Immunomodulatory properties of bovine caseins on innate immune cells

Richard Lalor B.A. M.Sc

Thesis Submitted for the Award of PhD

School of Biotechnology

Dublin City University

Supervisor: Associate Professor Sandra O'Neill

August 2019

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ ID No.:_____ Date: _____

Acknowledgements

Firstly, I would like to express a special thanks to my supervisor, assistant professor Sandra O'Neill. Thank you for introducing me to the fascinating topic of immunomodulation and allowing me the freedom to research questions using my own special brand of creativity. Your encouragement and mentoring has really influenced my growth as a scientist.

I would like to thank almost everyone in the school of Biotech, fellow postgrads, lectures and technical staff who made DCU welcoming and my time there very enjoyable and memorable. The animal work would not have been possible without the expertise and invaluable help from Carolyn and Damian. The kind words of wisdom, encouragement and general chat helped a lot too. A big thank you goes out to previous and current members of the O'Neill lab, Alli and Alessandria, who I started with, made me feel at home and inspired me. Anna and Arlene who helped shape my research to what it is today, and got me through some challenging times when nothing was working. I will always look back fondly at the time we shared together.

A special thank you also goes out to my family and friends. All the social activities with Andy, Dan and Jay helped make the 4 years fly by. To my parents, I am so thankful for your love, kindness, support and a lot of patience during the last 4 years. Your unwavering belief in me kept me going. It wouldn't have been possible without all you guys.

Table of Contents

List of figures

List of abbreviations

%:	Percent
°C:	Degrees celsius
α:	Alpha
APC:	Antigen presenting cell
Arg:	Arginine
β:	Beta
BCA:	Bicinchoninic acid
BMDC:	Bone-marrow derived dendritic cells
BMMφ:	Bone-marrow derived macrophage
BSA:	Bovine serum albumin
c:	Complete
Ca:	Calcium
CAS:	Casein
CD:	Cluster of differentiation
CLR:	C-type lectin receptors
CTLA:	Cytotoxic T-lymphocyte-associated protein
DC:	Dendritic cell
DMEM:	Dulbecco's modified eagle's medium
ELISA:	Enzyme-linked immunosorbent assay
FACS:	Fluorescence-activated cell sorter
FCS:	Fetal calf serum
FITC:	Fluorescein isothiocyanate
g:	Gram
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF:	Granulocyte-macrophage cell stem factor
gMFI:	Geometric mean fluorescence intensity

hr:	Hour
IFN:	Interferon
IκBα:	Inhibitor of kappa B
IKK:	IκB kinase
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
JAK:	Janus kinase
κ:	Kappa
LOX:	Loxoribine
LPS:	Lipopolysaccharide
M:	Molar
m:	Milli
M1:	Classically activated macrophages
M2:	Alternatively activated macrophages
mAb:	Monoclonal antibody
M-CSF:	Macrophage colony stimulating factor
MGL:	Macrophage galactose-type lectin
MHC:	Major histocompatibility complex
min:	Minute
Mφ:	Macrophages
MR:	Mannose receptor
n:	Nano
Na	Sodium
NFκB:	Nuclear factor kappa B
p:	Pico
PAGE:	Polyacrylamide gel electrophoresis
PAMP:	Pathogen associated molecular pattern
PBMC:	Peripheral blood mononuclear cell

PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PD:	Programmed death
PGE:	Prostaglandin
PGN:	Peptidoglycan
PI:	Propidium iodide
PMA:	Phorbolmyristate acetate
PRR:	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RELM	Retnla resistin like
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RPMI:	Roswell park memorial institute
s:	Second
SD:	Standard deviation
SDS:	Sodium dodecyl sulfate
SOCS:	Suppressor of cytokine signaling
STAT:	Signal transducer and activator of transcription
T_H:	T helper cell
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TMB:	3,3',5,5'-Tetramethylbenzidine
Treg:	T-regulatory cell
µ:	Micro
v/v:	Volume to volume
WP:	Whey protein
γ:	Gamma
YM-1:	chitinase-like 3

Abstract

Richard Lalor

Immunomodulatory properties of bovine caseins on innate immune cells

The field of nutraceutical research has rapidly expanded as more evidence suggests that functional foods like milk have positive health impacts beyond their nutritional value. The consumption of proteins and peptides derived from milk have been shown to display an array of bioactive properties that could be helpful in the management of many western diseases such as inflammatory, cardiovascular and metabolic. Immunomodulatory nutraceuticals have gained special attention due to their therapeutic potential for the amelioration of chronic inflammatory disorders as patients seek alternatives to drugs which often have side effects which can outweigh their benefits. Macrophages and dendritic cells are both key players in the induction, propagation and resolution of inflammatory responses, and are known to actively contribute to the pathogenesis of many inflammatory diseases. As such, these cells were chosen in this study to investigate the effects of bovine milk derived compounds on inflammatory processes. Sodium caseinate exhibited immunomodulatory properties, which were attributed to the kappa-casein subunit. Kappa-casein primed novel suppressive murine macrophages ($CD54^{high}$, $CD206^{high}$, $CD40^{high}$, $SOCS1^{high}$ & $SOCS3^{high}$) and semi-immature dendritic cell ($CD209^{low}$, $CD40^{low}$, $SOCS1^{high}$ & $SOCS3^{high}$) phenotypes that have not been previously described. It inhibited the induction of pro-inflammatory cytokines in both cell types by targeting the NFκB signal transduction pathways in a mechanism that may involve the upregulation of SOCS1 and SOCS3. These results were transferable in human derived macrophages. All kappa-casein induced phenotypes significantly suppressed the production of IL-2 from $CD4^{+}$ T-cell *in-vitro* & in *in-vivo*, a key cytokine required for effector T-cell responses. These immunomodulatory effects are attributed to a novel fragment of kappa-casein. Given the powerful immuno-modulatory effects exhibited by kappa-casein and our understanding of the immune pathology associated with inflammatory diseases, this fragment has potential as an oral nutraceutical to manage diseases such as inflammatory bowel disease and therefore warrants further investigation.

Chapter 1 – Introduction

Nutraceutical is a term derived from "nutrition" and "pharmaceutical" that is applied to products that are isolated from herbal, dietary supplements and functional foods such as dairy, cereals and beverages which beyond nutritional value possess physiological benefits to improve health or prevent chronic diseases (Kalra 2003; Zhao 2007). The nutraceutical industry has received considerable interest due to the safety and therapeutic effects exhibited by these products and are becoming more important in today's society as consumers are increasingly more health conscious. Consequently, these industries are rapidly expanding with a net value of $ 230.9 billion globally in 2018 which is projected to reach $ 336.1 billion by 2023 (Nutraceuticals: Global Markets 2018).

Functional food derived bioactive peptides has been a rapidly expanding sector within the nutraceutical industry. Bioactive proteins and peptides are defined as specific proteins or protein derivatives that have a positive impact on bodily functions which may ultimately influence health (Kitts and Weiler 2003). They have been derived from numerous sources including milk, cheese, yoghurt, fish, and soybeans among others and can vary in size from a small peptide 3 amino acids in length to full-sized proteins. Milk, in particular, has the greatest potential to be used commercially as a source of these bioactive nutraceuticals as the production and consumption of milk products has increased (O'Connor 2009) and bioactive proteins and peptides derived from milk display an array of bioactive properties including anti-tumour, anti-microbial, anti-oxidant, opioid, ACE-inhibitory and immunomodulatory activity (Rutherford-Markwick *et al.*, 2005; Savijoki *et al.*, 2006; Madureira *et al.*, 2007; Dziuba *et al.*, 2009).

Immunomodulation is necessary to control the consequences of a deregulated immune system. Strategies to effectively modulate the immune response have been extensively

sought out to combat inflammatory diseases and disorders. The most frequently used strategies involves the use of drugs that slow the progression of specific diseases, however they can often have unforeseen and potentially harmful side effects which can outweigh their benefits (Nongonierma & FitzGerald 2015). In this context, bioactive proteins represent a viable alternative to the use of drugs for the management of inflammation, as they have been shown to stimulate or inhibit certain immune functions, generally have low toxicity, are easily degraded and tend not to accumulate in bodily tissues (Gokhale & Satyanarayanajois 2014; Agyei *et al.,* 2016).

Inflammatory bowel diseases (IBD) that affect the gastrointestinal (GI) tract like crohn's' disease (CD) and ulcerative colitis (UC) whose incidence and prevalence are increasing worldwide (Molodecky *et al.,* 2012), are prime targets for the use of bioactive immuno-modulatory proteins and peptides as they are administered orally, a non-invasive natural route that delivers the bio-actives to the inflammatory site. They are characterized by severe inflammation of the GI tract and associated with prolonged activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and subsequent increased synthesis of pro-inflammatory mediators like the cytokine tumour necrosis factor-α (TNF-α) in both conditions (Strober & Fuss 2011) proposed to by mainly produced by antigen-presenting cells (APC) and T-cells (Cobrin & Abreu 2005).

Milk derived casein bioactive proteins and peptides have been demonstrated to ameliorate experimental models of IBD in mice and human pilot studies of UC (Requena *et al.,* 2008; Lopez-Posadas *et al.,* 2010; Hvas *et al.,* 2016). Studies, in particular *in-vitro* studies, have also demonstrated that these bioactive peptides have an immuno-modulatory effect on the inflammatory capacity of antigen presenting cells, namely macrophages and dendritic cells (DC) (Monnai & Otani 1997; Mikkelsen *et al.,* 2005; Cheng *et al.,* 2015; Li *et al.,* 2017),

two cell types heavily implicated in the initiation and propagation of IBD (Steinbach & Plevy 2014). While these studies examine how effective casein (CAS) bioactive proteins and peptides are at suppressing macrophages and dendritic cells inflammatory responses, there is a dearth of research on the mechanism by which these molecules act on these cell types. Therefore, more studies are required to understand the activation status, and to define the cellular phenotype which not only affects their immediate effector functions but can also heavily influence their ability to initiate and propagate adaptive immune responses (Takeda *et al.,* 2003; Pasare & Medzhitov 2004). This research will further examine the use of immuno-modulatory milk casein based bioactive proteins, advancing our understanding of the impact they have on key innate immune cells potentially leading to the discovery of new viable alternative to the use of pharmaceuticals in influencing health for the prevention and treatment of chronic diseases.

1.1 Functional foods and nutraceuticals

Functional foods while similar to conventional foods have been demonstrated to exhibit physiological benefits which can reduce the risk of chronic disease beyond their basic nutritional functions (Food and Agriculture Organization of the United Nations 2008). Functional foods such as yoghurt and fermented milk when ingested were shown to promote bowel regularity and modulate GI tract immune responses (Tamang 2010) while several epidemiologic studies have strongly correlated high dietary intake of certain fruits and vegetables with reduced risk of developing chronic diseases, some of the beneficial effects being attributed to their anti-oxidant properties (Balsano & Alisi 2009). Nutraceuticals defined as ‘a fragment of a food that provides medicinal and health benefits

for the prevention or treatments of a chronic disease' have been derived from both plant and animal functional foods. The supplementation of diets with nutraceuticals derived from functional foods have been shown to ameliorate various human diseases including hypertension, cardiovascular disease, obesity and type II diabetes (Bagchi *et al.* 2010). The popularity of the functional food and nutraceutical industries are increasing as consumers are becoming more health conscious and seek viable alternatives to the use of often expensive, high-tech disease treatment approaches currently employed in developed countries which can have unforeseen or undesirable side effects in the treatment of chronic conditions. The nutraceutical industry is an evolving entity that offers novel opportunities to merge scientific discovery with growing consumer interest which is rapidly expanding with a market value predicted to reach $336.1 billion by 2023 (Nutraceuticals: Global Markets 2018). Investment in research and development to find and verify health claims of beneficial nutraceutical products represents a large sector of the industry. Bovine milk is a prominent source of many bioactive proteins and peptides derivatives with an array of observed nutraceutical properties that have been shown to have a positive impact in human diseases (Mohanty *et al.*, 2015).

1.2 Bovine milk

Milk is composed of water, protein, fat, carbohydrates and trace elements (Fox 2009) and while the constituents are the same for mammals, the concentration of each constituent varies from species to species. In bovine milk, water is the main constituent, comprising of 87.4-90.7% of the total mass with carbohydrates as the second most prevalent component, comprising of mostly lactose which makes up 4-5% of the total milk composition. Milk fat

contributes to approximately 3.5-4.5% of milk and is mostly contained in fat globules composed of triglycerides, phospholipids, sterols, fatty acids that can contain the fat-soluble vitamins A, D, E and K. Protein contributes to 3-4% of milk constituents while trace elements including salts and minerals make up the final 0.7-0.8% (Food Standards Australia and New Zealand 2006). Research over the past twenty years has begun to identify milk components, milk proteins and in particular peptide derivatives that beyond their nutritional value have potential bioactivity as nutraceuticals.

1.3 Milk proteins

Proteins are complex macromolecules made up from specific sequences of 20 possible amino acids, covalently linked together by peptide bonds forming polypeptides (Rosenberg 1996). The amino acid sequences of these polypeptides make up the primary structure of proteins. These polypeptides can interact via hydrogen binding to from secondary structures called alpha helices and beta pleated sheets which refer to the 3 dimensional shapes the amino acid chains display. The overall 3-dimensional structure of a polypeptide is referred to as tertiary structure, which is primarily influenced by interactions between the attached functional groups of the amino acids within the protein. Many proteins are made up of a single polypeptide chain and only have 3 levels of structure. However, some proteins are made up of multiple polypeptide chains referred to as subunits, which can be held together by hydrophobic interactions, hydrogen bonding and Van der Waals forces, forming a proteins quaternary structure (Rosenberg 1996; Banga 2006).

Milk proteins have been studied for over two centuries (Fox and Mcsweeney 2003) and there are two major classes of proteins in milk: whey and CAS proteins which can be

separated based on their solubility at pH 4.6 at 20°C. Whey proteins include α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), immunoglobulins, lactoferrin and transferrin which constitute 20% of the total protein in bovine milk (Zayas 1997). CAS is composed of four subunits of protein: α_{s1}-, α_{s2}-, β-, and κ-CAS which constitute about 80% of the total protein in bovine milk (Walstra *et al.,* 1999). α_{s1}- and α_{s2}-CAS are moderately hydrophobic phosphoproteins which constitutes for ~50% of the total CAS in bovine milk (Huppertz *et al.,* 2018). α_{S1}-CAS is a 199 amino acids polypeptide with 8 phosphate residues, 7 of which residues near the middle of the protein, in a cluster. This cluster is also negatively charged making it a highly hydrophilic domain. α_{S2}-CAS consists of 207 amino acids with phosphorylated serine residues which unlike the other CAS subunits are not uniformly phosphorylated, the number varying from 10 to 13 (Farrell *et al.,* 2004). α_{s2}-CAS appears to occur primarily as a monomer (Snoeren *et al.,* 1980). It contains both high and low hydrophobic regions, with anionic clusters which are though to be related to its calcium binding properties (Toma and Nakai, 1973). The structure of α_{S2}-CAS, like α_{S1}-CAS, is suggested to be a natively unfolded coil, existing in a pre-molten globule state (Farrell *et al.*, 2009).

β-casein is composed of 209-amino acids, a strongly amphipathic phosphoprotein which constitutes up to 35% of the caseins in bovine milk (Huppertz *et al.,* 2018). The distribution of charge is responsible for the amphipathic properties with the N-terminus residues containing all the net charge of the molecule, have low hydrophobicity, while the C-terminal section contains many non-polar residues and is characterized by little charge and high hydrophobicity. κ-CAS accounts for the rest of the total CAS but displays some unique features. It is the smallest of the CAS, consisting of 169-amino acids and is the only glycosylated CAS subunit, with multiple isoforms showing different degrees of

glycosylation coexisting in milk (Dziuba & Minkiewicz 1996). κ-CAS is also predominantly hydrophilic, particularly the C-terminal portion which is phosphorylated and glycosylated, further increasing hydrophilicity (Huppertz *et al.,* 2018). Molecules of κ-CAS can have up to 9 glycans, containing galactose, N-acetylgalactosamine, and N-acetylneuraminic acid or sialic acid (Vreeman *et al.,* 1986). The structure of κ-CAS has been proposed to acquire a polyproline II helical confirmation interspersed with bends (Syme *et al.,* 2002).

All CAS subunits show a tendency to self-associate but also a propensity to associate with other CAS subunits assembling into unique spherical colloidal micelle-like structures. The micelle is held together and stabilized by unique physicochemical properties, the framework of which is thought to be formed by two dimers of α_{s1}-CAS, linked together by either a α_{s2}- or κ-CAS subunit. Two dimers of β-CAS can then associate with this framework, completing what could be considered to be the basic CAS particle (Kumosinski *et al.,* 1994; Farrell *et al.,* 2003). CAS micelles are important for the binding of minerals with low solubility like calcium and magnesium (de Kruif & Holt 2003). The anionic clusters of α_s- and β-CAS bind to the relatively insoluble minerals, while the calcium insensitive κ-CAS stabilizes the other CAS against calcium induced loss of solubility, providing steric and electrostatic repulsion between micelles and preventing aggregation. In addition to their role as mineral transporters, these CAS subunits have also been shown to exhibit bioactive properties, defined as specific proteins or protein fragments that have a positive impact on body functions or conditions and may ultimately influence health (Kitts & Weiler 2003).

1.4 Bovine milk as a source of bioactive compounds

Bovine milk proteins and peptide derivatives have been reported to have various bioactive properties (Dziuba *et al.,* 2009). Several clinical trials have reported a link between the consumption of dairy products and a reduced risk of heart diseases which was linked to the angiotensin converting enzyme (ACE) inhibitory activity of whey and CAS protein and its protein derivatives released during digestion (Givens *et al.,* 2014; Fernandez-Fernandez *et al.,* 2017). Lactoferrin, a minor whey protein was shown to display antimicrobial activities by inducing membrane disruption, thus preventing pathogen growth. (Cederlund *et al.,* 2011; Takeuchi *et al.,* 2014). Studies in humans have demonstrated that the ingestion of whey proteins has been reported to reduce oxidative stress which is important to human health as the buildup of oxidant species can lead to cellular damage (Sheikholeslami & Ahmadi 2012). CAS and whey proteins have also been shown to exhibit anti-inflammatory properties, reducing T-cell activition markers in low-grade inflammation associated with obese human subjects (Holmer-Jensen *et al.,* 2011).

1.5 Innate immunity and immuno-modulatory properties exhibited by casein bioactive proteins and peptides

The importance of a properly functioning and well-balanced immune system to maintaining health has become strikingly evident. Immune modulation via dietary supplementation strategies involving the use of bioactive nutraceuticals may contribute to the maintenance of immune homeostasis in a healthy population. Immunomodulation via bioactive proteins or peptides occurs when they initiate/regulate immunological responses and cellular functions which can result in the suppression or stimulation of either the innate (functioning

of macrophages, DCs, monocytes and granulocytes) and/or adaptive (T or B lymphocyte activation and proliferation, antibody production and cytokine expression) immune responses.

The immune system can be divided into innate and adaptive immunity, representing two arms of the immune system that are closely related to one another. The innate immune response is the first line of defense against infections and mounts a response when triggered by the recognition of a highly conserved molecular pattern or motif called a pathogen-associated molecular pattern (PAMP) via germ line-encoded pattern recognition receptors (PRRs) present on the extracellular milieu or in endosomal compartments of host cells (Kawai & Akira 2009). These PAMPs are recognised by cells of the innate immune system including macrophages, DCs, and monocytes which are distributed throughout the tissues in the body but also epithelial cells, endothelial cells, and fibroblasts which all play a major role in pathogen recognition during the innate immune response (Akira *et al.,* 2006). The binding of PAMPs by specific PRR triggers the activation of signal transduction pathways that result in the production and release of a diverse array of pro-inflammatory signalling mediators by the host cell which modify vascular endothelial permeability, and recruit immune cells to combat the microbial infection (Kawai & Akira 2009).

Although neutrophils are primarily the first cells to respond to an infection and injury (within a few hours), macrophages, DCs and monocytes are also key players in detecting and mounting a robust inflammatory response as they also phagocytose foreign bodies. During this recognition and response process, effector molecules with biocidal effects like reactive oxygen or nitrogen species are produced by these cells (Kohchi *et al.,* 2009), as well as immuno-stimulatory modulators like cytokines which heavily influence the inflammatory process and thus have a greater effect on controlling the early phase of

infections (Serbina *et al.,* 2003; Dunay *et al.,* 2010; Kim *et al.,* 2011). Moreover, to mount a robust immune response to antigens, effective crosstalk between innate and adaptive immune cells must occur. Macrophages, DCs and monocytes are prominent APCs of the innate immune system which drive subsequent adaptive immune responses. Following phagocytosis, APCs process foreign bodies and present antigens to T-cells on markers like major histocompatibility complex (MHC) II. The antigen-MHC complex is the main stimulatory signal (signal 1) presented by APCs to T-cells which engages with the T-cell receptor (TCR)-CD3 complex. Co-stimulatory receptors bind their T-cell counterparts (signal 2) such as CD80 and CD86; in the presence of immuno-stimulating factors (signal 3) like cytokines which influence which type of the effector T-cell response is elicited (Reis e Sousa 2006). The presence of these three signals is a requirement for effective T-cell stimulation (Reis e Sousa 2006; Green *et al.,* 2009), lowering the threshold needed for T-cell activation and the subsequent production of interlukin (IL) -2, a cytokine crucial to T-cell expansion (Lenschow *et al.,* 1996). However, a reduction in signaling strength, via the lack of co-stimulatory molecule interaction or the presence of inhibitory ligands can sequester T-cell responses by inhibiting IL-2 production and subsequent T-cell proliferation, which are important mechanisms used to maintain homeostasis and tolerance to self-antigens (Slavik *et al.,* 1999; Okazaki and Honjo 2006).

Improper resolution or activation of the inflammatory response can result in the persistence of effector cells and their pro-inflammatory mediators which can become deleterious for the host, leading to the development of chronic inflammatory diseases. Considering that many human diseases are immune-related, there in lies great potential and demand for the development of immunomodulatory nutraceuticals like bioactive protein and peptides as possible therapeutic to treat these diseases. CAS, its subunits and enzymatic derivatives of

these proteins have been extensively studied because of their potent immunomodulatory properties. Carr *et al.,* demonstrated that intact αs_1-CAS enhanced the mitogen-stimulated proliferation of murine splenic T-cell responses *in-vitro* (Carr *et al.,* 1990). Similarly to αs_1-CAS, Wong *et al.,* demonstrated that intact β-CAS significantly enhanced the mitogen-induced proliferation of T- lymphocytes and B lymphocytes *in-vitro* (Wong *et al.,* 1996). In contrast to αs_1- and β-caseins, κ-CAS exerted suppressive effects on murine and rabbit lymphocyte proliferation induced by a range of T-cell mitogens (Otani & Hata 1995). However, conflicting reports suggested that κ-CAS promoted proliferation of murine spleen cells, in the absence of extraneous mitogens (Yun *et al.,* 1996). The major immuno-suppressive effects exhibited by κ-CAS were attributed to the glycomacropeptide (GMP) component (Otani *et al.,* 1995). GMP consists of a portion of the C-terminal component of κ-casein that is usually glycosylated and can contain a high amount of N-acetylneuraminic acid residues. GMP was shown to inhibit proliferative responses in the spleens of mice and rabbit Peyer's patch cells (Otani *et al.,* 1995).

1.6 Benefits of using bioactive proteins in immunomodulation.

Research into the use of immuno-modulatory bioactive protein-based nutraceuticals has gained interest due to their potential use as a dietary intervention strategy in the treatment of many immune related diseases. These molecules do not exhibit the unwanted side effects that are commonly associated with traditional chemical pharmacologic drugs (Nongonierma & FitzGerald 2015). Currently, there are more than 60 approved protein-based therapeutics available on the market, with approximately 140 more in clinical trials (Gokhale & Satyanarayanajois 2014).

Bioactive based-proteins and peptides have several traits which makes them suitable for use as therapeutic agents. These traits include structural diversity, high activity and wide spectrum of action, including some multifunctional properties. Bioactive peptides are naturally occurring biologics and in contrast to synthetic substances are degraded into their component amino acids without the production of intermediate toxic metabolites. Peptides are readily degraded, with generally short half-lives, combined with their larger size avoids their accumulation in bodily tissues compared to smaller chemical molecules. Moreover, bioactive based-proteins and peptides are generally small enough to allow efficient delivery/adsorption and ensures a low likelihood of triggering undesirable immune responses (Hancock & Sahl 2006; Marx 2005; Mason 2010; Agyei *et al.*, 2016).

1.7 Inflammatory bowel disease and inflammatory signalling as a target for milk derived bioactive peptides

Conditions affecting the GI tract represent prime targets for the use of bioactive immuno-modulatory proteins and peptides as they can be easily administered orally, a non-invasive route that delivers the bio-actives to the inflammatory site. IBD, specifically CD and UC are GI autoimmune diseases whose incidence and prevalence are increasing worldwide (Molodecky *et al.,* 2012). IBD is considered an immune-mediated disease that involves a complex interplay between host genetics and environmental influences, including intolerance to the natural microbiota of the gut (Abraham & Cho 2009). Immunological results from several human studies combined with data from experimental animal models of the diseases indicate that microbial antigen exposure is heavily implicated in the initiation, perpetuation, and amplification of IBD (Lodes *et al.,* 2004; Abreu *et al.,* 2005;

Kiesler *et al.*, 2015). IBD is characterized by severe inflammation of the GI tract and is associated with prolonged activation of inflammatory signalling pathways, often the result of the stimulation of the toll-like receptors (TLR), PRR heavily involved in microbial antigen recognition (Figure 1.1).

The NFκB pathway is one of the most prominent signalling pathways, playing a central role in immunological processes by inducing expression of a variety of genes involved in inflammatory responses and cell survival (Baldwin 1995). In a steady state prior to activation, NFκB is retained in the cytoplasm, sequestered by inhibitor of kappa B (IκB) proteins (Baeuerle 1998). However, when a cell encounters inflammatory stimuli including; inflammatory cytokines, PAMPs, heavy metals or oxidative stress, signalling cascades result in the activation of IκB kinase (IKK) complexes which phosphorylate the NFκB inhibitor protein IκBα, targeting them for polyubiquitination and subsequent proteosomal degradation (Brown *et al.*, 1993). Once degraded, IκBα no longer sequesters the NFκB proteins which translocate to the nucleus and initiate transcription of target genes (Karin & Ben-Neriah 2000).

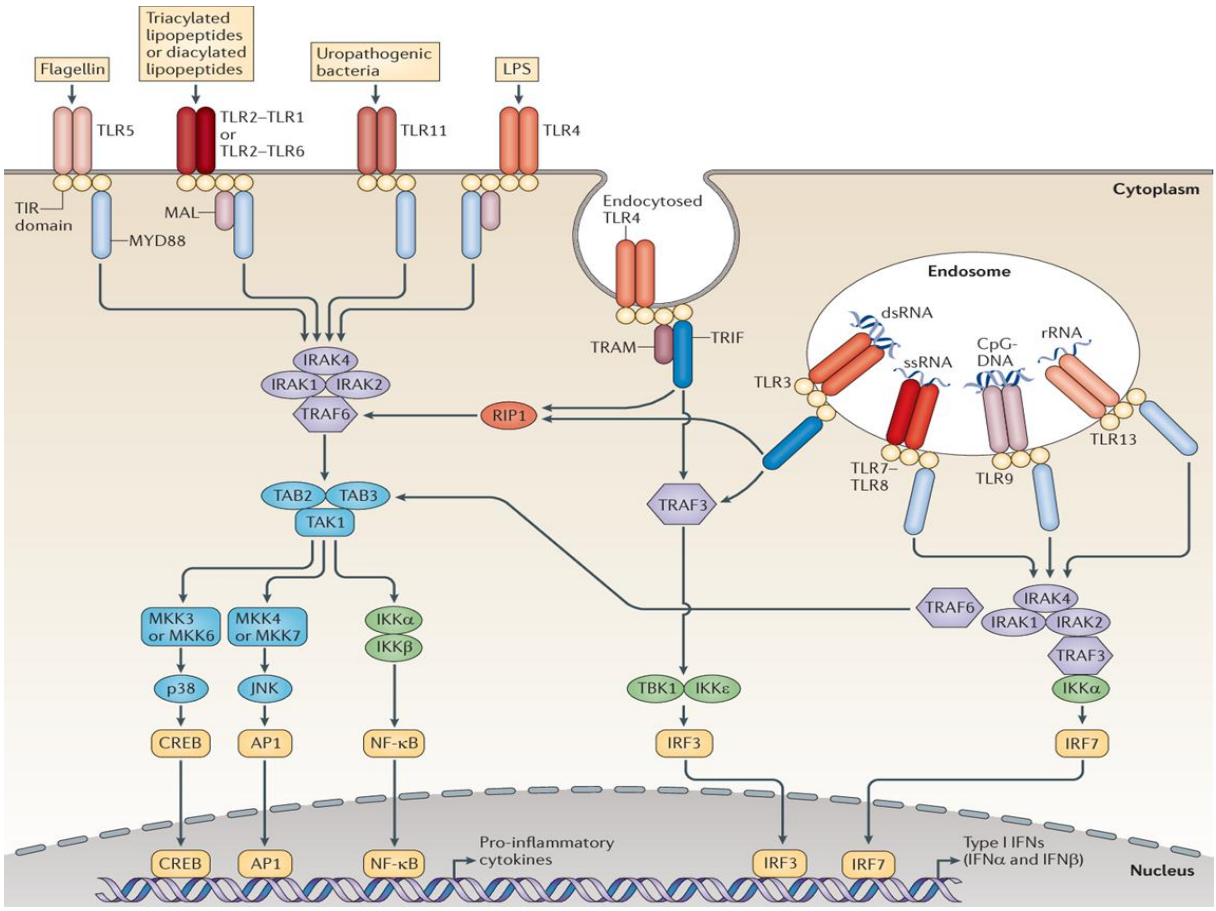

Figure 1.1 TLR induced Inflammatory signalling cascades. Upon recognition, TLR signalling is initiated by ligand-induced dimerization of the TLR receptors. Subsequently, the Toll IL-1-resistance (TIR) domains of TLRs engage with either the signalling adaptor molecules; myeloid differentiation primary-response protein 88 (MYD88) and MYD88-adaptor-like protein (MAL), or TIR domain containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). This engagement stimulates downstream signalling pathways that involve interactions between IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs), that lead to the activation of the mitogen-activated protein kinases (MAPKs) JUN N-terminal kinase (JNK) and p38. These signalling cascades culminate in the activation of the transcription factors; NFκB, interferon-regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1). Both extracellular and intracellular TLR signalling processes converge to produce pro-inflammatory cytokines, while endosomal TLRs also induce type I interferon (IFN). In late phase activation, TLR4 can translocate from the plasma membrane to endosomes where it switches signalling from MYD88 to TRIF, allowing for the production of induce type I IFN. Figure adapted from O'Neill *et al.*, 2013.

Although NFκB is considered the main regulator of the inflammatory response, there are several other pathways involved such as the mitogen-activated protein kinase (MAPK) pathway. There are 3 primary groups of MAPK; extracellular regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNK). Much like NFκB, they can be activated by a variety of stimuli. ERK 1/2 pathway is mainly activated by mitogens and growth factors, but also inflammatory cytokines and PAMPs (Chan & Riches 2001; Guha *et al.,* 2001). A series of the signal transduction cascades can result in the phosphorylation and activation of ERK 1/2 protein which in turn further propagate the signalling cascade upon translocation to the nucleus (Roskoski 2012). ERK 1/2 proteins phosphorylate various other transcription factors, including members of the activator protein-1 (AP-1) transcription factor family (Monje *et al.,* 2005).

Similarly to ERK 1/2, JNK and p38 MAPK can be activated by growth factors, pro-inflammatory cytokines, PAMPs and oxidative stress. Upon phosphorylation, they can elicit downstream effects on transcription factors such as cAMP response element-binding protein (CREB), activating transcription factor-1 (ATF1), signal transducers and activators of transcription-1 (STAT-1) and members of the AP-1 transcription factor family (Sabapathy *et al.,* 2004; Zarubin & Han 2005).

These inflammatory signalling pathways are upregulated in IBD and are responsible for the subsequent increased synthesis of an array of pro-inflammatory stimulatory factors (Strober & Fuss 2011). The inflammatory process is heavily influenced by these factors, the most prominent and well-studied of which are cytokines. Cytokines encompass a diverse group of secreted poly-peptides, which the majority are involved in orchestrating inflammatory cell-cell signalling processes. Early acute inflammatory responses are associated with the induction of the cytokines; TNF-α, IL-1β and IL-6 while IL-2, IL-4, IL-10, IL-12, IL-13,

TNF-α and IFN-γ are observed in longer chronic inflammatory responses (Feghali & Wright 1997). These cytokines signal in an autocrine and paracrine fashion, activating or regulating cellular functions and directing cells towards a specific inflammatory response.

TNF-α is a cytokine that significantly contributes to both acute and chronic inflammation (Feghali & Wright 1997). Not normally detectable in healthy individuals, TNF-α levels only become elevated in the serum and tissue during inflammatory and infectious conditions (Robak *et al.,* 1998) and its serum levels can correlate with the severity of certain infections (Kwiatkowski *et al.,* 1990). Activated cells of the monocyte/macrophage lineage and T-lymphocytes are the primary producers of TNF-α (Parameswaran & Patial 2010). A primary function of TNF-α is to alter the blood flow, vascular permeability, endothelial cell shape and expression of adhesion molecules, enhancing the capacity of inflammatory cells to infiltrate to the site of inflammation (Jersmann *et al.,* 2001). TNF-α also propagates the inflammatory response through the induction of other pro-inflammatory cytokines such as IL-6 (Tseng *et al.,* 2010) and can prolong the survival of activated inflammatory cells, thus sustaining and perpetuating further TNF-α production in an autocrine manner, increasing the induction of TNF-α associated pro-inflammatory factors (Lombardo *et al.,* 2007).

IL-12 is a cytokine secreted by a variety of hematopoietic cell types; however the majority is produced by APCs, typically DCs and macrophages (Heufler *et al.,* 1996). Although a cytokine normally only associated with longer chronic infection, IL-12 is an important immuno-stimulatory modulator at bridging the innate and adaptive immune systems as it induces naïve T-cells to differentiate into T_H1 cells, a T-helper subset that characterized by the production of IFN-γ and critical to the control of intracellular pathogens (Heufler *et al.,* 1996). However, T-cells activated in the presence of IL-4 acquire a T_H2 phenotype,

generating IL-4, IL-5, and IL-13 producing cells effective in the clearance of parasitic infections while activation in the presence of IL-6, TGF-β, and IL-23 results in cells expressing the T_H17 phenotype characterized by the production of IL-17 and are proposed to be involved in host protection against extracellular bacteria and some fungi (Tesmer *et al.*, 2008).

It has been proposed that the elevated production of IL-12 & TNF superfamily ligands by APCs are primarily responsible for the induction of excessive T_H1 T-cell responses observed in CD (Cobrin & Abreu 2005), while the T_H2 dominant T-cell response in UC is dependent on IL-13 primarily derived from NK T-cells (Targan & Karp 2005). IL-23 also has been demonstrated to play a more prominent role in IBD, as it is heavily involved in the induction of T_H17 T-cells, which are observed in both CD and UC (Cobrin & Abreu 2005; Ueno *et al.*, 2018). The long-term effects of dysregulated chronic inflammation caused by disorders like IBD have been linked to the development of cancers in the GI tract (Scarpa *et al.*, 2014). Given the influence innate APC immuno-stimulatory factors have on the initiation and propagation of inflammatory responses in IBD and chronic inflammatory disorders; we focused on the key innate APCs, namely macrophages and dendritic cells (Bates & Diehl 2014; Gren & Grip 2016).

The immune system is a complex and tightly regulated system, with feedback loops and regulatory agents involved in the dampening of the inflammatory responses to maintain this delicate balance. Inflammatory responses are robust, swift and self-limiting through anti-inflammatory mediators like the cytokines IL-10 or via intracellular regulatory proteins like suppressor of cytokine signaling (SOCS). IL-10 is a potent anti-inflammatory cytokine induced by the activation of the STAT3 transcription factor. IL-10 can also be produced by an array of immune cells following stimulation by TLR ligands such as LPS via the

activation of the transcription factors AP-1 and NFκB normally associated with pro-inflammatory signaling. However its production is delayed in comparison to pro-inflammatory genes such as TNF-α that are also induced by LPS (Donnelly *et al.,* 1995). This is thought to be essential for inhibiting the overexpression of TNF-α during inflammatory processes. The exact mechanism by which IL-10 exerts its anti-inflammatory effects is yet to fully elucidated, but there is mounting evidence that suggests IL-10 upregulates SOCS proteins, which inhibits the phosphorylation of IκBα, sequesting the nuclear translocation of NFκB and subsequent production of inflammatory cytokines (Driessler *et al.,* 2004; Hovsepian *et al.,* 2013).

SOCS are a family of intracellular regulatory proteins which are mainly induced by the activation of pathways involved in the expression of cytokines, acting as a negative-feedback loop to curtail excessive cytokine production (Yoshimura *et al.,* 2007). SOCS proteins also play a dynamic role in development and differentiation of cells, for example the ratio of SOCS1 to SOCS3 can influence the phenotype and inflammatory response of macrophages when challenged with a stimulant (Yoshimura *et al.,* 2012). The main inhibitory function of SOCS proteins is to target JAK/STAT pathways via binding of a kinase inhibitory region (KIR) domain on SOCS to JAK, thereby blocking the activation of cytokine signaling (Tamiya *et al.,* 2011). Two of the most well studied SOCS proteins are SOCS1 and SOCS3, both of which inhibit JAK activity through their KIR domain. SOCS1 is often associated with the inhibition of T_H1 signalling, the inflammatory cytokines TNF, IL-12, NFκB and TLR signaling (Yoshimura *et al.,* 2007). SOCS3 acts similarly having been also shown to negatively regulate the cytokines IL-6, IL-10, and IL-2 (Carow and Rottenberg 2014). Studies examining the inhibitory activities of SOCS3 have demonstrated its over-expression mitigated the production of the APC associated inflammatory cytokines

namely; IL-6, TNF, IL-1β in murine models of inflammatory disorders and IBD (Shouda *et al.,* 2001; Li *et al.,* 2012), and intracellular protein therapy with SOCS3 was shown to abrogate acute inflammatory responses induced by staphylococcal enterotoxin B and LPS (Jo *et al.,* 2005).

1.8 The role of macrophages in inflammatory disorders

Macrophages are derived from monocytic progenitor's that originate from the bone marrow. When monocytes infiltrate into peripheral tissues from the blood, they can differentiate into macrophages depending on the local environmental signals (Martinez *et al.,* 2006; Yang *et al.,* 2015). Macrophages act as sentinel antigen presenting cells (APCs) which respond to local stimuli, essential for the early induction of pro-inflammatory mediators and leukocyte recruitment, but are also involved in the resolution of inflammation, wound healing/ repair, homeostasis and tissue remodeling (Koh & DiPietro 2011; Ortega-Gómez *et al.,* 2013). Macrophages are generally organized into two distinct phenotypes: ''classically activated'' M1 or ''alternatively activated'' M2 (Figure 1.2). These macrophage phenotypes often mirror the T_H1/T_H2 T-cells paradigm, as T_H1 phenotypes are often associated with cellular immunity & pro-inflammatory responses while T_H2 phenotypes are generally associated with humoral immunity and anti-inflammatory responses (Mills *et al.,* 2000).

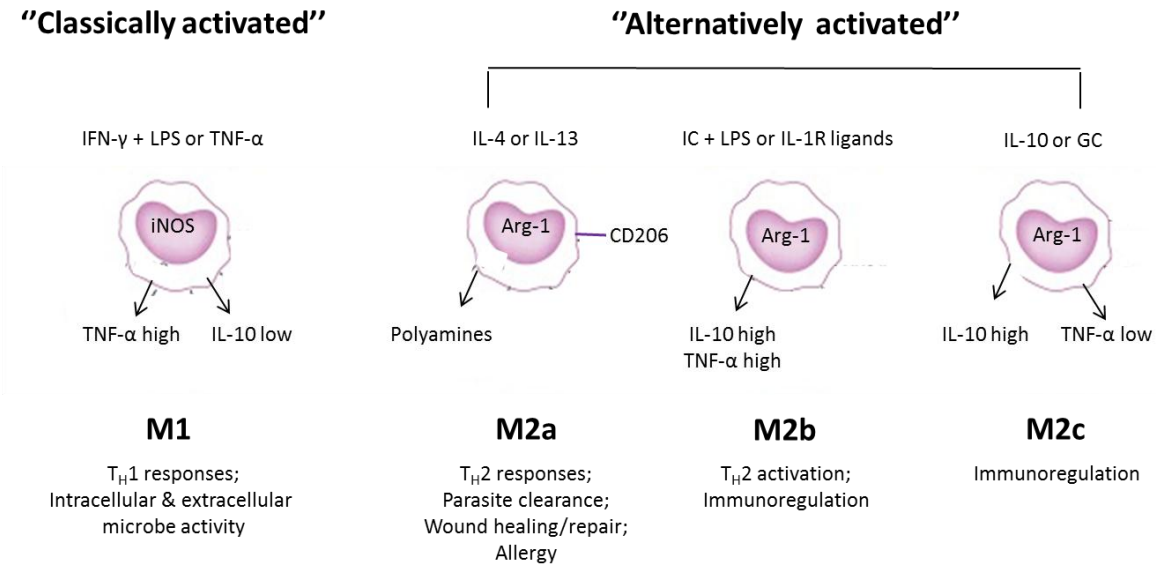

Figure 1.2 M1/M2 macrophage paradigm. M1 macrophages are induced by PAMP signals like LPS and their activation is enhanced by the pro-inflammatory cytokines interferon-gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α). M1 macrophages play an important role in innate defense against intracellular pathogens and are usually associated with T_H1 responses. M2 macrophages are subdivided to accommodate similarities and differences between IL-4 and IL-13 induced M2a, immune complex (IC) & TLR ligand or IL-1R ligand induced M2b, and IL-10 or glucocorticoid (GC) induced M2c. M2a macrophages have been implicated in wound healing/repair, parasite clearance, and allergy while M2b & M2c macrophages are often associated with immune-regulation and the suppression of immune responses. Figure adapted from Martinez & Gordon 2014.

M1 macrophages are pro-inflammatory and cause damage to the host and invading microbes. They are typically induced by the recognition of antigens by specific motifs called PAMPs, damage associated molecular patterns (DAMPs) or in response to other inflammatory stimuli. TLRs are a class of PRRs and a major contributor to M1 polarization, inducing a pro-inflammatory response through the activation of the NFκB, and MAP kinase signaling pathways (Takeda & Akira 2004; Laird *et al.*, 2009). Pro-inflammatory T_H1 associated cytokines such as interferon (IFN) -γ and TNF-α can enhance M1 macrophage microbicide proficiency and increase their capacity to secrete pro-

inflammatory mediators such as TNF-α (Duffield 2003). Along with increased production and secretion of pro-inflammatory cytokines, M1 macrophages also have increased expression of inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and the co-stimulatory receptors; cluster of differentiation (CD)80, CD86 and CD40 (Martinez, & Gordon 2014).

