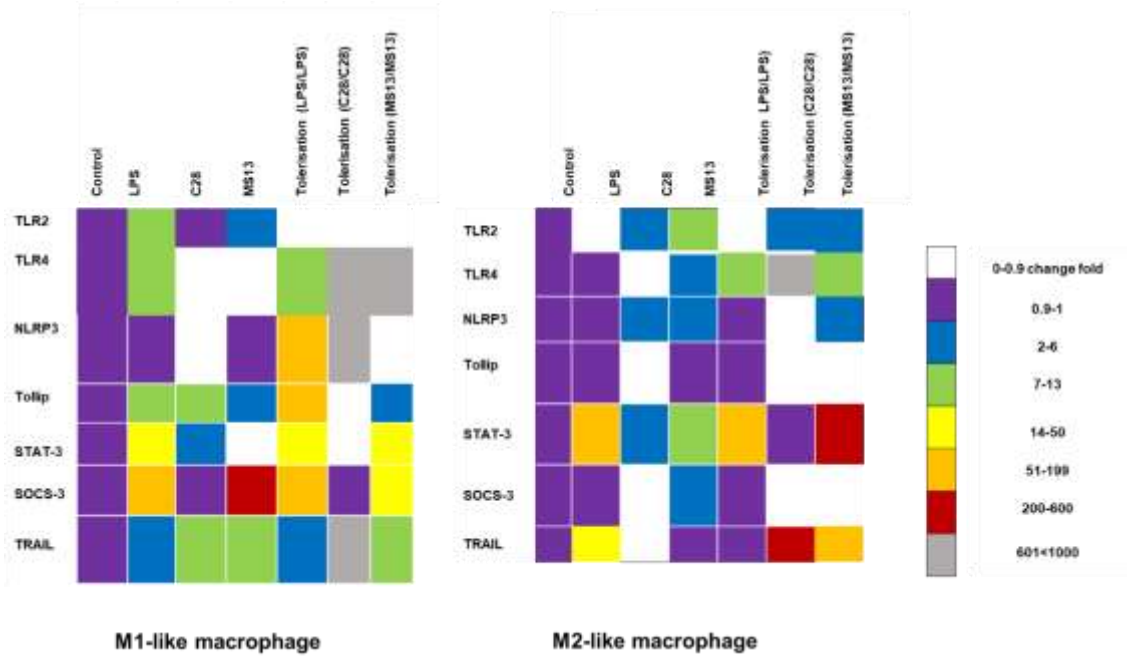

Figure 6.3: Probiotic bacteria and E.coli K12-LPS induced the expression of the immune regulatory molecules in macrophage subsets upregulation or suppression

6.8: The role of lactobacilli in the modulation of K12-LPS-induced endotoxin tolerance in M1 and M2 –like macrophages.

The comparison between the endotoxin tolerance by K12-LPS and the role of probiotic bacteria C28 and MS13 in tolerisation is discussed in this chapter. Lactobacilli modulation of cytokine production induced by endotoxin tolerance, are macrophage subset dependent (**Figure 6.4**). The modulation of M1 endotoxin tolerisation by C28 resulted in the upregulation of IL-18 and IL-23. These findings suggest a role of C28 through M1-like macrophages in the promotion of the Th1, hence immune protection against intracellular-resident pathogens, and as well as both pro- and anti-carcinogenic immune-responses. IL-18 increases Th1-cell cytokine production (IFN-*γ* and granulocyte macrophage (GM)-CSF), while suppressing the production of the anti-inflammatory cytokine IL-10 (Benveniste, 2014). O'Shea *et al.*, (2015) studied the role of IL-2, IL-12, IL-15, IL-21, and IL-23 in enhancing the production of IL-18; IL-12 in particular, dra-

matically synergises and enhances IL-18 activity as a result of enhancement of each other's receptors, normally expressed at very low levels. As such, IL18 and IL-12 synergistically activate NKs, hence strengthening protection to intracellular pathogens and tumour surveillance. Under certain circumstances, IL-18 can also induce Th2 or Th17 cytokines (Mak & Saunders, 2006). STAT3 promotes the IL-23-mediated pro-carcinogenic immune responses whereas inhibiting IL-12-dependent anti-tumor immunity (Kortylewski *et al.*, 2009). Christoffersen *et al.*, 2014), found that different strains of Lactobacillus and strains of Escherichia and Salmonella elevated iNOS expression (a marker of M1 macrophage phenotype) more so than strains of Enterococcus, Lactobacillus and Lactococcus, indicating a strain-selective capability to modulate macrophage plasticity in favour of M1 macrophages.