Extensive studies have also found that macrophages can play a significant role in the suppression of inflammation, tissue repair and growth (Martinez & Gordon 2014). These macrophages are often referred to as alternatively activated M2 macrophages. M2 macrophages can be divided into distinct phenotypes with different functions and this is dependent upon the initial stimulus. M2a macrophages represent a population of M2 macrophages with regulatory, wound/repair and effector functions. IL-4 is their primary stimulant, which induces arginase activity; a hallmark of M2 activation, via Arg-1 gene expression driven by the transcription factor signal transducer and activator of transcription (STAT) 6, (Muraille *et al.,* 2014). The upregulation of the mannose receptor; cluster of differentiation (CD) 206, is also a hallmark of M2a activation (Martinez & Gordon 2014). IL-4 and IL-13 are signature cytokines of the T_H2-type immune response which is associated with the induction of M2a macrophages. Tissue injury, helminth infection and allergy all induce T_H2 responses and are associated with the induction of M2a macrophages (Kreider, *et al.,* 2007; Wynn & Vannella 2016; Jiang & Zhu 2016). M2a macrophages are inefficient at antigen presentation, produce low levels of pro-inflammatory cytokines and have a low bactericidal capacity (Edwards *et al.,* 2006). Their role in host defense and adaptive immunity remains somewhat enigmatic as M2a macrophages can indirectly exert regulatory effects on immune responses by secreting polyamines that can influence cytokine production and clonal expansion of lymphocytes (Cordeiro-da-Silva *et al.,* 2004).

M2b and M2c macrophage phenotypes are associated with immuno-regulation, and the suppression of immune responses. M2b macrophages are induced by stimulation with immune complexes (IC) in the presence of TLR ligands, while M2c macrophages are induced by IL-10 or glucocorticoids. Upon activation they secrete high levels of IL-10, the production of which is the most important and reliable characteristic of regulatory macrophages (Mantovani *et al.,* 2004). The phosphorylation and activation of STAT3 is involved in the induction and polarisation of M2 phenotypes, and is heavily implicated in increased IL-10 production from M2 macrophages (Li *et al.,* 2013). As IL-10 is a potent anti-inflammatory cytokine, these macrophages inhibit the production and activity of various pro-inflammatory cytokines such as IL-12... Regulatory macrophages have also been reported to be a key cell type involved in the development of regulatory T-cells (Treg) that can inhibit immune responses (Cao *et al.,* 2010). Regulatory macrophages are often observed during the later stages of adaptive immune responses, to dampen and limit inflammatory processes (Mosser 2003).

However, while *in-vitro* studies have contributed to our understanding of the M1/M2 paradigm (Figure 1.1), macrophages can share M1/M2 phenotype characteristics and there often can be a lack of defined subsets in disease states (Vogel *et al.,* 2013; Italiani *et al.,* 2014). In the synovial tissue of patients with rheumatoid arthritis (RA), macrophages share M1 and M2 characteristics, producing an array of pro-inflammatory mediators including TNF-α, IL-1β & reactive oxygen species among others but also generate IL-1Ra, IL-10, TGF-β, which are anti-inflammatory. However gene analysis of macrophages in the mouse collage induced arthritis (CIA) models would suggest that these macrophages are more M1 polarized (Li, Hsu & Mountz 2012). Strategies to selectively deplete these M1 dominant inflammatory macrophages was demonstrated to prevented the development of arthritis, as

well as ameliorating established arthritis in mice (Li *et al.,* 2012). Furthermore, anti-TNF-α therapies reduced the number of infiltrating synovial macrophages and inhibited the activity of inflammatory macrophages (Taylor *et al.,* 2000; Ehrenstein *et al.,* 2004)

Macrophages also exhibit altered phenotypes that promote and propagate inflammation in IBD (Maloy & Powrie 2011; Yang *et al.,* 2014; Gren & Grip 2016). Studies of dysregulated intestinal inflammation in mice demonstrated the recruitment of inflammatory monocytes that differentiate into macrophages that produce high levels of TNF-α (Zigmond *et al.,* 2012; Tamoutounour *et al.,* 2012). Similarly, in humans with crohn's disease, $CD14^+$ macrophages displayed elevated respiratory burst activity and pro-inflammatory cytokines release in response to TLR stimulation. These hyper-responsive macrophages were shown to dominate the inflamed mucosa and mesenteric lymph nodes of patients with crohn's disease, unlike the resident macrophages of healthy controls which are unresponsive to TLR stimulation (Rugtveit *et al.,* 1997; Kamada *et al.,* 2008). In IBD patients, the accumulation of $CD14^+$ cells was also observed in the mesenteric lymph nodes; immunological sites where APCs migrate to when activated to elicit T-cell responses (Baba *et al.,* 2013). Moreover, macrophages derived TNF-α in IBD, has been implicated in the recruitment and activation of pathogenic effector T-cells, and significantly disrupts epithelial barrier function, exacerbating inflammation which has been shown to be ameliorated by anti-TNF-α therapy (Targan *et al.,* 1997; Rutgeerts *et al.,* 2005; Atreya *et al.,* 2011; Lissner *et al.,* 2015).

1.9 The role of dendritic cells in inflammatory disorders

DCs are another heterogeneous population of immune cells derived from monocytes that infiltrate throughout the peripheral tissues from the blood (León *et al.,* 2005). Similar to macrophages, DCs are sentinel APCs which continuously sample the local environment using a variety of PRR (Steinman & Idoyaga 2010). DCs are however considered to be more efficient at antigen-presentation and controlling/initiating adaptive immune responses compared to macrophages (Lutz & Schuler 2002).

DCs are generally phenotypically categorized based on their level of maturity. Phenotypic maturation is attained when DCs are activated by an antigen resulting in the upregulation of surface costimulatory molecules such as CD80, CD83, and CD86 along with the MHCII molecule (Reis e Sousa 2006). Prior to stimulation DCs exist in an 'immature' (iDC) state, characterised by the low expression of the MHCII and co-stimulatory molecules (Banchereau and Steinman, 1998, Banchereau *et al.,* 2000). DCs in this state are thought to be involved in the maintenance of immune hemostasis/tolerance by impeding the activation of adaptive immune cells. iDCs utilize a plethora of inhibitory mechanisms including the expression of ligands that result in T-cell anergy or the differentiation of immune-suppressive Tregs (Pardoll 2012). Upon stimulation however, iDCs take up a "classical" activation status and mature, upregulating MHCII, cell surface co-stimulatory markers, and produce immune-stimulatory cytokines like IL-12/ or suppressive cytokines like IL-10 depending on the stimulus (Reis e Sousa 2006; Dowling *et al.,* 2008). Signalling through NFκB and MAPK pathways are highly involved in the initiation of this DC activation (Nakahara *et al.,* 2006, Dowling *et al.,* 2008). DCs can also be rendered in a ''semi mature'' state (smDCs) that may lack all the required phenotypic maturation markers or immune-stimulatory signaling molecules like cytokines, needed to elicit a T-cell response.

smDCs have been shown to release immune-suppressive cytokines like IL-10 & TGF-β (Rutella *et al.,* 2006), have increased expression of programmed cell death ligands (PD-L) and have been demonstrated to stimulate the expansion of Treg populations (Harden & Egilmez 2012).

Given their capacity to initiate a cascade of immune responses, DCs have been recognized as key players in several inflammatory disorders. DCs in a steady state from healthy individuals display iDC characteristics and are hypo-responsive to a selection of TLRs (Dillon *et al.,* 2010), while those isolated from inflamed sites of IBD patients display exaggerated inflammatory activity in response to TLR ligands like LPS (Baumgart et *al.,* 2009). Studies have demonstrated that DCs in the mucosa of patients with crohn's disease acquired classically activated pro-inflammatory phenotypes, displaying elevated levels of PRR and co-stimulatory molecules compared to healthy controls (Hart *et al.,* 2005). The accumulations of these activated DCs were also observed in the mesenteric lymph nodes of patient with active crohn's disease and were demonstrated to spontaneously induce T_H1 and T_H17 T-cells *in-vitro* (Sakuraba *et al.,* 2009), compared to steady state DC which promote the induction of Tregs (Iliev *et al.,* 2009). Similarly, inflammatory DC prevalent in the inflamed tissues of RA patients were potent stimulators of T_H1 T-cell responses associated with the disorder (Santiago-Schwarz *et al.,* 2001). In experimental mouse models of IBD, DCs that secrete high levels of TNF-α which have been shown to increase epithelial barrier permeability, resulting in inflammation and injury similar to that observed in ulcerative colitis (Garrett *et al.,* 2007). In other inflammatory disorders, TNF-α, IL-23 and IL-12 secreted by inflammatory DCs is thought to be heavily implicated in the recruitment, induction and activation of T_H1 and T_H17 responses in T-cells, which in turn sustain and amplify the disease by producing cytokines associated with the chronic inflammatory

condition (Zaba *et al.,* 2009; Yawalkar *et al.,* 2009). TNF-α neutralization was shown to attenuate inflammatory DC activity and reduce their prevalence, ameliorating the condition (Marble *et al.,* 2007).

1.10 The role of monocytes in inflammatory disorders

Peripheral blood mononuclear cells (PBMCs) are a heterogeneous population of several different types of immune cell types in the blood including; among others, lymphocytes (B-cells, T-cells and NK-cells) and monocytes (Autissier *et al.,* 2010). CD14 is abundantly expressed on the surface of human monocytes and is used as a marker. During homeostatic conditions, monocytes represent a population of short lived innate immune cells, accounting for 5–12% of total PBMCs, which circulate in the blood and replenish tissue resident macrophages and dendritic cells (Autissier *et al.,* 2010). Upon infection or in response to inflammatory stimuli, monocytes numbers increase and infiltrate to the site of inflammation (Meuret *et al.,* 1974). Similar to macrophages and DCs, monocytes are equipped with PPRs and scavenger receptors, capable of recognizing PAMPs and remove microorganisms via phagocytosis. They produce a broad array of immune-stimulatory effector molecules such as IL-1β, IL-6 and TNF-α which are heavily involved in the early inflammatory response (Dunay *et al.,* 2010). However, monocytes also migrate to sites of injury, clearing apoptotic bodies, debris and producing anti-inflammatory cytokines like IL-10 and TGF-β, facilitating the regeneration of the injured tissues (Ogle *et al.,* 2016). In humans, 3 subsets of monocytes in the blood have been described, based on their expression of CD14/CD16 and seemingly different functions (Ziegler-Heitbrock *et al.,* 2010).

Classical monocytes ($CD14^{++}$ $CD16^{-}$), are phagocytic, exhibit high peroxidase activity, and preferentially produce high levels of anti-inflammatory IL-10 and low levels of pro-inflammatory TNF-α in response to LPS. Gene expression profiling analyses have determined that these monocytes express genes mainly involved in angiogenesis and wound healing (Wong *et al.,* 2011), and after more associated with the resolution/dampening of inflammatory responses/wound repair. Intermediate monocytes ($CD14^{+}$ $CD16^{+}$) have reduced phagocytic and peroxidase activity, but a higher capacity to produce pro-inflammatory cytokines such as TNF-α and IL-1β in response to LPS (Cros *et al.,* 2010). Intermediate monocytes are more akin to APCs, having higher expression levels of MHCII and co-stimulatory molecules required for mediating antigen presentation and T-cell activation (Wong *et al.,* 2011). The final subset, non-classical monocytes ($CD14^{+}$ $CD16^{++}$), can be distinguished by their smaller size and granularity. They have been demonstrated to contribute to inflammation and inflammatory processes, via the release of immune-stimulatory cytokines like IL-1β and TNF-α in response to DNA and RNA particles but have a limited ability to produce reactive oxygen species and inflammatory cytokines in response to microbial PAMPs (Cros *et al.,* 2010). Non-classical monocytes are mainly involved in patrolling of the vasculature, expressing genes involved in cytoskeletal rearrangement.

While monocytes play a pivotal role in the innate immune defense, hemostasis and wound repair, several clinical studies have correlated an increase in the numbers of inflammatory intermediate monocyte ($CD14^{+}$ $CD16^{+}$) populations with the severity of some human inflammatory diseases such as; RA and IBD (Kawanaka *et al.,* 2002; Grip *et al.,* 2007). Significant increases in non-classical monocytes ($CD14^{+}$ $CD16^{+}$) levels have also been described in chronic inflammatory conditions of obesity (Rogacev *et al.,* 2010). Monocytes

isolated from patients with crohn's disease were found to respond similarly to monocytes from healthy controls, when treated with microbial stimulants regarding their phagocytic capacity and production of reactive oxygen species. However, increases in the release of IL-1β and a reduced production of IL-10 were observed in the monocytes isolated from patients with crohn's disease (Schwarzmaier *et al.,* 2013). Intermediate monocytes were also demonstrated to produce higher level of pro-inflammatory cytokines (Fukui *et al.,* 2018) and have an increased capacity to propagate and expand inflammatory T-cell responses *in-vitro* (Rossol *et al.,* 2012) in other inflammatory disorders.

1.11 Immunomodulatory effects exhibited by casein bioactive proteins on macrophages and DCs

Milk derived CAS bioactive proteins and peptides have been demonstrated to ameliorate experimental models of IBD in mice and human pilot studies of UC (Requena *et al.,* 2008; Lopez-Posadas *et al.,* 2010; Hvas *et al.,* 2016). Studies in particular have focused upon these bioactive peptides and their immuno-modulatory effect on APCs such as macrophages and DCs. In murine macrophages, αs_1-CAS reduced phagocytic function and suppressed the production of reactive oxygen and nitrogen species in response to inflammatory stimuli in a dose-dependent manner (Otani & Futakami 1994; Otani & Futakami 1996). Moreover, αs_1-CAS derived peptides were shown to inhibit matrix metalloproteinase 9 activity, an enzyme involved in the induction of inflammatory cytokines (Juillerat-Jeanneret *et al.,* 2011; Chatterton *et al.,* 2013). In contrast to αs_1-CAS however, β-CAS was shown to enhance the production of oxidant species (Otani & Futakami 1994) and significantly increased the production of pro-inflammatory cytokines

from macrophages (Wong *et al.,* 1996). κ-CAS also reduced phagocytic function and suppressed the production of reactive oxygen and nitrogen species in response to inflammatory stimuli in murine macrophages (Otani & Futakami 1994; Otani & Futakami 1996).

Monnai & Otani reported that GMP induced the production of an IL-1 receptor antagonist (IL-1RA); blocking the immune-potentiating ability of IL-1 produced by macrophages following activation with LPS (Monnai & Otani 1997). More recently, GMP and GMP derived hydrolytates were shown to inhibit LPS mediated inflammatory responses in macrophage cell lines, by attenuating NFκB activation via upregulation of heme oxygenase-1 (Li *et al.,* 2017). Endotoxin binding activity by GMP was also implicated in the reduced inflammatory response to LPS by GMP treated murine macrophages (Cheng *et al.,* 2015). In contrast to the suppressive effects exerted by GMP in mouse models, GMP enhanced the proliferation and phagocytic activity of human macrophage-like cells (Li and Mine 2004) and induced the expression of TNF-α, IL-1β and IL-8 in monocytes in a concentration dependent manner (Requena *et al.,* 2009).

Despite the pronounced immunomodulatory effects exhibited by the CAS subunits, the intact whole CAS protein has only been shown to affect murine B lymphocyte proliferation *in-vitro* (Otani *et al.,* 1992) and exhibited anti-inflammatory properties on macrophage-like cell lines, by suppressing LPS mediated TNF-α release (Bamdad *et al.,* 2017). Extensive research on CAS, its subunits and their derivatives has clearly demonstrated their potential use as immunomodulatory compounds that suppress inflammatory processes in macrophages and DCs. However, while these studies have highlighted their immuno-modulatory capacity, there is a dearth of research on the mechanism and by which these compounds dampen the inflammatory responses.

1.12 Summary

Functional food derived bioactive nutraceuticals has been a rapidly expanding sector as supplementation of diets with nutraceuticals have been shown to ameliorate various human diseases including hypertension, cardiovascular disease, and obesity induced type II diabetes (Bagchi *et al.*, 2010). Considering that many diseases are immune-related, there lies great potential and demand for the development of immunomodulatory nutraceuticals as possible therapeutics to treat these diseases. A great body of evidence indicates that bovine milk-derived proteins have the potential to modulate immune function in a number of species (Gill *et al.,* 2000). CAS in particular have been shown to exert many immunosuppressive qualities on cells of the innate immune system, especially APCs like macrophages and DCs, which are heavily implicated in the development of chronic inflammation and inflammatory diseases like IBD (Cobrin & Abreu 2005). Mechanisms such as the induction of IL-10 or SOCS that suppress cytokine signaling may prevent the development of excessive and potentially destructive inflammation. CAS proteins and peptide derivatives have displayed immunomodulatory bioactivity which could upregulate and enhance these regulatory mechanisms which contribute to their capacity to attenuate inflammatory processes. Understanding the mechanisms by which these compounds exert their suppressive effects may be of enormous benefit, potentially enabling the development of therapeutics which can regulate intestinal inflammation, and inflammatory condition like IBD.

1.13 Objectives

The overall aim of this project is to examine the potential of CAS and its derivatives as potential nutraceuticals for the treatment of disease such IBD. This will be achieved by understanding it bioactive properties on cells of the innate immune system that are critical to the immunopathology associated with IBD. More specifically the project will:

- Characterise murine macrophage phenotype and function following stimulation with bovine derived CAS (Chapter 3).

- Determine if single or multiple CAS subunits can mimic the effect of whole CAS on murine macrophage phenotype and function and determine the capability of these macrophages to modulate T-cell development (Chapter 4)

- Characterise the effects of κ-CAS on DC maturation and the capacity of these cells to prime T-cell responses (Chapter 5).

- Determine if the results obtained in murine macrophages and dendritic cells are transferrable to human monocytes and macrophages (Chapter 6).

Chapter 2 – Materials and methods

2.1 Materials

2.1.1 Animals & Cell Lines

Product	Catalogue #	Company
C57BL/6JCrl (C57BL/6)	027	Charles River (Kent, UK)
B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII)	004194	Charles River (Kent, UK)
NCTC clone 929 (L929)	ATCC CCL-1	LGC Standards (Teddington, UK)
P3X63Ag8.653 (X63)	ATCC CRL-1580	LGC Standards (Teddington, UK)

2.1.2 Cell Culture

Product	Catalogue #	Company
DMEM	12491-015	Invitrogen (Paisley, UK)
Fetal calf serum (FCS)	10270-106	Gibco, Invitrogen (Paisley, UK)
Human AB serum	A25761	Gibco, Invitrogen (Paisley, UK)
L-Glutamine	G7513	Sigma-Aldrich (Wicklow, Ireland)
Phosphate buffer saline (PBS)	14190	Gibco, Invitrogen (Paisley, UK)
Penicillin/Streptomycin	1570-063	Gibco, Invitrogen (Paisley, UK)
RPMI 1640	31870-074	Invitrogen (Paisley, UK)
2-Mercaptoethanol	63689	Sigma-Aldrich (Wicklow, IRE)

2.1.3 Antigens, inhibitors & stimulants

Product	Catalogue #	Company
Alpha casein (α-CAS)	C6780	Sigma-Aldrich (Wicklow, IRE)
AS1517499	1992	Axon Medchem (Groningen, NL)
BAY117821	3132	Axon Medchem (Groningen, NL)
Beta casein (β-CAS)	C6905	Sigma-Aldrich (Wicklow, IRE)
CpG oligonucleotides (CPG)	TLRL-1585	Invivogen (Toulouse, FR)
GW9662	M6191	Sigma-Aldrich (Wicklow, IRE)
Hydrolysated sodium caseinate (hNaCAS)	-	Teagasc, Moorepark (Cork, IRE)
Hydrolysated whey protein (hWP)	-	Teagasc, Moorepark (Cork, IRE)
Ionomycin	I0634	Sigma-Aldrich (Wicklow, IRE)
Kappa casein (κ-CAS)	C0406	Sigma-Aldrich (Wicklow, IRE)
JAK2 peptide phosphorylated at Tyr(1007) (pJAK2)	-	GenScript (Piscataway, USA)
Lipopolysaccharide (LPS) (E. coli)	ALX-581-007	Alexis Biochemicals (Lausanne, CHE)
Loxiribine (LOX)	TLRL-LOX	Invivogen (Toulouse, FR)

LY-294 002 hydrochloride	L9908	Sigma-Aldrich (Wicklow, IRE)
Mouse anti-CD3	MAB4851	R&D Systems (Minneapolis, CAN)
Peptidoglycan (PGN) (E. coli)	TLRL-PGNEB	Invivogen (Toulouse, FR)
Phorbol 12-myristate 13-acetate (PMA)	P1585	Sigma-Aldrich (Wicklow, IRE)
Sodium caseinate (NaCAS)	-	Teagasc, Moorepark (Cork, IRE)
Tin Protoporphyrin IX dichloride (SnPPIX)	SC-203452	Santa Cruz Biotechnology (Heidelberg, DEU)
Whey protein fraction (WPF)	-	Teagasc, Moorepark (Cork, IRE)
WP1066	2316	Axon Medchem (Groningen, NL)
Zinc Protoporphyrin-9 (ZnPPIX)	SC-200329	Santa Cruz Biotechnology (Heidelberg, DEU)

2.1.4 Commercial Kits

Product	Catalogue #	Company
Annexin V-FITC Apoptosis Detection Kit	APOAF	Sigma-Aldrich (Wicklow, IRE)
Bicinchoninicacid (BCA) protein assay kit	23255	Thermo Scientific (Leicestershire, UK)
Human $CD4^+$ isolation kit	130-096-533	Miltenyi Biotec (Surrey, UK)
Human $CD14^+$ isolation kit	130-050-201	Miltenyi Biotec (Surrey, UK)
Human IL-2 ELISA kit	88-7025-88	eBiosciences (Hatfield, UK)
Human IL-4 ELISA kit	88-7046-88	eBiosciences (Hatfield, UK)
Human IL-5 ELISA kit	88-7056-88	eBiosciences (Hatfield, UK)
Human IL-10 ELISA kit	88-7106-88	eBiosciences (Hatfield, UK)
Human IL-13 ELISA kit	88-7439-88	eBiosciences (Hatfield, UK)
Human TNF-α ELISA kit	88-7346-88	eBiosciences (Hatfield, UK)
Human IFN-γ ELISA kit	88-7316-88	eBiosciences (Hatfield, UK)
Mouse $CD4^+$ T-cell isolation kit	130-104-454	Miltenyi Biotec (Surrey, UK)
Mouse IL-1β ELISA kit	DY401	R&D Systems (Minneapolis, CAN)
Mouse IL-2 ELISA kit	88-7024-88	eBiosciences (Hatfield, UK)
Mouse IL-4 ELISA kit	88-7044-88	eBiosciences (Hatfield, UK)
Mouse IL-5 ELISA kit	88-7054-88	eBiosciences (Hatfield, UK)
Mouse IL-10 ELISA kit	431412	Biolegend (London, UK)
Mouse IL-13 ELISA kit	88-7137-88	eBiosciences (Hatfield, UK)
Mouse TNF-α ELISA kit	88-7324-88	eBiosciences (Hatfield, UK)
Mouse IFN-γ ELISA kit	88-7314-88	eBiosciences (Hatfield, UK)
Mouse M-CSF ELISA Kit	MMC00	R&D Systems (Minneapolis, CAN)
Mouse GM-CSF ELISA Kit	432204	Biolegend (London, UK)
Promofluor protein labelling kit	PKPFLK488P10	Promokine (Heidelberg, DEU)
RNA isolation kit	11828665001	Roche Diagnostics (West Sussex, UK)
Transcriptor first strand cDNA synthesis kit	04379012001	Roche Diagnostics (West Sussex, UK)

2.1.5 Reagents

Product	Catalogue #	Company
2-Mercaptoethanol	63689	Sigma-Aldrich (Wicklow, IRE)
3,3′,5,5′-Tetramethylbenzidine dihydrochloride hydrate	T8768	Sigma-Aldrich (Wicklow, IRE)
Accutase detachment solution	A1110501	Thermo Scientific (Leicestershire, UK)
Acetone	27023	Sigma-Aldrich (Wicklow, IRE)
Acrylamide/Bis-acrylamide, 30% solution	A3699	Sigma-Aldrich (Wicklow, IRE)
Agarose	BIO-4125	Bioline (London, UK)
Ammonium persulfate	A7460	Sigma-Aldrich (Wicklow, IRE)
Bovine serum albumin (BSA)	A7906	Sigma-Aldrich (Wicklow, IRE)
Calcium chloride	383147	Sigma-Aldrich (Wicklow, IRE)
Cell culture grade water	W3500	Sigma-Aldrich (Wicklow, IRE)
Citric acid	C1857	Sigma-Aldrich (Wicklow, IRE)
Dimethyl sulfoxide (DMSO)	276855	Sigma-Aldrich (Wicklow, IRE)
Ethanol	E7023	Sigma-Aldrich (Wicklow, IRE)
Ethylenediamine-tetraacetic acid	E9884	Sigma-Aldrich (Wicklow, IRE)
Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)	E3889	Sigma-Aldrich (Wicklow, IRE)
FACS Flow Sheath	342003	BD Biosciences (Oxford, UK)
Formaldehyde solution	F8775	Sigma-Aldrich (Wicklow, IRE)
Glucose	G8270	Sigma-Aldrich (Wicklow, IRE)
Glycerol	G8773	Sigma-Aldrich (Wicklow, IRE)
Glycine	G8898	Sigma-Aldrich (Wicklow, IRE)
HEPES	H3375	Sigma-Aldrich (Wicklow, IRE)
Histopaque-1083	10831	Sigma-Aldrich (Wicklow, IRE)
Hydrochloric acid	H1758	Sigma-Aldrich (Wicklow, IRE)
HyperLadder IV	BIO33029	Bioline (London, UK)
Immobilon Western Chemiluminescent HRP	WBKLS0100	Millipore (Massachusetts, USA)
Magnesium chloride	M8266	Sigma-Aldrich (Wicklow, IRE)
N,N,N′,N′-Tetramethylethylenediamine	T9281	Sigma-Aldrich (Wicklow, IRE)
PCR mastermix	M7505	Promega (Madison, USA)
Potassium chloride	P4504	Sigma-Aldrich (Wicklow, IRE)
Potassium dihydrogen phosphate	30407	Sigma-Aldrich (Wicklow, IRE)
Protease Inhibitor Cocktail	P8340	Sigma-Aldrich (Wicklow, IRE)
Resazurin salt	R7017	Sigma-Aldrich (Wicklow, IRE)
RIPA Buffer	R0278	Sigma-Aldrich (Wicklow, IRE)
Sodium acetate	S2889	Sigma-Aldrich (Wicklow, IRE)
Sodium azide	13412	Sigma-Aldrich (Wicklow, IRE)
Sodium bicarbonate	S5761	Sigma-Aldrich (Wicklow, IRE)
Sodium carbonate	S7795	Sigma-Aldrich (Wicklow, IRE)
Sodium chloride	S/3160	Sigma-Aldrich (Wicklow, IRE)
Sodium dodecyl sulphate (SDS)	L4390	Sigma-Aldrich (Wicklow, IRE)

Sodium phosphate		Sigma-Aldrich (Wicklow, IRE)
Sulfuric acid	435589	Sigma-Aldrich (Wicklow, IRE)
SYBRSafe DNA gel stain	S33102	Thermo Scientific (Leicestershire, UK)
Tetramethylbenzidine (TMB)	T3405	Sigma-Aldrich (Wicklow, IRE)
Trizma base	93352	Sigma-Aldrich (Wicklow, IRE)
Triton X-100	T8787	Sigma-Aldrich (Wicklow, IRE)
Trypan blue	T8154	Sigma-Aldrich (Wicklow, IRE)
Trypsin-EDTA solution	T4049	Sigma-Aldrich (Wicklow, IRE)
Tween 20	P1379	Sigma-Aldrich (Wicklow, IRE)

2.1.6 Equipment

Product	Catalogue #	Company
Analogue stirred water bath	NE4-22T	VWR (East Grinstead, UK)
Benchtop microcentrifuge	4214	MSC Co. Ltd. (Dublin, IRE)
BIOQUEL microflow class II ABS cabinet	ABS1200F	VWR (East Grinstead, UK)
Block heater	BBA series	MSC Co. Ltd. (Dublin, IRE)
Dual mini slab kit-gel electrophoresis apparatus	AE-6450	ATTO Bio-Instrument (Bunkyo, JPN)
FacsAria flow cytometer	-	BD Biosciences (Oxford, UK)
G-Box gel imaging system	-	Syngene (Cambridge, UK)
Haemocytometer	-	MSC Co. Ltd. (Dublin, IRE)
Hotplate stirrer	AGB1000	Jenway (Stone, UK)
Leica inverted microscope	DMIL	Leica Microsystems (Wetzlar, DEU)
Midi horizontal electrophoresis unit 658	Z338796	Sigma-Aldrich (Wicklow, IRE)
Nanodrop 1000	-	Thermo Fischer (Dublin, IRE)
Sigma 4K15 benchtop refrigerated centrifuge	10740	Sigma Centrifuges (Merrington, UK)
TECAN genios microplate reader	-	Tecan Group (Mannedorf, CHE)
Thermo Scientific CO_2 water jacketed incubator	3111	MSC Co. Ltd. (Dublin, IRE)
Vortex mixer	SA8	Stuart (Stone, UK)
West balance	BL120S	Sartons (Goettingen, DEU)

2.1.7 Disposables

Product	Catalogue #	Company
1.5 mL tubes	72.706.200	Sarstedt (Wexford, IRE)
15 mL tubes	62.554.502	Sarstedt (Wexford, IRE)
50 mL tubes	62.559.001	Sarstedt (Wexford, IRE)
12 well plates	83.3921	Sarstedt (Wexford, IRE)
24 well plates	83.3922.005	Sarstedt (Wexford, IRE)
96 well plates	83.3924.005	Sarstedt (Wexford, IRE)

5 mL pipette	86.1253.025	Sarstedt (Wexford, IRE)
10 mL pipette	86.1254.025	Sarstedt (Wexford, IRE)
25 mL pipette	86.1685.001	Sarstedt (Wexford, IRE)
Cell culture flask T75	83.3911.002	Sarstedt (Wexford, IRE)
Cover slips	MLS17-20	Lennox Ltd (Dublin, IRE)
FACS tubes	352054	Benson dickson (Dublin, IRE)
Petri dishes	82.1473.001	Sarstedt (Wexford, IRE)

2.1.8 Software

Product	Catalogue #	Company
FlowJo	-	Tree Star (Ashland, USA)
GraphPad prism	-	GraphPad (CA, USA)
ImageJ	-	SciJava consortium

2.2 Methods

2.2.1 Animals

4-6 week old C57BL/6JCrl or B6.Cg-Tg(TcraTcrb)425Cbn/J mice were purchased from Charles River, housed in DCU's Bioresource Unit, and kept under specific pathogen free conditions. All mice were maintained according to the guidelines of the Health Products Regulatory Authority (HPRA) and the DCU animal welfare body.

2.2.2 Casein and whey sample preparation

Lyophilised sodium caseinate (NaCAS) and whey (WP) were obtained from Teagasc, Moorepark. Lyophilised alpha (α-CAS), beta (β-CAS) and kappa (κ-CAS) caseins were purchased from Sigma-Aldrich. Samples were reconstituted in sterile cell culture grade PBS (Sigma-Aldrich) by gentle agitation and stirred gently for 15 min at room temperature to ensure complete dispersion. Reconstituted samples were centrifuged for 5 min at 5000 x

g and filtered through 0.2 μm cellulose acetate filters (GE Healthcare Life Sciences) to remove any undissolved remnants. Protein concentrations were determined using a BCA protein assay kit (See section 2.2.3) (Thermo Scientific) and used at the indicated concentrations.

2.2.3 BCA protein assay

The bicinchoninic acid (BCA) protein assay is used for quantitation of total protein in a sample. The principle of this method is that amino acid residues of proteins can reduce Cu^{2+} to Cu^{1+} in an alkaline solution, resulting in a purple color formation when bicinchoninic acid reacts with the reduced cation. The concentrations of samples were determined using the BCA protein assay kit (Thermo Scientific) according to manufacturer's guidelines. Briefly, BCA assays were carried out in 96 well plates (Sarstedt), with 10 μL of each sample loaded in triplicate. A serial dilution of bovine serum albumin ranging from 2000 μg to125 μg were used as standard controls. 200 μL of a 1:50 diluted 4% copper (II) sulfate pentahydrate solution in bicinchoninic acid solution were added to samples and standards and incubated at 37^{o}C for 30 min. The absorbance values at 562 nm were recorded using a TECAN genios microplate reader (Tecan Genios). The protein concentrations were determined by using the equation of the line derived from a standard curve of known serial diluted protein controls.

2.2.4 Murine granulocyte-macrophage colony-stimulating factor preparation

Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) required for the generation of bone marrow derived dendritic cells (BMDC) were obtained from P3X63Ag8.653 (X63) conditioned medium. X63 cells (LGC Standards), a mouse B lymphoblast cell line were seeded in a 75 cm^2 vented adherent flask at a cell density of 2 x 10^5 cells/mL in complete RPMI (Table 2.1) and cultured at 37^oC and 5% CO_2 for 7 days. Supernatants were collected, centrifuged at 300 x g for 10 min and filtered with 0.45µm filters (Sarstedt) to remove any un-pelleted cells or cellular debris. The concentrations of GM-CSF were determined by ELISA (See section 2.2.14) and stored at -80^oC until use.

Table 2.1 Compete RPMI formulation

Reagents	Concentration
RPMI 1640	90% (v/v)
FCS	10% (v/v)
Penicillin/Streptomycin	100 U/ml; 100 µg/ml
L-glutamine	2 mM
2-mercaptoethanol	50 µM

2.2.5 Murine macrophage colony-stimulating factor preparation

Murine macrophage colony-stimulating factor (M-CSF) required for the generation of bone marrow derived macrophages were obtained from NCTC clone 929 (L929) conditioned medium. L929 cells (LGC Standards) a murine adipose tissue fibroblast cell line were seeded at 5 x 10^5 cells per 75 cm^2 vented adherent flask (Sarstedt) in 55 mL of complete DMEM (Table 2.2) and cultured at 37^oC and 5% CO_2 for 14 days. Supernatants were collected, centrifuged at 300 x g for 10 min, filtered with 0.45 µm filters to remove any un-

pelleted cells or cellular debris. The concentrations of M-CSF were determined by ELISA (See section 2.2.14) and stored at -80^{o}C until use.

Table 2.2 Compete DMEM formulation

Reagents	Concentration
DMEM	90% (v/v)
FCS	10% (v/v)
Penicillin/Streptomycin	100 U/ml; 100 µg/ml
L-glutamine	2 mM
2-mercaptoethanol	50 µM

2.2.6 Generation of bone marrow derived dendritic cells

Bone marrow derived dendritic cells (BMDC) were differentiated using a previously described method (Lutz *et al*. 1999). C57BL/6JCrl or B6.Cg-Tg(TcraTcrb)425Cbn/J (Charles River) mice were sacrificed by cervical dislocation. Femurs and tibias were separated, the muscle tissues were removed and the bones were steralised in 70 % ethanol (v/v) for 10 s and subsequently washed in sterile PBS immediately afterwards. The bones were cut at both ends and the bone marrow cells were extracted by flushing with ice cold PBS using a 25-gauge needle (Sarstedt). The bone marrow cells were counted, centrifuged at 300 x g for 10 min and re-suspended at a cell density of 2×10^5 cells/mL in complete RPMI (Table 2.1) supplemented with 10% (v/v) X63 conditioned medium (See section 2.2.4). On day 3, an additional 10 mL of fresh complete medium with 10% X63 conditioned medium were added. On day 6, 9 mL of media were aspirated and replaced with 10 mL of fresh complete medium with 10% X63 conditioned medium. On day 8, 9 mL of media were transferred to a 50 mL tube, centrifuged at 300 x g and the pellets were re-suspended in 10 mL of fresh complete medium with 10% X63 conditioned medium

before being added back to the plate. On day 10, non-adherent cells were harvested. The purity of dendritic cells were analysed by flow cytometry (See section 2.2.15), with >95% of the population identified as dendritic cells on the basis of a positive expression for CD11c using a PE-Cy7 conjugated CD11c antibody (Figure 2.1).

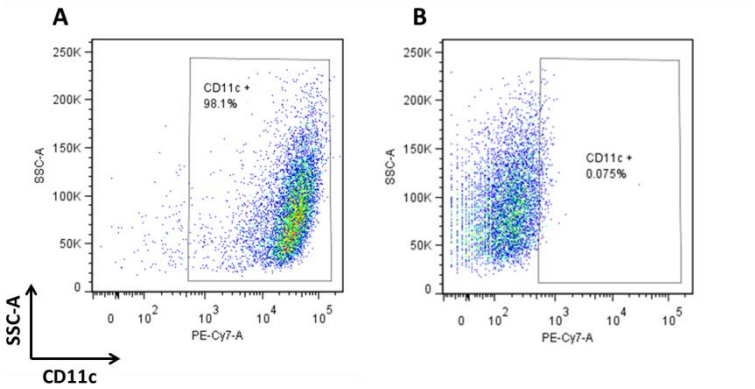

Figure 2.1. BMDC purity. BMDC purity were assessed after 10 days by measuring the expression of the extracellular dendritic cell marker; CD11c. Cells were deemed positive for CD11c using a PE-Cy7 conjugated anti mouse CD11c antibody (A), based on a gating strategy using an isotype control (B).

2.2.7 Generation of bone marrow derived macrophages

Bone marrow derived macrophages (BMMΦ) were differentiated using a previously described method (Weischenfeldt & Porse 2008). C57BL/6JCrl (Charles River) mice were sacrificed by cervical dislocation. Femurs and tibias were separated and the bone marrow cells were extracted by flushing with ice cold PBS using a 25-gauge needle (Sarstedt). The bone marrow cells were counted, centrifuged at 300 x g for 10 min and re-suspended at a cell density of 2×10^6 cells/mL in complete RPMI (Table 2.1) supplemented with 30%

(v/v) L929 conditioned medium (See section 2.2.5). 10 mL of the cell suspensions were transferred to a 10 cm^2 sterile petri dish (Sarstedt) and cultured at 37°C and 5% CO_2. On day 3, media were aspirated, adherent cells were washed twice with PBS and fresh complete RPMI with 30% (v/v) L929 conditioned medium were added. On day 6, non-adherent cells were removed by washing 3 times with pre warmed PBS. To harvest macrophages, adherent cells were detached from petri dishes with 5 mL of accutase detachment solution (Invitrogen) for 5 min at room temperature. Macrophage purity was analysed by flow cytometry (See section 2.2.15), with >95% of the population identified as macrophages on the basis of double positive expression of both CD11b using a PE-Cy7 conjugated anti mouse CD11b antibody and F4/80 using an APC conjugated anti mouse F4/80 antibody. (Figure 2.2).

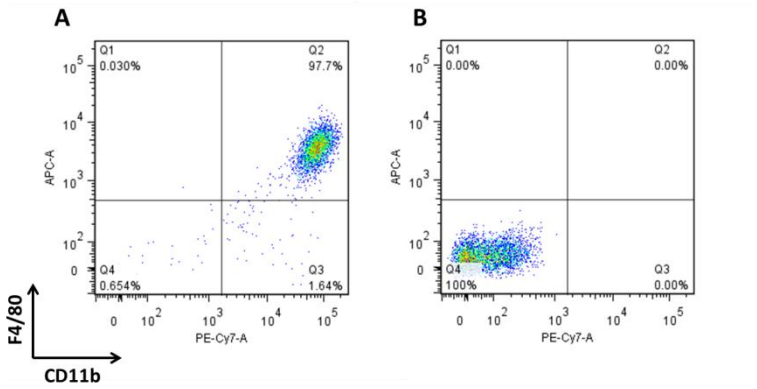

Figure 2.2. BMMφ purity. Macrophage purity was assessed after 6 days by measuring the expression of extracellular macrophage markers; CD11b using a PE-Cy7 conjugated anti mouse CD11b antibody and F4/80 using a APC conjugated anti mouse F4/80 antibody. Cells were deemed double positive for F4/80 and CD11b (A) based on a gating strategy using isotype controls (B).

2.2.8 BMDC and BMMΦ activation

Day 10 BMDC were harvested and re-suspended in complete RPMI medium (Table 2.1) supplemented with 1 % (v/v) X63 conditioned medium (See section 2.2.4). Cell number and viability were determined using trypan blue staining (See section 2.2.22) BMDC were seeded in flat bottomed 24 well plates (Sarstedt) at a cell density of 1 x 10^6 cells/mL. The cells were allowed to acclimate for 2 hr prior to any stimulation.

Day 6 BMMφ were harvested and re-suspended in complete RPMI medium (Table 2.1). Cell number and viability were determined using trypan blue staining (See section 2.2.22) BMMφ were seeded in flat bottomed 12 well plates (Sarstedt) at a cell density of 1 x 10^6 cells/mL. The cells were allowed to acclimate for 2 hr prior to any stimulation.

The cells were pre-cultured with PBS or the indicated concentrations of casein or whey stimulants 1 hr prior to the addition, of PBS or toll like receptor (TLR) agonists (Alexis Biochemicals); TLR4 against; lipopolysaccharide (LPS) (100 ng/mL), TLR2 against; peptidoglycan (PGN) (5 ug/mL), TLR7 against; loxoribine (LOX) (0.5 mM) or TLR9 against: synthetic oligonucleotides containing CpG motifs (CpG) (2 μM). The additions of TLR ligands alone or PBS were used as positive and negative controls respectively. At indicated time points, supernatants were collected and analysed for cytokine secretion by ELISA (See section 2.2.14). Cells were also analysed by flow cytometry for the detection of extracellular markers (See section 2.2.15). In some experiments, RNA was extracted to examine gene expression by polymerase chain reaction (PCR) (See section 2.2.17) or quantitative PCR (QPCR). Total proteins were also examined by western blot (see section 2.2.19).

To elucidate the signalling pathways and mechanism by which casein exerts its effects, cells were cultured with chemical antagonists of STAT6; AS1517499 (Axon medchem), STAT3; WP1066 (Axon medchem), NFκB; BAY117821 (Axon medchem), PPARγ; GW9662 (Sigma-Aldrich), PI3K; LY294002 (Sigma-Aldrich), and HO-1; Zinc-protoporphyin-9 (ZnPPIX) (Santa cruz biotechnology) or Tin-protoporphyin-9 (SnPPIX) (Santa cruz biotechnology) at the indicated concentrations, 30 min prior to addition of caseins. Cells were also cultured where indicated with a peptide inhibitor of suppressor of cytokine signalling (SOCS); pJAK2 (GenScript) at stated concentrations, 24 hr prior to the addition of caseins. 1 hr after the addition of caseins, cells were stimulated with or without LPS (100 ng/mL) for 3, 6 or 18 hr and supernatants were collected for cytokine analysis (See section 2.2.14) or RNA was extracted from cells to examine gene expression by PCR (See section 2.2.17).