Other leading observations of this study, include the role of C28 in M2-like macrophages, upregulating both pro-inflammatory and anti-inflammatory cytokines by K12-LPS tolerised M2-like macrophages. These findings suggest the role of probiotic bacteria in polarisation of M2-like macrophage towards M2b subset macrophages, determined by a distinct cytokine profile (TNF-α, IL-1β, IL-18, IL-23, IL-12, IL-6, and IL-10). This agreed with Mosser and his group, who classified M2-macrophages into three subsets depending on the methods of activation and cytokine production (Benoit, Desnues & Mege, 2008; Edwards *et al.*, 2006; Martinez *et al.*, 2008). Moreover, the activation of M2b macrophages by either LPS or IL-1 has been demonstrated to result in a distinct cytokine profile of TNFα, IL-23, IL-1, IL-6, and IL-10 (Mantovani *et al.*, 2004; Martinez & Gordon, 2014). This study proposes that probiotics help shape the anticancer immune response and the modulation of macrophage subsets. The present findings seem to be consistent with other research, which revealed an im-

munomodulatory role of probiotic bacteria *in vitro* and *in vivo* against chemically induced CRC. Hradicka et al., (2020) studied the cocktail of *lactobacilli strains in vitro and on the macrophage subsets; the probiotic treatment effectively* decreased multiplicity, volume, and total tumour numbers and restored colon length. Again, indicative that similar strains are capable of exhibiting both anti-inflammatory and anti-tumour responsiveness, determined by both physiological and pathophysiological context.

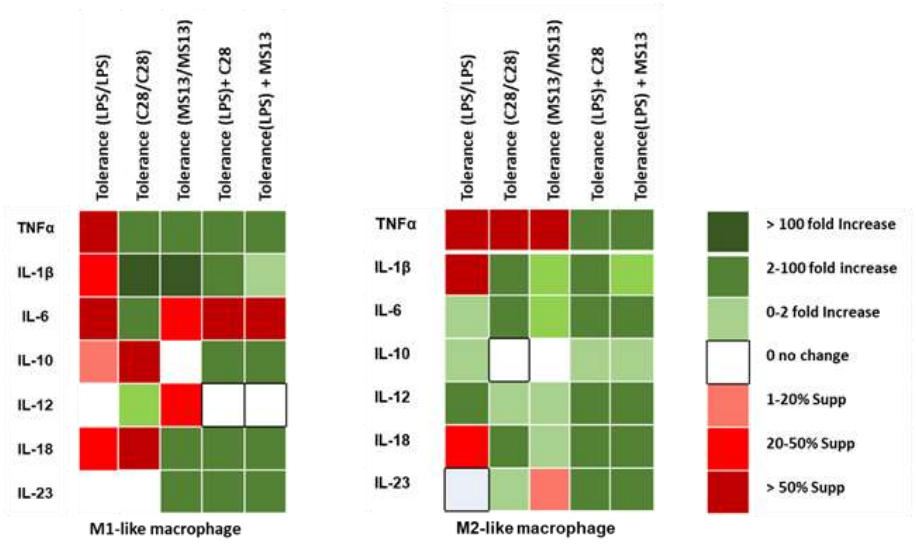

Figure 6.4: LPS, C28 and MS13 tolerisation/repeat stimulation induces differential cytokine expression profiles, is dependent on probiotic strain and macrophage subset

6.9: Probiotic bacteria differentially modulate Intestinal epithelial barrier function in a homeostatic or inflammatory environment

The intestinal epithelium acts as the central cell border between the gut lumen and the lamina propria. It is recognised for its potency against an attack of luminal bacteria that may be accumulated with a bacterial cell density of more than 10^{14} CFU/ml. This barrier typically mediates tolerance to commensal bacteria but initiates the pro-inflammatory signalling pathway in response to pathogenic bacteria. On the contrary, in IBD, together with an augmented permeability, the barrier fails to understand such initial recognition, possibly a result of

lack of defence molecules, displaying no response before the bacteria attack the basolateral side (Elphick et al., 2008). The exact signalling procedures are still unclear. Prior studies, in typical *in vitro* monocultures of Caco-2 epithelial cell line have indicated the importance of the normal mucosa with integral tight junctions, which become compromised in IBD. Such characteristics however, do not necessarily reflect the pathophysiological modifications proceeding in an inflamed area of IBD.