2.2.9 BMDC or BMMφ: $CD4^+$ T-cells co-culture

C57BL/6JCrl (Charles River) mice were sacrificed by cervical dislocation. Spleens were extracted and spleenocytes obtained by passage of the spleen through a 40 μm filter (Sarstedt) using the plunger from a sterile 1 mL syringe (Sarstedt). $CD4^+$ T-cells were isolated from spleenocytes using a negative selection $CD4^+$ isolation kit (Stemcell) and were only used if the purity were determined to be > 95% $CD4^+$ by flow cytometry (Figure 2.3).

Pre-stimulated cells were washed three times with PBS and co-cultured with $CD4^+$ T-cells at a ratio of 1:10 for BMDCs or 1:4 for BMMφ in complete RPMI media (Table 2.1) in 24 well plates pre-coated overnight with anti-CD3 (1 μg/mL) (R & D systems). After 72 hr

and supernatants were collected and analysed for cytokine secretion by ELISA (See section 2.2.14). To elucidate if receptors were involved in cell-cell signalling during co-cultures, where indicated cells were pre-incubated with blocking antibodies (all from R & D systems) for anti-CD40 (20 µg/mL) or anti-OX40L (20 µg/mL) for 30 min in PBS prior to co-culture.

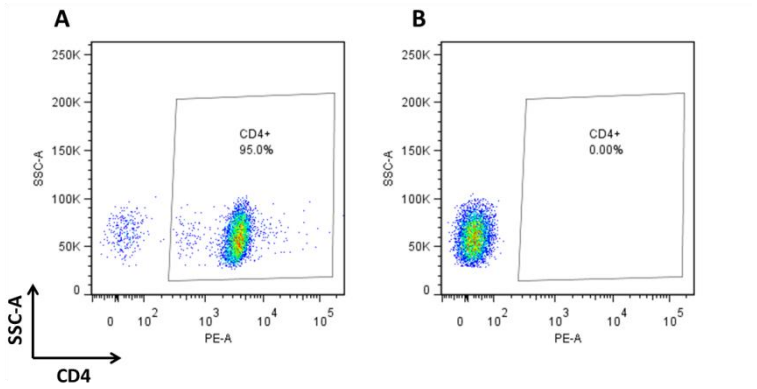

Figure 2.3 CD4$^+$T cell purity from spleenocytes. CD4$^+$T cell purity were assessed by measuring the expression of extracellular CD4$^+$ T cell markers; CD4. Cells were deemed positive for CD4 using a PE conjugated anti mouse CD4 antibody (A) based on a gating strategy using an isotype control (B).

2.2.10 *In-vivo & in-vitro OVA* T-cell priming assay

For *in-vivo* and *in-vitro* ovalbumin (OVA) T-cell priming assays, BMDCs were isolated from B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII) mice (See section 2.2.6). On day 10 BMDCs 1 x 10^6 cells were stimulated *in-vitro* with PBS or indicated concentration of caseins in the presence of OVA peptide (100 nM) (Sigma-Aldrich) for 24 hr. After stimulations, cells were washed with sterile PBS.

For *in-vivo* T-cell priming, 3 x 10^5 cells were delivered over the sternum of OTII mice by subcutaneous injection. Mice were culled after 7 days by cervical dislocation. Skin draining lymph nodes were extracted and single cell suspensions of cells were obtained by passage of the lymph nodes through a 40 μm filter using the plunger from a sterile 1 mL syringe. Draining lymph nodes cells were counted and seeded at 1 x 10^6 cell/mL in 24 well plate and stimulated with PBS, OVA (500 nM) or with PMA (25 ng/ml) in wells pre-coated with anti-CD3 (1 μg/ml) (R & D systems). After 72 hr, supernatants were collected and analysed for cytokine secretion by ELISA.

For *in-vitro* T-cell priming, BMDCs were co-cultured at a 1:10 ratio with $CD4^+$ T-cells isolated from the spleen (See section 2.2.9) of OTII mice in complete RPMI media (Table 2.1) in 24 well plates pre-coated overnight with anti-CD3 (1 μg/mL). After 72 hr, supernatants were collected and analysed for cytokine secretion by ELISA (See section 2.2.14).

2.2.11 Human PBMC and $CD14^+$ isolation form human buffy coat

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of healthy donors obtained from the Irish blood transfusion service (St James's hospital, Dublin) by density gradient centrifugation using Histopaque-1083 (Sigma-Aldrich). Buffy coat bloods were mixed 1:1 with sterile PBS. An equal volume of diluted blood were gently layered on top of 15 mL of Histopaque-1083 and centrifuged at 800 x g for 30 min with the accelerator and break switched off to prevent any mixing of separated layers. The blood components separated according to their relative density. PBMCs form a separated layer from blood cells which were isolated from the solution using a pasteur pipette (Sarstedt). PBMCs were

washed twice with PBS before total number of cells and viability were determined using trypan blue staining (See section 2.2.22). $CD14^+$ monocytes were isolated from PBMCs using a magnetic activated cell sorting positive selection $CD14^+$ isolation kit (Miltenti) and were only used if the purity were determined to be > 90% $CD14^+$ by flow cytometry (Figure 2.4).

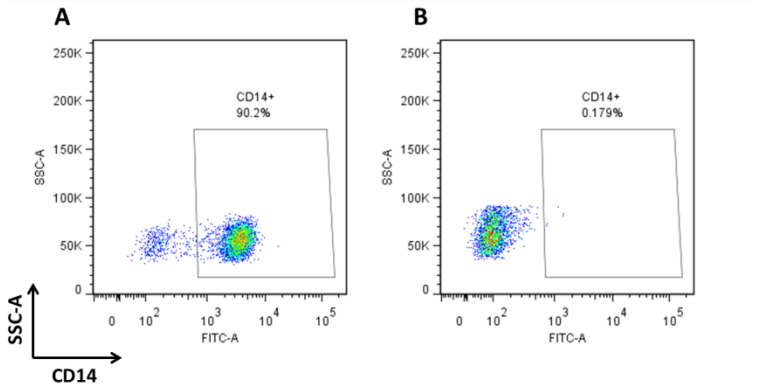

Figure 2.4 $CD14^+$ monocyte purity from PBMCs. $CD14^+$ monocyte purity was assessed by measuring the expression of extracellular CD14. Cells were deemed positive for CD14 using a FITC conjugated anti human CD14 antibody (A) based on a gating strategy using an isotype control (B).

2.2.12 Generation of monocyte derived human macrophages

Monocyte derived human macrophages (hMφ) were differentiated using a previously described method (Saghaeian-Jazi *et al.* 2016). $CD14^+$ monocytes were isolated from PBMC as previously described (See section 2.2.11). The monocytes were counted, centrifuged at 300 x g for 10 min and re-suspended at a cell density of 1×10^6 cells/mL in complete RPMI (Table 2.1) supplemented with 10% (v/v) human AB serum (Invitrogen) instead of FCS. 20 mL of the cell suspensions were transferred to a 75 cm^2 vented adherent flask (Sarstedt) and cultured at 37°C and 5% CO^2 for 14 days. The culture media were

renewed every 3 days. Non-adherent cells were removed on day 7 by aspirating all media, followed by 3 washes with PBS. To harvest hMφ, adherent cells were detached from culture flasks with 10 mL 0.25% trypsin-EDTA solution (Sigma-Aldrich) at room temperature. When the cells were detached, trypsin-EDTA solutions were neutralized by adding twice the volume of media.

2.2.13 Human PBMC, monocyte and macrophage activation

Isolated PBMC, $CD14^+$ monocytes or hMφ were re-suspended in complete RPMI medium (Table 2.1) supplemented with 10% (v/v) human AB serum (Invitrogen) instead of FCS. Cell number and viability were determined using trypan blue staining (See section 2.2.22). Cells were seeded in flat bottomed 12 well plates (Sarstedt, Ire) at a cell density of 1×10^6 cells/mL. The cells were allowed to adhere and acclimate for 2 hr before any stimulation. Cells were stimulated with indicated concentrations of caseins 1 hr prior to the addition of or without LPS (100 ng/mL) (Enzo life sciences) or PMA (20 ng/ml) (Sigma-Aldrich) and Ionomycin (1 μM) (Sigma-Aldrich). After 18 hr, supernatants were collected and analysed for cytokine secretion by ELISA (See section 2.2.14) and the expression of extracellular markers were detected by flow cytometry (See section 2.2.15).

2.2.14 Enzyme-linked immunosorbent assay (ELISA)

Supernatant were collected at indicated time points and the secretion of cytokines were measured using commercially available sandwich ELISA kits, according to the manufacturer's instructions (Biolegend, eBioscience). Briefly, 96 well plates (Sarstedt)

were coated with capture antibody overnight at 4^{o}C. The plates were washed three times with wash buffer (PBS with 0.05% (v/v) Tween-20) to remove unbound capture antibody and incubated with assay diluent (PBS with 10% (v/v) FCS) for 1 hr at room temperature to inhibit non-specific binding. After blocking, the pates were washed three times with wash buffer. Samples and serial diluted standards of known concentration were plated in triplicate on the 96 well plates and left for 2 hr at room temperature or overnight at 4^{o}C. The plates were washed three times to remove unbound sample and biotinylated detection antibody were added for 1 hr at room temperature. The plates were washed again three times to remove unbound detection antibody and a horse radish peroxidase conjugated streptavidin labeled antibody were added to bind bound biotinylated detection antibody. The plates were finally washed five times to remove unbound horse radish peroxidase conjugated streptavidin labeled antibody and incubated in the dark for thirty min with TMB substrate resulting in a colorimetric change. The reactions were terminated by the addition of 0.16 M Sulfuric acid and the absorbance values at 450 nm were recorded using a TECAN genios microplate reader (Tecan Genios). The concentrations were determined by comparison to the standard curve derived from the serial diluted standard controls.

2.2.15 Flow cytometry

Cells were harvested, washed twice and re-suspended in ice cold flow cytometry buffer (Table 2.3) at a cell density of <1x10^{6} cells/100 µL. Cells were incubated with 5 µl of fluorochrome labelled antibodies (Table 2.4) for 30 min at 4^{o}C in the dark. After incubation, the cells were washed three times with 1 mL of flow cytometry buffer to remove any unbound fluorochrome labelled antibodies. After final wash, cells were re-

suspended in 500 μL of flow cytometry buffer and processed within 30 min on the flow cytometer FACs Aria (Becton Dickinson). Data was analysed using FlowJo software (Treestar). For gating, cell duplicates were excluded using forward scatter area (FFC-A) and height (FFC-H) (Figure 2.5A). Cell debris was then excluded using forward and side scatter (SSC) (Figure 2.5B). Unlabeled and fluorocrhome labelled isotype control antibodies were used to check background flourencence and non-specific staining of cells (Figure 2.5C). Fluorochrome labelled antibodies spcific to a target marker on cells are detected as a fluresent signal (Figure 2.6D), with increases in signal strength indicative of increased binding of the specific antibody to its target. The average strength of the signal detected on each cell is calculated by FlowJo software and was recorded as the geometrical mean fluresence intensity (gMFI). The gMFI is a mean or average, which indicates the central tendency and was used as it is less affected by outliers compared to the MFI (Wu *et al.*, 2016). The gMFI results were tabulated and can also be displayed in a histogram format showing the fluresence intensity of the cell populations (Figure 2.6E).

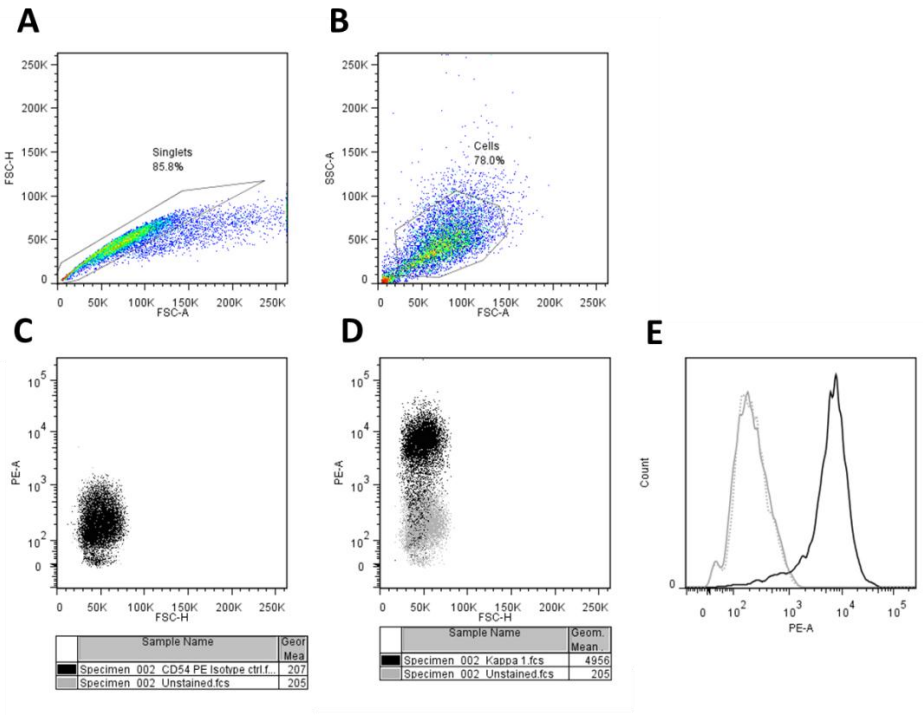

Figure 2.5. Flow cytometry gating strategy. Singlet cells gated based on area and height (A). Debris and dead cells removed based on size and granularity (B). Scatter plots display unstained (gray) and isotype (black) labelled cells (C) or unstained (gray) and positively labelled cells (black) (D). A representative histogram plot of the 3 popultaions combined (E); unstained (gray), isotype (dotted gray) and positive cells (black).

Table 2.3 Flow cytometry buffer formulation

Reagents	Concentration
PBS	98% (v/v)
FCS	2% (v/v)
EDTA	1mM

Table 2.4 Flow cytometry antibodies

Antibody	Fluorochrome	Clone	Catalogue #	Company
CD4	PE	RM4-4	116005	Biolegend
CD11b	Pe-Cy7	M1/70	101215	Biolegend
CD11c	Pe-Cy7	N418	117317	Biolegend
CD14	FITC	M5E2	301803	Biolegend
CD40	PE	3/23	124609	Biolegend
CD54 (ICAM1)	PE	YN1/1.7.4	12-0541	Ebioscience
CD80	PE	16-10A1	104707	Biolegend
CD86	FITC	GL1	105005	Biolegend
CD252 (OX40L)	PE	MRI	12-5905	Ebioscience
CD206 (MMR)	APC	141707	C068C2	Biolegend
Dectin-1	Alexa fluor 488	GE2	MCA4661A	AbD serotec
F4/80	APC	BM8	123115	Biolegend
MGL	Alexa fluor 488	ER-MP23	MCA2392A	AbD serotec

2.2.16 Cell Binding Assay

Caseins and bovine serum albumin (BSA) were fluorescently labelled using the Promofluor labelling kit in accordance with manufacturer's instructions (Promokine). The labelling process resulted in the formation of a stable covalent amide linkage of a FITC-488 florescent dye to the protein. To preform cell binding assays, 1 x 10^5 cells/0.1 mL were seeded in a 96 well plate. Cells were incubated with indicated concentrations of labelled caseins or BSA for 45 min at 4^{o}C. Cells were then washed in ice cold PBS and re-suspended in 500 µL of flow cytometry buffer (Table 2.3). The cells were then processed within 30 min on the flow cytometer FACs Aria (Becton Dickinson) and data were analysed using FlowJo software (Treestar). Labelled BSA were used as a non-specific binding control. To determine if the binding were calcium dependent, cells were pre-incubated with EDTA (10mM) (Sigma-Aldrich) for 45 min at 37^{o}C and 5% CO^2.

2.2.17 RNA extraction, cDNA synthesis & PCR

Total RNA were extracted from cultured cells using a RNA isolation kit (Roche Diagnostics) according to manufacturer's guidelines. Briefly, cells were harvested, washed with PBS and lysed by the addition of lysis buffer with RNase inhibitors. The lysate-PBS mixtures were transferred to high pure filter tubes, containing glass fiber fleeces in a column. The RNA becomes immobilized in the glass fiber fleeces and DNase was added to digest genomic DNA. The columns were washed twice with ethanol based buffers to remove impurities while retaining the RNA. Finally, RNA was eluted from the column in RNase and DNase free water. All solutions and buffers were supplied in the kit.

RNA levels were measured using a Nanodrop machine and cDNA were synthesized from the isolated RNA using a transcriptor first strand cDNA synthesis kit (Roche Diagnostics) according to manufacturer's guidelines. Briefly, 1 μg of RNA were used with random primers and were maintained at 20^{o}C for 10 min as an initial step followed by 55^{o}C for 30 min, and finally 85^{o}C for 5 min to yield 1 μg of cDNA.

Synthesized cDNA were used as a template for PCR using primers specific for *Arg 1*, *Ym-1, iNOS, RELM α,* and *β-actin* (Table 2.5). Samples were maintained at 95^{o}C for 1 min as an initial step followed by 60^{o}C for 30 seconds, and finally 72^{o}C for 1 min. These amplification cycles were carried out 40 times. The process were preceded by a denaturation phase at 95^{o}C for 5 min and a final extension phase of 72^{o}C for 5 min. PCR products were electrophoresed on 1% agarose gels with SYBRSafe (Invitrogen) as gel stain.

Table 2.5 PCR Primer sequences

Gene name	Forward primer	Reverse primer
Arg-1	ATGGAAGAGACCTTCAGCTAC	GCTGTCTTCCCAAGAGTTGGG
Ym-1	TCACAGGTCTGGCAATTCTTCTG	TTTGTCCTTAGGAGGGCTTCCTC
iNOS	CCCTTCCGAAGTTTCTGGCAGCAGC	GGCTGTCAGAGAGCCTCGTGGCTTTGG
RELMα	TCCCAGTGAATACTGATGAGA	CCACTCTGGATCTCCCAAGA
B-actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG

2.2.18 QPCR

Primer probes (all from Roche Diagnostics) with a primer efficiency of 2 were used to analyse gene expression, for the mouse genes; *Arg-1*, *SOCS1* and *SOCS 3* (Table 2.6). The housekeeping genes *GAPDH* (NM_008084.2) were used as an internal standard. Experiments were carried out in triplicate with each reaction containing 50 ng of cDNA, 2 µL of primer probe and 10 µL of primer probe master mix containing a 6-carboxyfluorescein labelled enzyme (Roche Diagnostics). Reaction volumes were brought up to a final volume of 20 µL with PCR grade H_2O (Roche Diagnostics). Gene expression were analysed using a Light Cycler 96 (Roche Diagnostics). Samples were maintained at 95°C for 10 s as an initial step, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 60 s. Pfaffl's methods were used to determine relative gene expression (Pfaffl 2001).

Table 2.6 QPCR Primer sequences

Gene name	Forward primer	Reverse primer
Arg-1	AACACGGCAGTGGCTTTAACC	GGTTTTCATGTGGCGCATTC
SOCS1	GAGTGGTTGTGGAGGGTGAG	TGAGAGGTGGGATGAGGTC
SOCS3	ATTTCGCTTCGGGACTAGC	AACTTGCTGTGGGTGACCAT
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG

2.2.19 Protein extraction

Cells from control or stimulated cells at indicated time points were washed twice with TBS (Table 2.7) and total proteins were extracted by lysing cells in 70 µL of radio-immunoprecipitation assay buffer (RIPA) (Sigma-Aldrich), supplemented with protease and inhibitor cocktail (Sigma-Aldrich). Lysates were vigorously vortexed before centrifugation at 10,000 x g for 10 min at 4^{o}C. The supernatants were transferred to new tubes and protein concentrations were determined by BCA (see section 2.2.3).

Table 2.7 TBS (pH 7.6) formulation

Reagents	Concentration
Tris	20 mM
NaCl	150 mM

2.2.20 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were performed based on a previously established method (Laemmli 1970) using a mini-gel system (ATTO Bio-Instrument, Bunkyo, Japan). SDS-PAGE were used to separate out proteins on a gel based on their molecular weight. Firstly, resolving gels were prepared (Table 2.8), with TEMED only being added just prior to pouring. When poured, isopropanol (Sigma-Aldrich) was layered on top of the resolving. Stacking gels were then prepared (Table 2.8), again ensuring TEMED only being added prior to pouring. Once the resolving gel had polymerised, isopropanol was decanted from the gels. Stacking gels were then poured on top of the polymerized resolving gel and a spacer combs were inserted. Once polymerized, the combs were removed and the gels were transferred to the electrophoresis chamber

which was filled with running buffer (Table 2.9). 20 µg of protein samples (See section 2.2.19) were prepared in loading buffer (Table 2.9), and boiled for 2 min on a heating plate to denature prior to loading. Samples and a molecular weight marker (LI-COR) were loaded on the gels and electrophoresis was performed at 150 V for 1 hour. Upon completion, the gels were removed from the electrophoresis chamber, washed in dH_2O and the 5% stacking gels were removed.

Table 2.8 Stacking & resolving gels formulation

5% Stacking gels		10% resolving gels	
Reagents	**Volume (mL)**	**Reagents**	**Volume (mL)**
30% Acylamide	670 µL	30% Acylamide	4.62 mL
1M Tris-HCL pH 6.8	625 µL	1M Tris-HCL pH 8.8	3.8 mL
dH2O	2.7 mL	dH2O	5.6 mL
10% SDS	50 µL	10% SDS	150 µL
10% APS	50 µL	10% APS	75 µL
TEMED	25 µL	TEMED	25 µL

Table 2.9 Running buffer formulation

Reagents	Concentration
Tris	25 mM
Glycine	192 mM
SDS	0.2% (v/v)

Table 2.10 Loading buffer formulation

Reagents	Concentration
Tris	0.625 M
Glycerol	50% (v/v)
SDS	10% (v/v)
Bromophenol blue	0.1% (v/v)
2-mercaptoethanol	5% (v/v)

2.2.21 Western blot

Protein samples which had been run on an SDS-PAGE gel were transferred to a PVDF membrane using the iBLOT (Lifesciences) as per manufactures instructions. Membranes were blocked for 1 hr with 1% skimmed milk (v/v) (Marvel) in TBS (Table 2.7) to block non-specific binding of antibodies with the membrane. After blocking, the membranes were washed with 0.01% Tween 20 (v/v) (Sigma-Aldrich) in TBS and incubated overnight at 4^{o}C with primary antibody (Table 2.11) in 1% skimmed milk (v/v) (Marvel) in TBS. The membranes were washed again 3 times before the addition of an HRP conjugated secondary antibody in 1% skimmed milk (v/v) in TBS for 1 hour at room temperature. The membranes were washed again 3 times before being visualized with a chemiluminescent HRP substrate (Millipore), on a G-Box imaging system (Syngene). Protein bands were quantified using ImageJ analysis software (SciJava consortium). The levels of protein were normalised to a control protein, β-actin and the levels of protein are shown as fold increases over the untreated levels.

Table 2.11 Western blot antibodies

Antibody	Dilution	Catalogue #	Company
IκBα	1:10000	ab32518	Abcam
SOCS3	1:1000	2932	Cell Signaling
β-actin	1:1000	643802	Biolegend

2.2.22 Cell counting

Cell counts were performed using trypan blue (Sigma-Aldrich). Cells were diluted in trypan blue by a factor of 1:50 for PBMCs or 1:3 for all others cells, and allowed to sit for 5 min. With a cover-slip in place on a haemocytometer (Jenway), the diluted cells were pipetted

beneath the cover slip ensuring that the entire surfaces of the counting grids were covered. By counting the numbers of cells on the etched grids, the total numbers of cells were determined using the following equation: (Average number of cells on grid of haemocytometer) x (Dilution factor) x 10^4 = cell number/mL

2.2.23 Cytotoxicity

Resazurin assays were used to determine cytotoxicity of compounds using a previously described method (Riss *et al.* 2013). Cells were treated with respective stimulations, at a cell density of 1 x 10^5 cell/100 µL in 96-well plates. At the end time point, 20 µl of 0.15 mg/mL resazurin salt (Sigma-Aldrich) in PBS were added to each well and incubated for an additional 6 hr at 37°C and 5% CO_2. The absorbance values were recorded at a 560 nm excitation / 590 nm emission filters using a TECAN genios microplate reader (Tecan Genios). Cytotoxic effects were measured and compared to vehicle stimulated controls.

Similarly, the cytotoxic effects of stimulants on cells were also measured using the Annexin V-FITC apoptosis detection kit I (BD Biosciences). Briefly, cells were treated with respective stimulations, harvested at the end time point, washed twice in cold PBS and incubated in ice cold flow cytometry buffer (Table 2.3) with annexin V and propidium iodide (PI) for 15 min. Cells were then immediately analysed by flow cytometry (See section 2.2.15).

2.2.24 Statistics

All data sets were analysed for normality prior to statistical testing using the Shapiro-Wilk test in Prism® 7.1 software (GraphPad software Inc), and all data were deemed to be normally distributed ($p > 0.05$). For statistical analysis comparing two groups, the Student's t test was used. Where multiple comparisons were made, one-way ANOVA was used when there was only one independent variable or two-way ANOVA if there were two or more independent variables. In all statistical tests, $p < 0.05$ were deemed significant.

Chapter 3 – NaCAS exhibits immunosuppressive properties, inducing an M2-like phenotype in macrophages.

3.1 Introduction

Milk is a potent source of immuno-regulatory agents. Previous studies have shown that bovine milk contains numerous components that can affect immune function (Gill *et al.,* 2000). The majority of these immunomodulatory protein fractions are hydrolysates of milk proteins that occur naturally after enzymatic digestion (Gill *et al.,* 2000). Milk proteins are divided into two main groups; the casein (CAS) fraction and the serum fraction. The CAS fraction consists of a heterogeneous group of phosphorylated proteins, classified as α-, β-, and κ-CAS (Palumbo 1972). κ-CAS is also the main glycoprotein of cow's milk containing o-glycosidically linked polysaccharides (Jollès & Fiat 1979). The serum fraction includes a-, β-lactoglobulin, immunoglobulins, albumin, and enzymes in a lower proportion (McWhitney 1988). Many of these milk proteins and hydrolysates are derived from their enzymatic cleave and have been shown to display immuno-modulatory properties; binding and influencing cellular function by enhancing (Migliore-Samour & Jolles, 1988) and suppressing (Otani & Hata, 1995) lymphocyte proliferative responses, enhancing natural killer cell activity (Migliore-Samour & Jolles 1988), inducing neutrophil locomotion (Elitsur & Luk, 1991), enhancing macrophage phagocytic capacity (Meisel *et al.,* 1997) and abrogating TLR mediated responses (Mukhopadhya *et al.,* 2014; Kiewiet *et al.,* 2017).

There is mounting evidence which suggests that the number of individuals who suffer from chronic inflammatory conditions has increased, with an estimated prevalence among developed countries of 5 to 7 % (El-Gabalawy *et al.,* 2010). Strategies to effectively modulate the immune response have been extensively sought out to combat inflammatory diseases and disorders. The balance between appropriate inflammatory and anti-inflammatory responses is crucial to maintenance of homeostasis and the prevention of

harmful chronic conditions. The improper activation of inflammation is a major contributory factor in chronic conditions such as inflammatory bowel diseases (IBD), which are characterized by prolonged activation of NF-κB and subsequent increased synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Strober & Fuss 2011) mostly derived from APC like macrophages (Cobrin & Abreu 2005). Research into the use of naturally occurring bioactive proteins as immuno-regulatory agents for the management of inflammation, has been proposed as a viable alternative to drugs which can produce intermediate toxic metabolites when degraded, resulting in unwanted side effects.

Macrophages were chosen to investigate the potential anti-inflammatory properties of bovine milk derived compounds as they represent a population of innate immune cells, with multiple phenotypes known to be involved in the induction, propagation and resolution of inflammatory responses, homeostasis and can actively contribute to the pathogenesis of many inflammatory diseases (Kumar *et al.,* 2013; Winn *et al.,* 2013). Previous studies had observed intact CAS and CAS derived hydrolysates exhibited anti-inflammatory properties, by suppressing LPS mediated TNF-α in macrophages (Bamdad *et al.,* 2017). However, there is a derth of knowledge as to the phenotype of macrophage induced by CAS as depending on the stimulus, macrophages can acquire pro/anti-inflammatory phenotypes termed as classically activated M1 or alternatively activated M2 macrophages. M1 macrophages are characterized by their capacity to produce reactive oxygen species and pro-inflammatory cytokines (Duffield 2003). They promote T_H1 responses and are implicated in the immune response against intracellular pathogens, and tumors (Mosser & Edwards 2008). M2a macrophages are involved in metazoan parasites containment, tissue remodeling and the promotion of T_H2 response. They are characteristically low in the secretion of pro-inflammatory cytokines (Mosser 2003) and produce ornithine and

polyamines (Gordon & Taylor 2005). M2a macrophages express a wide array of C-type lectin receptors (CLRs) including; CD206, MGL and dectin-1 (Martinez *et al.,* 2009). Additionally, genes such as *Arg-1, RELMα* and *YM-1*, and the transcription factor STAT6 also being signatures of M2 macrophage phenotypes (Raes *et al.*, 2005). Regulatory M2b/c macrophages are characterized by the production of high levels of IL-10 and have been shown to have a significant role in the suppression of inflammation (Jones 2000) and immune tolerance (Gordon & Taylor 2005). As macrophage plasticity enables their phenotypic characteristics and functions to change based on different stimuli, they present a promising target for intervention strategies using immuno-therapeutics to abrogate inflammatory processes to combat inflammatory disorders.

3.2 Experimental design

In this chapter we sought to further investigate the immunosuppressive effects CAS exerts on macrophages by characterising the type macrophage it induces. Firstly, the ability of intact sodium caseinate (NaCAS) to suppress LPS mediated cytokine production in a concentration dependent manner was determined. BMMΦ were pre-treated with intact NaCAS (1mg/mL-0.25 mg/mL) for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL). M1 stimulant; IFNγ (20 ng/mL), M2a stimulants; IL-4 & IL-13 (20 ng/mL) or M2c stimulant; PGE_2 (5μM) were used as macrophage differentiating controls. PBS was used as an unstimulated control. Following 24 hr stimulation, supernatants were analysed for the secretion of TNF-α and IL-10 by ELISA.

To investigate if NaCAS primed macrophages towards an M1 or M2 phenotype, we examined the upregulation of phenotype related genes. BMMφ were treated as above, and RNA was extracted to measure *Arg-1*, *Ym-1, RELM α, iNOS* and *β-actin* gene expression. We examined the possible involvement of STAT signalling in the suppression of cytokines and induction of M2 genes. BMMφ were treated as above with chemical antagonists of STAT6 (1 μM) or STAT3 (1 μM) for 30 minutes prior to stimulation with NaCAS. After 24 hours RNA was extracted from cells to examine *Arg-1* gene expression by PCR. The levels of TNF-α and IL-10 were also measured.

To further characterise the phenotype of macrophage primed by NaCAS, we investigated what receptors were upregulated. BMMΦ were pre-treated as above, and flow cytometric analysis was used to measure differences in the expression of the cell surface markers; CD54, OX40L, CD206, MGL and Dectin-1. Given that M2 macrophages upregulate CLRs that bind glycosylated proteins in a Ca^{2+} dependant manner, we examined the binding

efficiency of NaCAS on BMMΦ and the involvement of CLRs in mediating this binding as the κ-CAS subunit of NaCAS is glycosylated (Jollès & Fiat 1979). NaCAS and BSA (a non-specific control) were fluorescently labelled with FITC-488 label. BMMΦ were stimulated with the labelled NaCAS and BSA (0.25 mg/mL) and analysed by flow cytometry. To investigate whether the binding was Ca^{2+} dependant, cells were pre-incubated with EGTA (10 mM) for 30 min prior to the addition of labelled NaCAS.

3.3 Results

3.3.1 NaCAS does not reduce macrophages viability

In order to ensure that the immuno-modulatory effects of NaCAS on macrophages were not due to cytotoxic effects on cells, BMMφs were pre-treated for 2.5 hr with NaCAS (1 mg/mL) prior to stimulation with and without LPS (100 ng/ml) for 24 hr. PBS and LPS alone were used as positive controls. 4 % PFA were used as negative controls. The results demonstrated that the doses used for NaCAS (Figure 3.1) did not exhibit any significant cytotoxic effect on BMMφs *in-vitro* compared to controls and as such were used at the same concentrations or lower for all subsequent experiments.

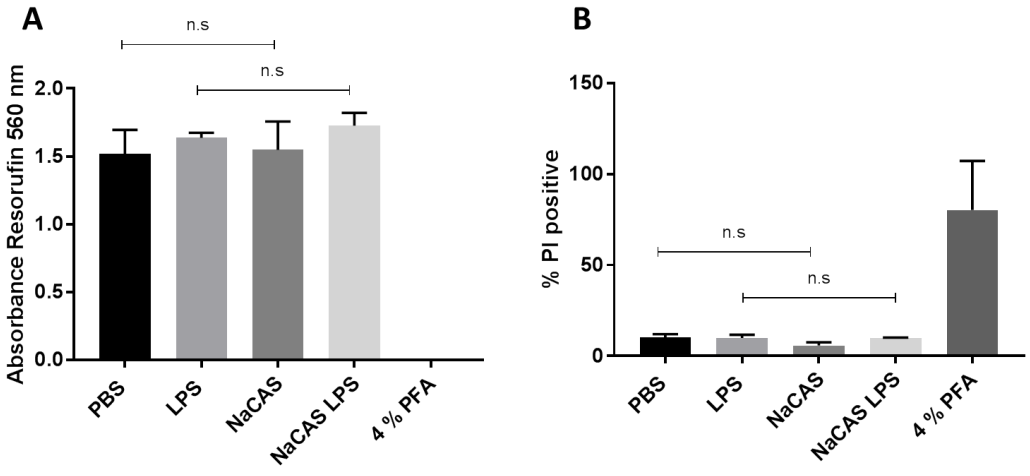

Figure 3.1 NaCAS does not affect cell viability. Resazurin (A) and PI apoptosis (B) assays were performed on BMMφ pre-treated for 2.5 hr with NaCAS (1 mg/mL) prior to stimulation with and without LPS (100 ng/ml) for 24 hr. PBS and LPS alone were used as positive controls while 4 % PFA was used as negative controls. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons tests. Ns; no significant difference compared to positive control groups.

3.3.2 NaCAS suppresses LPS induced TNF-α and IL-10 in BMMΦ.

To determine if NaCAS suppressed or enhanced TNF-α (M1-like) and IL-10 (M2-like) secretions (Figure 3.2), 1 x 10^6 BMMφ from C57BL/6 mice were stimulated with NaCAS for 2.5 hr in a dose dependant manner (0.05 mg/mL – 1 mg/mL), followed by stimulation with or without LPS (100 ng/mL) for 18 hr. PBS was used as a control. The supernatants were analysed for the secretion of TNF-α and IL-10. The basal levels of TNF-α were below the detectable range compared to the addition of stimulants alone (Data not shown). Moreover, no differences in basal levels of IL-10 were observed from the addition of stimulants alone (Data not shown). NaCAS significantly supressed the secretion of TNF-α (Figure 3.1A** , $p \leq 0.01$ *, $p \leq 0.05$) and IL-10 (Figure 3.1B *, $p \leq 0.05$) in response to LPS, the effect of which increased with increases in the concentration of NaCAS used. We determined that at higher concentrations, NaCAS exerted a greater suppressive effect on LPS induced TNF-α and IL-10 cytokine secretions in BMMφ.

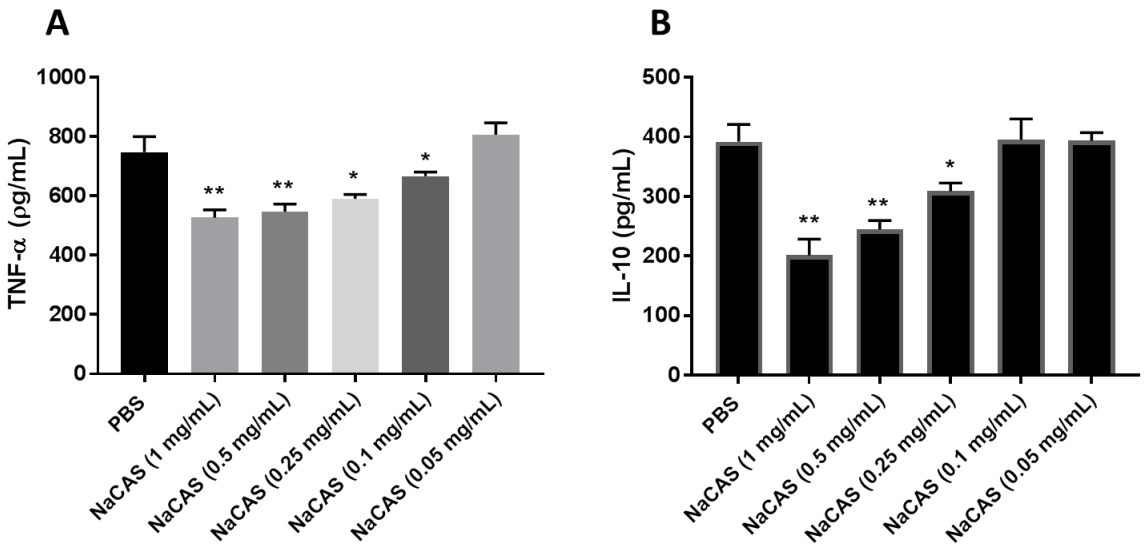

Figure 3.2 LPS induced TNF-α and IL-10 is suppressed by NaCAS in a dose response. BMMφ were pre-treated with NaCAS (0.05 mg/mL – 1 mg/mL) for 2.5 hr, followed by stimulation in the presence or absence of LPS (100 ng/mL) for 18 hr. Supernatants were removed and TNF-α (A) and IL-10 (B) were measured by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$ compared to PBS control group.

3.3.3 NaCAS induces genes associated with M2 macrophage phenotypes in BMMφ

Having established that NaCAS supressed LPS induced cytokine secretions of TNF-α and IL-10 from BMMφ, we sought to further characterise the phenotype of NaCAS activated macrophages, by comparing its phenotype to differentiated M1, M2a and M2c macrophages. BMMφ derived from C57BL/6 mice were pre-treated with NaCAS (1 mg/mL), M1 stimulant; IFN-γ (20 ng/mL) & LPS (100 ng/mL), M2a stimulant; IL-4 (20 ng/mL) or M2c stimulant; PGE_2 (5μM) as macrophage differentiating controls. PBS was used as a negative control. After 18 hr stimulation, BMMφ were isolated for RNA extraction to measure *Arg-1*, *Ym-1, RELM α, iNOS* and *β-actin* gene expression.

Our results demonstrated that NaCAS induced *Arg-1*, *Ym-1* and *RELM α*, genes associated with M2 macrophage phenotypes (Figure 3.3A), however only Arg-1 was determined to significantly upregulated when subjected to densetometic analysis (Figure 3.3B) No induction of iNOS was observed, a marker of M1 activation, except for the relevant control, suggesting that NaCAS selectively induces an M2-like macrophage phenotype.

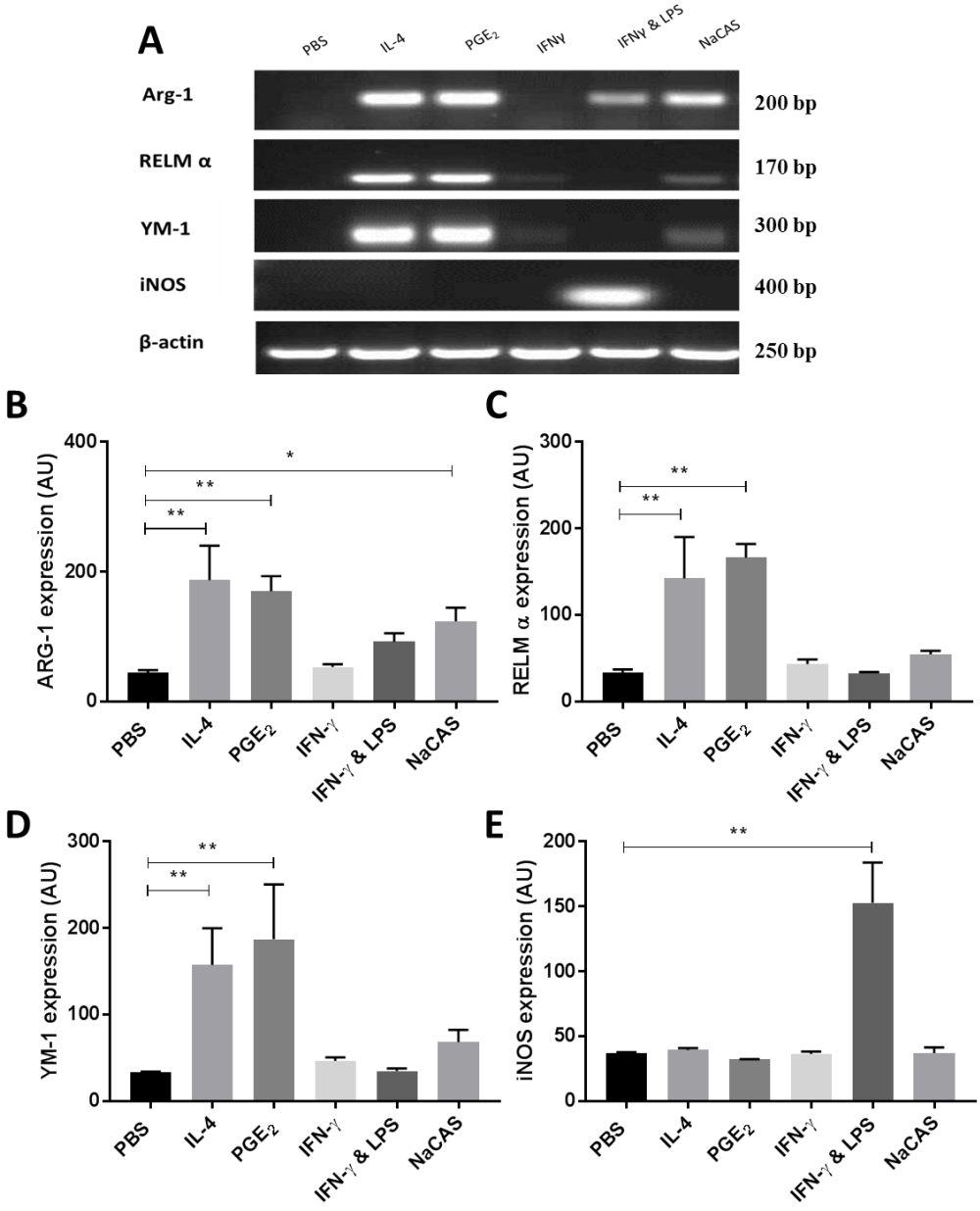

Figure 3.3 NaCAS treated BMMφ express M2 markers. BMMφ were treated with NaCAS (1 mg/mL), M1 stimulant; IFNγ (20 ng/mL) & LPS (100 ng/mL), M2a stimulant; IL-4 (20 ng/mL) or M2c stimulant; PGE_2 (5μM) for 18 hr. PBS was used as a negative control. Subsequently, BMMφ were harvested for RNA extraction to measure *Arg-1*, *RELM α, Ym-1*, *iNOS* and *β-actin* gene expression. The expression of genes were visulaised on agrose gel (A). The expression of *Arg-1* (B), *RELM α* (C), *Ym-1* (D), and *iNOS* (E) were evaluated by densitometry. Results are expressed as mean ±SD of 3 independent experiments. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$ compared to PBS control group.