Additionally, a single cell line can never represent the complex interactions of different cell types during inflammation (Leonard et al., 2010). Caco2 cell line is commonly established as a model of the normal, healthy intestinal mucosa. However, as this typically includes only enterocytes, it cannot mimic the complex links with other cells, namely immune cells. These interactions could be essential for the epithelial barrier role (Leonard et al., 2010). In chapter 5 of this study, Caco2 cells were used to develop *in vitro* the effects of two different strains of probiotic bacteria C28 and MS13 in mimicking the potential treatment for inflammation of epithelial cells, compared with healthy conditions. The model of the interaction of gut epithelial cells with immune cells (M2-like or M1-like macrophages), represents normal homeostasis (Caco-2/M2) or a chronic inflammation model (Caco-2/M1), representative of the inflammatory environment in Crohn's Disease. THP-1 cell line and Caco-2 have been widely used; however, the use of THP-1 derived macrophages, representing both homeostatic and inflammatory environments, to study intestinal epithelial barrier function in health and disease, has not. It is important to note however, that these cells are cancer-derived and may/may not reflect the pathophysiological changes in the state of inflammation. Probiotic bacteria C28 and MS13 regulate these changes in intestinal epithelial cells. Secondly, these probiotic bacteria regulate the in-

flammatory environment resulting from secretion of a pro-inflammatory cytokines such as TNFα and IL-1β, in monoculture. This overproduction of TNFα and IL-1β by uncontrolled immune cells, such as mucosal macrophages, contributes to the initiation of tissue harm in IBD (Strober and Fuss, 2011), and is reflected in this study. Expression of hBD-2, ZO-1, IL-10, and TNF-α has proved to be a useful profile in investigating the potentially beneficial or detrimental effects of probiotic bacteria on mucosal barrier integrity or immunity. MS13, by augmenting hBD-2, ZO-1 and IL-10 mRNA expression, is beneficial in a homeostatic environment, whereas in a pro-inflammatory environment, defined by excess TNFα and IL-1β, MS13 suppressed hBD-2 expression, hence may detrimentally affect mucosal anti-microbial defences.

Chapter: 7

Conclusions and future work

In summary, the probiotic strains MS13 and C28 have displayed a potential to be both pro-inflammatory and suppressive, effectively activating and negatively regulating pro-inflammatory and anti-inflammatory cytokines and barrier-associated molecules which is dependent on environment (homeostatic or inflammatory) and macrophage subset functional phenotype.

C28 and MS13 enhance pro-inflammatory cytokine TNFα and IL-1β in both M1 and M2 macrophages, whereas C28 suppresses M1-IL-10 and MS13 suppresses M2-IL-10. Thus, both probiotic strains elevate the M1 TNFα/IL-10 ratio, whereas C28 alone enhances M2 TNFα/IL-10 ratio. This is suggestive that both probiotics induce a pro-inflammatory status in M1 and M2 macrophages and indicate that these bacteria may even manipulate M2 plasticity to change to a more conventional M1 phenotype. In the presence of the inflammatory stimulus, LPS, however, both strains further enhanced this pro-inflammatory cytokine ratio in both macrophage subsets. Investigation of IL-1β expression however, demonstrated both C28 and MS13 to augment IL-1β and the inflammasome molecule, NLRP3 in M2 macrophages and to differentially modulate M1-IL-1β/NLRP3 **[Refer to Chapter 3]**.