3.3.4 STAT6 is partially involved in the suppression of Arg-1 and IL-10 in BMMφ by NaCAS

Given that NaCAS stimulated BMMφ had been shown to have a phenotype closely resembling that of an alternatively activated M2 phenotype, we sought to examine if NaCAS induced this phenotype via similar mechanisms. The two most prominent transcription factors involved in M2 macrophage activation are STAT6 & STAT3 (Nelms *et al.,* 1999; O' Farrell *et al.,* 1998). Arg-1 production, the hallmark of M2 polarization has been shown to be driven by the transcription factor STAT6, induced by IL-4 & IL-13 receptor signalling (Muraille *et al.,* 2014) but the increased phosphorylation and activation of STAT3 has also been implicated (Li *et al.* 2013).

Transcription factor inhibition experiments were performed to antagonise phenotype polarisation and to deduce if these transcription factors were involved in the induction of M2 like phenotypes in BMMΦ by NaCAS. BMMφ derived from C57BL/6 mice were pre-treated with chemical antagonists of STAT6; AS1517499 (1 μM) (Axon medchem, AN) or STAT3; WP1066 (1 μM) (Axon medchem, AN) or PBS as a control for 30 min. Cells were subsequently stimulated with NaCAS (1 mg/mL) for 2.5 hr and treated with or without LPS (100 ng/mL) for 18 hr. Non LPS treated BMMφ were harvested for RNA extraction to measure *Arg-1* and *β-actin* gene expression (Figure 3.4A). The supernatants were analysed for the secretion of TNF-α (Figure 3.4C) and IL-10 (Figure 3.4D).

Here we once again demonstrated that NaCAS induced *Arg-1*, a prominent gene associated with M2 macrophage phenotypes (Figure 3.4A). STAT6 antagonism was observed to exacerbate *Arg-1* expression (Figure 3.4B) and reduced the suppressive effects exerted by NaCAS on LPS induced IL-10 secretion. STAT3 antagonism resulted in a small but not significant reduction in the relative expression of Arg-1 (Figure 3.4B). The suppression of

TNF-α was unaffected by STAT3 antagonism while an increase in the suppression of IL-10 was observed (Figure 3.4D).

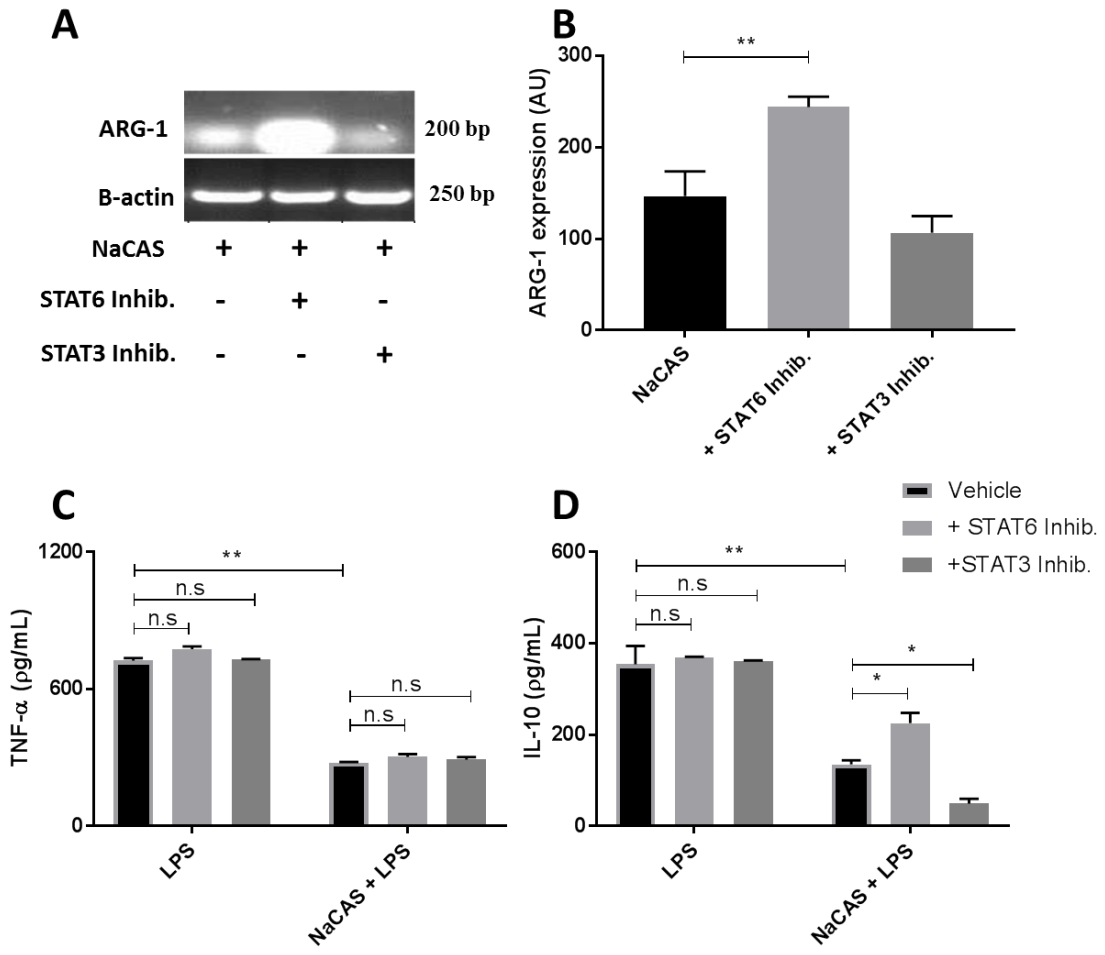

Figure 3.4 STAT6 is partially involved in Arg-1 and IL-10 suppression by NaCAS. BMMφ were pre-treated with or without chemical antagonists of AS1517499 (1 μM) (Axon medchem, AN), WP1066 (1 μM) (Axon medchem, AN) or PBS for 30 minutes. Cells were subsequently stimulated with NaCAS (0.5 mg/mL) for 2.5 hr and treated with or without LPS (100 ng/mL) for 18 hr. Non-LPS treated BMMφ were isolated for RNA extraction to measure *Arg-1* and *β-actin* gene expression (A) and denstometirc analysis was used to measure relative expression (B). Supernatants of LPS treated BMMφ were analysed for TNF-α (C) and IL-10 (D) by ELISA. Results are expressed as mean ±SD of at least 3 independent experiments. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$ compared to control group.

3.3.5 NaCAS induces a novel BMMΦ subtype with a mixed M1 and M2-like receptor repertoire

In order to confirm and further characterise the M2a-like phenotype induced by NaCAS, we compared the cell surface marker expression of NaCAS treated BMMΦ to differentiated M1, M2a and M2c macrophages. BMMφ derived from C57BL/6 mice were pre-treated with NaCAS (1 mg/mL) for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL) for 18 hr. M1 stimulant; IFNγ (20 ng/mL), M2a stimulants IL-4 (20 ng/mL) and M2c stimulants; PGE_2 (5μM) were used as macrophage differentiating controls. PBS was used as an undifferentiated control. Cells were subsequently washed and stained with fluorochrome-labelled monoclonal antibodies to the M2a cell surface markers; CD206, MGL, Dectin-1, or M1 associated; CD54 in preparation for cytometric analysis by flow cytometry. BMMφ stimulation with NaCAS resulted in a significant increases in M2a associated CD206 (Figure 3.5A ** , $p \leq 0.01$) and MGL (Figure 3.5B * , $p \leq 0.05$). However, NaCAS did not induce any increased expression of Dectin-1 (Figure 3.5C) compared to PBS control. Interestingly, NaCAS also significantly increased CD54 (Figure 3.5D ** , $p \leq 0.01$), a marker normally associated with classical M1 activation.

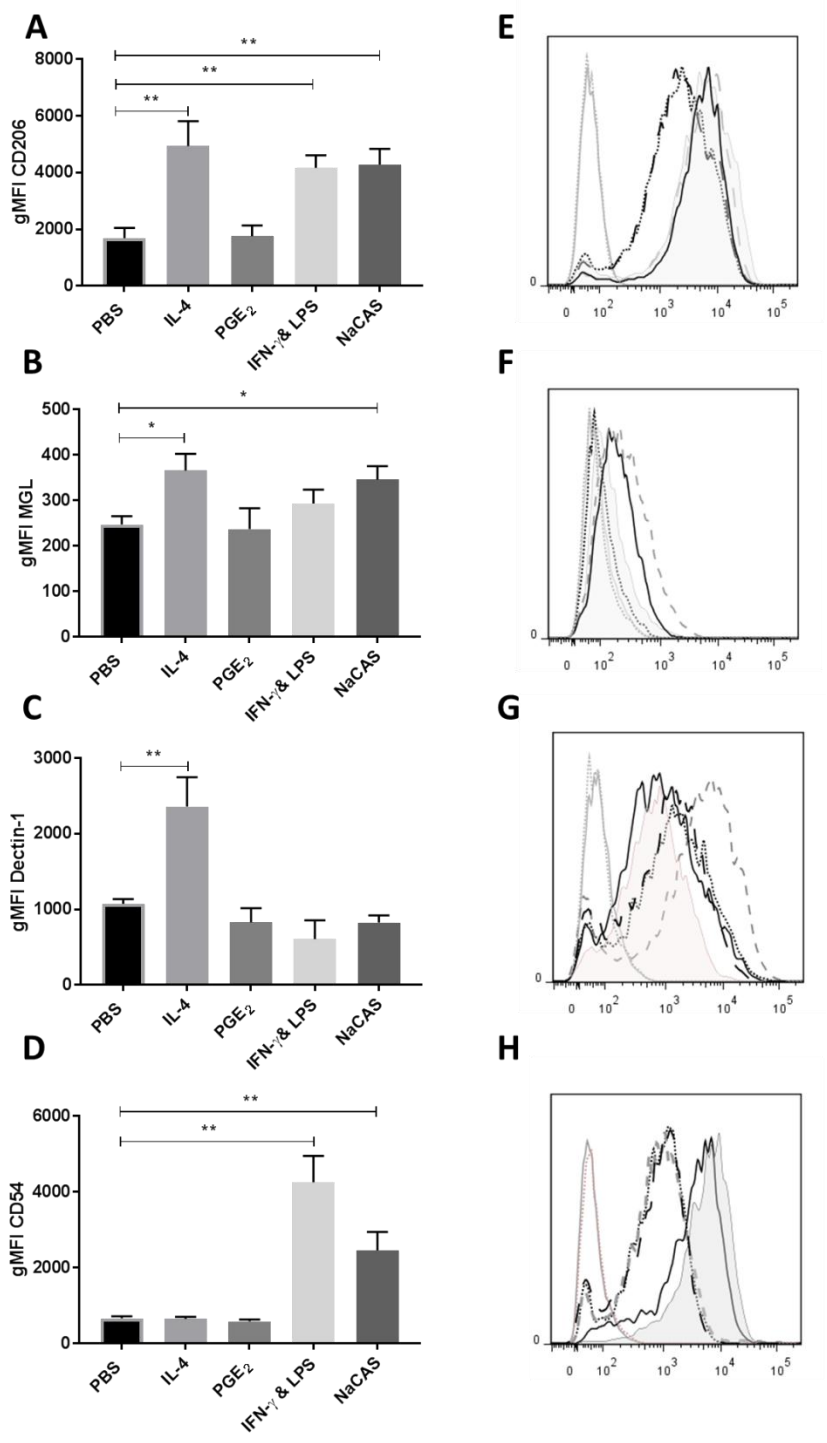

Figure 3.5 NaCAS induced receptors associated with both M1 & M2 macrophages. BMMφ were pre-treated with NaCAS (1 mg/mL) for for 24 hr. M2 stimulants IL-4 (20 ng/mL) or PGE_2 (5μM) and M1 stimulants IFNγ (20 ng/mL) & LPS (100ng.mL) were used as macrophage differentiating controls. PBS was used as a negative control. Subsequently, cells were washed and stained for 30 min with specific antibodies or with an isotype matched control and analysed by flow cytometry (FACSAria, BD, UK). Results were analysed using FlowJo software and are expressed

as the gMFI ±SD of three independent experiments (A-D). Histograms show representative data of the surface expression of CD206 (E), MGL (F), Dectin-1 (G) and CD54 (H); unstained (gray line), isotype (dotted grey line), PBS (black dotted line), IL-4 (broken thick gray line), PGE_2 (broken thick black line), IFN-γ & LPS (tinted gray histogram), and NaCAS (black line). P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$ compared to PBS control group.

3.3.6 BMMφ specifically binds NaCAS in a Ca^{2+} dependent manner

Given that NaCAS is a glycosylated protein and having demonstrated the upregulation of the CLR; CD206, a scavenger receptor which can engage in calcium dependant binding of carbohydrates on proteins (Sorvillo *et al.,* 2012; Hoving *et al.,* 2014), we examined the binding efficiency of NaCAS by BMMΦ and whether any bind was calcium dependant, possibly implicating CD206. BMMφ derived from C57BL/6 mice were pre-treated with or without EGTA (10 mM) for 10 min, followed by stimulation with FITC labelled NaCAS (0.25 mg/mL) for 30 min, subsequently washed twice and analysed by flow cytometry. FITC labelled BSA (0.25 mg/mL) was used as a non-specific protein control. Un-labelled NaCAS was used as a negative control. NaCAS showed significant binding to BMMφ (Figure 3.6A * , $p \leq 0.01$) compared to BSA. This binding was significantly reduced (Figure 3.6B * , $p \leq 0.05$) by pre-treatment with EGTA; an inhibitor of Ca^{2+} dependent binding, however the binding was not completely blocked to levels comparable with unstained control.

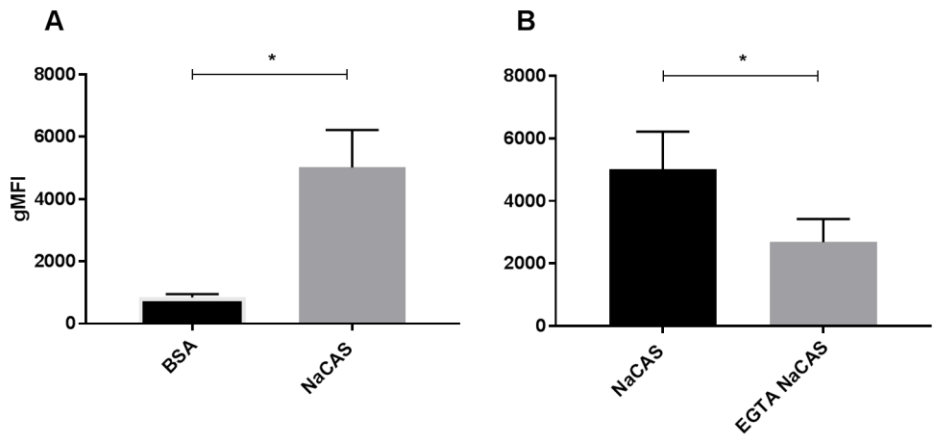

Figure 3.6 NaCAS is specifically bound by BMMφ in a Ca^{2+} dependant manner. BMMφ were stimulated with FITC labelled NaCAS (0.25 mg/mL) or FITC labelled BSA (0.25 mg/mL) for 30 min before being analysed by flow cytometry (A). Cells were also pre-treated with or without EGTA (10 mM) for 10 min prior to stimulation with labeled NaCAS (0.25 mg/mL) (B). Un-labelled NaCAS was used as a negative control. Results were analysed using FlowJo software (Treestar, USA) and are expressed as gMFI ±SD of 3 independent experiments. P-values were calculated using student's t test. * , $p \leq 0.05$

3.4 Discussion

Our data suggests that intact NaCAS suppressed LPS induced cytokine release from macrophages similar to previous studies (Bamdad *et al.,* 2017). However, we also present novel evidence which suggest that NaCAS induced an M2a-like phenotype in macrophages as we observed the induction of M2 related genes; *Arg-1*, *RELMα* and *YM-1* (Figure 3.3). *Arg-1* production is a hallmark of M2 polarization and has been shown to be driven by the transcription factor STAT6, induced by IL-4 & IL-13 receptor signaling (Muraille *et al.,* 2014). Other signaling molecules besides IL-4 & IL-13 are known to play a role in M2 polarization including STAT3 phosphorylation (Li *et al.,* 2013). However, while STAT6 and STAT3 were not shown to be prominently involved in the induction of *Arg-1,* and inhibition of LPS induced TNF-α by NaCAS, other transcription factors have been implicated in other studies. Liao *et al.,* demonstrated that Kruppel-like factor 4 coordinates with STAT6 to induce M2 genes such as *Arg-1, YM-1*, *RELMα* and attenuates M1 genes such as *TNF-α* and i*NOS* by sequestration of co-activators necessary for NF-κB activation (Liao *et al.,* 2011). Hypoxia-inducible factor 2α was also shown to be involved in the induction of *Arg-1* expression and the M2 state (Takeda *et al.,* 2010), while interferon regulatory factor 4 (IRF) negatively regulates TLR signaling in a MyD88 independent manner, driving M2 activation (Satoh *et al.,* 2010). Bone morphogenetic protein 7 was demonstrated to induce M2 polarization *in-vitro* via activation of the Phosphoinositide 3-kinase (PI3K) pathway (Rocher *et al.,* 2013).

We also observed an upregulation of CLRs CD206 (Figure 3.5A) and MGL (Figure 3.5B), other hallmarks of M2a macrophage phenotypes (Stein *et al.,* 1992). Interestingly, dectin-1, another receptor generally associated with M2a primed macrophages was not upregulated

(Figure 3.5C). Interestingly, NaCAS was observed to increase the surface expression of the CD54 (Figure 3.5D). CD54 is part of a group of receptors that mediate cell-cell or cell-extrcellular matrix attachment (Wautier *et al.,* 1990). However the induction of CD54 in macrophages has been generally associated with pro-inflammatory M1-like activation (Hubbard & Giardina 2000), while *iNOS*; a gene associated with M1 phenotypes was not found to be upregulated by NaCAS stimulation (Figure 3.3). Despite their functional role as scavenger (CD206) and adherence receptors (CD54), which upon ligation initiate intracellular signalling, these receptors have been also demonstrated to be heavily involved in cell-cell communication and signal transduction (Brill *et al.,* 2004; Aldridge *et al.,* 2016). This data would suggests that NaCAS induces a novel mixed M2-like macrophage phenotype which does not adhere to the described M1/M2 paradigm commonly cited in the literature (Martinez & Gordon 2014).

We also revealed that NaCAS was more significantly bound by macrophages then the protein control; BSA, in a Ca^{2+} dependent manner. This would suggest that NaCAS was specifically bound by a complex, in a mechanism which requires Ca^{2+}. Thus the recognition and binding of NaCAS is not non-specific and possibly implicating a role for receptors in mediating this process.

This study shows, like others showed that NaCAS supresses TLR induced cytokine expression (Mukhopadhya *et al.,* 2014; Kiewiet *et al.,* 2017; Bamdad *et al.,* 2017). However this is the first study to show that NaCAS induced an M2a like phenotype in macrophages, expressing the M2a related genes; *Arg-1, YM-1* & *RELM-α* and CLRs; CD206 & MGL, the induction of which was shown to be STAT6 and STAT3 independent. We have also shown that NaCAS is specifically bound by macrophages, and this binding was found to be Ca^{2+} dependent. As NaCAS is a protein composed of; α_{s1}-, α_{s2}-, β- & κ-

CAS subunits, further studies would need to be carried out to identify if a single subunit is responsible for the suppression of TLR responses and the induction of this M2-like macrophage phenotype.

Chapter 4 – κ-CAS induces a suppressive M2-like macrophage phenotype

4.1 Introduction

Macrophages are key mediators of innate and adaptive immunity which actively contribution to the initiation and propagation of host defence, inflammation, immuno-regulation and tissue repair (Mosser & Edwards 2008; Hume 2008; Wynn *et al.,* 2013; Mantovani *et al.,* 2013). Macrophages are distributed throughout the bodily tissues exhibiting effecter functions that enable them to activate or dampen immune responses by the release of immuno-stimulatory factors such as cytokines and as antigen presenting cells (APC) which can present antigen *in-situ,* driving adaptive immunity (Martinez & Gordon 2014). Thus a bioactive nutraceutical with immuno-modulatory properties which affects macrophages would be of great interest due to their prominent role in both innate and adaptive immunity.

Local micro-environmental stimuli drive macrophages to acquire distinct functional and morphological characteristics which polarize them into classically activated M1 or alternatively activated M2 macrophages (pro- and anti-inflammatory, respectively) (Biswas *et al.,* 2012). The M2 macrophage phenotype is composed of a functionally diverse group of subtypes rather than being a unique activation state. Accordingly, M2 macrophages can be further divided into M2a, M2b, M2c subtypes with distinct gene expression profiles. The M2a subtype is elicited by the T_H2 associated cytokines IL-4 & IL-13 and is often observed in fungal and helminth infections. M2b is elicited by IL-1R ligands, immune complexes and LPS whereas M2c is elicited by IL-10, TGF-β and glucocorticoids (Martinez *et al.,* 2008; Rőszer 2015). However, these M2 subtypes can be difficult to define as macrophages may retain a plasticity of function and phenotype depending on the stimulus at a given time (Cassetta *et al.,* 2011).

Many studies have shown that M2-like macrophages phenotypes can be induced by non-classical stimuli. For example; Endothelin-1, a pro-fibrotic peptide molecule released by endothelial cells was shown to induce M2-like phenotypic characteristics (Soldano *et al.,* 2016) while helminth derived tegmental proteins and excretory/secretory products were also shown to induce M2-like phenotypes in macrophages (Donnelly *et al.,* 2008; Adams *et al.,* 2013). Given that macrophages exhibit a plasticity of function which can be polarised based on expose to external stimuli, a bioactive nutraceutical with immuno-modulatory properties which affects a macrophages phenotype and subsequent functionality would be of interest due their prominent role in both inflammatory processes and immune-suppression.

In the previous chapter we observed the induction of an M2-like phenotype by NaCAS, expressing the M2 related gene; *Arg-1*, the extracellular CLR receptor; CD206 and also exhibited a reduced pro-inflammatory cytokine response to the classical activation stimulant; LPS. NaCAS is comprised of 4 different protein subunits: α_{s1}-, α_{s2}-, β- & κ-CAS (Dalgleish & Law 1988). Previous studies have identified these subunits and hydrolysate derivatives of these subunits to exhibit immuno-modulatory properties. The α_{s1}-CAS subunit was shown to stimulate the maturation of monocytes into dendritic cells (Vordenbaumen *et al.,* 2011; Dominguez-Melendez *et al.,* 2012). α_{s1}-, β- and κ- CAS derived peptides were demonstrated to exhibit ACE-inhibitory activity (Yamaguchi *et al.,* 2009; Weimann *et al.,* 2009). The κ-CAS derived glycomacropeptide (GMP) also exhibited protective effects, increasing the IL-1Rα antagonist (Wu *et al.,* 2011; Ashare *et al.,* 2005), and reducing the upregulation of iNOS and IL-1 (Daddaoua *et al.,* 2005). Additionally, GMP was found to counteract dextran-sulphate-sodium (DSS)-induced colitis in rats,

suppressing inflammatory cytokines, with an emphasis on the role mononuclear phagocytes like macrophages contributed in this process (Lopez-Posadas *et al.*, 2010).

It is unknown if a single subunit or all the subunits of CAS are involved in the induction of this M2-like phenotype. This study addresses this lack of knowledge and helps in identifying the immunomodulatory subunit that induces M2-like phenotypes in macrophages, the mechanism by which this occurs and investigates what impact this induced phenotype has on the wider immune response.

4.2 Experimental design

This chapter focuses on identifying the subunit of CAS responsible for the induction of the M2 phenotype, it's interaction with macrophages and the impact these cells have on wider inflammatory process. Having determined that casein supressed LPS mediated cytokine responses in macrophages, we investigated whether this was the result of a single or multiple subunits. BMMφ were pre-incubated with α, β and κ-CAS (1 mg/mL) subunits for 2.5 hr prior to the addition of LPS (100 ng/mL) for 18 hr. The effects of increasing concentration (0.1 – 2 mg/mL), multiple time points (3, 6 & 18 hr) and time of exposure (casein treatment 2.5 hr prior, at the same time or 2.5 hr post LPS stimulation) were also assessed. As we observed the suppression of TLR4 mediated cytokine responses, we chose to investigate if these suppressive effects would also be observed in multiple other TLR pathways. Cells were treated as outlined previously, but stimulated with a TLR2 ligand; PGN (5 μg/mL), a TLR7 ligand; LOX (0.5 mM) and a TLR9 ligand; CpG (2 μM).

Exposure of macrophages to PAMPs such as LPS results in the activation of the NFκB transcription factor, which orchestrates the expression of genes that induce the production of pro-inflammatory cytokines (Sharif *et al.,* 2007). Given that we observe a reduction in cytokine production, we investigated the involvement of the NFκB signalling pathway. BMMφs were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr prior to stimulation with LPS (100ng/ml). Control BMMφ were treated with PBS or κ-CAS or LPS alone. Cells were harvested after 15 min LPS stimulation, and IκB-α protein levels was determined in whole-cell lysates by Western blot analysis.

To further characterise the type macrophage phenotype induced by the casein subunits, we investigated what genes and extracellular rectors were upregulated. Cells were treated with caseins (1 mg/mL) for 18 hr. Following stimulation cells were washed and either lysed for

RNA or stained with fluorochrome-labelled monoclonal antibodies to the surface markers and analysed by flow cytometry. RNA was reverse transcribed to cDNA and subsequently anlaysed for the M2 related genes; Arg-1, RELMα and YM-1. β-actin was used as a housekeeping gene expression control. For the measurement of SOCS1 and SOCS3 gene expression, cells were treated with κ-CAS (1 mg/mL) over a time course, total RNA was extracted and after reverse transcription, the cDNA was analysed by qPCR. RNA expression was normalized to GAPDH control gene expression and showed relative to PBS control.

The impact these cells had on wider inflammatory process was also assessed, by examining their interaction and priming of adaptive responses. BMMφs treated with κ-CAS (1 mg/mL) of 18 hr, washed and subsequently co-cultured for 72 hr with $CD4^+$ T-cells isolated from naïve mice in the presence of anti-CD3 (1 μg/ml). Supernatants were analysed for the production of IL-2, IL-13, IL-10 and IFN-γ. $CD4^+$ T-cells were also analysed for the anergic extracellular surface markers; CTLA and PD-1 by flow cytometry.

4.3 Results

4.3.1 κ-CAS is the subunit responsible for the suppression of LPS induced cytokine responses in macrophages.

From the previous chapter NaCAS was observed to have an inhibitory effect on the induction of TNF-α & IL-10 upon stimulation with the bacterial antigen LPS. Given that NaCAS is comprised of multiple subunits; α, β and κ caseins (Dalgleish & Law 1988), we examined if a single or multiple casein subunits were responsible.

BMMφ derived from C57BL/6 mice were pre-incubated with α, β and κ caseins (1 mg/mL) for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL) for 18 hr. Mφs treated with PBS (negative), caseins (negative) or LPS (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α and IL-10. κ-CAS was shown to significantly inhibited the secretion of TNF-α (Figure 4.1A** , $p \leq 0.01$) and IL-10 (Figure 4.1B **, $p \leq 0.01$) in response to LPS. While α- and β-CAS also significantly supressed the secretion of IL-10 in response to LPS (Figure 4.1B **, $p \leq 0.01$), no suppressive effects were observed on LPS induced TNF-α. Furthermore, α- and β-CAS stimulations alone significantly enhanced the secretion of TNF-α from BMMφ (Figure 4.1A **, $p \leq 0.01$).

The significant induction of basal levels of TNF-α by α and β-CAS treatment alone would suggest that these proteins induced and activated M1-like macrophages phenotypes, while no TNF-α was induced by κ-CAS treatment alone. Moreover, κ-CAS significantly inhibited the ability the production of cytokines induced by LPS stimulation, similar to previous results obtained from the intact NaCAS protein.

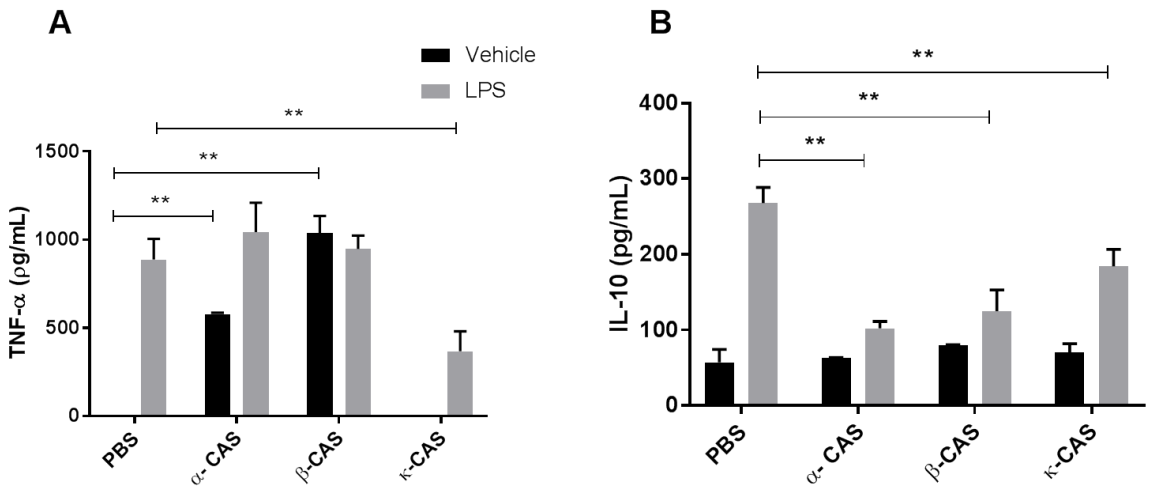

Figure 4.1 LPS induced TNF-α and IL-10 was suppressed by κ-CAS. BMMφ were pre-treated with α-, β- and κ-CAS (1 mg/mL) at for 2.5 hr. Following pre-treatment, cells were stimulated in the presence or absence of LPS (100 ng/mL) for 18 hr. Supernatants were analysed for the secretion of the cytokines TNF-α (A) and IL-10 (B) by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons test. ** , $p \leq 0.01$ compared to PBS control group.

4.3.2 κ-CAS induces M2 related genes in macrophages.

Having previously established that NaCAS induced M2 related genes in BMMφ, we sought to investigate whether the κ-CAS component also induced this M2 like phenotype. BMMφ derived from C57BL/6 mice were pre-treated with NaCAS (1 mg/mL) or κ-CAS (1 mg/mL. PBS was used as a negative control. After 18 hr stimulation, BMMφ were isolated for RNA extraction to measure *Arg-1*, *Ym-1, RELM α, iNOS* and *β-actin* gene expression. Our results demonstrated that κ-CAS similarily to NaCAS also induced *Arg-1*, *Ym-1* and *RELM α*, genes associated with M2 macrophage phenotypes (Figure 4.2A), however only Arg-1 was determined to significantly upregulated when analysed by densetometry (Figure 4.2C * , $p \leq 0.05$), which was confirmed by qPCR analysis (Figure 4.2B * , $p \leq 0.05$). Furthermore,

no induction of iNOS was observed, a marker of M1 activation, suggesting that the κ-CAS component of casein is responsible for selective induction of a M2 like phenotype in BMMφ.

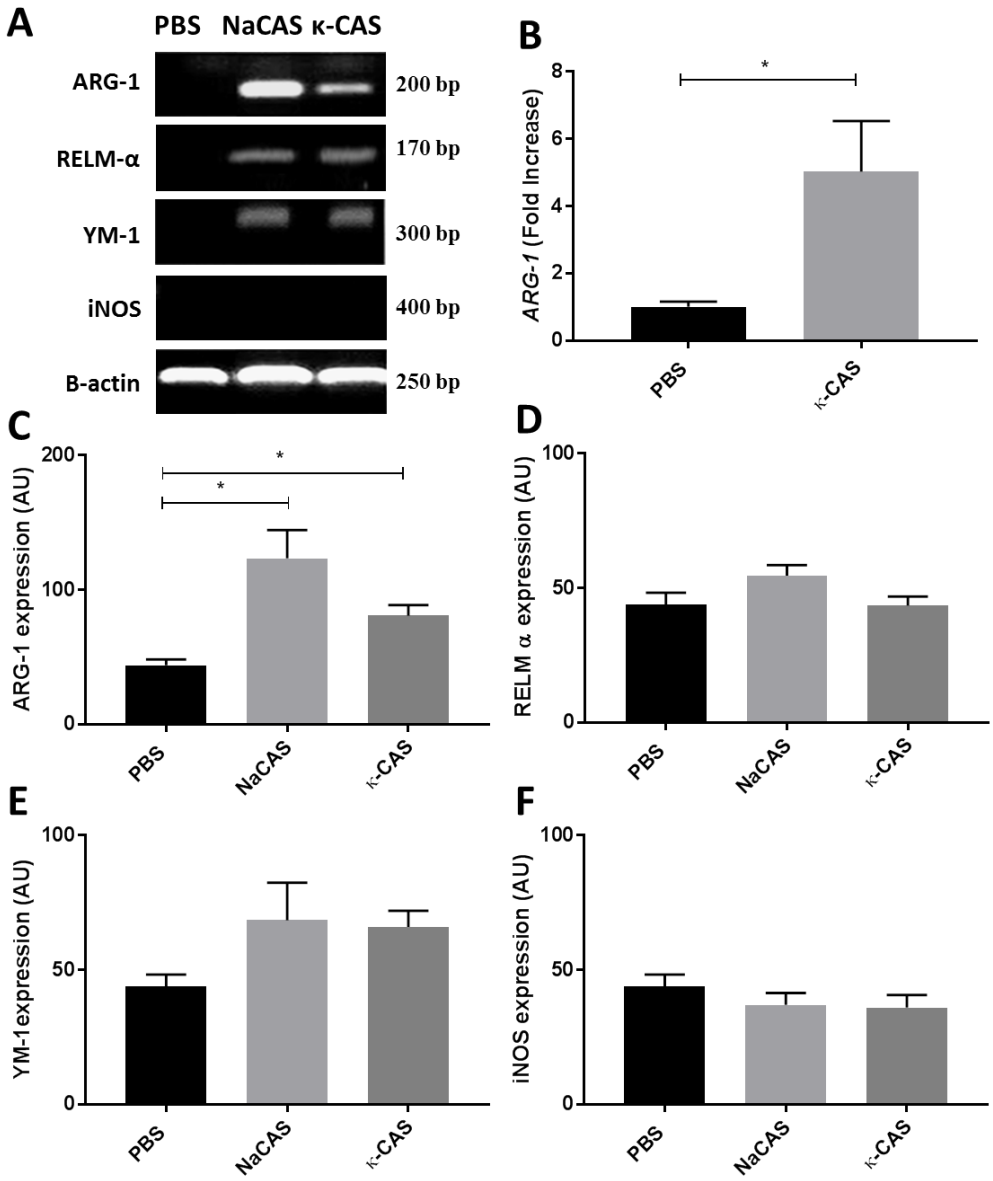

Figure 4.2 κ-CAS treated BMMφ also express M2 markers. BMMφ were pre-treated with NaCAS (1 mg/mL) or κ-CAS (1 mg/mL) for 18 hr. PBS was used as a negative control. BMMφ were harvested for RNA extraction to measure Arg-1, RELM α, Ym-1, iNOS and β-actin gene expression. The expression of genes were visulaised on agrose gel (A). Increases in the expression of Arg-1 was measured by qPCR (B) and densitometric analysis examining the relative expression

from conventional PCR were elavuvated for Arg-1 (C), RELM α (D), Ym-1 (E), and iNOS (F). Results are expressed as mean ±SD of at least 3 independent experiments. P-values were calculated using student's t-test when comparing between two groups or ANOVA multiple comparisons test for multiple groups. * , $p \leq 0.05$, compared to PBS control group.

4.3.3 The suppression of LPS induced cytokine responses in macrophages by κ-CAS increases with increased concentration, but differs over time.

Having determined that κ-CAS significantly suppressed the production of TNF-α and IL-10 in LPS stimulated BMMΦ, we next sought to assess if the cytokine suppression correlated with the concentration of κ-CAS & if the phenomenon occurred at earlier time points. BMMφ derived from C57BL/6 mice were incubated with varying concentrations of κ-CAS for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL) for the indicated amount of time. BMMφs treated with PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α and IL-10. The levels of TNF-α were below the detectable range for negative controls (data not shown). No significant differences in the levels of IL-10 were observed for negative controls (data not shown). κ-CAS was observed to significantly inhibited the secretion of TNF-α at all concentrations (0.1 – 2 mg/mL), in a concentration dependant manner in response to LPS at 3 (Figure 4.3A ** , $p \leq 0.01$) and 6 hr (Figure 4.3C ** , $p \leq 0.01$). However the degree of suppression diminished at lower concentrations after 18 hr (Figure 4.3E ** , $p \leq 0.01$ *, $p \leq 0.05$), with the lowest concentration (0.1 mg/mL) no longer significantly suppressing TNF-α. This would suggest that κ-CAS exerts greater inhibitory effects on TNF-α production at earlier time points. In contrast, κ-CAS exhibited no inhibitory effects on IL-10 secretion at the earlier time point of 3 hr (Figure 4.3B). However, over time κ-CAS was observed to significantly attenuated IL-10 secretion in a

concentration dependent manner (0.25 – 2 mg/mL) after 6 hr (Figure 4.3D * , $p \leq 0.05$), with the suppressive effects extending to lower concentrations (0.1 - 1 mg/mL) after 18 hr (Figure 4.3F ** , $p \leq 0.01$ *, $p \leq 0.05$). Interestingly, after 18 hr, this suppression was reversed at the highest concentration of 2 mg/mL.

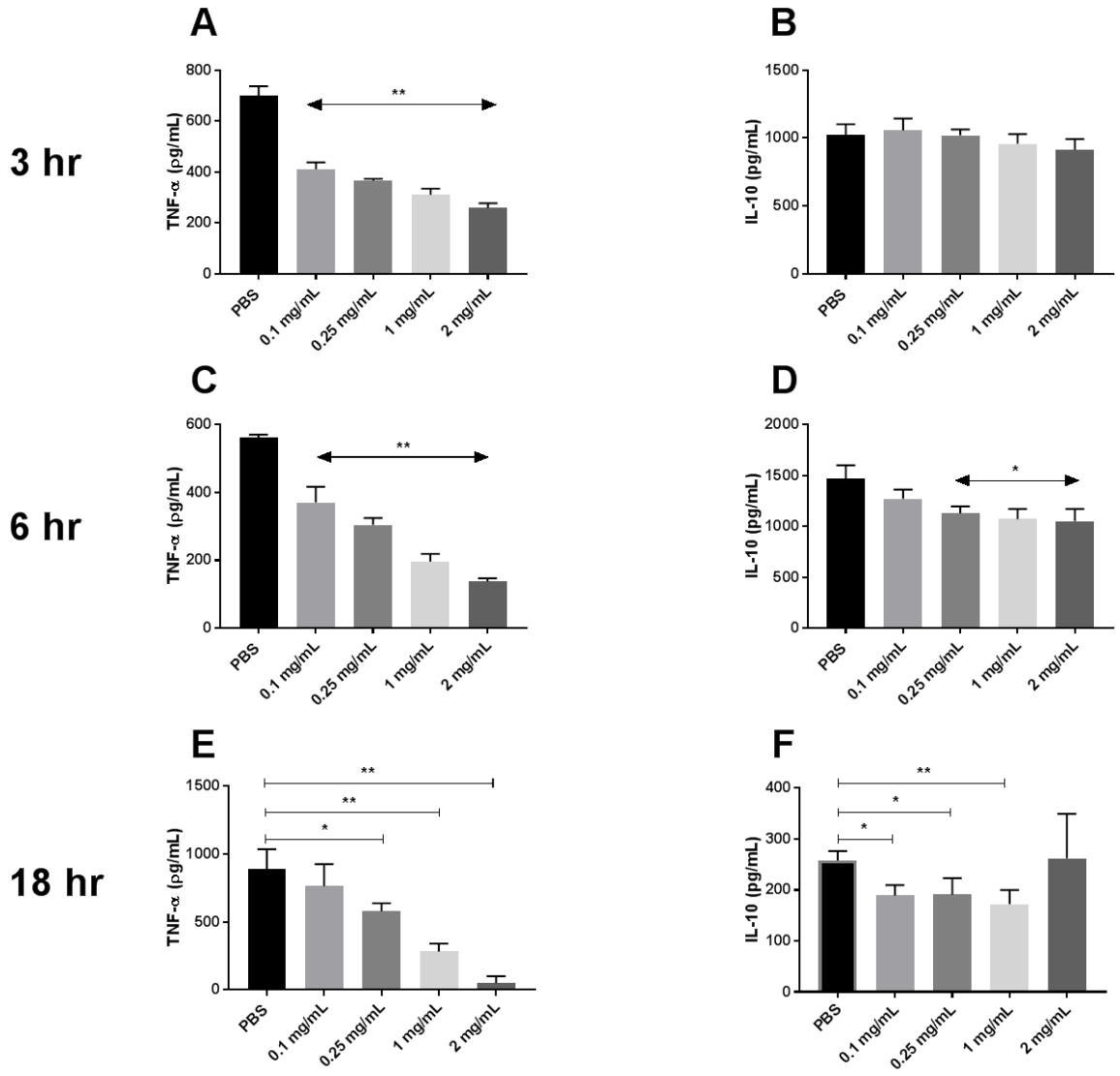

Figure 4.3 Suppressive effects of κ-CAS on LPS induced TNF-α & IL-10 over time. BMMφ were pre-treated with κ-CAS at indicated concentrations for 2.5 hr. Following pre-treatment, cells were stimulated in the presence or absence of LPS (100 ng/mL) for 3 hr (A, B), 6 hr (C, D) or 18 hr (E, F). Supernatants were analysed for the secretion of the cytokines TNF-α (A, C, E) and IL-10 (B, D, F) by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-

values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$, compared to PBS control group.

4.3.4 κ-CAS supresses TNF-α and IL-10 secretion prior and post LPS treatment in macrophages.

Considering κ-CAS suppressive effects were concentration dependent and differed based on early or later cytokine responses, we sought to investigate what impact the time of exposure to κ-CAS had on this phenomena. BMMφs were treated with κ-CAS (1 mg/mL) for 2.5 hr prior (-2.5 hr), simultaneously as (0 hr), or 2.5 hr after (+2.5 hr) stimulation with LPS (100 ng/mL). BMMφs treated with PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. Cells were incubated for 18 hr from time of LPS stimulation and the supernatants were analysed for the secretion of TNF-α and IL-10 by ELISA. The levels of TNF-α were below the detectable range for negative controls (data not shown). No significant differences in the levels of IL-10 were observed for negative controls (data not shown). κ-CAS significantly suppressed the production of TNF-α (Figure 4.4A ** , $p \leq 0.01$) and IL-10 (Figure 4.4B ** , $p \leq 0.01$) in LPS stimulated BMMφs when added prior to (-2.5 hr) LPS stimulation as shown previously. However, BMMφ were equally as suppressed when treated with κ-CAS simultaneously (0hr) or after (+2.5 hrs) LPS stimulation. No significant differences in suppression were detected between exposure times. Therefore, the suppression LPS induced cytokines by κ-CAS shows no dependence on time of exposure.

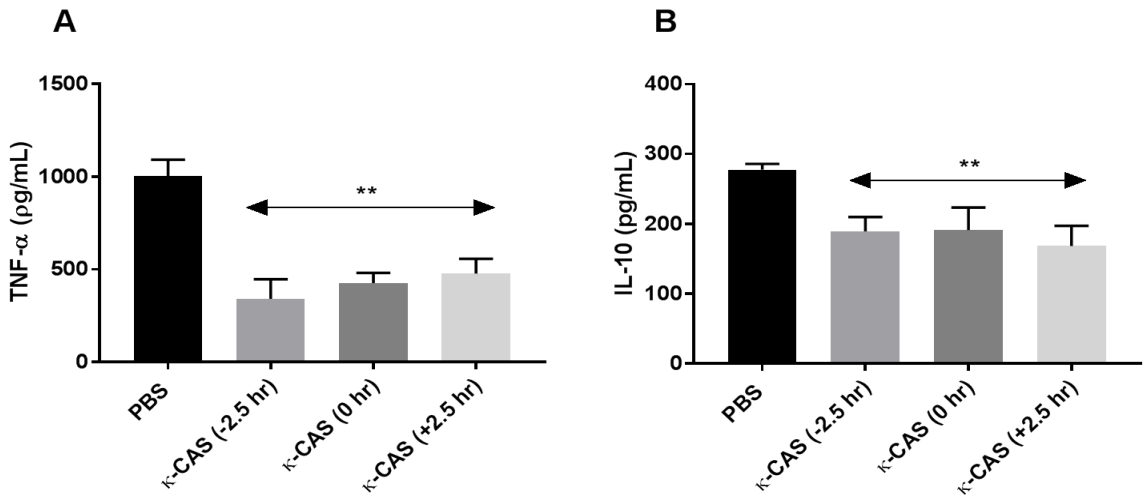

Figure 4.4 κ-CAS suppresses TNF-α & IL-10 production post and prior LPS stimulation. BMMφ were treated with κ-CAS (1 mg/mL) 2.5 hours before, same time as, or 2.5 after hours after LPS (100 ng/mL) stimulation. Supernatants were analysed for the secretion of the cytokines TNF-α (A) and IL-10 (B) by ELISA after 18 hr. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons test. ** , $p \leq 0.01$ compared to PBS control group.

4.3.5 κ-CAS suppresses cytokine secretion from an array of TLR agonists.

κ-CAS was observed to significantly attenuate TLR4 mediated responses. There are 11 known mammalian TLRs which are categorised into groups based on the agonists they recognise (Vidya *et al.*, 2017). For these experiments we chose agonists of TLR 2, 7 and 9, to broadly represent the TLR groups, to examine if the suppressive effect exhibited by κ-CAS were specific for TLR4 or targeted multiple TLRs. All TLRs, except TLR3, can signal through MyD88 (MyD88 dependent signaling), which recruits a cascade of accessory proteins to activate the NFkB, AP-1 & IRF pathways, ultimately stimulating the transcription of inflammatory cytokines. However, while, TLR3 acts independently of

MyD88, instead signaling through TRIF, the pathway leads to the activation of the same transcription factors; NFκB, AP-1 & IRFs (Liu *et al.*, 2017, Barton & Kagan 2009).

BMMφ derived from C57BL/6 mice were pre-incubated with κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with or without TLR ligands for 18 hr. Cells treated with PBS (negative), κ-CAS (negative) or TLR ligands (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α. PGN (5 μg/mL), a major surface component of gram-positive bacteria was used as an agonist of TLR2. LOX (0.5 mM), a guanosine analog, was used to activate TLR7. The synthetic oligonucleotide CpG (2 μM) that contains unmethylated CpG dinucleotides was used as a TLR9 agonist. For cytokines, the PBS and κ-CAS treatments alone were below the detectable range (data not shown). κ-CAS was observed to significantly suppress the secretion of TNF-α (Figure 4.5A ** , $p \leq 0.01$) and IL-10 (Figure 4.5B *, $p \leq 0.05$; **, $p \leq 0.01$) in response to all TLR agonists. Thus we determined that κ-CAS abrogates multiple TLR pathway signaling.

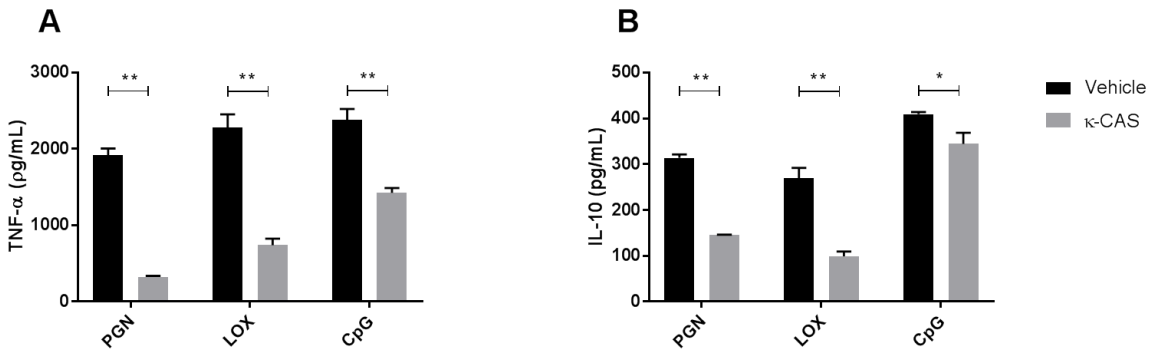

Figure 4.5 κ-CAS targets multiple TLRs in BMMφs. BMMφ were pre-treated κ-CAS (1 mg/mL) at for 2.5 hr. Following pre-treatment, cells were stimulated in the presence or absence of PGN (5 μg/mL), LOX(0.5 mM) or CpG (2 μM) for 18 hr. Supernatants were analysed for the secretion of the cytokines TNF-α (A) & IL-10 (B) by ELISA. Results are expressed as mean ±SD of 2 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons tests. * , $p \leq 0.05$ ** , $p \leq 0.01$ compared to PBS control group.

4.3.6 κ-CAS abrogates NFκB activation.

NF-κB has long been considered the prototypical pro-inflammatory signaling pathway, largely based on the role it plays in the induction of pro-inflammatory genes resulting in the production of cytokines, chemokines, and adhesion molecules upon activation (Liu *et al.*, 2017; Lawrence 2009). In a resting state, inhibitory IκB proteins complex with and sequester NFκB family members in the cytoplasm, rendering NFκB transcription factors inactive. When TLR signaling occurs, these inhibitory proteins become phosphorylated by an active IκB kinase complex, which results in the IκB proteins being degraded. Without the inhibitory proteins, NFκB is enabled to translocate into the nucleus and begin expressing pro-inflammatory signaling (Liu *et al.*, 2017; Ferreiro & Komives 2010).

Having seen the suppressive effects exhibited by κ-CAS on the induction of TLR induced of cytokine responses, we sought to investigate if NFκB signaling was involved in this

phenomenon by examining the degredation/if any of IkB proteins. BMMφ derived from C57BL/6 mice were pre-incubated with κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL) for 15 min. PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as a control. PBS and κ-CAS alone showed no significant differences in intensity, indicating that no degradation of the IκB-α protein had occurred (Fig. 4.6A lanes 1, 2 & 3, 4). The time point of optimal IκB-α degradation was deduced to be at 15 min after LPS treatment (data not shown), which led to a significant reduction in the levels of IκB-α protein (Fig. 4.6A lanes 5, 6). However, while cells treated prior to LPS with κ-CAS still lead to a reduction in IκB-α protein compared to non LPS treated controls (Fig. 4.6A, lanes 7, 8), densitometric analysis of blots revealed that there was a significant difference between this and the LPS only treated control (Fig. 4.6B *, $p \leq 0.05$). This data infers that κ-CAS may partially abrogate NFκB signaling.

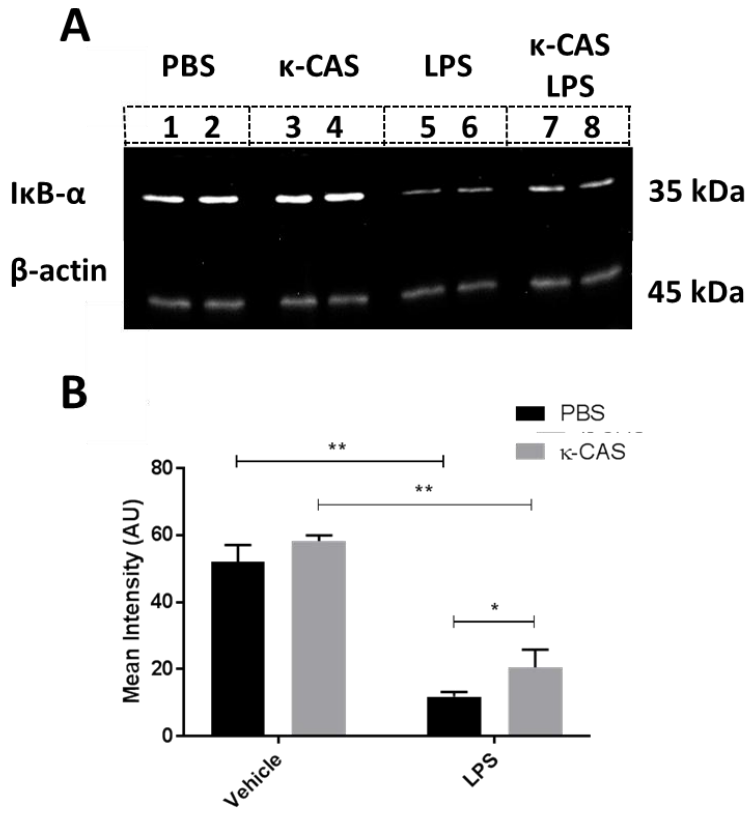

Figure 4.6 κ-CAS suppresses LPS mediated IκB-α degradation in Mφ. BMMφs were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr prior to stimulation with LPS (100ng/ml). Control BMMφ were treated with PBS or κ-CAS or LPS alone. (A) Cells were harvested after 15 min LPS stimulation, and IκB-α protein levels was determined in whole-cell lysates by Western blot analysis. A representative blot is shown. The cells were treated with PBS (1, 2), κ-CAS (3, 4), LPS (5, 6), & κ-CAS and LPS (7, 8). (B) Densitometric analysis was performed on all immunoblots, and IκB-α protein levels were expressed in arbitrary units relative to β-actin control. Results are expressed as mean intensity ±SD of 3 independent experiments. P-values were calculated using ANOVA multiple comparisons test *, $p \leq 0.05$; ** , $p \leq 0.01$.

4.3.7 κ-CAS up-regulates the transcription SOCS1 & SOCS3.

As the NF-kB pathway was shown to be abrogated by κ-CAS, we hypothesized that this compound either directly targets signaling molecules further upstream of the TLR signaling or by the induction of negative regulators of the TLR pathway. SOCS1 & SOCS3 are two known prominent negative regulators of the TLR and cytokine signaling (Duncan *et al.,* 2017; Naka *et al.,* 2005). Furthermore, both SOCS1 & SOCS3 have recently been shown to be heavily involved in macrophage polarization and function (Zhou *et al.,* 2017; Wilson 2014; Whyte *et al.,* 2011).

Given that κ-CAS was shown to induce the polarization of macrophages towards a M2-like phenotype and the exertion of suppressive effects on TLR induced of cytokine responses, we sought to investigate if κ-CAS induced SOCS1 or SOCS3. BMMφ derived from C57BL/6 mice were incubated with κ-CAS (1 mg/mL) for indicated times. PBS treated BMMφs were used a negative as controls. κ-CAS significantly enhanced the expression of SOCS1 (Figure 4.7A *, $p \leq 0.05$) and SOCS3 (Figure 4.7A *, $p \leq 0.05$; ** , $p \leq 0.01$). However, while both SOCS molecules were significantly upregulated, SOCS3 upregulation occurred at earlier time points, beginning to significantly increase after 30 min, while no significant increases of SOCS1 were observed until 2 hr. Furthermore, the observed significant increases of SOCS3 were greater than that of SOCS1. The levels of expression for both SOCS molecules remained significantly upregulated at 6 hr but returned to baseline after 12 hr suggesting that κ-CAS induces SOCS1 & 3 at early time points.

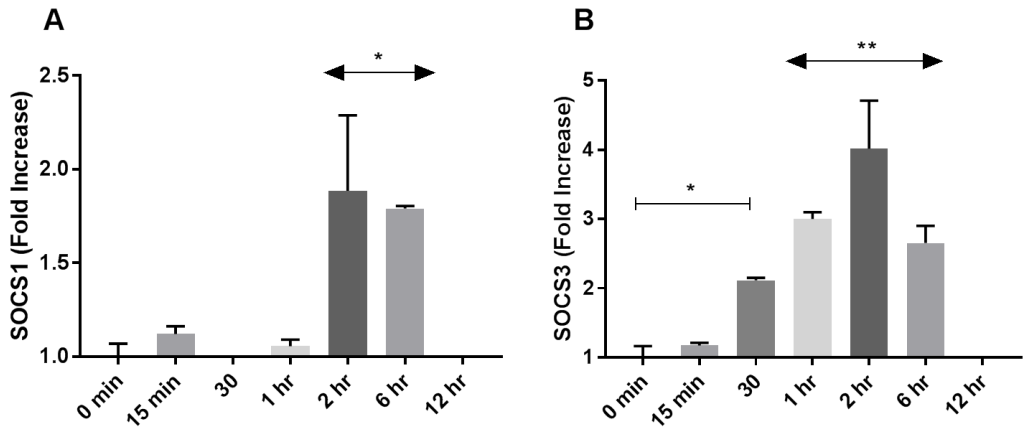

Figure 4.7 κ-CAS induces SOCS1 & SOCS3. BMMφs were incubated with and without κ-CAS (1 mg/mL) for the indicated times. Total RNA was extracted and after reverse transcription, the cDNA was analysed by qPCR for SOCS1 (A) & SOCS3 (B). RNA expression was normalized to GAPDH control gene expression and showed relative to PBS control. Figures are representative of 2 independent experiments in dublicate. P-values were calculated using ANOVA multiple comparisons test *, $p \leq 0.05$; ** , $p \leq 0.01$ compared to PBS control group.

4.3.8 κ-CAS induced SOCS is possibly implicated in the suppression of LPS induced TNF-α.

SOCS1 & SOCS3 were both shown to be up-regulated by κ-CAS at early time points and are known to be key inhibitory molecules of the TLR4 signaling pathway. Furthermore, SOCS1 & SOCS3 has been identified as a key determinant of differential M2 macrophage activation (Zhou *et al.,* 2017; Wilson 2014; Whyte *et al.,* 2011). Given that we observe the induction of M2 markers by κ-CAS we investigated the involvement of SOCS1 in the suppression of cytokine responses by κ-CAS. Previous studies have utilised a synthetic peptide antagonist of SOCS1/3; pJAK2 (Ahmed *et al.,* 2015; Waiboci *et al.,* 2007), which we also used to inhibit SOCS1 and SOCS3 activity. BMMφ derived from C57BL/6 mice were pre-incubated with indicated concentrations of the SOCS1 inhibitor; pJAK2 for 24 hr

prior to κ-CAS (1 mg/mL) treatment. Cell were subsequently stimulated after 2.5 hr with or without LPS (100 ng/mL) for 18 hr. BMMφs treated with PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α. The levels of TNF-α were below the detectable range for negative controls (data not shown). No significant differences were observed between vehicle levels from the addition of the SOCS1 and SOCS3 inhibitor; pJAK2. As previously observed, κ-CAS, significantly inhibited the production of TNF-α (Figure 4.8 ** , $p \leq 0.01$). SOCS1 antagonism was not observed to reverse this significant inhibition at any concentration. However, pJAK2 was shown to significantly increase the levels of LPS induced TNF-α by κ-CAS treated BMMφ at a concentration of 80 μM (Figure 4.8 ** , $p \leq 0.01$). No further increases in SOCS inhibitor were used as significant increases in basal levels were detected after 80 μM (data not shown).

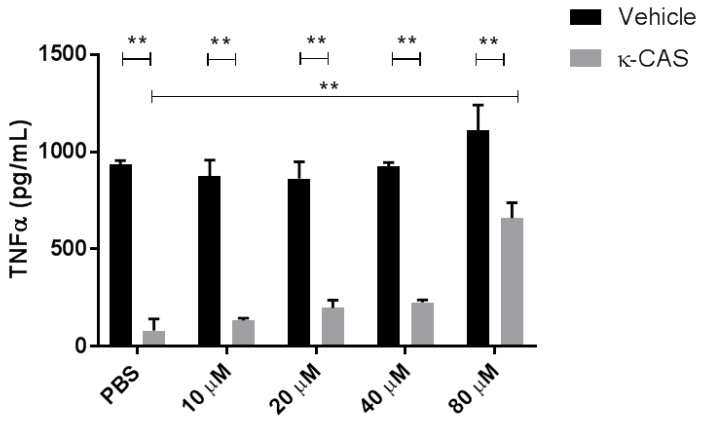

Figure 4.8 The suppression of LPS induced TNF-α is partially SOCS dependent. BMMφ were pre-treated with pJAK2 at indicated concentration for 18 hr. Subsequently, cells were incubated with κ-CAS (1 mg/mL) 2.5 hours before, LPS stimulation for 18 hr. Supernatants were analysed for the secretion of the cytokines TNF-α by ELISA. Results are expressed as mean ±SD of 2 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons tests. ** , $p \leq 0.01$ compared to PBS control group.

4.3.9 Proteolytic hydrolysis of κ-CAS by cells is responsible for activity.

As several peptides derived from the proteolysis of κ-CAS have been shown to exert immuno-suppressive activity, notably GMP or its hydrolysate derivatives (Cheng *et al.*, 2015; Requena *et al.*, 2009; Mikkelsen *et al.*, 2005; Li & Mine 2004), we next examined if intact κ-CAS or a fragment accounted for the observed activity. We used a protease inhibitor cocktail to block the possible hydrolysis of κ-CAS by cell proteases. BMMφ derived from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL) for the indicated amount of time. A protease inhibitor cocktail (1:200), containing; 4-(2-aminoethyl)-benzenesulphonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A and E-64 was added 30 min prior to any treatment. BMMφs treated with PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α and IL-10. The levels of TNF-α were below the detectable range for negative controls (data not shown). The blocking of protease activity in BMMφ was shown to reverse the suppressive effects κ-CAS exhibited on LPS induced TNF-α (Figure 4.9A) & IL-10 (Figure 4.9B) production. This would indicate that a fragment released from the proteolytic cleavage of κ-CAS by cells is responsible for the immune-suppressive effects.

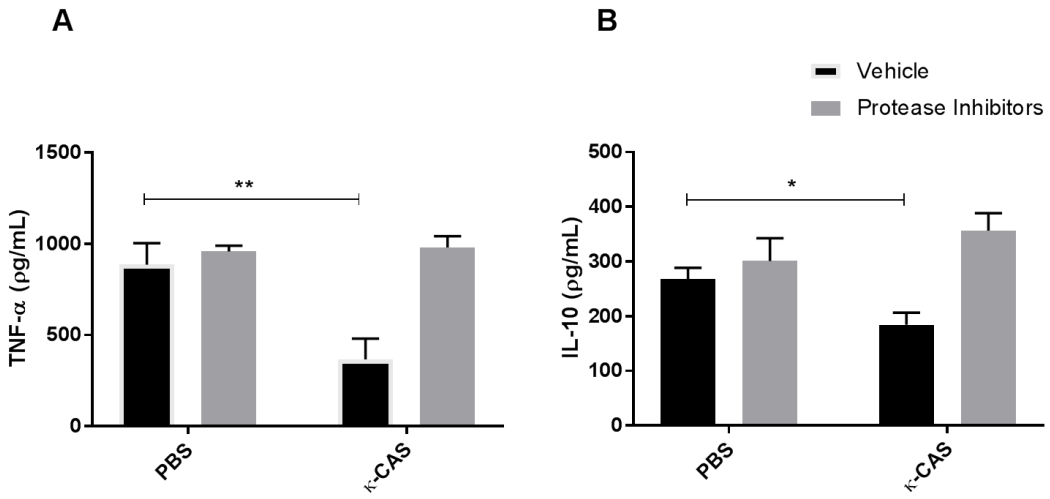

Figure 4.9 Effect of protein hydrolysis on κ-CAS activity. BMMφ were pre-treated with a protease inhibitor cocktail (1:200 v/v) and subsequently incubated with or without κ-CAS (1 mg/mL) for 2.5 hr. Cells were subsquently stimulated in the presence or absence of LPS (100 ng/mL) for 18 hr. Supernatants were analysed for the secretion of TNF-α (A) & IL-10 (B) by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$ compared to PBS control group.

4.3.10 BMMφ treated with κ-CAS exhibit a mixed M1/M2 receptor repertoire & and selectively upregulate co-stimulatory molecules.

Having previously observed the induction of M2 like phenotype markers from NaCAS treated BMMφ, we investigated if κ-CAS induced a similar receptor repertoire. BMMφ were pre-treated with κ-CAS (1 mg/mL) for 18 hr. PBS was used as an unstimulated control. Cells were subsequently washed and stained with fluorochrome-labelled monoclonal antibodies to the surface markers and analysed by flow cytometry. BMMφ stimulation with κ-CAS resulted in significant increases in M2a associated CD206 (Figure 4.10 A** , $p \leq 0.01$). However, we observed no significant differences with the M2 associated MGL and Dectin-1 receptors (data not shown). Similar to the results we had

previous obtained for NaCAS, κ-CAS also significantly increased CD54 (Figure 4.10D ** , $p \leq 0.01$), a marker normally associated with classical M1 activation. Interestingly, while no significant differences were observed for the co-stimulatory markers; CD80 or CD86 (Data no shown), CD40 (Figure 4.10B *, $p \leq 0.05$) and OX40L (Figure 4.10C *, $p \leq 0.05$) were shown to be significantly increased.

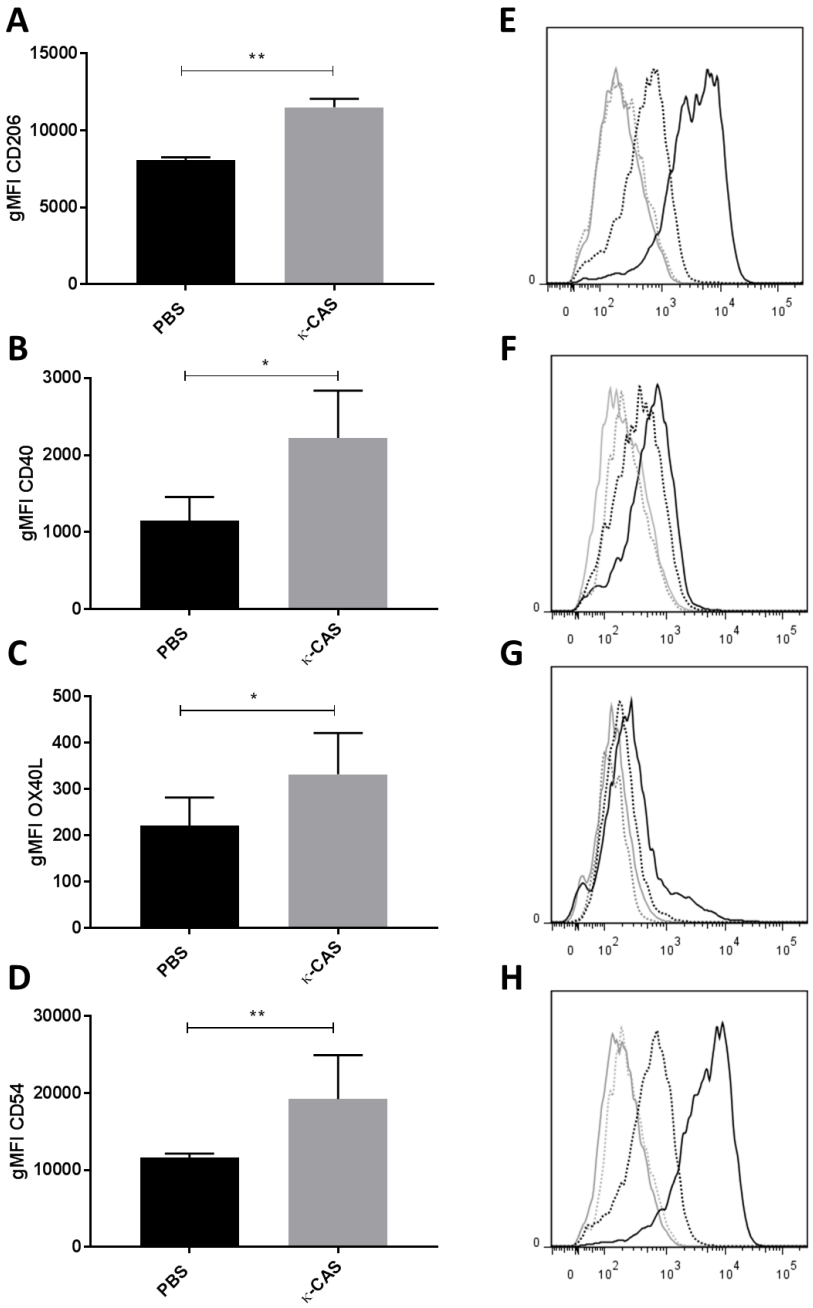

Figure 4.10 κ-CAS induced a mixed M1 & M2 receptor repertoire and selectively upregulated co-stimulatory molecules. BMMφ were pre-treated with κ-CAS (1 mg/mL) for for 24 hr. PBS was used as a negative control. Subsequently, cells were washed and stained for 30 min with specific antibodies or with an isotype matched control and analysed by flow cytometry (FACSAria, BD, UK). Results were analysed using FlowJo software (Treestar, USA) and are expressed as the gMFI ±SD of at least 3 independent experiments (A-D). Representative histograms show the surface expression of CD206 (E), CD40 (F), OX40L (G) and CD54 (H); unstained (gray line), isotype (dotted grey line), PBS (black dotted line) and κ-CAS (black line). P-values were calculated using student's t tests. ** , $p \leq 0.05$, ** , $p \leq 0.01$ compared to PBS control group.

4.3.11 κ-CAS induced BMMφ functionally alter cytokine secretion from $CD4^+$ T-cells.

Macrophages participate in many aspects of the innate immune response. They are heavily involved in the phagocytosis and clearance of extracellular pathogens, the generations of microbicides, wound healing/repair, and the production of immuno-modulatory cytokines (Mosser & Edwards 2008; Mantovani *et al.,* 2013; Wynn *et al.,* 2013). In addition, macrophages can also present antigen to responsive T-cells, participating directly in the generation of adaptive immune responses (Hume 2008).

Considering κ-CAS significantly suppressed cytokine production, but upregulated the costimulatory markers CD40 & OX40L expression in BMMφ, we investigated the impact these cells had on wider inflammatory process, by examining their interaction and priming of adaptive responses. While previous studies have examined the effects κ-CAS and its hydrolysate derivatives have on T-cell activity directly, the ability of κ-CAS stimulated macrophages to modulate T-cells has not been previously shown. BMMφs from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for 18hr. BMMφ were treated with PBS (positive) as a control. Cells were washed and subsequently co-cultured with $CD4^+$ T-cells at a ratio of 1:4 on plates pre-coated with anti-CD3 (1 μg/well) for 72 hr. $CD4^+$ T-cells

alone (negative) were used a control. BMMφs primed with κ-CAS induced significantly less IFN-γ (Fig. 4.11A *, $p \leq 0.05$) and IL-2 (Fig. 4.11D **, $p \leq 0.01$) compared to control BMMφ primed with PBS. Interestingly, κ-CAS treated BMMφ increased $CD4^+$ T-cell induced IL-13 compared to PBS control, however the levels were not determined to be significant (Figure 4.11B p=0.07). We also observed a non-significant decrease in the levels of IL-10 as well (Figure 4.11C). This would indicate that BMMφs exposed to κ-CAS suppress T_H1 responses *in-vitro*.

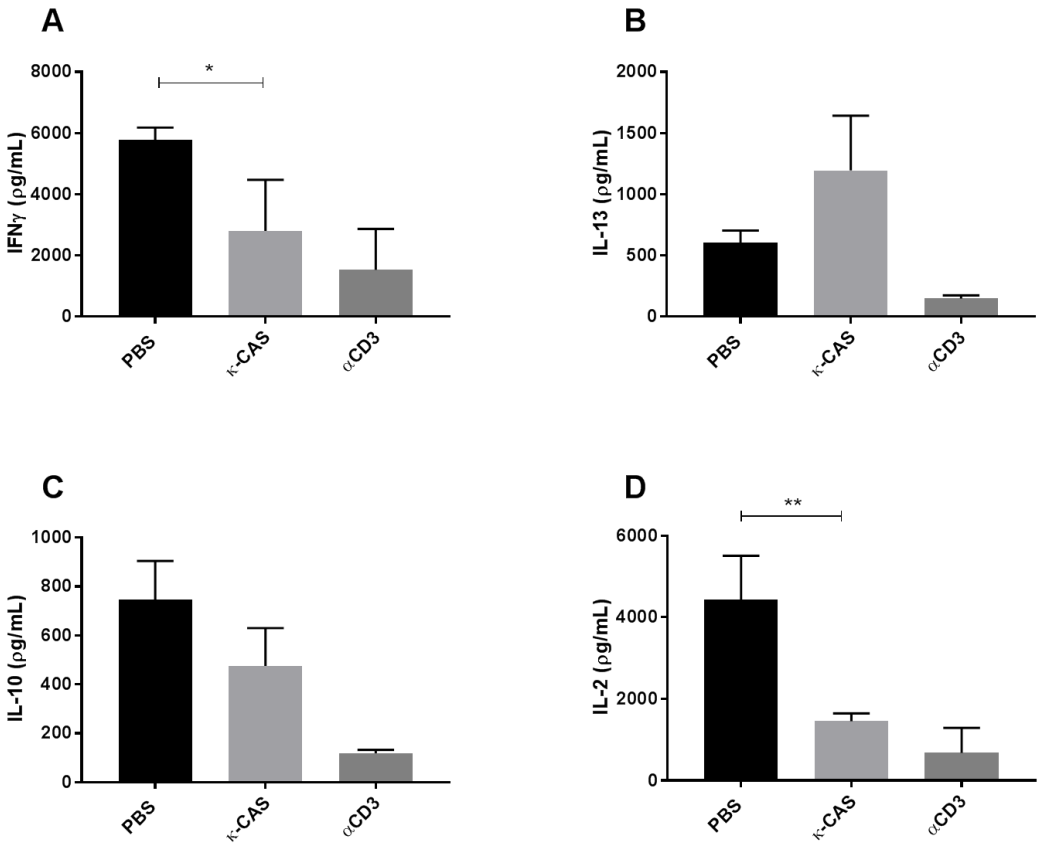

Figure 4.11 κ-CAS treated BMMφ functionally alter cytokine secretion from $CD4^+$ T-cells *in-vitro*. BMMφs were pre-treated with κ-CAS (1 mg/mL) for 18hr. Control BMMφ were treated with PBS. κ-CAS treated and PBS macrophages were washed and subsequently co-cultured with $CD4^+$ T-cells at a ratio of 1:4 on plates pre-coated with anti-CD3 (1 μg/well). $CD4^+$ T-cells not cultured with macrophages were used a negative control. After 72 hr, supernatants were analysed for the

cytokines IFN-γ (A), IL-13 (B), IL-10 (C) and IL-2 (D) by ELISA. Results are expressed as mean ±SD of at least 3 independent experiments in dublicate. P-values were calculated using ANOVA multiple comparisons test *, $p \leq 0.05$; ** , $p \leq 0.01$ compared to PBS control group.

4.3.12 κ-CAS treated macrophages do not induce markers of anergy when co-cultured with $CD4^+$ T-cells.

T-cell anergy is a tolerance mechanism by which lymphocytes become functionally inactivated following an antigen encounter, remaining in a hypo-responsive state for an extended period of time (Schwartz 2003). T-cell anergy is associated with the poor production of cytokines, most prominently IL-2, as it is the key cytokine associated with T-cell proliferation (Wells *et al.,* 2001) and required for the effective induction of T-cell responses. CTLA4 and PD1 are two cell surface markers enhanced on anergic T-cells (Okazaki and Honjo 2006, Wells *et al.,* 2001, Butte *et al.,* 2007).

Given that we observed the suppression of T-cell induced IL-2 by κ-CAS treated BMMφ, we investigated if this was the result of anergy. BMMφs from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for 18hr. BMMφ were treated with PBS were used as a control. Cells were washed and subsequently co-cultured with $CD4^+$ T-cells at a ratio of 1:4 on plates pre-coated with anti-CD3 (1 μg/well). $CD4^+$ T-cells alone on plates pre-coated with anti-CD3 (1 μg/well) were used as an anergic control (Zheng *et al.,* 2009). After 72 hr incubation, cells were washed and stained with fluorochrome-labelled monoclonal antibodies for the surface markers CTLA4 or PD-1 and analysed by flow cytometry. No significant increases in either of the anergic cell surface markers; CTLA (Figure 2.12A) or PD-1 (Figure 4.12B) were observed compared to PBS controls. This would suggest that the T-cells induced by κ-CAS BMMφs are not anergic.

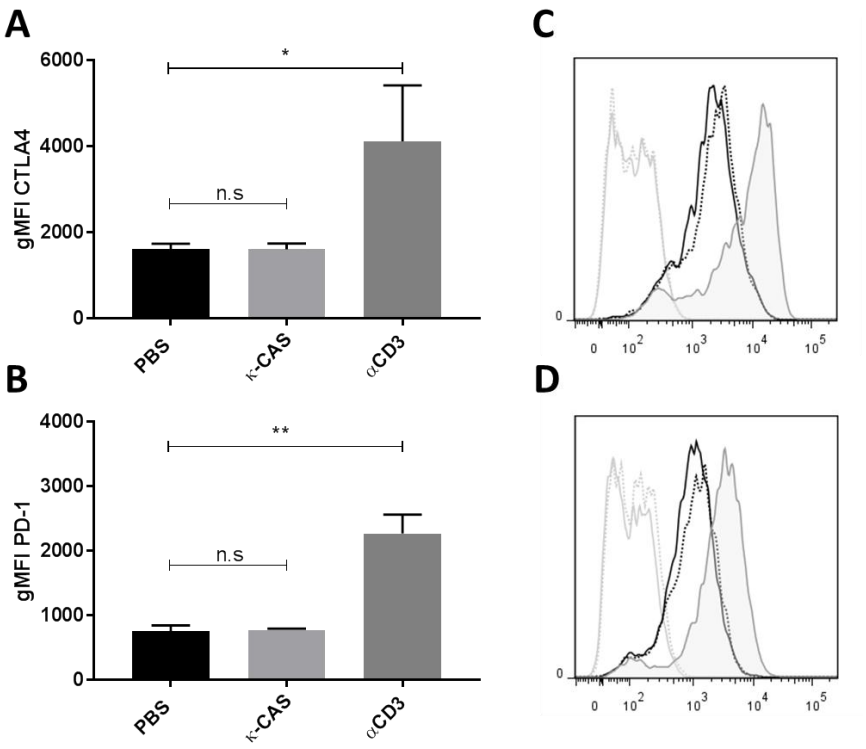

Figure 4.12 CD4$^+$ T-cells cultured with κ-CAS treated BMMφ do not display anergic markers. BMMφs were pre-treated with κ-CAS (1 mg/mL) for 18hr. Control BMMφ were treated with PBS. κ-CAS treated and PBS macrophages were washed and subsequently co-cultured with CD4$^+$ T-cells at a ratio of 1:4 on plates pre-coated with anti-CD3 (1 μg/well). CD4$^+$ T-cells not cultured with macrophages were used an anergic control. After 72 hr, cells were analysed for the anergic extracellular surface markers CTLA4 (A) or PD-1 (B) by flow cytometry (FACSAria, BD, UK). Results were analysed using FlowJo software (Treestar, USA) and are expressed as the gMFI ±SD of 2 independent experiments. Representative histograms show the surface expression of CTLA4 (C) and PD-1 (D); unstained (gray line), isotype (dotted grey line), PBS (black dotted line), κ-CAS (black line), and anti-CD3 alone (tinted gray histogram). P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$, ** , $p \leq 0.01$ compared to PBS control group.

4.4 Discussion

In the previous chapter we showed that NaCAS induced a M2-like phenotype in macrophages and suppressed LPS mediated cytokine responses. Here we sought to determine if we could identify the bioactive subunit responsible for the induction of this novel M2-like phenotype. We demonstrated that the κ-CAS subunit alone was shown to prime macrophages towards a M2-like phenotype expressing the M2 related gene markers; *Arg-1, RELMα* and *YM-1* (Martinez & Gordon 2014). κ-CAS treated macrophages also upregulated the expression of CD206, a C-type lectin receptor associated with M2 macrophage phenotype priming (Mantovani *et al.,* 2013). However CD54, an extracellular adhesion receptor associated with antigen presenting cell activation and usually upregulated in pro-inflammatory M1 macrophage phenotypes was also shown to be induced by κ-CAS (Hubbard & Giardina 2000; Sheikh & Jones 2008; Murray *et al.,* 2014). Yet recent studies have demonstrated that CD54 deficiency induces M1 macrophage polarization, while overexpression promoted M2 polarization (Gu *et al.,* 2017).

κ-CAS like the intact protein NaCAS, was shown to impair the ability of macrophages to elicit the release of the key inflammatory cytokine mediators; TNF-a and IL-10 in response to LPS. κ-CAS treated macrophages also were rendered hypo-responsive to a range of other TLR ligands. However the suppressive effect differed over time. TNF-α was significantly supressed at early points at all concentrations, and during later time points at higher concentrations. In contrast, no suppression of IL-10 was observed at any concentration during early time points. However, κ-CAS did significantly attenuate IL-10 production at later time points, except at the highest concentration of 2 mg/mL, where no significant reduction was observed. Also, κ-CAS was shown to exhibit its suppressive effects independent of when the TLR ligand was added suggesting that κ-CAS does not

compete with LPS for binding and that κ-CAS does not exerting its effects through the TLR. The observed reduction in cytokine responses were determined not to be the result of loss in viability as κ-CAS was shown not to affect the viability of macrophages as determined by Annexin V staining and resazurin assays (Appendix A).

Multiple other studies attribute the inhibitory activity of κ-CAS to the C-terminal fragment; GMP and its derivatives (Wu *et al.,* 2011; Ashare *et al.,* 2005; Daddaoua *et al.,* 2005; Otani & Monnai 1993). More recently, GMP and its derivatives were found to supress TLR4 meditated responses in macrophages by attenuating the activation of NFκB signalling (Cheng *et al.,* 2015). The mechanism by which this occurred was found to be due to the upregulation of heme oxygenase-1, which when inhibited resulted in the restoration of inflammatory cytokine release and NFκB activity (Li *et al.,* 2017). While we attained similar results with κ-CAS, which was also shown to abrogate LPS mediated inflammatory cytokine release and NFκB activation, the inhibition of heme oxygenase-1 did not restore inflammatory cytokine release (Appendix B). Other studies examining the effects of enzymatic digestion has on GMP determined that proteolytic treatment with trypsin did not significantly affect its activity. Requena *et al.,* also demonstrated that inhibiting the possible hydrolysis of GMP by cell proteases had no effect on activity (Requena *et al.,* 2009), unlike κ-CAS, which we demonstrated lost its suppressive effects. Given these differences, we can deduce that the results we obtained for κ-CAS are unlikely to be due to GMP but another novel fraction of κ-CAS with immune-modulatory activity.

SOCS proteins play a prominent role in maintaining homeostasis by preventing the overexpression of harmful pro-inflammatory cytokine responses (Yoshimura *et al.,* 2007). κ-CAS was shown to supress TLR mediated pro-inflammatory cytokine responses and NFκB activation. κ-CAS also induced SOCS1 and SOCS3, two prominent negative

regulators of the TLR pathway. SOCS3 is one of the main inhibitory proteins involved in the auto-regulatory response to pathogen induced inflammation (Yoshimura *et al.,* 2012). Previous studies have shown that over expression of SOCS3 in macrophages was shown to suppress LPS-mediated TNF-a (Cacalano *et al.,* 2006). Nair *et al.,* demonstrated that a phosphorylated SOCS3 can interact with the IκBα-NFκB complex, which inhibits the phosphorylation of IkB-α and subsequent activation of macrophages during stimulation with LPS (Nair *et al.,* 2011). However, there are is some conflicting evidence which suggests that SOCS3 has little or can even enhance the effects of TLR responses in macrophages (Liu *et al.,* 2008). SOCS1 also plays a key role in the negative regulation of cytokine receptor and TLR mediated signalling and has been shown to be upregulated in M2 macrophages (Whyte *et al.,* 2011). Studies have demonstrated that LPS induced NFκB activation and consequent production of pro-inflammatory cytokines are inhibited in cells overexpressing SOCS1 (Kinjyo *et al.,* 2002; Nakagawa *et al.,* 2002). SOCS1 has been demonstrated to target multiple adaptor proteins involved in the TLR and NFκB signalling pathway including; IRAK1 (Nakagawa *et al.,* 2002), Mal/TIRAP (Mansell *et al.,* 2006), the p65 subunit of NFκB (Ryo *et al.,* 2003) and JAK2 (Kimura *et al.,* 2005). Although the sequestration of κ-CAS induced SOCS1 and SOCS3 activity by the peptide inhibitor did not reverse the suppression of LPS induced TNF-α, we did observe a significant increase in the levels of TNF-a produced. This would suggest that SOCS1 or SOCS3 may be implicated in this process.