With regards the potential to tolerise M1 and M2 macrophage subsets, LPS/LPS homotolerisation protocol effectively suppressed M1 and M2 pro-inflammatory cytokines however, the M2 subset maintained responsiveness to LPS (up-regulation of TLR4) and augmented IL-10. Both C28/C28 and MS13/MS13 homotolerisation protocols effectively failed to tolerise or suppress macrophage subset functionality. These repeat-stimulation protocols resulted in macrophage activation, whereby M2 macrophages were pro-inflammatory and, in the case of C28, may activate NK cells. M1 macrophages were also pro-inflammatory and in addition, C28 is indicative of inducing both activation of

NKs and Th1 whereas MS13 may activate Th17-mediated responses. Heterotolerisation protocols, in contrast, were both activatory/pro-inflammatory but were clearly indicative of driving both Th1/Th17 responses in a macrophage-subset-specific manner. LPS/C28 heterotolerisation resulted in activation, which predisposed towards M1-Th17 and M2-Th1/Th17. LPS/MS13 resulted in macrophage activation predisposing towards M1-Th17 and M2-Th1/NK **[Refer to Chapter 4]**.

Intestinal epithelial cells demonstrated a more variable responsiveness to these probiotic strains, dependent on environment (homeostatic/inflammatory). In homeostatic conditions, both C28 and MS13 re-inforce barrier functionality (C28 augments ZO-1, whereas MS13 augments ZO-1, hBD-2 and IL-10). Inflammatory environments (presence of TNFα or IL-1β) generally up-regulate hBD-2 and IL-8, with TNFα auto-activating its own expression. In the presence of TNFα, C28 suppresses IL-8, TNFα and IL-10, whereas MS13 suppresses IL-8, TNFα, IL-10 and hBD-2 and augments ZO-1. In contrast C28 suppressed IL-1β-induced hBD-2 and augmented IL-8, ZO-1 and TNFα, whereas MS13 suppressed IL-1β-induced hBD-2 and ZO-1 and augmented IL-8 and IL-10. Thus, dependent on mucosal environment, these probiotic strains have the capability of driving both barrier integrity/functionality and pro-/anti-inflammatory responses **[Refer to Chapter 5]**.

As a consequence of these summarised data presented above, it is possible to draw several conclusions from this investigation:

Conclusions:

1) Live C28 and MS13 directly activate pro-inflammatory responses in both M1 and M2 macrophage subsets that are further reinforced in the presence of LPS. Thus, rather than the hypothesised regulatory nature, these probiotic strains are immune-activatory, predisposing to a direct induction of pro-inflammatory mediators or manipulating macrophage plasticity to more inflammatory M1-like phenotype.
2) Upon direct exposure to macrophages, instead of inducing the expected endotoxin-tolerance suppression of inflammatory responses, MS13 and C28 introduced by repeat stimulation as a consequence of homotolerisation or heterotolerisation protocol with LPS, effectively activated both M1 and M2 macrophage subsets to express a cytokine profile indicative of driving both innate pro-inflammatory responses as well as being activatory for NK cells and Th1/Th17 cells. Such responses induced by these probiotics are pivotal to immune defence against viral infection or immune tumour surveillance.
3) Live C28 and MS13 beneficially modulate epithelial cell functionality in a homeostatic environment (augment barrier integrity – ZO-1; antimicrobial activity – hBD-2 and mucosal tolerance – IL-10).
4) Live C28 and MS13 differentially modulate epithelial cell molecules ZO-1, hBD-2, IL-8, TNFα (both pro-inflammatory and immune-activatory) and IL-10 (immune-suppressive), dependent on the inflammatory environment being predominated by TNFα or IL-1β. Thus, have the capability to either re-inforce mucosal tolerance and barrier functionality or either activate appropriate innate defences or over-activate destructive pro-inflammatory responses.

Further work:

These results and conclusions drawn are indicative of the need for extensive further investigation to be undertaken in order to reach a point whereby these probiotic strains may be recommended for adoption as clinically relevant modulators of the host immune system. Several issues have presented themselves, as a consequence of development of this project and that of others presented in the scientific literature:

1) These probiotics may activate pro-inflammatory responses in macrophages, yet suppressive responses in epithelial cells, representative of the gut mucosal barrier. It is imperative to fully investigate the role of these probiotic strains in modulating cellular interactions between epithelial cells and macrophages in a trans-well co-culture system of the gastro-intestinal mucosa. These data can then be used to justify expansion of co-culture model to investigate the effects on T cell subset responses, incorporating peripheral blood-derived T cells.
2) Although the original train of thought was that these probiotic strains represented potential negative-regulators of destructive mucosal inflammatory responses, results presented in this thesis are indicative of their immune-activatory nature, pre-disposing to anti-viral and anti-tumour responses. Future work can focus on probiotic modulation of i) *in vitro* TLR-mediated antiviral responses of macrophages, ii) *in vivo* anti-tumour responses in rat models of colorectal cancer (CRC) (Hradicka et al., 2020) and iii) *ex vivo* CRC explant tissue immune proteomic profiling.