The state of activation and maturation of APCs like macrophages determines their ability to interact with naïve T-cells, influencing the type of immune response that is initiated (Banchereau & Steinman 1998). Given that κ-CAS upregulated the co-stimulatory molecules CD40 and OX40L involved in T-cell activation (Grewal & Flavell 1996; Murata

et la., 2000), we examined what impact these cells had on the development of the adaptive immune response using $CD4^+$ T-cell priming studies. During normal activation, APC and T-cell engagement leads to T-cell activation, promotion of IL-2 secretion and proliferation. Alternatively, activation through anergic pathways results in poor production of IL-2, the loss of proliferation and renders T-cells hypo-responsive (Wells *et al.* 2001). Our results demonstrated that κ-CAS primed macrophages significantly suppressed T_H1 associated cytokines, notably IFN-γ. Moreover, we also observed a significant reduction in the production of IL-2. However, these cultured $CD4^+$ T-cells did not display either of the prominent extracellular surface markers associated with anergy; CTLA4 and PD-1 (Buchbinder & Desai 2016). Previous studies observed that intact κ-CAS and GMP significantly inhibited the mitogen-induced proliferative response of mouse spleen lymphocytes and Peyer's patch cells (Otani & Hata 1995; Otani *et al.,* 1995). Our results would suggest that exposure to κ-CAS interferes with the ability of macrophages to prime naïve T-cells towards a pro-inflammatory immune response given the lack of IL-2 required for the induction of a robust adaptive response (Bachmann & Oxenius 2007).

In summary, this study demonstrated that κ-CAS induces a suppressive M2-like phenotype in macrophages, which are hypo-responsive to TLR induced cytokine production, via the abrogation of the NFκB pathway. This may in part be due to the upregulation of two prominent negative regulators of the TLR pathway; SOCS3 and SOCS3. These suppressive M2-like macrophages prime T-cell to induce significantly less T_H1 associated pro-inflammatory cytokines and IL-2, sequestering the ability of these cells to elicit an adaptive immune response. Given the powerful immune-modulatory effects exhibited by κ-CAS on macrophages, key cells involved in the initiation and control of inflammation (Mantovani *et*

al., 2013), further study may lead to the development of κ-CAS as a novel immune therapeutic for the treatment of inflammatory diseases.

Chapter 5 – κ-CAS suppress DC maturation and T-cell priming capacity

5.1 Introduction

In the previous chapter we presented evidence of how κ-CAS primed macrophages acquired an alternatively activated M2-like suppressive phenotype, which were hypo-responsive to the release of inflammatory cytokines in response to TLR stimulation and suppressed T_H1 associated $CD4^+$ T-cell cytokine responses *in-vitro*. Dendritic cells (DCs) like macrophages are antigen presenting cells (APCs), but are considered more critical to the mediation between innate and adaptive immunity, often termed as professional APCs. While macrophages present antigen *in-situ*, upon antigen uptake, DCs become activated, mature and migrate to the lymph nodes to present antigens to the T-cells resulting in the systematic initiation of an adaptive immune response. Thus a bioactive nutraceutical with immuno-modulatory properties which affects DCs would of great interest due to their prominent role in both innate and adaptive immunity.

DCs are a heterogeneous population of phagocytes distributed throughout the body. DCs present in tissues and mucosal sites survey for pathogen or danger associated molecular patterns using an array of recognition receptors such as the toll-like receptor (TLRs), C-type lectin receptors, NOD-like receptors (NLRs), and others (Pulendran & Maddur 2015). These receptors not only enable DCs to sense and respond to external pathogens and environmental antigens but also internal danger signals and molecules generated during tissue injury (Bianchi 2007). Depending on the stimulus, DCs can produce a variety of anti/pro-inflammatory signaling molecules including; cytokines, chemokines and cell signaling receptors (Patil *et al.*, 2010; Thaiss *et al.*, 2011). DCs are well established as the most prominent APCs (Banchereau & Steinman 1998) due their expression of higher levels of the co-stimulatory molecules; CD80, CD86, MHC II and CD40, required for efficient

antigen presentation upon activation (Steinman *et al.,* 1999; Dudek *et al.,* 2013). Thus, DCs are considered as highly effective cells at both initiating and propagating inflammatory responses (Iwasaki & Medzhitov 2010).

In a steady state, DCs have been shown to be less responsive to inflammatory stimuli and produce anti-inflammatory mediators, thought to responsible for the promotion and maintenance of regulatory T-cell populations required for homeostasis (Scott *et al.,* 2011). However, abnormal inflammatory responses can alter their function into cells that promote and propagate inflammation. DC migration and acumination is observed in chronic intestinal inflammatory diseases (Silva 2009). These DCs have been shown to be hyper-responsive to TLR stimulation, secrete high levels of pro-inflammatory cytokines like TNF-α, IL-23 and IL-12 which are crucial for the induction of T_H1/T_H17 inflammatory T-cell responses observed in intestinal inflammatory diseases (IBD) (Baumgart *et al.,* 2009; Sakuraba *et al.,* 2009; Zaba *et al.,* 2009; Yawalkar *et al.,* 2009). In experimental mouse models of IBD, DCs have been demonstrated to secrete high levels of TNF-α which have been shown to increase epithelial barrier permeability, resulting in inflammation and injury similar to that observed in ulcerative colitis (Garrett *et al.,* 2007).

Given the prominent role DCs play in gastrointestinal inflammatory conditions and as innate professional APCs, which are good sources of IL-12p70, a cytokine critical in driving T_H1 adaptive immune responses (Lichtenegger *et al.,* 2012); a complete analysis of the DC phenotype induced by a κ-CAS was performed. A limited number of studies have previously examined the impact κ-CAS had on DC function and activation status. Mikkelsen *et al.,* demonstrated that κ-CAS reduced the capacity of DCs to produce the pro-inflammatory cytokines; IL-1β, IL-10, IL-12 and TNF-α upon LPS stimulation. GMP; the hydrolysed derivative of κ-CAS was also shown to reduce LPS induced TNF-α and IL-1β

production from DCs (Mikkelsen *et al.*, 2005). Herein we investigate the ability of κ-CAS to modulate DC maturation and function and what impact κ-CAS treated DCs have in driving the adaptive immune responses.

5.2 Experimental design

Given the immunomodulatory effects κ-CAS exerted on TLR mediated responses in macrophages (Chapter 4), and studies previously demonstrating the capacity of κ-CAS to suppress LPS mediated cytokine responses in DCs (Mikkelsen *et al.,* 2005), we firstly compared the ability of κ-CAS to modulate cytokine production in DCs stimulated with a panel of TLR ligands. Mouse BMDCs were pre-incubated with κ-CAS (1 mg/mL) for 2.5 hr prior to the addition of LPS (100 ng/mL) for 18 hr. The effects of increasing concentration (0.1 – 2 mg/mL), multiple time points (3, 6 & 18 hr) and time of exposure (κ-CAS treatment 2.5 hr prior, at the same time or 2.5 hr post LPS stimulation) were also assessed. Cells were also treated as outlined previously, but stimulated with a TLR2 ligand; PGN (5 μg/mL), a TLR7 ligand; LOX (0.5 mM), TLR9 ligand; CpG (2 μM) or PMA (20 ng/mL) to investigate the κ-CAS exerted on other TLR and NFκB signalling pathways.

Exposure of DCs to PAMPs such as LPS activates the NFκB transcription factor, which influences the maturation and subsequent inflammatory response of DCs (Rescigno *et al.,* 1998). To examine what effect κ-CAS exhibited on NFκB signalling, BMDCs were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr prior to stimulation with LPS (100ng/ml). Control BMDCs were treated with PBS or κ-CAS or LPS alone. Cells were harvested after 15 min LPS stimulation, and IκB-α protein levels was determined in whole-cell lysates by western blot analysis.

To further characterise the effects κ-CAS exerted on DC maturation and to elucidate the signaling mechanisms involved, cells were treated with κ-CAS (1 mg/mL) for 18 hr. Following stimulation cells were washed and stained with fluorochrome-labelled monoclonal antibodies to the surface markers and analysed by flow cytometry. For the measurement of SOCS1 and SOCS3 gene expression, cells were treated with κ-CAS (1

mg/mL) over a time course, total RNA was extracted and after reverse transcription, the cDNA was analysed by qPCR. RNA expression was normalized to GAPDH control gene expression and showed relative to PBS control.

Lastly, the effect of κ-CAS on DC function was characterised by its ability to alter their T-cell priming capacity *in-vitro* and *in-vivo*. BMDCs were treated with κ-CAS (1 mg/mL) & OVA peptide (100 nM) for 18 hr. After treatments, cells were washed with sterile PBS. For *in-vitro* T-cell priming, treated BMDCs were co-cultured at a 1:10 ratio with $CD4^+$ T-cells isolated from the spleen of B6.Cg-Tg(TcraTcrb)425Cbn/J (OTII) mice in wells pre-coated with anti-CD3 (1 μg/mL). After 72 hr, supernatants were collected and analysed for cytokine secretion by ELISA. For *in-vivo* T-cell priming, 3×10^5 treated BMDCs were delivered over the sternum of OTII mice by subcutaneous injection. Mice were culled after 7 days by cervical dislocation. Skin draining lymph nodes were extracted and single cell suspensions of cells were obtained by passage of the lymph nodes through a 40 μm filter using the plunger from a sterile 1 mL syringe. Draining lymph nodes cells were counted and seeded at 1×10^6 cell/mL and stimulated with PBS, OVA (500 nM) or with PMA (25 ng/ml) in wells pre-coated with anti-CD3 (1 μg/ml). After 72 hr, supernatants were collected and analysed for cytokine secretion by ELISA.

5.3 Results

5.3.1 κ-CAS suppresses LPS induced pro-inflammatory but not anti-inflammatory cytokines in DCs.

Having previously demonstrated that κ-CAS significantly suppressed the production of TNF-α and IL-10 in LPS stimulated BMMΦ, we sought to assess if κ-CAS affected cytokine production in other innate cell types, specifically BMDCs. BMDCs derived from C57BL/6 mice were incubated with κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation in the presence of absence of LPS (100 ng/mL) for 3 (Figure 5.1A) or 18 hr (Figure 5.1B). BMDCs treated with PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α, IL-10 and IL-12p70. No significant differences in the levels of TNF-α, IL-10 or IL-12p70 were observed for negative controls at all time points (data not shown).

κ-CAS was observed to significantly suppress 59.75 ± 3.36% of the secretion of TNF-α 3 hr post LPS stimulation (Figure 5.1A ** , $p \leq 0.01$). Moreover, a non significant increase of 27.34 ± 11.72 % in LPS induced IL-10 was detected between κ-CAS and PBS treated BMDCs (Figure 5.1B). The levels of IL-12p70 at 3 hr were below the detectable range for all conditions (data not shown). Similarly to earlier time points, κ-CAS was shown to significantly attenuate 71.72 ± 6.17 % of TNF-α production (Figure 5.1C ** , $p \leq 0.01$) and non significantly increased the sectretion IL-10 by 34.15 ± 21.85 % (Figure 5.1D) 18 hr post LPS stimulation. κ-CAS was also shown to significantly suppress 66.07 ± 2.83 % of LPS induced IL-12p70 production (Figure 5.1E ** , $p \leq 0.01$) at this later time point. This would suggest that κ-CAS selectively exerts inhibitory effects on pro-inflammatory cytokine production at early and late time points, while simultaneously increasing the production of IL-10.

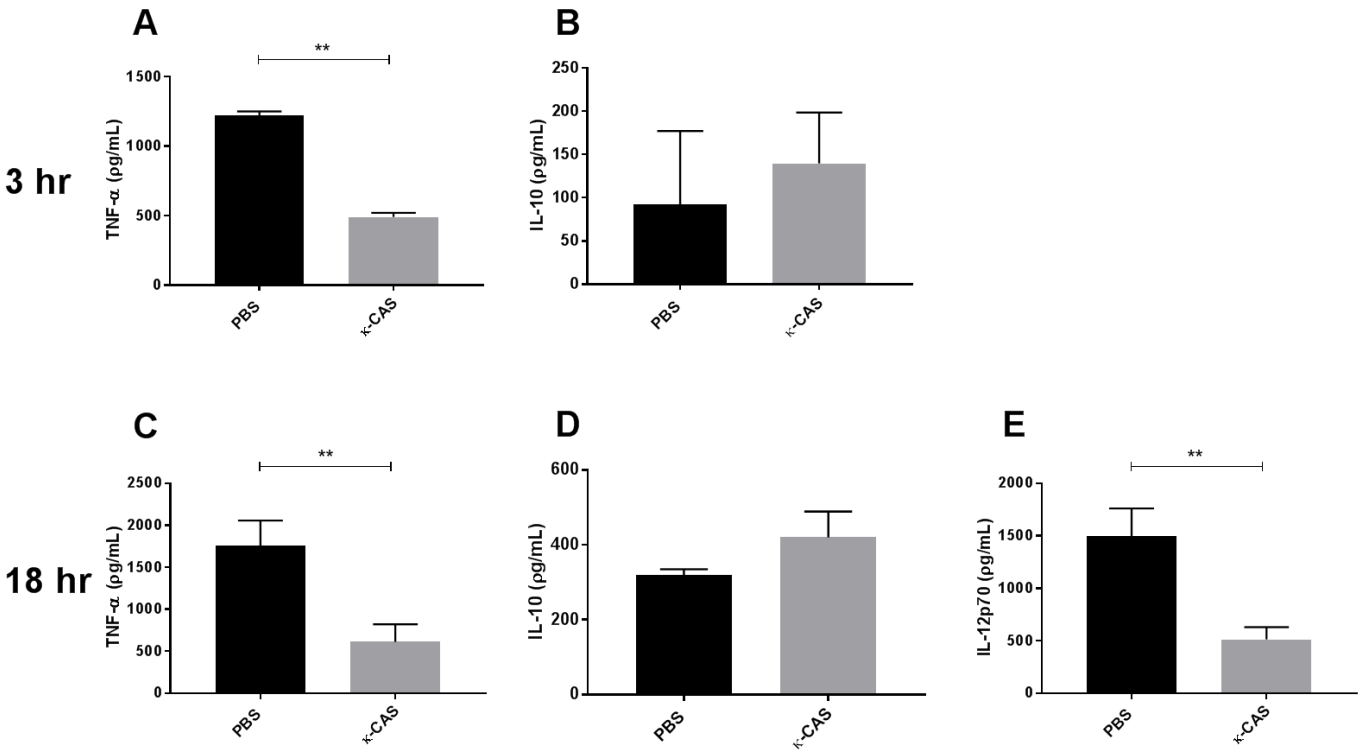

Figure 5.1 Effects of κ-CAS on LPS induced cytokine secretion in DCs. BMDC were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr. Following pre-treatment, cells were stimulated in the presence or absence of LPS (100 ng/mL) for 3 hr (A, B), or 18 hr (C, D, E). Supernatants were analysed for the secretion of the cytokines TNF-α (A, C), IL-10 (B, D) or IL-12p70 (E) by ELISA. Results are expressed as mean ±SD of at least 3 independent experiments in triplicate. P-values were calculated using two-tailed student's t-test. ** , $p \leq 0.01$ compared to PBS control group.

5.3.2 κ-CAS suppresses TNF-α & IL-12p70 production post and prior to LPS stimulation in BMDCs.

To investigate what impact the time of exposure DCs had to κ-CAS on the suppression of cytokines, BMDCs were treated with κ-CAS (1 mg/mL) for 2.5 hr prior (-2.5 hr), simultaneously as (0 hr), or 2.5 hr after (+2.5 hr) stimulation with LPS (100 ng/mL). BMDCs treated with PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. Cells were incubated for 18 hr from time of LPS stimulation and the supernatants were analysed for the secretion of TNF-α and IL-10 by ELISA. No significant differences in the levels of TNF-α or IL-12p70 were observed for negative controls (data not shown).

κ-CAS significantly suppressed the production of TNF-α (Figure 5.2A ** , $p \leq 0.01$) and IL-10 (Figure 5.2B ** , $p \leq 0.01$) in LPS stimulated BMDCs when added prior to (-2.5 hr) LPS stimulation as shown previously. However, BMDCs were equally as attenuated when treated with κ-CAS simultaneously (0hr) or after (+2.5 hrs) LPS stimulation. No significant differences in suppression were detected between exposure times. Therefore, this data would suggest that κ-CAS can exert it effects without pre-stimulation and does not compete with LPS for binding to TLR4.

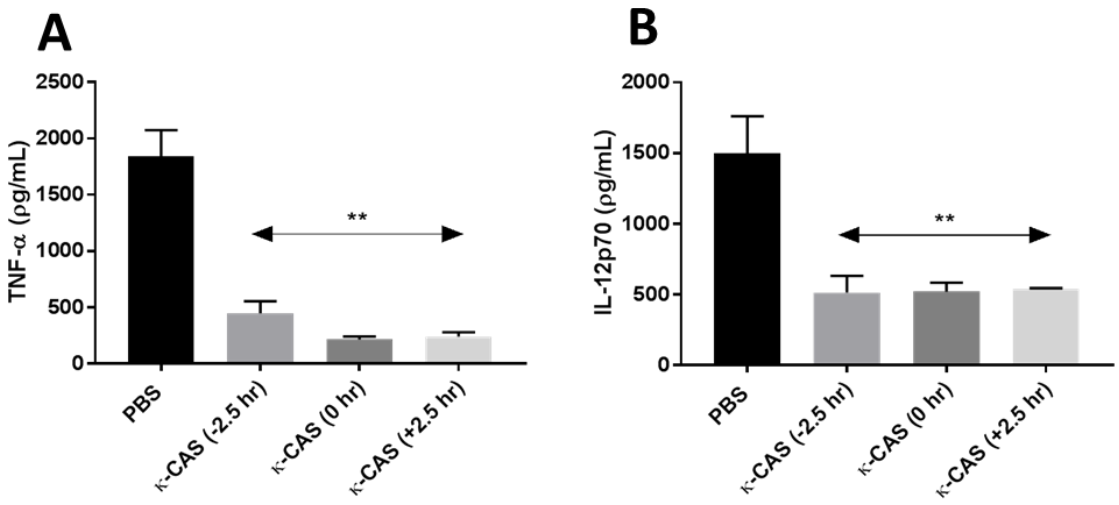

Figure 5.2 κ-CAS suppresses TNF-α & IL-12p70 secretion prior to and post LPS activation. BMDCs were treated with κ-CAS (1 mg/mL) 2.5 hours before, same time as, or 2.5 after hours after LPS stimulation. Supernatants were analysed for the secretion of the cytokines TNF-α (A) and IL-12p70 (B) by ELISA. Results are expressed as mean ±SD of at least 3 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons test. ** , $p \leq 0.01$ compared to PBS control group.

5.3.3 κ-CAS suppresses pro-inflammatory cytokine release by DCs in multiple TLR pathways.

TLR activation leads to recruitment of a cascade of accessory proteins which activate the NFκB, AP-1 & IRF pathways, ultimately stimulating the transcription of inflammatory cytokines (Liu *et al.,* 2017, Barton & Kagan 2009). Given that κ-CAS was observed to significantly supress TLR4 mediated responses in DCs, we next the investigated if other TLRs were also affected. For these experiments we chose agonists of TLR 2, 7 and 9, to broadly represent the TLR groups.

BMDCs derived from C57BL/6 mice were pre-incubated with κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with or without TLR ligands for 18 hr. Cells treated with PBS

(negative), κ-CAS (negative) or TLR ligands (positive) alone were used as controls. The supernatants were analysed for the secretion of TNF-α (Figure 5.3A) and IL-12p70 (Figure 5.3B). PGN (5 μg/mL), a major surface component of gram-positive bacteria was used as an agonist of TLR2. LOX (0.5 mM), a guanosine analog, was used to activate TLR7. Synthetic oligonucleotide CpG (2 μM) that contains unmethylated CpG dinucleotides was used as the TLR9 agonist. No significant differences in basal TNF-α or IL-12p70 were detected between PBS and κ-CAS treatments alone (data not shown).

κ-CAS was observed to significantly suppress the secretion of TNF-α (Figure 5.3A ** , $p \leq 0.01$) in response to all TLR agonists. Moreover, κ-CAS also significantly suppressed TLR7 induced IL-12p70 (Figure 5.3A ** , $p \leq 0.01$), but failed to have the same effect against TLR 2 and TLR9 agonists. However, the levels of TLR2 and TLR9 induced IL-12p70 were decreased by κ-CAS. Thus we determined that κ-CAS abrogates multiple TLR signaling pathways in DCs.

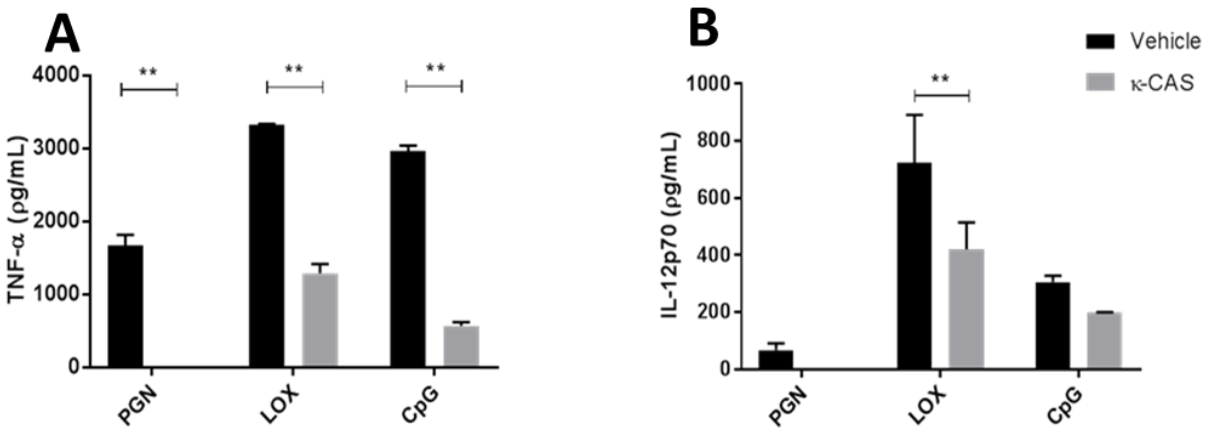

Figure 5.3 κ-CAS targets multiple TLRs in DCs. BMMφ fwere pre-treated κ-CAS (1 mg/mL) at for 2.5 hr. Following pre-treatment, cells were stimulated in the presence or absence of PGN (5 μg/mL), LOX(0.5 mM) or CpG (2 μM) for 18 hr. Supernatants were analysed for the secretion of the cytokines TNF-α (A) & IL-10 (B) by ELISA. Results are expressed as mean ±SD of 2 independent experiments in triplicate. P-values were calculated using ANOVA multiple comparisons tests. ** , $p \leq 0.01$ compared to TLR ligand alone stimulated control group.

5.3.4 κ-CAS suppresses PMA induced TNF-α release in DCs.

Having demonstrated that κ-CAS suppressed TLR induced pro-inflammatory cytokine secretions in DCs, we next examined if κ-CAS also abrogated the production of pro-inflammatory cytokines from a non-TLR agonist. Phorbol 12-myristate 13-acetate (PMA) is a chemical compound known to induce the activation of protein kinase C (PKC) signaling. PMA-PKC signaling is implicated in the regulation of NFκB activity by mediating the IKK via the activation of MAPKs (Holden *et al.,* 2008; Sun & Yang 2010). Similar to TLR signaling, the activation of PKC by PMA in DCs has been shown to activate NFκB, resulting in the production of the pro-inflammatory cytokine TNF-α (Song *et al.,* 2015).

BMDCs derived from C57BL/6 mice were pre-cultured with PBS or κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with PMA (20 ng/mL) for 18 hr. The supernatants were analysed for the secretion of TNF-α and IL-12p70. κ-CAS was observed to significantly suppress the secretion of TNF-α (Figure 5.4 * , $p \leq 0.05$) in response PMA. The levels of IL-12p70 induced by PMA were below the detectable range. Thus we determined that κ-CAS also abrogates non-TLR induced TNF-α cytokine signaling in DCs.

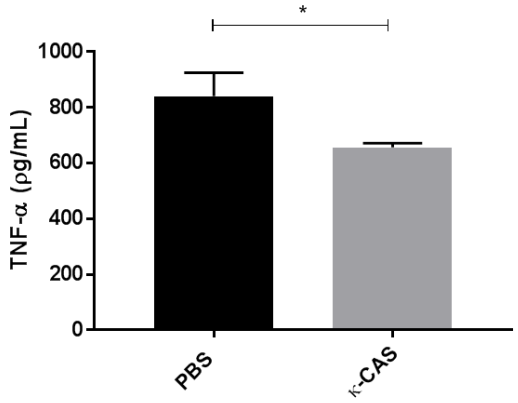

Figure 5.4 Effects of κ-CAS on PMA induced TNF-α secretion in DCs. BMDC were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr. PBS treated cells were used a control. Following pre-treatment, cells were stimulated with PMA (20 ng/mL) for 18 hr. Supernatants were analysed for the secretion of TNF-α by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using two-tailed student's t-test. * , $p \leq 0.05$ compared to PBS control group.

5.3.5 κ-CAS attenuates NFκB activation in DCs.

Having observed the suppressive effects exhibited by κ-CAS on the induction of both TLR and PMA induced cytokine responses; we sought to investigate if NFκB signaling was involved in this phenomenon by examining the degredation/if any of IκB inhibitory

proteins. BMDCs derived from C57BL/6 mice were pre-incubated with κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with or without LPS (100 ng/mL) for 15 min. PBS (negative), κ-CAS (negative) or LPS (positive) alone were used as controls. The western blots revealed that PBS (Fig. 5.5A lanes 1, 2, 3) and κ-CAS (Fig. 5.5A lanes 4, 5, 6) alone showed no significant differences in intensity, indicating that no degradation of the IκB-α protein had occurred when measured by densitometric analysis (Fig. 5.5B). After 15 min LPS treatment (Fig. 5.4A lanes & 7, 8, 9) a significant level of IκB-α degradation was observed compared to PBS & κ-CAS treatments alone (Figure 5.5B * , $p \leq 0.05$). However, DCs pre-treated prior to LPS with κ-CAS (Fig. 5.5A, lanes 10, 11, 12) no longer showed significant reductions in the levers of IκB-α protein compared to both non-LPS treated controls (Fig. 5.4B). This data infers that κ-CAS may partially abrogate NFκB signaling.

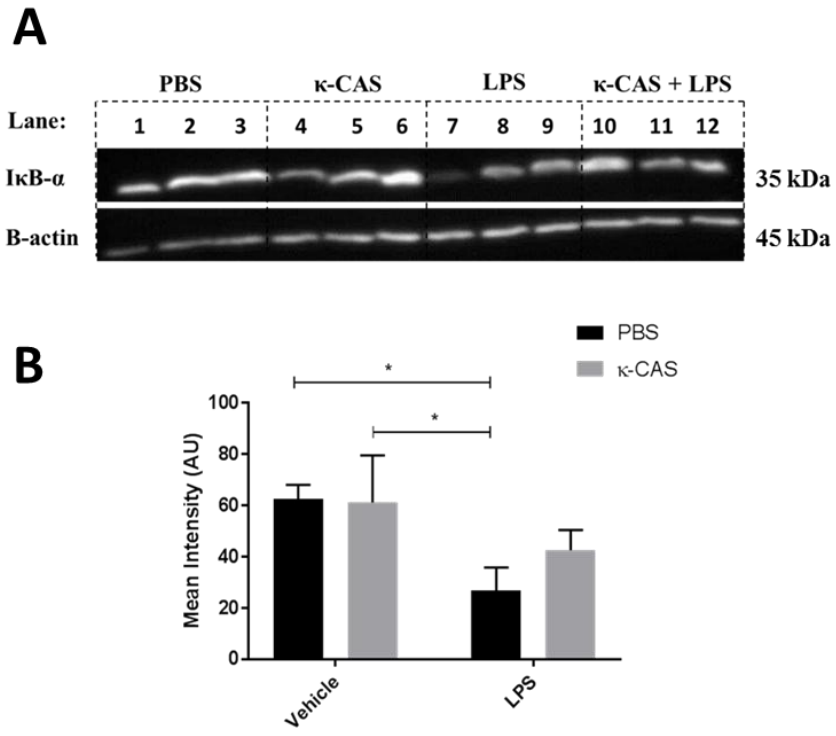

Figure 5.5 κ-CAS suppresses LPS mediated IκB-α degradation in DCs. BMDCs were pre-treated with κ-CAS (1 mg/mL) for 2.5 hr prior to stimulation with LPS (100ng/ml). (A) DCs were

harvested 15 min post LPS challenge, and the protein levels of IκB-α were determined in whole-cell lysates by western blot analysis. The cells were treated with PBS (1, 2, 3), κ-CAS (4, 5, 6), LPS (7, 8, 9) or κ-CAS and LPS (10, 11, 12). The protein levels of β-actin were used as a housekeeping control. (B) Densitometric analysis was performed and IκB-α protein levels were expressed in arbitrary units. Results are expressed as mean intensity ±SD of the 3 experiments. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$ compared to PBS or κ-CAS vehicle control group.

5.3.6 κ-CAS up-regulates the transcription *SOCS1* & *SOCS3*.

Having shown the suppressive effects κ-CAS exerts on NFκB activation and pro-inflammatory cytokine responses, we sought to investigate if κ-CAS induced *SOCS1* or *SOCS3*, prominent negative regulators of NFκB and cytokine signaling in DCs (Kubo *et al.*, 2003). BMDCs derived from C57BL/6 mice were incubated with κ-CAS (1 mg/mL) for indicated times. PBS treated BMDCs were used a negative as controls. κ-CAS significantly enhanced the expression of *SOCS1* (Figure 4.7A **, $p \leq 0.01$) and *SOCS3* (Figure 4.7A *, $p \leq 0.05$; ** , $p \leq 0.01$) after 6 hr exposure. However, while the fold increase in SOCS expression remained significantly upregulated after 2 hr, there was a decrease to baseline levels of expression for both genes after 6 hr.

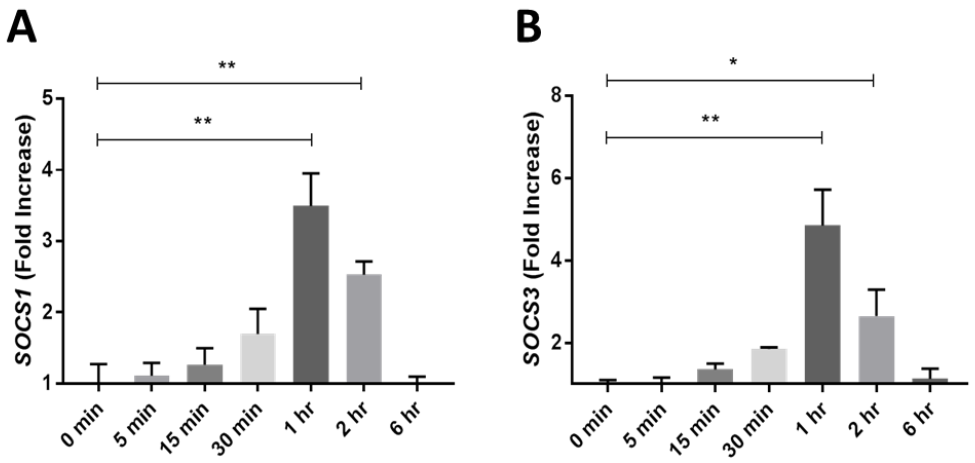

Figure 5.6 κ-CAS upregulates the expression of *SOCS1* & *SOCS3* in DCs. BMDCs were incubated with and without κ-CAS (1 mg/mL) for the indicated times. Total RNA was extracted and after reverse transcription, the cDNA was analysed by qPCR for *SOCS1* (A) & *SOCS3* (B). RNA expression was normalized to GAPDH control gene expression and shown as mean fold increase ±SD relative to PBS control. Figures are representative of at least 2 independent experiments in dublicate. P-values were calculated using ANOVA multiple comparisons test. *, $p \leq 0.05$; ** , $p \leq 0.01$ compared to PBS stimulated control.

5.3.7 κ-CAS downregulates activation markers, adhesion and co-stimulatory receptors on DCs.

Having previously demonstrated that κ-CAS induced the expression of activation and co-stimulatory markers in Mφ (Chapter 4), we investigated if this phenomena was also observed in DCs. BMDCs derived from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for 18 hr. PBS was used as an un-activated control. Cells were subsequently washed and stained with fluorochrome-labelled monoclonal antibodies to the surface markers and analysed by flow cytometry.

BMDCs stimulation with κ-CAS resulted in a significant decrease in the expression of the adhesion receptors CD54 (Figure 5.7 A* , $p \leq 0.05$) and CD209 (Figure 5.7 B* , $p \leq 0.05$).

κ-CAS was also shown to significantly reduced the expression of the co-stimulatory receptors CD80 (Figure 5.7 C* , $p \leq 0.05$) and OX40L (Figure 5.7 D ** , $p \leq 0.01$) However, no significant differences were observed for the expression of the co-stimulatory markers; CD86, CD40 and MHCII (data not shown).

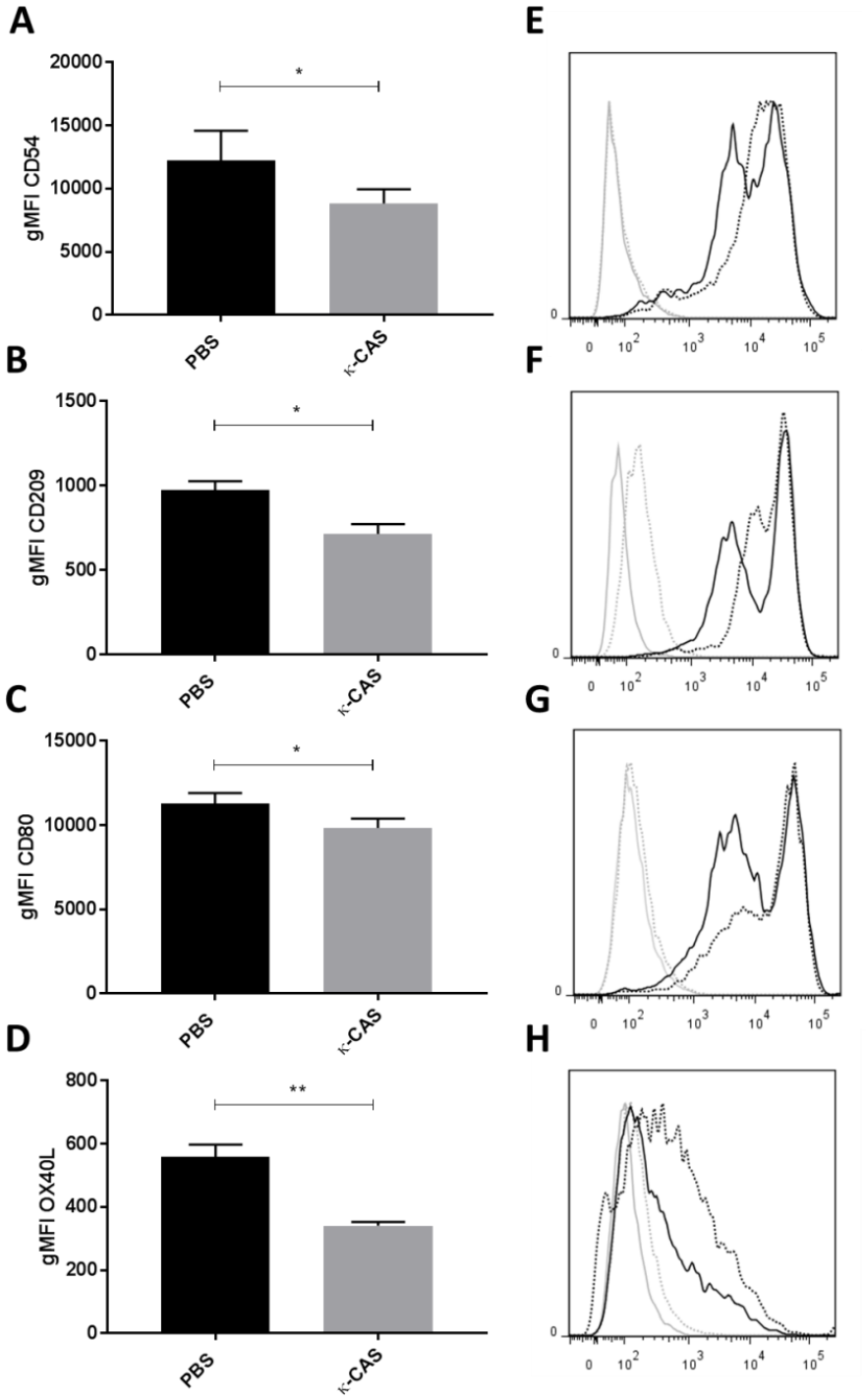

Figure 5.7 κ-CAS downregulates co-stimulatory & adhesion receptors. BMDCs were pre-treated with κ-CAS (1 mg/mL) for 18 hr. PBS was used as a negative control. Subsequently, cells were washed and stained for 30 min with specific antibodies or with an isotype matched control and analysed by flow cytometry (FACSAria, BD, UK). Results were analysed using FlowJo software (Treestar, USA) and are expressed as the gMFI ±SD of at least 3 independent experiments (A-D). Representative histograms show the surface expression of CD54 (E), CD209 (F), CD80 (G) and OX40L (H); unstained (gray line), isotype (dotted grey line), PBS (black dotted line) and κ-CAS (black line). P-values were calculated using two-tailed student's t-test. ** , $p \leq 0.01$ * , $p \leq 0.05$ compared to PBS control.

5.3.8 κ-CAS alters the ability of DCs to prime T-cell responses *in-vitro & in-vivo.*

Considering κ-CAS significantly suppressed cytokine production, activation, adhesion and costimulatory receptor expression, its effects on the ability of DCs to prime OVA specific T-cell responses in transgenic OVA sensitised mice was assessed. BMDCs derived from C57BL/6-Tg(TcraTcrb)425Cbn/Crl (OTII) mice were treated with OVA peptide (100 nM) in the presence or absence of κ-CAS (1 mg/mL) for 18 hr.

For *in-vitro* studies, DCs were subsequently washed and co-cultured with $CD4^+$ T-cells from naïve OTII mice at a 1:10 ratio in 96 well plates pre coated with anti-CD3 (1 μg/mL) for 72 hr. PBS primed DCs induced IFN-γ, IL-13, and IL-2 production from $CD4^+$ T-cells *in-vitro* (Figure 5.8). BMDCs primed with κ-CAS significantly suppressed OVA-specific IL-2 production *in-vitro* in a concentration dependent manner (0.1 mg/mL – 1 mg/mL) (Figure 5.8C ** , $p \leq 0.01$ * , $p \leq 0.05$) compared to control DCs primed with PBS. Moreover, κ-CAS treated DCs significantly increased the secretion of IL-13 from $CD4^+$ T-cells (Figure 5.8B ** , $p \leq 0.01$) at only higher concentrations (1 mg/mL). However significant no differences in the levels of IFN-γ (Figure 5.8A) were detected from *in-vitro* co-cultures.

For *in-vivo* experiments, DCs were subcutaneously injected over the sternum of naïve OTII mice. After 7 days, draining skin lymph nodes were removed for re-stimulation *ex-vivo* with PBS, OVA (500 nM) or with PMA (20 ng/mL) and anti-CD3 (1 μg/mL). After 72 hr, T-cell priming was assessed via measuring supernatants for the cytokines IFN-γ, IL-13, IL-10 and IL-2. PBS primed DCs induced IFN-γ, IL-13, IL-10 and IL-2 production from $CD4^+$ T-cells *in-vivo* from skin draining lymph node cells in response to OVA stimulation, although the levels were low in comparison to PMA and anti-CD3 stimulated cells (Figure 5.9). BMDCs primed with κ-CAS significantly suppressed OVA-specific IL-2 production (Figure 5.9D *, $p \leq 0.05$) compared to control DCs primed with PBS. In contrast to *in-vitro* results, skin draining lymph node cells from κ-CAS treated DC recipient mice exhibited reduced levels of IFN-γ (Figure 5.9A), IL-13 (Figure 5.9B), and IL-2 (Figure 5.9C), however these reductions were not deemed significant. Interestingly, these cells stimulated with PMA and anti-CD3 produced significantly less IFN-γ (Figure 5.9A **, $p \leq 0.01$) and IL-13 (Figure 5.9B **, $p \leq 0.01$) compared to cells from PBS treated control DC recipient mice.

This data would indicate that while there were differences between *in-vitro* and *in-vivo* T-cell priming assays, κ-CAS suppressed DC mediated OVA specific T-cell responses, by abrogating the production of IL-2, a cytokine essential for propagation of adaptive immune responses, but also had a general non-specific suppressive effect on T-cell responses as well.

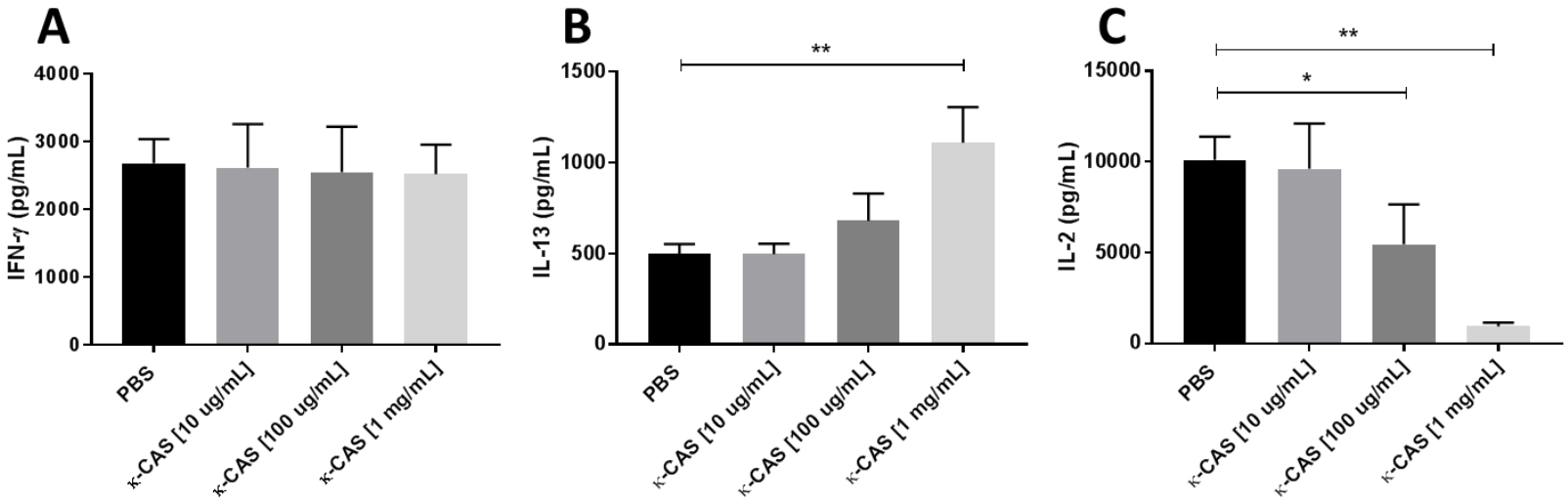

Figure 5.8 κ-CAS alters the ability of DCs to prime T-cell responses *in-vitro*. BMDCs from OTII mice were cultured with OVA (100 nM) in the presence of PBS or indicated concentrations of κ-CAS overnight. Cells were subsequently washed and co-cultured with $CD4^+$ T-cells from naïve OTII mice at a 1:10 ratio in 96 well plates pre coated with anti-CD3 (1 μg/mL). After 72 hr supernatants were analyzed for the cytokines; IFN-γ (A), IL-13 (B), and IL-2 (C) by ELISA. Results are expressed as mean ±SD of three individual wells from three individual mice. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$ ** , $p \leq 0.01$ compared to PBS control.

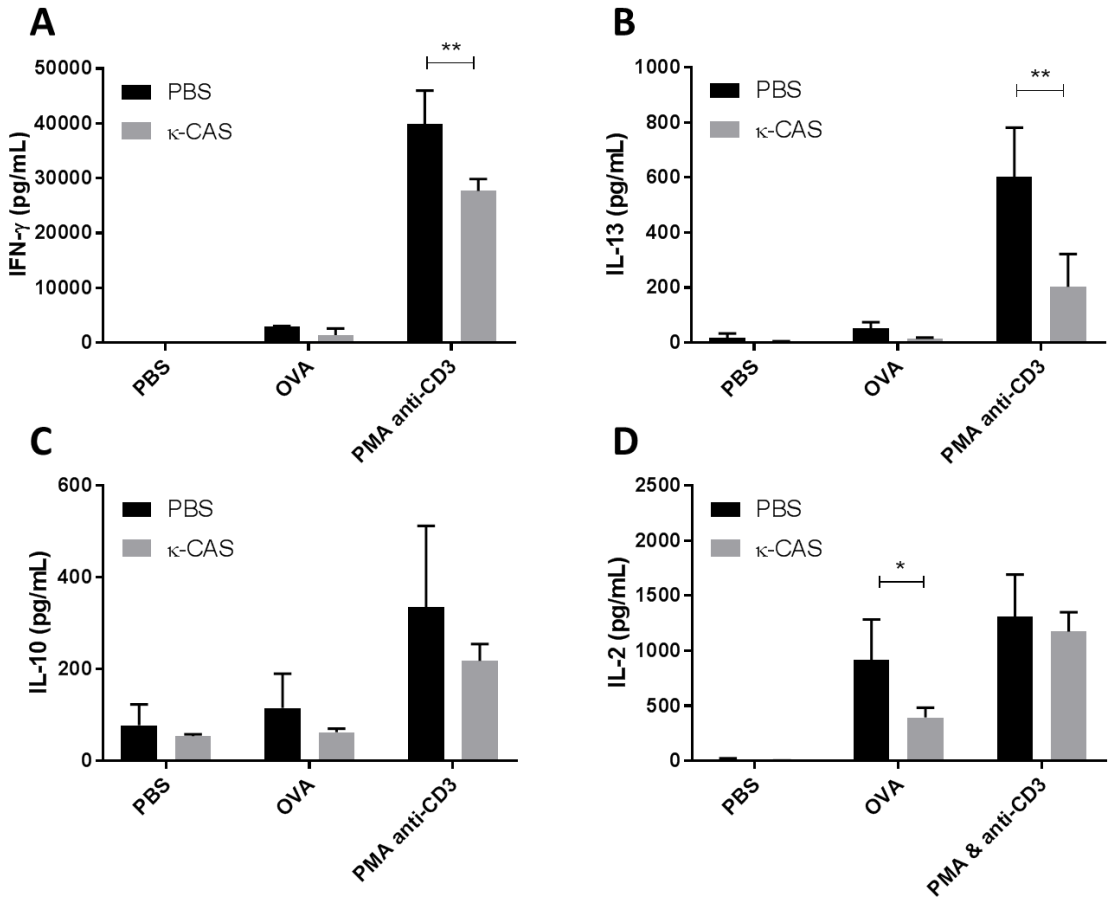

Figure 5.9 κ-CAS alters the ability of DCs to prime T-cell responses *in-vivo*. BMDCs from OTII mice were cultured with OVA (100 nM) in the presence of PBS or κ-CAS (1 mg/mL) overnight. Cells were subsequently washed and subcutaneously injected over the sternum of naïve OTII mice. After 7 days, draining skin lymph nodes were removed for re-stimulation *in-vitro* with PBS, OVA (500 nM) or with PMA (20 ng/mL) and anti-CD3 (1 μg/mL). After 72 hr, supernatants were analyzed for the cytokines; IFN-γ (A), IL-13 (B), IL-10 (C) and IL-2 (D) by ELISA. Results are expressed as mean ±SD of three individual wells from three individual mice. P-values were calculated using ANOVA multiple comparisons tests. * , $p \leq 0.05$ ** , $p \leq 0.01$ compared to PBS treated group.

5.4 Discussion

While a limited number of previous studies have presented evidence on the suppressive potential of κ-CAS and GMP (a κ-CAS derived bioactive peptide) on LPS mediated cytokine release from DCs (Mikkelsen *et al.,* 2005), there is a dearth of research examining the ability of κ-CAS to modulate DC maturation, and function and what impact κ-CAS treated DCs have in driving the adaptive immune responses.

In this study the effects of κ-CAS on DCs responsiveness to the maturation inducer and pro-inflammatory stimulant; LPS was examined. κ-CAS was shown to selectively reduce the secretions of the pro- inflammatory cytokines; TNF-α and IL-12p70 at all time points similar to the observations of previous studies (Mikkelsen *et al.,* 2005). κ-CAS also rendered DCs hypo-responsive to a panel of other TLR ligands with significant decreases in pro-inflammatory cytokine production. In contrast, we demonstrated that cytokine suppression by κ-CAS was selective, given that no significant decrease in the production of IL-10 was observed, a cytokine traditionally associated with an anti-inflammatory or regulatory response (Moore *et al.,* 2001). The discrepancy may be accounted for by differences in the concentrations of κ-CAS used. We had previously demonstrated (chapter 4) that lower concentrations of κ-CAS (0.1 mg/mL), similar to that used by Mikkelsen *et al.,* suppressed IL-10 production in BMMφ (Figure 4.3F). κ-CAS treatment was also shown to significantly suppress the production of TNF-α from PMA; a non TLR stimulant that activates and matures DCs via the upregulation of protein-kinase C (PKC) signaling (Cejas *et al.,* 2005; Lindner *et al.,* 2007; Song *et al.,* 2015).

The timing of exposure of DCs to κ-CAS did not affect the suppressive response to TLR activation which would indicate that κ-CAS does not require pre-stimulation and therefore

does not block TLR ligands from binding, but rather attenuates inflammatory signalling pathways internally.

NFκB activation is a crucial signaling pathway involved in the induction of pro-inflammatory genes (Liu *et al.,* 2017; Lawrence 2009). TLRs and PMA stimulation of DCs has been shown to activate through this pathway, inducting the production of pro-inflammatory cytokines like TNF-α (Song *et al.,* 2015; Sakai *et al.,* 2017). We demonstrated that the LPS mediated activation of NFκB in DC was attenuated by κ-CAS. This suppressive effect exerted by κ-CAS on LPS mediated degradation of the NFκB inhibitor protein; IκBα could explain the observed decrease in pro-inflammatory cytokines demonstrated in this study. Previous studies have also demonstrated that IκBα blockage disables LPS induced maturation of DCs (Rescigno *et al.,* 1998).

SOCS proteins have emerged as critical modulators of cytokine-mediated processes. κ-CAS also significantly induced SOCS1 and SOCS3, two prominent negative regulators of the TLR pathway. Both SOCS proteins bind and inhibit the janus kinases (JAKs); intracellular, non-receptor tyrosine kinases that signals via the JAK-STAT pathway critical to the initiation of NFκB signalling (Banerjee *et al*., 2017). SOCS3 is a prominent inhibitory protein, normally induced in response to inflammatory stimuli in a negative feedback loop that is essential in the resolution of inflammation (Yoshimura *et al.,* 2012). The upregulation of SOCS1 has been shown to restrict pro-inflammatory IL-12 production & signaling in DCs (Evel-Kabler *et al.,* 2005).

Due to their central role in initiating adaptive immune responses the effect of κ-CAS on DCs priming capacity was also examined. This was confirmed in the T-cell priming studies, which demonstrated that κ-CAS treated DCs significantly suppressed $CD4^+$ T-cell

induced IL-2 both in *in-vitro* and *in-vivo* models, however did not display a prominent extracellular surface marker associated with anergy; CTLA4 (Buchbinder & Desai 2016). DCs T-cell priming capability is dependent on their maturation state. In order for this process to occur, the antigen-MHC complexes (signal 1) and co-stimulatory receptors (signal 2) provided by DCs must engage with their complimentary T-cell counterparts. These initial signals determine the magnitude and fate of an antigen-specific T-cell response. Moreover, cytokines (signal 3) are thought to be required for optimal T-cell activation, differentiation and clonal expansion (Curtsinger *et al.,* 1999; Curtsinger & Mescher 2010). However, if any of these signals are suppressed, T-cell activation, production of IL-2, and subsequently proliferation can be hampered (Banchereau & Steinman 1998). We demonstrated that the co-stimulatory molecules; CD80 and activation/adhesion receptors; CD54 & CD209 were downregulated in response to κ-CAS which are important for the successful activation and differentiation of naïve $CD4^+$ T-cells. CD80 is a well know co-stimulatory molecule. CD54 is an extracellular adhesion molecule. While there is no single universally accepted hallmark of APC activation, there is supporting evidence for the use of CD54 upregulation as a marker for assessing APC activation (Sheikh & Jones 2008). CD54 has also been shown to be implicated in the promotion of lymphocyte adhesion (Van Seventer et al., 1990) and its high expression on DCs is correlated with its antigen-presentation properties (McCarthy *et al.,* 1997). Cell-cell contact between DCs and T-cells is an integral component of antigen-presentation and the involvement of CD54 in this process has been demonstrated by antibody-blocking studies, which show that CD54 is necessary for DC clustering of T-cells (Scheeren *et al.,* 1991), stimulating allogeneic T-cell responses (Xu *et al.,* 1992; Sheikh & Jones 2008) and for the migration of DCs to regional lymph nodes (Ma *et al.,* 1994). Moreover, antibody blocking

of CD54 and LFA-1; an adhesion molecule binding receptor, has been shown to significantly prolong allograft survival in tissue transplantation models (Ozer & Siemionow 2001). CD209 is a C-type lectin receptor highly expressed by DCs involved in the internalization of antigens and DC trafficking (Geijtenbeek *et al.,* 2002). Moreover, it has been implicated in the early interaction between DCs and naive T-cells. CD209 has been demonstrated to bind the extracellular adhesion molecules CD50 and CD102 on T-cells, thereby promoting the adhesion of DCs to naive T-cells (Geijtenbeek *et al.,* 2000). Therefore, the down regulation of these cell-cell contact receptors could hamper T-cell: DC interactions.

SOCS 1 and SOCS 3 which were both upregulated by κ-CAS, have been identified as an important negative regulators of antigen presentation by DCs. SOCS 3-transduced DCs have been demonstrated to exhibit low expression levels of co-stimulatory receptors and immuno-stimulatory cytokines like IL- 12p70 and a decreased ability to induce T-cell proliferation (Li *et al.,* 2006). *Fasciola hepatica* derived tegument proteins has been shown to enhance SOCS3 expression in DCs, which also display a decreased capacity to prime T-cells (Vukman, Adams & O'Neill 2013). Studies have also demonstrated that the silencing of the SOCS1 gene resulted in enhanced antigen presentation by DCs and subsequently the induction of enhanced pro-inflammatory T-cell responses (Shen *et al.,* 2004; Hanada *et al.,* 2005).

These results would suggest that exposure of DCs to κ-CAS renders them hypo-responsive to inflammatory signals, reduces their co-stimulatory and adherent receptor repertoire expression which may interfere with the inflammatory capabilities of DCs. We could also deduce that κ-CAS treated DCs acquire an immature-like phenotype which impairs their function and ultimately their capacity to induce and propagate T-cell responses. Given the

powerful modulatory effect κ-CAS exerts on DCs, a cell type heavily involved in initiation of adaptive immune responses, a greater understanding its exact mechanisms may lead to the development of novel immune-modulatory nutraceutical therapeutic for the treatment of inflammatory diseases, by sequestering the initiation of T-cell responses, a critical process in the propagation of inflammatory responses.

Chapter 6 – κ-CAS activates human monocytes, suppresses their T-cell priming capacity and renders human macrophages hypo-response to TLR stimulation.

6.1 Introduction

Peripheral blood mononuclear cells (PBMC) are nucleated cells in the blood, which consist of 2 major sub-populations including; lymphocytes (T-cells, B-cells and NK-cells) and monocytes. Monocytes are the precursor cells to macrophages and DCs (Geissmann *et al.*, 2010) but also play a pivotal role in the innate immune defense against pathogens via phagocytosis, processing and presentation of antigens and the release of inflammatory effector molecules (Wong *et al.*, 2011). Studies have demonstrated the infiltration of pro-inflammatory monocytes and the accumulation of their macrophage progeny at the sites of inflammation in patients with inflammatory disorders. They are shown to display increased respiratory burst activity and pro-inflammatory cytokine release (Rugtveit *et al.*, 1997; Kamada *et al.*, 2008). Moreover, the aberrant production of TNF-α from macrophages in the inflamed mucosa of inflammatory bowel disease (IBD) patients has been shown to significantly contribute to the disruption of the epithelial barrier function, exacerbating intestinal inflammation (Lissner *et al.*, 2015).

CAS subunits and their hydrolysate derivatives have been shown to exhibit immuno-modulatory properties in human PBMC populations. The treatment of PBMCs with bovine CAS has been previously used in studies that have investigated T-cell proliferative responses to cow's milk protein allergy (CMPA), measured from clinically reactive and tolerised CMPA patients to α-, β- and κ-CAS subunits (Hoffman *et al.*, 1997; Sletten *et al.*, 2007). Moreover, a limited number studies using $CD14^{+}$ monocytes isolated from PBMCs demonstrated that α- CAS, and the κ-CAS derived GMP peptide, activated MAPK and IκB/NFκB signal transduction pathways, inducing monocytes to produce TNF-α, IL-1β, IL-

6 and GM-CSF (Requena *et al.,* 2009; Vordenbäumen *et al.,* 2011); cytokines associated with the propagation of inflammatory and autoimmune disorders (Hamilton 2002; Turner *et al.,* 2014). An enzymatic digest of β-CAS was also shown to act as a selective monocyte/macrophage chemoattractant, inducing the migration and infiltration of monocytes/macrophages (Kitazawa *et al.,* 2007). In contract Aihara *et al.,* demonstrated that a CAS derived peptide fragment abrogated monocyte adhesion; a mechanism required by monocytes to infiltrate to sites of inflammation, via attenuation of the JNK pathway, suggesting a distinct anti-inflammatory effect (Aihara *et al.,* 2009).

In previous chapters we observed the induction of suppressive/immature phenotypes of murine macrophages and dendritic cells (DC) by κ-CAS, which were hypo-responsive to pro-inflammatory stimuli and failed to induce robust T-cell responses. Apart from allergic responses in CMPA, there is a dearth of research on the effects of the whole κ-CAS subunit on PBMCs and herein we sought to determine if the suppressive effects κ-CAS exerted on murine macrophages and DCs were translatable in human cells using PBMC populations and monocytes; the progenitor cell of macrophages and DCs.

6.2 Experimental design

Having observed the immunosuppressive effects κ-CAS exhibited on murine macrophages and DCs, we firstly sought to determine if these observations were translatable to humans by firstly comparing the ability of κ-CAS to modulate cytokine production in human PBMCs. PBMCs were isolated from human buffy coat blood packs sourced from the Irish Blood Transfusion Service, St James' Hospital, Dublin, using density gradient centrifugation. Multiple blood donors were used for these studies. PBMCs were plated at $1x10^6$ cells per mL and treated with κ-CAS (1 mg/mL) for 2.5 hr prior to the addition of LPS (100 ng/mL) or PMA (20 ng/mL) and ionomycin (1 μM). For some experiments, $CD14^+$ monocytes were isolated from PBMCs by magnetic activated cell sorting. Human macrophages were differentiated from $CD14^+$ cells over 14 days with human serum. Human macrophages, $CD14^+$ cells and $CD14^+$ depleted PBMCs were treated as outlined above. In some instances, to elucidate the mechanism by which TNF-α was induced by κ-CAS treatment of $CD14^+$ cells, $CD14^+$ cells were incubated with BAY117821 (10 μM); a chemical antagonists of the NFκB pathway (Ghashghaeinia *et al.,* 2011) for 30 min prior to κ-CAS treatment.

Given that PBMCs are composed of a mixture of immune cell populations, binding studies were performed to determine which cell type's interacted with κ-CAS. PBMCs were seeded at $1x10^5$ cells per 100 μL in 96 well plates, and incubated with 10 or 20 μg/mL of FITC-488 labelled κ-CAS for 45 min at 37 °C. Cells were subsequently washed in ice cold PBS before being analysed by flow cytometry. As a control for non-specific binding, cells were incubated with 20 μg/mL of FITC-488 labelled BSA.

From studies conducted in our mouse models, we have shown that macrophages and DCs treated with κ-CAS exhibited a reduced capacity to induce robust T-cell responses in mice.

To investigate if this could be replicated with human monocytes, $CD14^+$ cells were incubated with PBS or κ-CAS overnight before being washed and co-cultured at a 1:10 ratio with $CD4^+$ T-cells in plates coated with anti-CD3 (1 μg/mL). After 72 hr supernatants were analysed for the production of cytokines by ELISA. Non-adherent cells were also washed and analysed by flow cytometry for viability or the expression of extracellular anergy markers.

6.3 Results

6.3.1 κ-CAS induces the production of TNF-α from PBMCs.

From the previous chapters we demonstrated that κ-CAS treatment suppresses pro-inflammatory cytokine production from murine macrophage and DCs after LPS challenge. We examined if this phenomenon would be translatable to humans using human blood buffy coat derived PBMCs. PBMC isolated by gradient centrifugation were seeded at $1x10^6$ cell/mL and stimulated with κ-CAS (1 mg/mL) 2.5 hr. PBS treated PBMCs were used as a control. Cells were subsequently stimulated in the presence or absence of LPS (100 ng/mL) (Figure 6.1A) or PMA (20 ng/mL) and ionomycin (1 mM) (Figure 6.1B). κ-CAS treatment alone induced a significant induction of TNF-α by PBMCs (Figure 6.1A * , $p \leq 0.05$, Figure 6.1B * , $p \leq 0.05$). No significant differences were detected between LPS stimulated PBMCs that were pretreated with PBS or κ-CAS. However, we observed a significant increase in the production of TNF-α between PMA & ionomycin stimulated PBMCs that were pretreated with κ-CAS compared to PBS control (Figure 6.1B ** , $p \leq 0.01$).

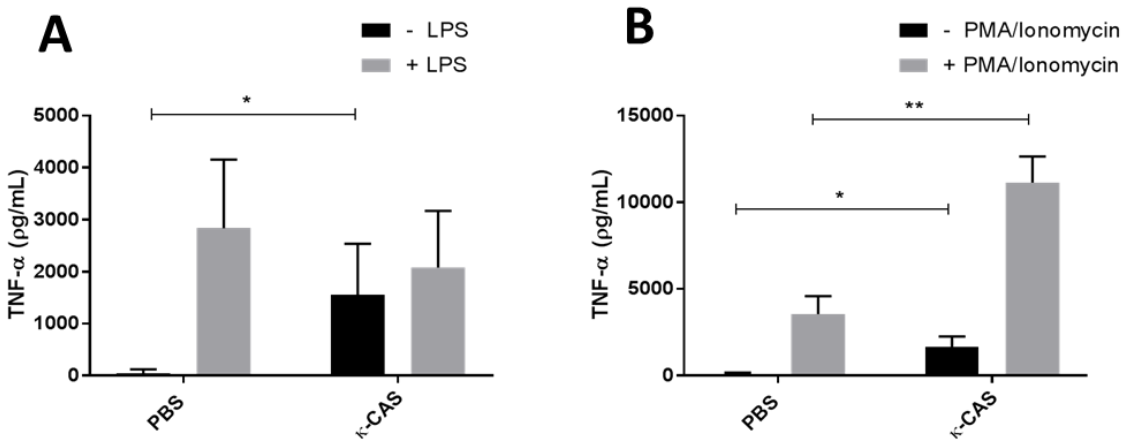

Figure 6.1 Effects of κ-CAS on LPS & PMA/Ionomycin induced TNF-α in PBMCs. PBMCs were treated with κ-CAS (1 mg/mL) for 2.5 hr. Cells were subsequently stimulated in the presence or absence of LPS (100 ng/mL) (A) or PMA (20 ng/mL) and Ionomycin (1 μM) (B) for 18 hr. Supernatants were analysed for the secretion of the cytokines TNF-α by ELISA. Results are expressed as mean ±SD of 4 independent experiments from 5 individual donors for LPS treated samples or 3 independent experiments from 3 individual donors for PMA/ionomycin samples. P-values were calculated using were calculated using ANOVA multiple comparisons tests. ** , $p \leq 0.01$, * , $p \leq 0.05$ compared to PBS control group.

6.3.2 The majority of monocytes bind κ-CAS

Given that κ-CAS was observed to induce TNF-α from PBMC, a mixed population of different immune cell types, we next examined which PBMC population κ-CAS interacted with. PBMCS were isolated from human buffy coats by gradient centrifugation and seeded at $1x10^5$ cells/100 μL in a 96 well plate. Cells were treated with 488-FITC labelled κ-CAS (10 or 20 μg/mL) for 45 mins and were subsequently analysed by flow cytometry. 488-FITC labelled BSA (20 μg/mL) was used a non-specific binding control. The binding of κ-CAS to the whole PBMC population was measured and deemed to be specifically bound at 20 μg/mL compared to BSA control (Figure 6.2A * , $p \leq 0.05$). κ-CAS was bound by 18.6 %

± 8 of the total PBMC population (Figure 6.2B), and of the 2 main immune cell populations found in PBMCs, 73.7 % ± 2.6 of monocytes and 6.9 % ± 1 of lymphocytes were shown to bind κ-CAS (Figure 6.2C).

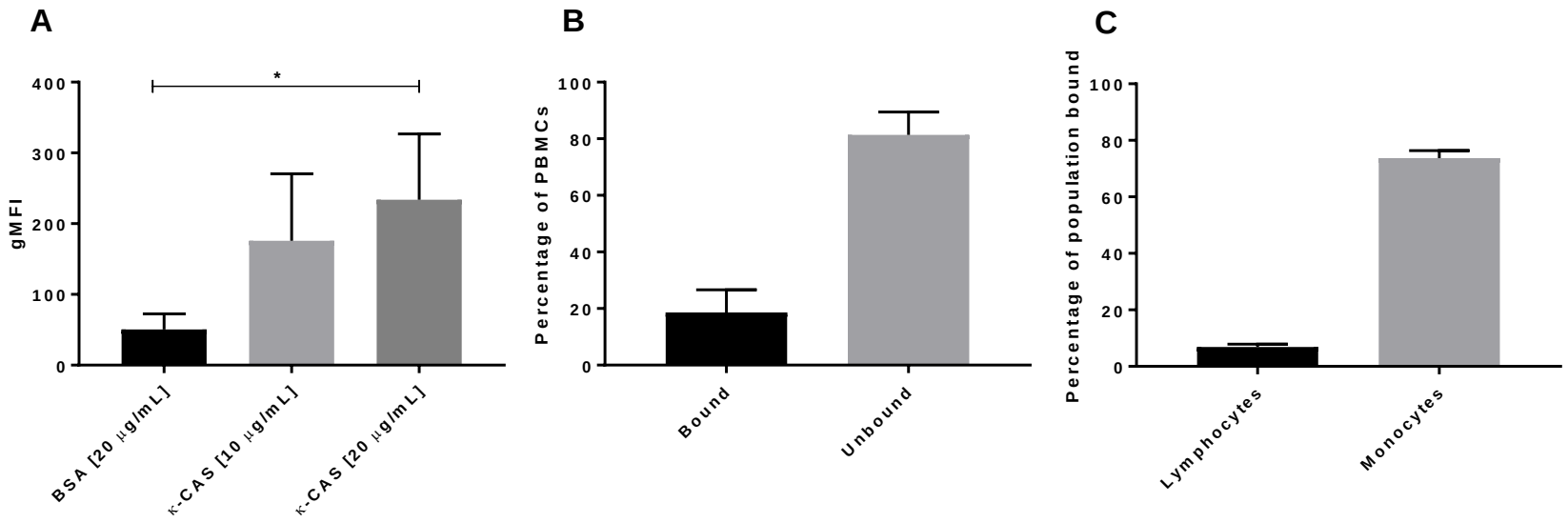

Figure 6.2 Monocytes bind κ-CAS. PBMCs were stimulated with 488-FITC labelled κ-CAS (10 – 20 μg/mL) or 488-FITC labelled BSA (20 μg/mL) for 45 min before being analysed by flow cytometry. The binding of κ-CAS to PBMCs as a whole population at 10 μg/mL and 20 μg/mL was shown. P-values were calculated using ANOVA multiple comparisons test. * , $p \leq 0.05$ compared to BSA control group. Results were analysed using FlowJo software (Treestar, USA) and are expressed as the gMFI ±SD of 3 independent experiments from 3 individual donors. (A). PBMCs that bound FITC labelled κ-CAS (20 μg/mL) as a percentage of the total PBMC population was analysed from 3 individual donors (B). The percentage of lymphocytes or monocytes in the PBMC population that bound FITC labelled κ-CAS (20 μg/mL) was assessed from 3 individual donors (C).

6.3.3 $CD14^+$ monocytes responsible for the induction of TNF-α from κ-CAS treatment in PBMCs.

As the majority of monocytes bind κ-CAS, we investigated if these cells were involved in the induction of TNF-α when treated with κ-CAS. $CD14^+$ monocytes were isolated from PBMCs by magnetic activated cell sorting. The isolated $CD14^+$ cells were stimulated with κ-CAS (1 mg/mL) 2.5 hr in the presence or absence of LPS (100 ng/mL) (Figure 6.3A). CD14 depleted PBMCs were also stimulated with κ-CAS (1 mg/mL) 2.5 hr, but in the presence or absence of PMA (20 ng/ml) and Ionomycin (1 μM) (Figure 6.3B). PBS treated cells were used a control. Supernatants were analysed for the production of TNF-α. κ-CAS treatment alone induced a significant induction of TNF-α by $CD14^+$ monocytes (Figure 6.3A * , $p \leq 0.05$, Figure 6.3B * , $p \leq 0.05$). No significant differences were detected between LPS stimulated $CD14^+$ cells that were pre-treated with PBS or κ-CAS. Moreover, no significant differences were observed in the production of TNF-α between PMA & ionomycin stimulated CD14 depleated PBMCs that were pretreated with κ-CAS compared to PBS control (Figure 6.3B ** , $p \leq 0.01$). This would suggest that $CD14^+$ monocytes are responsible for the induction of TNF-α in response to κ-CAS treatment.

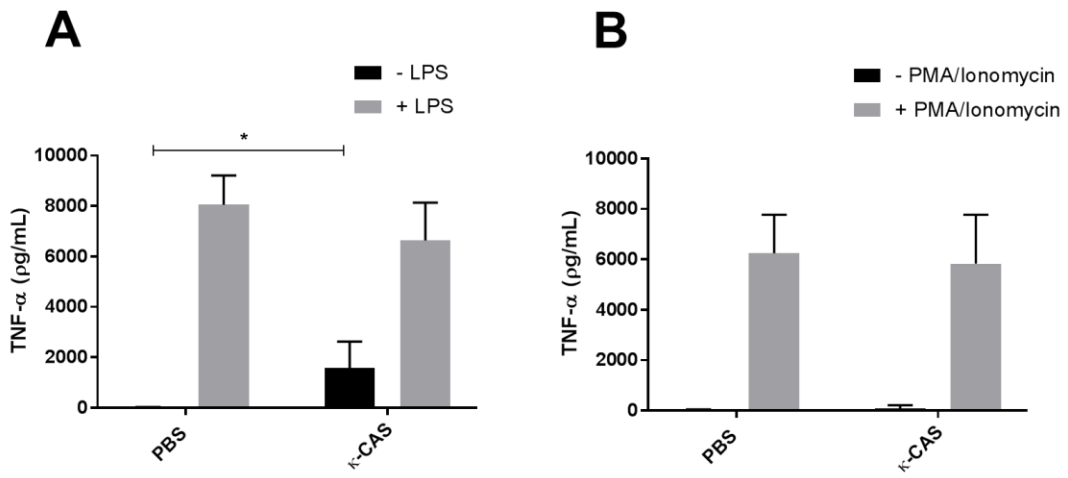

Figure 6.3 κ-CAS treated CD14$^+$ monocytes induce TNF-α in PBMCs. CD14$^+$ monocytes (A) or CD14$^+$ monocyte depleted PBMCs (B), were treated with κ-CAS (1 mg/mL) for 2.5 hr. CD14$^+$ monocytes were subsequently stimulated in the presence or absence of LPS (100 ng/mL). CD14$^+$ monocyte depleted PBMCs were stimulated in the presence or absence PMA (20 ng/mL) and Ionomycin (1 μM). After 18 hr, supernatants were analysed for the secretion of TNF-α by ELISA. Results are expressed as mean ±SD of 3 independent experiments from 4 individual donors for LPS treated samples or 3 independent experiments from 3 individual donors for PMA/ionomycin samples. P-values were calculated using were calculated using ANOVA multiple comaprisons tests. * , $p \leq 0.05$ compared to PBS control group.

6.3.4 NFκB pathway involved in the induction of TNF-α by κ-CAS treated CD14$^+$ monocytes.

NFκB has long been considered the most prominent pathway involved in pro-inflammatory signaling, mainly due to its role in the induction of pro-inflammatory genes, which culminates in the production of a range of cytokines and chemokines upon activation (Liu *et al.,* 2017; Lawrence 2009) Having observed the induction of TNF-α; a pro-inflammatory cytokine by CD14$^+$ monocytes treated with κ-CAS, we sought to investigate if NFκB signaling was involved in this phenomenon. CD14$^+$ monocytes were isolated from PBMCs

by magnetic activated cell sorting. The isolated $CD14^+$ cells were pre-treated with a chemical antagonist of NFκB; BAY117821 (10 μM) for 30 min prior to stimulation with or without κ-CAS (1 mg/mL) overnight. Supernatants were analysed for the production of TNF-α. $CD14^+$ monocytes significantly induced the secretion of TNF-α in response to κ-CAS treatment alone as previously observed. However, pre-treatment with the chemical antagonist of NFκB activation significantly reduced the levels of TNF-α produced by κ-CAS treatment. This data would indicate that the induction of TNF-α by κ-CAS in $CD14^+$ cells signals through the NFκB pathway.

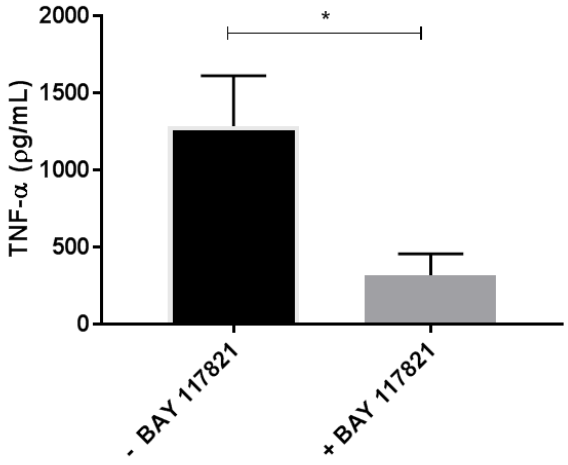

Figure 6.4 NFκB involved in κ-CAS induced TNF-α from $CD14^+$ monocytes. $CD14^+$ monocytes were pre-treated with the NFκB inhibitor; BAY117821 (10 μM) for 30 min and subsequently treated with κ-CAS (1 mg/mL) for 18 hr. Supernatants were analysed for the secretion of TNF-α by ELISA. Results are expressed as mean ±SD of 2 independent experiments from 3 individual donors. P-values were calculated using two-tailed student's t-test. * , $p \leq 0.05$ compared to PBS control.

6.3.5 κ-CAS suppresses $CD14^+$ monocyte induced IL-2 from $CD4^+$ T-cells in co-culture.

Having demonstrated the increases in pro-inflammatory cytokine production κ-CAS exhibits on $CD14^+$ cells, their ability to modulate $CD4^+$ T-cell responses was investigated. $CD14^+$ monocytes were isolated from PBMCs by magnetic activated cell sorting. The isolated $CD14^+$ cells were stimulated with κ-CAS (1 mg/mL) overnight. PBS treated cells were used as a control. The cells were subsequently washed and co-cultured at a 1:10 ratio with $CD4^+$ T-cells from allogenic or allergenic donors on plates coated with anti-CD3 (1 μg/mL) for 72 hr. Supernatants were analysed for the production of cytokines. $CD14^+$ monocytes pre-treated with κ-CAS showed no significant differences for the induction of IFN-γ (Figure 6.5 A-B), IL-13 (Figure 6.5 C-D), or IL-10 (Figure 6.5 E-F) from $CD4^+$ T-cells in allogenic or allergenic co-cultures. However, the levels of IL-2 for both allogenic or allergenic co-cultures were significantly reduced (Figure 6.5 E-F * , $p \leq 0.05$).

.

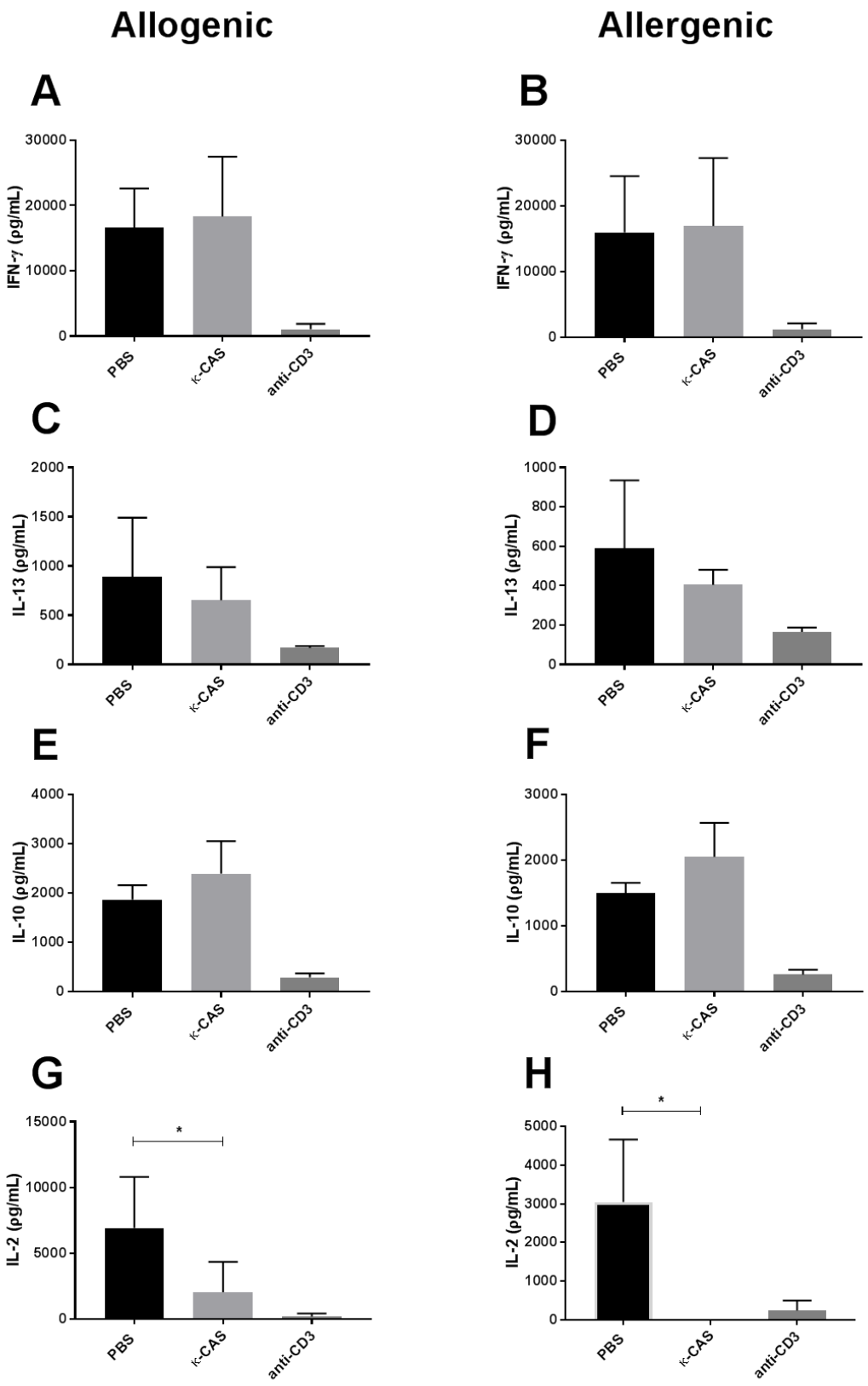
Allogenic
Allergenic
A
B
C
D
E
F
G
H
IFN-γ (pg/mL)
IL-13 (pg/mL)
IL-10 (pg/mL)
IL-2 (pg/mL)
PBS
κ-CAS
anti-CD3
*

Figure 6.5 κ-CAS alters the capacity of CD14$^+$ monocytes to prime T-cell responses. CD14$^+$ monocytes were stimulated with PBS or κ-CAS (1 mg/mL) overnight. Cells were subsequently washed and co-cultured with allogenic (A, C, E, G) or allergenic (B, D, F, H) CD4$^+$ T-cells at a 1:10 ratio in 96 well plates pre coated with anti-CD3 (1 μg/mL). After 72 hr supernatants were analyzed for the cytokines; IFN-γ (A, B), IL-13 (C, D), IL-10 (E, F) and IL-2 (G, H) by ELISA. Results are expressed as mean ±SD of 3 independent experiments from 4 donors for allogenic samples and 3 independent experiments from 3 donors for allergenic samples. P-values were calculated using ANOVA multiple comparisons test. ***** , $p \leq 0.05$ compared to PBS control.

6.3.6 CD4$^+$ T-cells co-cultured with κ-CAS treated CD14$^+$ monocytes do not display markers of anergy.

To examine if the reduction in IL-2 observed was due to anergy or apoptosis, the expression of anergic markers; CTLA4 or apoptosis, was assessed following co-culture. CD14$^+$ monocytes were isolated from PBMCs by magnetic activated cell sorting. The isolated CD14$^+$ cells were stimulated with κ-CAS (1 mg/mL) overnight. PBS treated cells were used as a control. The cells were subsequently washed and co-cultured at a 1:10 ratio with CD4$^+$ T-cells from allogenic or allergenic donors on plates coated with anti-CD3 (1 μg/mL) for 72 hr. Non-adherent cells were isolated and analysed for surface marker expression of CTLA4 and viability. No significant differences in the expression of the extracellular receptor; CTLA4 (Figure 6.5A) or viability (Figure 6.5B) were observed between T-cells co-cultured with PBS or κ-CAS treated CD14$^+$ cells.

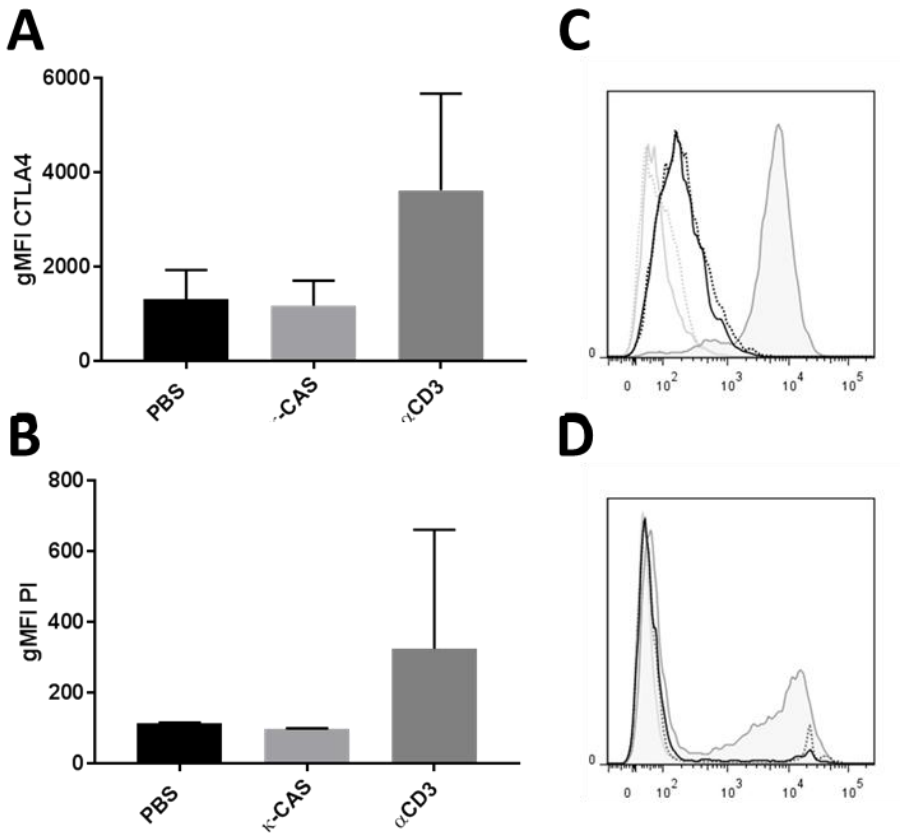

Figure 6.6 κ-CAS treated CD14$^+$ monocytes do not induce anergy markers in CD4$^+$ T-cells. Non-adherent cells were harvested after allogenic co-culture, washed and incubated for 30 min with antibodies for the detection of the extracellular anergy receptor marker; CTLA4 (A) or the apoptotic marker; propidium iodide (PI) (B) and analysed by flow cytometry. Results were analysed using FlowJo software and are expressed as the gMFI ±SD of 2 independent experiments from 2 donors. Representative histograms show the surface expression of CTLA4 (C) or uptake of the PI (D); unstained (gray line), isotype (dotted grey line), PBS (black dotted line), κ-CAS (black line) and anti-CD3 (tinted gray histogram).

6.3.7 κ-CAS suppresses LPS induced TNF-α from human macrophages.

In previous chapters we demonstrated κ-CAS capacity to modulate murine macrophages cytokine production in response to TLR ligands. We next examined if a similar effect was observed in humans using human monocyte derived macrophages. $CD14^+$ cells were isolated from PBMCs by magnetic activated cell sorting. Human macrophages were differentiated from $CD14^+$ cells over 14 days with human serum. Macrophages were pre-treated with κ-CAS (1 mg/mL) 2.5 hr prior to stimulation with and without LPS (100 ng/mL) for 18 hr. PBS treated cells were used a control. Supernatants were analysed for the production of TNF-α. The addition of κ-CAS alone did not induce TNF-α, unlike that observed for $CD14^+$ monocytes. Moreover, κ-CAS significantly inhibited the secretion of TNF-α in response to LPS compared to PBS control (Figure 6.7 **, $p>0.001$).