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List of Publications

Conferences

Poster presentation

Al-Abdulwahid, L., Beal, J., & Foey, A.D. (2016).Live probiotic bacteria modulate immune response of THP-1 macrophage. PUPSMD Annual Graduate Research Event, St Mellion, Cornwall, UK, 2016.

Al-Abdulwahid, L., Beal, and J. & Foey, A.D. (2016): Live probiotics activate cytokines production in presence or absence of E.coli strain K12 lipopolysaccharides (LPS). Innate Immunity Symposium. BSI & NVVI Joint Conference,6th-9th December, Liverpool, UK.

Al-Abdulwahid, L., Beal, J. & Foey, A.D. (2017). Selective ability of probiotics to prime or tolerise distinct macrophage subsets in a strain-specific manner. International Cytokine & Interferon Society (ICIS), Kanazawa-Japan.

Platform presentation

Lactobacillus plantarum (C28) and *Lactobacillus salivarius* strain (MS13) induced production of IL-1β and IL-18 differentially from M1 and M2-like macrophages. Annual Research Event 2018 for Postgraduate Research, at Plymouth.

Appendices

General material

1 Tissue culture reagents and material

Table 1-1: Tissue culture reagents and material

Product	Product code	Supplier
1, 25-(OH)2 Vitamin D3	D1530	Sigma-Aldrich, Poole, UK
Bovine serum albumin (BSA)	A8531-1VL	Sigma-Aldrich, Poole, UK
phorbol-12-myristate acetate (PMA)	P1585	Sigma-Aldrich, Poole,
L-Glutamine 200nM		Fisher Scientific UK Ltd
RMPI 1640	12-167F	Lonza Bioscience ltd, UK
Foetal Calf Serum	FCS-SA-10545/500	Labtech International Limited, East Sussex, UK
Escherichia coli K12-LPS	tlrl-peklps	Invivogen, UK
DMEM	BE12-614F/12	Lonza Bioscience ltd, UK
Dulbecco's Phosphate Buffered Saline	D5652-10X1L	Sigma-Aldrich, Poole, UK
dimethyl sulfoxide (DMSO)	D8418	Sigma-Aldrich, Poole, UK
Ethyl alcohol	E7023	Sigma-Aldrich, Poole, UK
Penicillin/streptomycin A	A 2213	Source Bioscienc
THP-1 cells line		ECACC, Salisbury, UK
Caco-2 cell line		ECACC, Salisbury, UK
Thincert cell culture insert-well	665640	Greiner Bio-one-Ltd, Stonehouse, UK
Cryopreservation Storage media 20%v/v FBS/10% v/v dimethyl sulfoxide (DMSO)/ DMEM or RPMI		
Cell culture costumes (Flasks, plates. Tips,pipettes)	Greiner Bio-one-Ltd,	Stonehouse, UK
Mrs broth	69966- 500g	Sigma-Aldrich Company Ltd
Bovine serum albumin (BSA)	A7906	Sigma-Aldrich, Poole,

2. ELISA

Table 2-1: ELISA reagents and Antibodies.

IL-10 Purified Rat Anti-Human capture	**554497**	**BD Pharmingen, UK**
IL-10 Biotin Anti-Human detect antibody	**554499**	**BD Pharmingen**
Purified anti-human anti-IL-10	**501402**	**Biolegend, London, UK**
IL-1β capture antibody	**MAB601**	**R&D Systems,**
IL-1β detection antibody	**BAF201**	**R&D Systems, Abingdon, UK**
IL-1β standard antibody	**1989**	**NIBSC, Hertfordshire,**
TNFα standard antibody	**12/154**	**NIBSC, Hertfordshire,UK**
TNFα anti-human capture antibody	**551220**	**BD Pharmingen, UK**
TNFα Biotin Mouse Anti-Human detection antibody	**554511**	**BD Pharmingen, UK**
IL-6 Purified Rat Anti-Human capture antibody	**554543**	**BD Pharmingen, UK**
IL-6 standard antibody	**89/548**	**NIBSC, Hertfordshire, UK**
IL-6 Purified Rat Anti-Human captureantibody	**554543**	**BD Pharmingen, UK**
IL-8 Purified Rat Anti-Huma Capture antibody	**554716**	**BD Pharmingen, UK**
IL-8 Biotin Anti-Human detection antibody	**554718**	**BD Pharmingen, UK**
IL-8 standard antibody	**89/520**	**NIBSC, Hertfordshire, UK**
L-18 Human Matched Antibody Pair	**BMS267/2MST**	**eBioscience, Thermo Fisher Scientific Inc, UK**
Human Total IL-18	**DY318-05**	**R&D systems abiotecnne brand**
IL-12 p35 Monoclonal Antibody (B-T21 (BT21)),	**14-7128-82**	**Thermo Fisher Scientific**

IL-12/IL-23 p40 Monoclonal (50ug)	**14-7125-81**	**Thermo Fisher Scientific**
IL-23 KIT	**88-7237-88**	**Thermo Scientific**
SULFORHODAMINE B	**230162-5G**	**Sigma-Aldrich Company Ltd**
PhosphateBufferedSaline: **powder 5 5 L of 10X** **Phosphate buffered saline tablets**	**sc-24947** **P4417**	**SANTA CRUZ BIOTECHOLOGY** **Sigma-Aldrich, Poole**
TMB Microwell Peroxidase Substrate Kit (component)	**50-76-00**	**Insight Biotechnology**
Nunc ELISA Plates Immunosorbent.	**10547781**	**Fisher Scientific, UK**
Tween 20	**P2287-100ML**	**Sigma-Aldrich, Poole, UK**
3-4-5-dimethyl-2.5 thiazol-2.5 diphe-nyltetrazolium bromide (MTT)		**(Sigma-Aldrich, Poole, UK))**

Western Blotting reagents were obtained from Sigma-Aldrich, Poole, UK

Lysis Buffer

Ripa buffer

Protease Inhibitor Cocktail

1:20 = for 200µl lysis buffer add 10µl

Phosphatase Inhibitor Cocktail

1:100 = for 200µl lysis buffer add 2µl

SDS-PAGE reagents

Acrylamide, electrophoresis grade

Bis- acryl amide (N.N –methylenebisacrylamide)

Tris (2-hydroxyethyl-2-methyl-1, 3-propanediol)

SDS (sodium dodecyl sulphate or sodium lauryl sulphate)

TEMED (N, N, N, N,-tertamethylene-ethylenediamine)

APS (Ammonium per sulphate)

2-mercaptoethanol

Glycerol

Bromophenol blue

Glycine

HCl (Hydrochloric acid)

Solution A (separating gel buffer)

Acrylamide Stock Solution, 100 ml 30% (w/v) acrylamide

(w/v) bis- acrylamide

Solution B (separating gel buffer):

1.125 M Tris base 68.14 g per 500 ml

0.3%w/v SDS 1.5 g per 500 ml

Dissolved in DW to 450 ml adjusted to pH 8.8 with HCl.

Top up to 500 ml with DW.

Solution C (Stacking buffer)

140 mM Tris 8.42 g per 500 ml

0.11%w/v SDS 0.55 g per 500 ml

Dissolve in DW to 450 ml. Adjust to pH 6.8 with

Concentrated HCl 100%. Top up to 500 ml with DW

10%w/v APS (0.1g APS dissolved into 1ml DW)

Electrophoresis buffer (Running buffer)

Tris 3 g

Glycine 14.4 g

SDS 1 g

Dissolved in DW 1L, pH 8.3

Transfer buffer stock (10X Exc. Methanol)

Tris base 30.3g

Glycine 144g

DW 1L.

Transfer buffer working solution

10X transfer buffer 100 ml

Methanol 200 ml

DW 700 ml

Washing solution

tris buffered saline with tween 20 (TBST)

TBS-Tween-20 (0.1% v/v)/TBS

10xTBS (tris buffered saline)

NaCl 80g

Tris 24.4g

DW Mix in 800ml of DW, adjusts to pH 7.6, and make up

To 1L with DW

TBS-Tween-20 (TBST)

10x TBS 40ml

DW 959ml

Tween-20 1ml

Blocking solution

TBST 100 ml

5% w/v BSA 5g

Stripping buffer

Mild stripping

15 g glycine

1 g SDS

10 ml Tween 20

Adjust pH to 2.2 Top up to 1 L with DW

Coomassie Gel stains (1L.)

Coomassie blue R-250 1.0 g

100%Methanol 450 ml

DW 450 ml

100 ml 100 % glacial acetic acid

De-staining Coomassie Gel stain solution (1L.)

Methanol 100% 100 ml

Glacial acetic acid 100% 100 ml

DW 800 ml **3**

Protein Ladder (Hyper Page pre-stained protein Marker, Bio line, UK).

3: Protein analysis

Table 3-1: 1 Protein analysis reagents western blotting solutions

Product	Product code	Supplier
Biotinylated Protein Ladder Detection	7727S	New England Biolabs, Herts, UK
Criterion Xt precast gel, 4-12% Bis-Tris	345-0123	Bio-Rad Laboratories
anti-NLRP3/NALP3,mAb(Cryo-2) 100ug	AG-20B-0014-	AdipoGenAG
Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (100X)	78442	Thermo Scientific, UK
Luminata Crescendo Western HRP sub-strate	WBLUR0100	Millipore
Micro BCA Protein Assay Kit	23235	Thermo FisherScientific, UK
Pierce Prestained Protein MW Marker	26612	Thermo FisherScientific, UK
PVDF Transfer Membrane	88518	Thermo Fisher Scientific, UK
Restore Western Blot Stripping Buffer	21062	Thermo FisherScientific , UK
RIPA Lysis and Extraction Buffer	89900	Thermo Fisher Scientific , UK
Water ,Molecular Biology Reagent	W4502-1L	Sigma-Aldrich, Poole, UK
Western Blotting Filter Paper	88620	Thermo Fisher Scientific , UK
anti-mouse IgG,HRP 1 mg		**AdipoGen AG**

4:RNA qualification

4.1: RNA qualification reagents

Product	Product code	Supplier
Bovine serum albumin (BSA)	A8531-1VL	Sigma-Aldrich, Poole, UK
Deoxynucleotide Mix,10mM	(plates, Tips and pipettes)	Fisher Scientific UK
Ethyl alcohol	E7023	Sigma-Aldrich, Poole, UK
GenElute mammalian total RNA extraction kit	RTN350-1KT	Sigma-Aldrich, Poole, UK
On-Column DNase I Digestion Set	DNASE70	Sigma-Aldrich, Poole,UK
High-Capacity RNA-to-cDNA™ kit	4387406	Thermo Fisher Scientific
SYBR® Green Master Mix	10658255	Applied Biosystems,Cheshire - UK
Water ,Molecular Biology Reagent	W4502-1L	Sigma-Aldrich, Poole,UK
Primers		Eurofins Genomics, Wolverhampton, UK

Table 4.2: Summary of primers used in this study and the amplicon product size

Target	Forward primer 5'	Size (base pair bp)	Reverse primer 3'	Size (base pair bp)	Product size bp
GAPDH	CTGCTCCTCCTGTTCGACAGT	21	CCGTTGACTCCGACCTTCAC	23	100
TNFα	ACATCCAACCTTCCCAAACG	20	TCGATGACAGCGCCGTAGCCT	22	151
IL-10	AGGAGGTGATGCCCCAAGCTGA	22	TCGATGACAGCGCCGTAGCCT	21	110
IL-1 β	GCCAATCTTCATTGCTCAAGT	20	AGCCATCATTTCACTGGCGA	20	73
IL-6	TGGCTGCAGGACATGACAAC	20	TGAGGTGCCCATGCTACATTT	20	100
IL-12p35	AGCCTCCTCCTTGTGGCT A	19	GCTGGGAGTACCCTGACAC	19	100
IL-23P19	TGCTGGATTGCAGAGCAGTAA	21	CTGGAGGAGTTGGCTGAGTC	20	100
TLR4	AGCCCTTCACCCCGATTC	18	TAGAAATTCAGCTCCATGCATTG	23	100
TLR2	GGCATGTGCTGTGCTCTGTT	20	GGAGCCAGGCCCACATC	17	100
NLRP3					
Tollip	TCTCATGCCGTTCTGGAAAAT	21	TCACATCACAAAATGCCATGAA	22	110
TRAIL	CTTGACCTGACCCCGAGATA	20	CCCACAGAGAAAGGAAGCAG	20	100