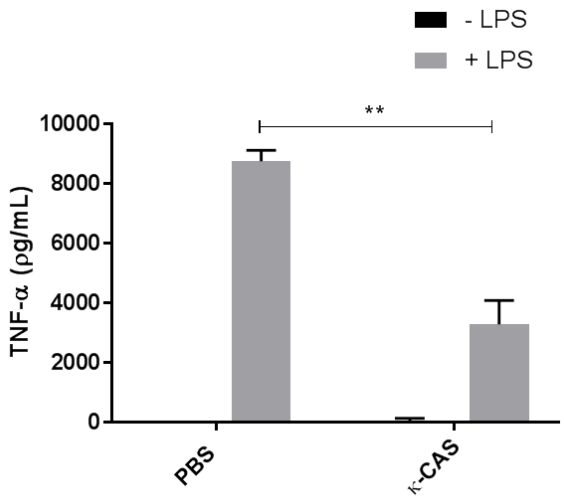

Figure 6.7 Effects of κ-CAS on LPS induced TNF-α from hMφ. hMφ derived from $CD14^+$ monocytes were treated with κ-CAS (1 mg/mL) for 2.5 hr. Cells were subsequently stimulated in the presence or absence of LPS (100 ng/mL) for 18 hr. Supernatants were analysed for the secretion of the cytokines TNF-α by ELISA. Results are expressed as mean ±SD from 3 individual donors. P-values were calculated using were calculated using ANOVA multiple comaprisons tests. ** , $p \leq 0.01$, compared to PBS control group.

6.4 Discussion

In the previous chapters we showed κ-CAS capacity to impair the secretion of pro-inflammatory cytokines; notably TNF-α, from murine macrophages and DCs in response to inflammatory stimuli. In this chapter, we sought to determine if our observations were transferable to human cells by investigating the response of PBMC populations to κ-CAS. Monocytes, a PBMC population of phagocytes involved in innate immune defense and the precursor cells to macrophages and DCs (Geissmann *et al.,* 2010) were determined to be the PBMC population that had the strongest affinity for binding κ-CAS. Unlike the suppressive effects observed in murine macrophages and DCs, κ-CAS did not suppress the induction of TNF-α from LPS treated monocytes. Moreover, κ-CAS treatment alone induced the secretion of TNF-α in a mechanism related to the activation of the NF-κB signal transduction pathway. Similarly, Requena *et al.,* demonstrated that GMP; a peptide derivative of κ-CAS, up-regulated the secretion of TNF-α, IL-1β and IL-8 in monocytes, dependent on the phosphorylation of IκB-α and the nuclear translocation of the NF-κB subunits p50 and p65 (Requena *et al.,* 2009). The release of TNF-α by monocytes treated with κ-CAS would be suggestive of their development towards a ‘’classically activated’’ inflammatory phenotype normally associated with the release of pro-inflammatory cytokines like TNF-α (Zarif *et al.,* 2016).

In addition to the secretion of pro-inflammatory mediators in response to stimuli, monocytes can also function as APCs; bridging the gap between innate and adaptive immunity. It has become increasingly more evident that monocytes play a prominent role in influencing inflammatory responses *in-vivo*, affecting the generation, expansion and polarization of T-cells they encounter at the site of inflammation *in-situ* (Evans *et al.,* 2007; Geissmann *et al.,* 2008; Evans *et al.,* 2009). The state of activation and maturation of APCs

determines their ability to interact with T-cells, influencing the type of immune response that is initiated (Banchereau & Steinman 1998). The release of the inflammatory cytokine TNF-α by monocytes treated with κ-CAS would be indicative of ‘’classical activation’’ (Zarif *et al.,* 2016). Evans *et al* demonstrated that *in-vitro* and *in-vivo* activated inflammatory monocytes promoted T-cell responses in $CD4^+$ T-cells (Evans *et al.,* 2009). Monocyte released TNF-α is thought to be implicated in the shaping of inflammatory T-cell responses, but monocyte/T-cell contact is also heavily involved (Evans *et al.,* 2009). While κ-CAS treated monocytes induced TNF-α, co-culture with T-cells resulted in a significant decrease in the secretion of IL-2, a cytokine centrally involved in the activation and propagation of robust effector T-cell responses (Boyman *et al.,* 2015). Hence, κ-CAS treatment of monocytes may present as a target to control excess cytokine production by T-cells, as aberrant T-cell activity is associated with a large number of chronic inflammatory disorders, including RA, MS, and IBD (Tesmer *et al.,* 2008; Waite *et al.,* 2011; Weaver *et al.,* 2012).

Macrophages are key innate immune cells involved in inflammatory processes. They can be divided into “alternatively activated” M2 macrophages that are associated with T_H2 T-cell activation, helminth defense, wound repair and immuno-suppression (Martinez *et al.,* 2006). In contrast, “classically activated” M1 macrophages produce pro-inflammatory cytokines like TNF-α and are involved in T_H1 T-cell activation and mediate acute inflammation. In the previous chapter we demonstrated using murine macrophages that κ-CAS induced a suppressive M2-like macrophage phenotype and attenuated the induction of key inflammatory cytokine mediators in response to LPS. Similarly, herein we observed human macrophages being rendered hypo-responsive, releasing significantly reduced levels of the pro-inflammatory cytokine TNF-α upon LPS stimulation. In contrast to monocytes,

κ-CAS treatment alone did not induce any basal levels of TNF-α from human macrophages, consistent with the observed results in murine macrophages. TNF-α is a monocyte-, macrophage- and T-cell-derived cytokine, and is considered to be a key mediator of inflammatory disorders such as RA, psoriasis and IBD (Plevy *et al.,* 1997; Bradley 2008). TNF-α has been shown to be heavily implicated in the disruption to epithelial barrier function (McGuckin *et al.,* 2009), and aberrant TNF-α production from macrophages was shown to significantly contribute to this disruption, exacerbating intestinal inflammation in IBD (Lissner *et al.,* 2015). Hence, κ-CAS treatment of macrophages may present as a promising target to control excess TNF-α cytokine production in inflammatory conditions.

Distinct cellular proteome differences are observed between macrophages, DCs and their monocyte precursors. Studies have confirmed that macrophages contain significantly higher levels of proteases and peptidases, involved in proteolysis, compared to monocytes (Rossman *et al.,* 1990; *Menard et al.,* 2000; Jin *at al.,* 2004). Furthermore, monocytes contained significantly higher levels of protease inhibitors, specifically leukocyte elastase inhibitor, compared with macrophages (Jin *at al.,* 2004). Considering that in previous chapters we established that κ-CAS required proteolytic cleavage by the cells to elicit its suppressive effects, the differences in proteases, peptidases and proteases inhibitors between monocytes and macrophages may result in differential cleavage and release of peptide fragments with immunomodulatory properties.

In summary, this study reported on the interaction of κ-CAS with $CD14^+$ monocytes, which induced their activation and production of TNF-α via the NFκB pathway. κ-CAS significantly reduced the capacity of these activated monocytes to prime T-cell induced IL-2, potentially sequestering the ability of these cells to elicit an effective adaptive immune response, similar to results obtained in murine DCs. We reported on the suppression of LPS

induced pro-inflammatory cytokine production by κ-CAS in human macrophages, similar to the results obtained from murine macrophages. Given that the immuno-modulatory effects exhibited by κ-CAS in mice were transferable in human cells; further study is warranted as our data suggest that κ-CAS has potential to be developed into a novel immune-suppressive nutraceutical for the management of human inflammatory diseases in humans.

Chapter 7 - Final discussion:

Research in the field of functional food derived nutraceutical's has rapidly expanded in recent times as more evidence emerges on their positive health impacts (Kitts and Weiler 2003). In particular, milk has a great potential to be used commercially as a source of bioactive nutraceuticals as its consumption and production has increased globally (O'Connor 2009) making milk as a source of nutraceutical's culturally acceptable and the abundance of material means bio-actives can be produced at low cost in large quantities. These bio-actives derived from milk display an array of bioactive health enhancing properties (Savijoki *et al.,* 2006; Madureira *et al.,* 2007; Dziuba *et al.,* 2009). The consumption of some of these bioactive proteins has been reported to be helpful in the management of many western diseases (Dhaval *et al.,* 2016). Considering that many of these diseases are immune-related, immunomodulatory nutraceuticals have garnered special attention from both academic and industrial researchers for their potential use as therapeutics for the amelioration of chronic inflammatory disorders like IBD (Bouglé & Bouhallab 2015).

This study sheds new light on immunomodulatory effects exhibited by κ-CAS, a milk derived CAS protein subunit, on APCs isolated from murine and human origin. The study has reported on a number of novel findings including the characterisation of suppressive M2-like macrophage and semi-immature DC phenotypes induced by κ-CAS in mice. We demonstrated that κ-CAS treated cells were rendered hypo-responsive to inflammatory stimuli, via the attenuation of the NFκB signalling pathway partially mediated by the enhanced expression of the negative regulators SOCS1 & SOCS3. κ-CAS stimulated murine APCs suppress or have a capacity to significantly reduce T-cell responses, a result which was transferable in human monocytes; progenitor cells of macrophages and DCs.

The following discussion will highlight key findings from the study and discuss future work.

7.1 κ-CAS induces a novel suppressive M2-like macrophage and semi-immature DC phenotype that are hypo-responsive to inflammatory stimuli.

Macrophage and DC activation, maturation and phenotypes are heavily influenced by exposure to exogenous stimuli (Hoshino *et al*., 1999; Martinez & Gordon 2014). Here, we demonstrated that κ-CAS induced a novel suppressive-like M2 phenotype as characterized by the expression of M2 related genes; *Arg-1, RELM-α* and *YM-1* and the M2 associated CLR CD206 in macrophages. M2 macrophages are generally subdivided into tissue injury, helminth infection and allergy associated M2a or anti-inflammatory M2b/c macrophages. They are induced by a variety of stimuli and are characterized by the induction of *Arg-1*, C-type lectin receptors (CLRs) and low expression of inflammatory mediators. The transcription factor STAT6 is a critical mediator in the induction of M2a phenotypes, while STAT3 is primarily responsible for the induction of M2b/c macrophages. In contrast, we demonstrated that neither STAT6 nor STAT3 were prominently involved in the induction of the M2-like phenotype induced by κ-CAS. In contrast, we demonstrated that STAT6 antaganism induced more Arg-1 and IL-10 production form NaCAS treated BMMϕ, suggesting that it may play a suppressive role in the induction of this novel phenotype. Moreover, this intermediate M2 state induced by NaCAS and κ-CAS does not conform to the M1/M2a/b/c categorization, more indicative of the phenotypes observed *in-vivo* or in disease states which often lack defined subsets, sharing M1/M2 phenotype characteristics (Vogel *et al.,* 2013; Italiani *et al.,* 2014), questioning the validity of the M1/M2 paradigm and a possible need now to expand and redefine macrophage phenotypes that include those

that are induced by antigens derived by pathogenic and non-pathogenic sources in the absence of more traditionally defined stimuli (Donnelly *et al.,* 2005; Figueroa-Santiago *et al.,* 2014).

CD54 an adhesion receptor generally associated with inflammatory M1 phenotypes (Hubbard & Giardina 2000; Murray *et al.*, 2014) was also shown to be upregulated by κ-CAS. CD54 expression is often seen upregulated at sites of inflammation, which encourages infiltration of many immune cell types and cell-cell signalling. Studies have also demonstrated that CD54 overexpression on macrophages promotes M2 polarization (Gu *et al.,* 2017) and is more a surrogate marker of antigen presenting cell activation rather than inflammatory status (Sheikh & Jones 2008). The majority of macrophages observed in inflammatory regions express CD54. However, more recently, CD54 on macrophages was shown to have immunosuppressive function on mesenchymal stromal cell at inflammatory sites, dampening the immune response (Espagnolle *et al.,* 2017). This would suggest that CD54 expression on macrophages can be indicative of inflammatory M1 macrophage activation but can have a regulatory role, the upregulation of which reinforces our observation that κ-CAS induces a novel regulatory macrophage phenotype that could have a substantive role in immuno-suppression.

DCs are generally phenotypically categorized based on their level of maturity. We demonstrated that κ-CAS reduced the expression of the co-stimulatory receptor CD80; as well as adhesion and activation markers CD209 and CD54. Given the association between antigen presenting cell activation and CD54 upregulation (Sheikh & Jones 2008) and the reduced co-stimulatory marker expression, we concluded that κ-CAS rendered DCs in a semi-immature state. DCs in a steady state prior to activation are termed immature and characterized by expressing low levels of cell surface co-stimulatory markers; CD80,

CD86, MHC II, CD40 and attenuated production of pro-inflammatory cytokines (Banchereau *et al.,* 2000, Dowling *et al*., 2008). Once activated, DCs take up a "classical" activation status and mature, upregulating cell surface co-stimulatory markers, and produce immune-stimulatory cytokines like IL-12/ or suppressive cytokines like IL-10 depending on the stimulus (Reis e Sousa 2006; Dowling *et al.,* 2008).

κ-CAS was also shown to down regulate the secretion of cytokines from both macrophages and DCs in response TLR and non-TLR ligands both prior to and following TLR and non-TLR stimulations. Here we demonstrated that the suppression of LPS induced responses by κ-CAS was shown to be mediated via a mechanism which attenuates the phosphorylation and degradation of the NFκB inhibitor protein IκBα, sequestering the activation of the NFκB signal transduction pathway. We propose that the upregulation of SOCS1 and SOCS3 by κ-CAS plays a role in suppressing the activation of NFκB resulting in the inhibition of cytokine secretion (Figure 7.1). While studies using plant derived bioactive metabolites have also been demonstrated to increase the expression of SOCS 1 and SOCS 3, which were shown to suppresses inflammatory signalling pathways (Shakibaei *et al.,* 2008; Zhang *et al.,* 2015), this is the first study to our knowledge that demonstrated a milk derived nutraceutical influencing SOCS protein expression and subsequent inflammatory signalling. Further studies have to be conducted to elucidate the mechanism by which κ-CAS induces the upregulation of these SOCS proteins. SOCS proteins are classically induced by signalling cascades from inflammatory stimuli as a negative feedback to dampen the response. However, the upregulation of SOCS have been demonstrated to be also mediated though receptor signalling like DC-SIGNR1, or growth factor receptor signalling which has been shown to induce the activation of the cAMP response element-

binding protein (CREB) pathways also involved in the upregulation of SOCS protein expression (Srivastava *et al.,* 2009; Chakrabarti *et al.,* 2017).

The SOCS inhibitor peptide used in our study indiscriminately binds to both SOCS 1 & SOCS 3 proteins via their shared kinase inhibitory regions, sequestering the inhibitory activity of both proteins (Ahmed *et al.*, 2015). Thus whether one or both SOCS proteins are involved in the inhibition of LPS induced cytokine production by κ-CAS has yet to be determined. Other more specific alternatives like knockout mice are not available as mice lacking SOCS1 develop a severe inflammatory syndrome within the first two weeks after birth (Marine *et al.,* 1999), while SOCS3-deficient mice fail to complete embryogenesis (Roberts *et al.,* 2001). Other alternatives like, chemical antagonist or gene silencing techniques (si-RNA) are available, which may help further define the role these SOCS proteins play in the immune-modulatory capacity of κ-CAS. However, while these techniques are more effective at targeting specific components, the work presented in this thesis used primary immune cells which required *in-vitro* differentiation, which could be influenced by gene silencing. The use of the macrophage like cell line; RAW267.4 could be used to further investigate the effects of SOCS 1 or SOCS3 proteins have on this process, although caution must be taken as cells lines can undergo chromosomal duplications, rearrangements, mutations, and epigenetic changes that alter their phenotypes, genes and receptor expression that may be involved.

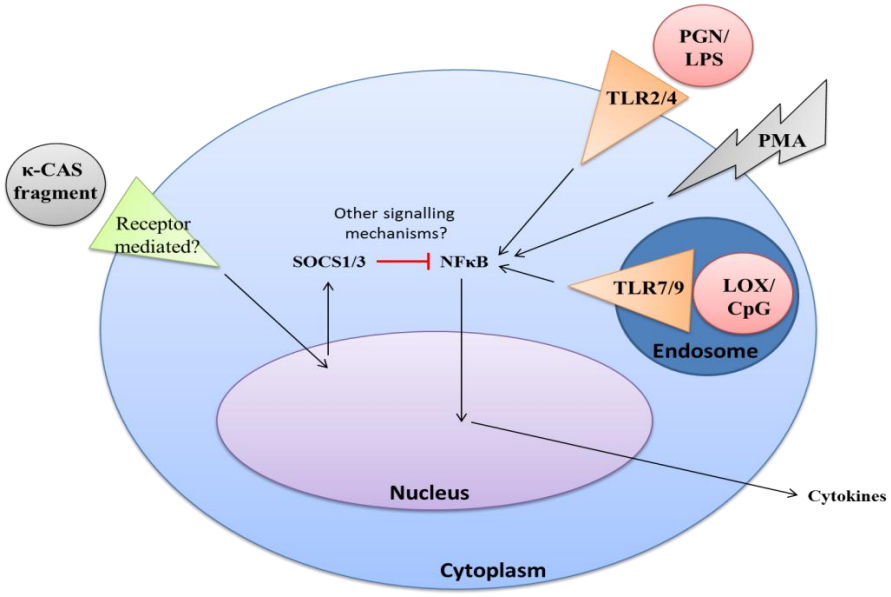

Figure 7.1 Possible mechanisms by which κ-CAS suppresses inflammatory cytokine secretions in macrophages and dendritic cells. κ-CAS induces the transcription of SOCS1 and SOCS3, which are implicated in the suppression of TLR and PMA induced NFκB signalling, thus impairing pro-inflammatory cytokine production and release. However, inhibitory mechanisms other than the upregulation of SOCS proteins may be involved, mediated by receptor binding or other recognition and signalling processes. κ-CAS; kappa casein, SOCS; suppressor of cytokine signalling, NFκB; nuclear factor kappa-light-chain-enhancer of activated B cells, TLR; toll like receptor, PGN; peptidoglycan, PMA; phorbol myristate acetate, LPS; lipopolysaccharide, LOX; loxoribine, CpG; unmethylated CpG dinucleotides. Figure adapted from Vukman 2013

While SOCS inhibitor peptides partially reversed κ-CAS induced suppression of LPS mediated TNF-α release in macrophages it is plausible that κ-CAS upregulates other inhibitory molecules which may act in synergy with SOCS1 & SOCS3 to target TLR and non-TLR signalling pathways in these cells which should be further investigated. Several other inhibitory molecules act to supress inflammation in a redundancy pathway that is heavily involved in the resolution of inflammatory responses such as the negative regulators including; docking protein 3 (DOK3), small heterodimer partner (SHP), B-cell lymphoma-3 encoded protein (BCL-3), that can sequester TLR and non TLR induced

activation of NFκB and subsequent release of TNF-α when upregulated in macrophages (Anwar *et al.,* 2013). Deubiquitinating enzyme A (A20); a zinc finger protein has been shown to inhibit TLR signalling and NFκB activation by several TLR and non-TLR stimuli, sequestering subsequent induction of inflammatory cytokines (Verstrepen *et al.,* 2014). We have yet to determine whether κ-CAS can suppress other members of the NFκB family (such as the p50 subunit) or any other interferon regulatory factors (IRF) transcription factors, such as IRF3, which are activated following TLR-ligand stimulation.

The mechanism by which κ-CAS initiates' intracellular signals that inhibit the aforementioned pathways remains to be elucidated. We revealed that κ-CAS was more significantly bound by cells then a protein control, in a Ca^{2+} dependant manner. This would infer that κ-CAS was specifically bound by a complex which requires Ca^{2+} rather than non-specific binding. An array of receptor including G-protein coupled receptors, among others mediates intracellular signalling upon ligation, in Ca^{2+} dependant manner (Mitra *et al.,* 2013). Co-precipitation could be used to determine if this process is receptor mediated, examining the interactions between the known protein; κ-CAS and the components with which it complexes; a possible receptor. However, this technique requires antibody recognition of the known protein. Given that a fragment of κ-CAS is responsible for the observed effects, recognition epitopes may be lost during proteolytic cleavage. Therefore, whether proteolytic cleavage of the subunit occurs internally or externally would also need to be examined further. Confocal laser scanning techniques could be used to track any internalisation or compartmentalisation which may occur.

The indirect inhibition or direct binding of the κ-CAS fragment to upstream signalling components would present as another possible alternative inhibitory mechanism exerted by κ-CAS which contributes to for the observed attenuation of the NFκB pathway and

subsequent inflammatory response. Previous studies have demonstrated that milk protein derived hydrolysates sequestered NFκB activation via targeting of p38 MAPK (Ming *et al.,* 2015; Song *et al.,* 2017). Further studies examining the phosphorylation and subsequent activation of the upstream signalling molecules MAP kinases and IKK complexes, involved in the activation of the inflammatory and NFκB signalling pathway (Hippenstiel *et al.,* 2000) should be investigated.

Studies using κ-CAS and its immune-modulatory role have mainly been focused on its interactions with cells of murine origin. The majority of studies using κ-CAS in humans models have been to investigate *in-vitro* T-cell proliferative responses in cow's milk protein allergy (Hoffman *et al.,* 1997; Sletten *et al.,* 2007). Some studies have investigated the effects of the κ-CAS derived GMP peptide on the monocyte-like cell line THP-1 cells or primary monocytes isolated from PBMCs (Requena *et al.,* 2009; Vordenbäumen *et al.,* 2011). We investigated the effect of κ-CAS on total PBMCs and the different sub-populations. From our mouse studies, we demonstrated that κ-CAS treatment significantly supressed the secretion of LPS induced pro-inflammatory cytokines like; TNF-α and IL-12p70 from macrophages and DCs. In contrast, κ-CAS treatment was shown to enhance TNF-α production from human PBMCs. We demonstrated that $CD14^{+}$ monocytes were responsible for the induction of TNF-α by PBMCs in a mechanism dependant on NFκB activation. Similarly, studies by Requena *et al.,* on human monocytes have shown that GMP induced the secretion of the inflammatory cytokines; TNF-α, IL-1β and IL-8 via the stimulation of MAPK and the phosphorylation of IκB-α, resulting in the nuclear translocation of the NFκB subunits; p50 and p65 (Requena *et al.,* 2009). However, despite the increased production of TNF-α; associated with inflammatory monocyte activation, κ-

CAS treated monocytes exhibited a reduced capacity to prime T-cell responses, similar to results obtained from murine macrophage and DC T-cell co-cultures.

7.2 κ-CAS treated macrophages supress T-cell responses while DCs have a reduced capacity to prime T-cells via the attenuation of IL-2.

DCs are generally considered to be the major drivers of $CD4^+$ T-cell responses; however, more evidence is accumulating that macrophages also play a prominent role in this process. The activation status of these cells not only affects their immediate innate effector function but can also heavily influence their ability to initiate and propagate adaptive immune responses (Takeda *et al.,* 2003; Pasare & Medzhitov 2004). Previous studies observed that intact κ-CAS and GMP significantly inhibited the mitogen-induced proliferative response of mouse spleen lymphocytes and Peyer's patch cells (Otani & Hata 1995; Otani et al., 1995). Given that κ-CAS was shown to induce novel phenotypes in murine macrophages and DCs and activated human $CD14^+$ monocytes, the capacity of these cells modulate T-cell responses cells was examined.

Macrophages treated with κ-CAS significantly attenuated $CD4^+$ T-cells from producing IFN-γ; a T_H1 associated pro-inflammatory cytokine, and IL-2; a cytokine critical for the initiation and propagation of robust T-cell responses (Bachmann & Oxenius 2007), in *in-vitro* co-cultures. In addition, κ-CAS treated DCs and monocytes co-cultured with $CD4^+$ T-cells *in-vitro* also exhibited an impaired ability to produce IL-2, however IFN-γ production from $CD4^+$ cells was not affected. Moreover, we conducted T-cell priming studies where κ-CAS or PBS treated DCs stimulated with OVA peptide were adoptively transferred over the sternum of transgenic mice pre sensitised to OVA. We demonstrated that OVA specific T-cells responses from the skin draining lymph nodes of these mice were supressed when

re-stimulated with OVA peptide *ex-vivo* compared to the results obtained from PBS treated DC recipient mice. While all cytokines were attenuated, IL-2 was the only cytokine significantly inhibited.

3 signals are required to elicit effective T-cell responses (Reis e Sousa 2006; Green *et al.,* 2009). The antigen-MHC complex is the main stimulatory signal (signal 1), co-stimulatory receptors bind their T-cell counterparts (signal 2) and the presence of immuno-stimulating factors (signal 3) like cytokines which influence which type of the effector T-cell response is elicited (Reis e Sousa 2006). All 3 signals lower the threshold needed for T-cell activation and the subsequent production of IL-2 (Lenschow *et al.,* 1996). Reduced signalling strength, via downregulation of co-stimulatory receptor interactions can result in the suppression of T-cell responses, which was observed for κ-CAS DCs. However, no significant decreases in MHCII or co-stimulatory receptor expression were exhibited by κ-CAS treated macrophages. In contrast, the co-stimulatory receptors CD40, OX40L were significantly upregulated which are generally associated with the induction of T-cell responses (Ozaki *et al.,* 1999; Croft *et al.,* 2009). Other possible receptors such as CD54 or CD209 could be responsible for the suppression of T-cell responses by κ-CAS activated macrophages. We demonstrated that CD54 an adhesion receptor was upregulated by κ-CAS in macrophages. CD54 is known to be involved in APC-T-cell communication (Vukman *et al.,* 2013) However, more recently CD54 expression on macrophages was shown to have immunosuppressive function at inflammatory sites, dampening the immune response (Espagnolle *et al.,* 2017). This would suggest that CD54 expression on macrophages can exhibit stimulatory and regulatory properties. κ-CAS treatment also upregulated the CLR CD206. Aldridge *et al.,* demonstrated CD206 upregulation on APCs supressed T-cell cytokine responses in in-vitro co-culture (Aldridge & O'Neill 2016). Further studies

examining the effects the upregulation these receptors by κ-CAS has on T-cell responses should be carried out. Neutralization antibodies to the receptors can inhibit receptor interactions, while receptor specific knockout mice can be used to verify any positive results for further validation.

The lack of inflammatory immuno-stimulatory factors produced by κ-CAS macrophages and DCs may account for the reduced T-cell responses. M2 macrophages are known to produce polyamines (Hasko *et al.,* 2000). Polyamines have been shown to exert regulatory effects on the immune responses inhibiting T-cell associated cytokines responses (Cordeiro-da-Silva *et al.,* 2004). DCs in a semi-mature activation state; expressing some costimulatory molecules but only low levels of inflammatory cytokines, such as TNF-α and IL-12 have also been reported to exert regulatory effects on T-cell responses (Lutz and Schuler, 2002). In contrast to κ-CAS treated macrophages and DCs, κ-CAS treated monocytes were shown to secrete high levels of inflammatory cytokines. However, in humans, monocytes and DC with high expression levels of costimulatory molecules and pro-inflammatory cytokine release have been demonstrated to suppress T-cell activation via the release of a combination of factors like indoleamine 2,3 deoxygenase (IDO) and IL-10 (Popov *et al.,* 2008; Von Bergwelt-Baildon *et al.,* 2006).

Low doses of IL-2 appear to mainly support the maintenance of Tregs which play a prominent role in restricting effector T-cells cells (Yu *et al.,* 2009; Klatzmann & Abbas 2015). Cytokines derived from macrophages and DCs like IL-10 & TGF-β have been demonstrated to stimulate the expansion of Treg populations (Harden & Egilmez 2012). However, while activated κ-CAS treated DCs secreted high levels of IL-10, we observed no upregulation of the extracellular receptor CTLA4; a marker expressed by Tregs or upregulation of anti-inflammatory cytokines like IL-10 by co-cultured $CD4^+$ T-cell.

Alternatively, activation through anergic pathways results in significantly reduced IL-2 induction, loss of proliferation and can renders T-cells hypo-responsive (Wells *et al.*, 2001). The presence of inhibitory ligands on antigen presenting cells can account for the inhibition of IL-2 and subsequent anergic T-cell response (Slavik *et al.*, 1999; Okazaki and Honjo 2006). However, $CD4^+$ T-cells cultured with κ-CAS treated DCs did not display prominent extracellular surface markers associated with anergy; CTLA4 and PD-1 (Buchbinder & Desai 2016). In future studies, other key markers of anergy should be investigated like T-cell proliferative responses and the enhancement of gene related to anergy in lymphocytes (GRAIL); an ubiquitin-protein ligase that plays a prominent role in T-cell anergy (Whiting *et al.*, 2011). Future work should be conducted to investigate if the suppressive effects exerted by κ-CAS treated macrophages, DCs and monocytes are due to cell-cell mediated interactions leading to an anergic state or the release of immuno-stimulatory factors which induce a suppressive T-cell phenotype.

This could have significant implication for the use of κ-CAS as a therapeutic, as it exhibits a duality of function. Our data demonstrated that κ-CAS attenuated the capacity of DC; key antigen presenting cells, from inducing IL-2 production from $CD4^+$ T-cells, sequestering their ability to elicit T-cell responses, affecting the induction of adaptive immune responses. Moreover, macrophages persist at sites of inflammation, involved in the recruitment of additional inflammatory cells in a positive feedback loop that further propagates the chronic inflammatory state. The alteration of these macrophages towards a suppressive phenotype by κ-CAS would also significantly attenuate the propagation and exacerbation of inflammatory responses at the sites of inflammation.

7.3 A fragment of κ-CAS can mimic the immuno-modulatory effects of whole κ-CAS.

We demonstrated that κ-CAS, a subunit of the milk protein; casein, exhibited immuno-modularity properties and moreover, a fragment of κ-CAS released by cell proteases, not the intact subunit was shown to be responsible for the observed effects. Several studies have reported immuno-modulatory properties arising from chymosin or pepsin cleavage of κ-CAS, resulting in the release of the ciliated C-terminal fragment; GMP and its derivatives (Wu *et al.,* 2011; Ashare *et al.,* 2005; Daddaoua *et al.,* 2005; Otani & Monnai 1993). Cheng *et al.,* demonstrated that GMP and a hydrolysed derivative inhibited LPS meditated inflammatory responses in macrophages via the suppression of the NFκB signalling pathway (Cheng *et al.,* 2015). Similarly, we demonstrated that κ-CAS also supressed LPS induced inflammatory cytokine release, via a mechanism that attenuates NFκB activation. However, while GMP was shown to exert its suppressive effect via the upregulation of heme oxygenase-1, which once inhibited, restored inflammatory cytokine release and NFκB activity (Li *et al.,* 2017), the inhibition of heme oxygenase-1 did not restore inflammatory cytokine release in κ-CAS treated macrophages (Appendix B). Moreover, Mikkelsen *et al.,* demonstrated that DCs stimulated with κ-CAS significantly supressed LPS induced TNF-α and IL-12p70, while GMP only attenuated TNF-α secretion. The degree of LPS mediated suppression of TNF-α by GMP was also significantly less than that of κ-CAS (Mikkelsen *et al.,* 2005). Furthermore, studies examining the modulatory effects of enzymatic digestion on GMP determined that proteolytic treatment with trypsin exhibited no significant effect on GMPs suppressive activity on pro-inflammatory cytokine release. However, we observed a reversal of the suppressive effects κ-CAS exerted on LPS stimulated macrophages when κ-CAS was treated with trypsin (Appendix C).

Another source of immunomodulatory κ-CAS fragments are casoxins, which have been shown to behave as opioid antagonists. Greeneltch *et al.,* demonstrated that opioid antagonism inhibited acute endotoxic shock via the suppression of TNF-α production, however opioid antagonism *in-vitro* had no direct effect on LPS induced TNF-α production in macrophages which would suggest that casoxins only prevent LPS-induced septic shock via indirect inhibition (Greeneltch *et al.,* 2004). Given these differences, we can deduce that the results obtained from our studies are unlikely to be due to GMP or casoxins but another fraction of κ-CAS with novel immuno-modulatory activities.

Further studies are required to identify the bioactive sequence responsible for the immune mdulatory activities observed in this study. Given that a cocktail of multiple protease inhibitors supresses the release and subsequent activity of the κ-CAS fragment, targeting of specific cell proteases could help identify which are involved. If the protease/proteases are elucidated, the resulting protein fragments can be characterised and isolated via high phase liquid chromatography, separating peptides based on their size and hydrophobicity (Lemieux *et al.,* 1991). Coupled to a mass spectrometer, it is also possible to determine the amino acid sequence of the detected fragments (Chen *et al.,* 1995). These are the techniques most often used for the identification and characterisation of bioactive protein fragments from enzymatic digests (Mamone *et al.,* 2003; Hernández-Ledesma *et al.,* 2004; Manso *et al.,* 2005; Jacobsen 2007). Depending on the size, the peptide could be synthesised by solid-phase peptide synthesis, allowing for further and more in-depth analysis of the bioactivity and mechanism to be investigated *in-vivo* or *in-vitro*.

7.4 Implications for the use of κ-CAS as a therapeutic for chronic gastrointestinal inflammatory conditions like IBD

Purposively deploying immune-modulating nutraceuticals in existing or new dietary products is an attractive opportunity to manage immune-related diseases. The uses of bioactive peptides as a therapeutic possess several advantages over classical pharmacological drugs. Bioactive peptides are naturally occurring biologics and in contrast to synthetic substances are degraded into their component amino acids without the production of intermediate toxic metabolites, a major problem associated with synthetic chemical drugs. Due to peptides being readily degraded, they generally have short half-lives which avoids their accumulation in bodily tissues. In addition, they are associated with lower manufacturing costs, high activity and greater stability (Jani *et al.,* 2012; Kaspar & Reichert 2013).

IBD that affect the gastrointestinal tract like CD and UC whose incidence and prevalence are increasing worldwide (Molodecky *et al.,* 2012), are prime targets for the use of bioactive immuno-modulatory proteins and peptides as they are administered orally, a non-invasive natural route that delivers the bio-actives to the inflammatory site. The oral route of entry for bioactive proteins and peptides can problematic due the highly acid environment of the stomach and expose to several proteolytic enzymes which may denature or sequester bioactivity (Madureira *et al.,* 2007) Many of these drawbacks can be overcome via encapsulation in liposomes, and micelles, which ensure protein stability and allows for the controlled release of the bioactive at the site of interest (Martins *et al.,* 2007).

Immunological results from several human studies and animal models indicate that microbial antigen exposure is heavily implicated in the initiation, perpetuation, and

amplification of IBD (Lodes *et al.,* 2004; Abreu *et al.,* 2005; Kiesler *et al.,* 2015). IBD is characterized by severe inflammation of the gastrointestinal tract (Strober & Fuss 2011). The pro-inflammatory cytokines such as IL-1β, IL-2, IL-12, IL-23, and IFN-γ, are associated with the initiation and progression of IBD. However, TNF-α is often cited as the master cytokine in this diseases pathogenesis (Murch *et al.,* 1993). It initiates cytotoxic, apoptotic, and acute-phase responses (Baumann & Gauldie 1994) and increases the secretion of other pro-inflammatory cytokines IL-1β, IL-6, perpetuating and exacerbating inflammation (Sanchez-Munoz *et al.,* 2008). The primary sources of TNF-α in IBD is proposed to be induced by inflammatory macrophages, but also differentiated T_H1 T-cells (Begue *et al.,* 2006). Macrophages and DCs isolated from IBD patients have also been demonstrated to have exaggerated inflammatory responses to the TLR ligand; LPS (Baumgart *et al.,* 2009). Study on the effectiveness of κ-CAS in models of IBD should be examined as we demonstrated that κ-CAS was a potent inhibitor of TLR and non-TLR induced TNF-α, among other inflammatory cytokines in murine macrophages and DCs, the primary cells associated with the induction of TNF-α in IBD. Anti-TNF-α therapy is currently one of the most prominent treatments used in IBD (Cohen 2017).

Due to the secretion of pro-inflammatory mediators, $CD4^+$ T cells are considered as a subset of cells that play a pivotal role in the development of IBD. IL-2, together with IFN-γ, is produced by activated T_H1 T-cell subsets and enhances inflammatory macrophage, natural killer (NK) cells and cytotoxic T-cell activity (Breese *et al.,* 1993). The presence of large numbers of activated T-cells in the involved mucosa of IBD patients suggests that IL-2 is likely to be playing at least some role in stimulating inflammation. Moreover, there is a positive correlation between clinical activity index (CAI) and $IL\text{-}2^+$ $CD4^+$ T-cells, thought to contribute to the pathogenesis of disease. (Ebrahimpour *et al.,* 2017). Our data

demonstrated that κ-CAS treated antigen presenting cells sequestered IL-2 production from $CD4^+$ T-cells, which would merit further examination in models of IBD as a potential therapeutic, reducing T-cell activity and the pathogenesis of the disease.

Further studies using murine models of IBD should be employed to deduce whether orally administered κ-CAS could alleviate the symptoms. Dextran sodium sulfate (DSS) is a well-studied model which induces severe murine colitis which closely resembles human UC (Okayasu *et al.*, 1990). The DSS colitis model would be of particular interest as the development of the intestinal inflammation is likely due to damage to the epithelial monolayer lining by DSS, allowing the dissemination of pro-inflammatory intestinal contents like bacteria that activate innate immune cells, like macrophages and DCs which in turn propagate and exacerbate the inflammatory response (Chassaing *et al.*, 2014).

7.5 Limitaions of the current study

There are limitaions in the current study at predicting the efficacy of κ-CAS to be used as a potencial theriputic for inflammatory conditions. Most of the research conducted examined the effects κ-CAS exhibited on cell populations in isolation. In an *in-vivo* environment, no cell population exists in isolation and the interaction between different cell types is essential for many biological processes, including immunity (Pasqual *et al.*, 2018). Moreover, cells in an *in-vivo* environment have been shown to exhibit differences in differentiation, maturation, response to stimuli, and cell-cell communication compared to *in-vitro* cultured cells (Antoni *et al.*, 2015).

In all experiemnts conducted, cells were directly exposed to κ-CAS. While *in-vitro* experiments can provide a detailed insight into the effects compounds exert, many factors

are not accounted for which may affect bioavailability that would heavily influence any compounds capacity to interact and induce similar effects to the same cell types in an *in-vivo* environment (Craik *et al.,* 2013). Given that oral ingestion would be envisioned as the route of adminiteration, some of these affects could be alluded to by use of enzymatic digest experiments with proteases and peptidases at pH's encountered during digestion to account for any changes to the compounds activity and stability. The ability of the compound to be absorbed at the intestinal line can also be simulated using Caco-2 cells as a model of the absorptive (Sambuy *et al.,* 2005). Chemical modification can be used to improve stability while formulation vehicles can protect compounds from enzymatic degredation and improve penetration/adsorbsion (Shaji & Patole 2008).

7.6 Conclusion

This study sheds new light on immunomodulatory effects exhibited by a fragment of κ-CAS. In summary, we report that this κ-CAS fragment induces a novel M2-like suppressive macrophage phenotype, maintains DCs in a semi-immature state, rendering both cell types hypo-responsive to NFκB dependant inflammatory signalling, and impairs their capacity to development adaptive immune responses. These results were also found to be transferable in human monocytes and macrophages.

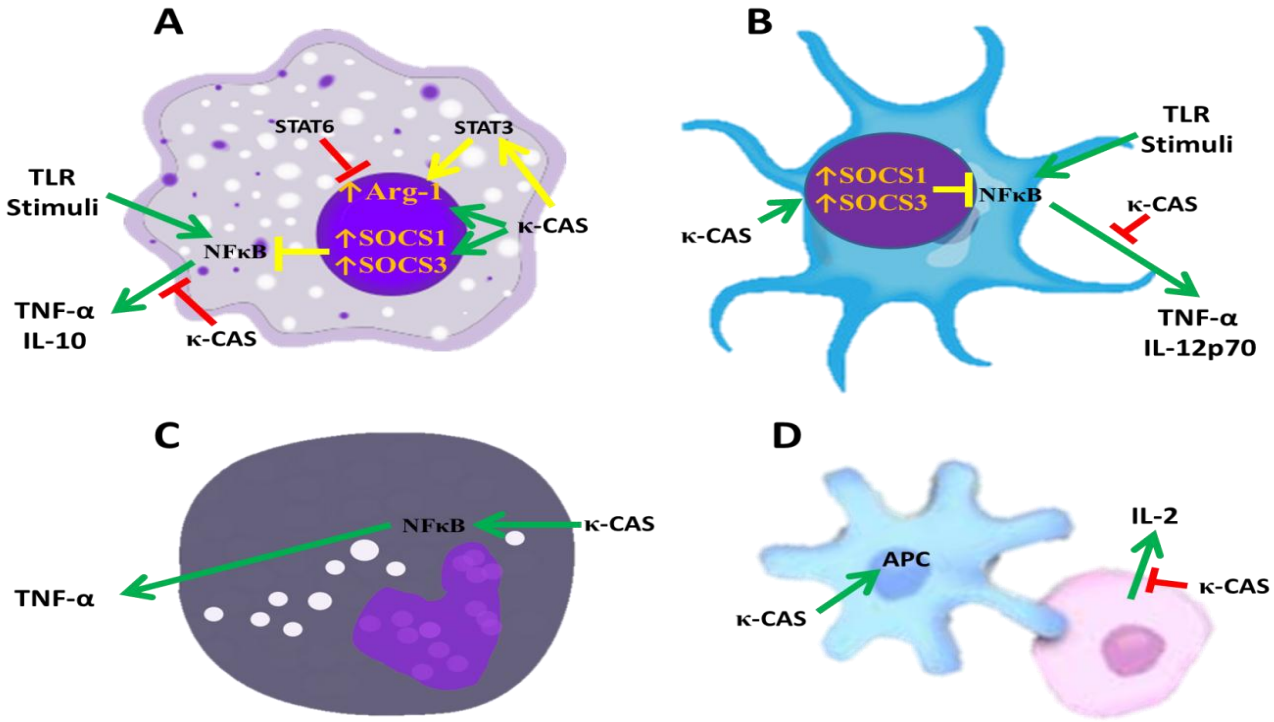

Figure 7.2 Immunomodulatory effects exerted by κ-CAS. NaCAS and κ-CAS induced M2 macrophage associated genes, notably Arg-1, potencially in a STAT3 dependant mechanism, while STAT6 was shown to exert regulatory effects on gene expression (A). κ-CAS abrogated TLR dependant induction of cytokines in macrophages (A) and DCs (B) by attenuating NFκB signalling, which is partially mediated by an increased induction of the negative regulators of cytokine signalling; SOCS1 and SOCS3. In contrast, κ-CAS induced the secretion of TNF-α from human monocytes in a NFκB dependant manner (C). However, κ-CAS reduced the capacity of all 3 cell types to induce T-cell repsonce, significantly attenulating their ability to induce IL-2 from $CD4^+$ T-cells (D).

Given the powerful immune-modulatory effects exhibited by this fragment of k-CAS and considering that many human diseases are immune-related, there lies great potential and demand for its development as a immunomodulatory nutraceutical and possible use as a novel immune therapeutic to treat inflammatory diseases like IBD. Interest for the use of nutraceuticals in the management of diseases is growing as patients seek alternatives to the use of drugs that slow the progression of specific diseases, as they can often have unforeseen and potentially harmful side effects which can outweigh their benefits (Nongonierma & FitzGerald 2015). In this context, a κ-CAS bioactive protein fragment represents a viable alternative to the use of such drugs, as protein based therapeutics generally have low toxicity, are easily degraded and tend not to accumulate in bodily tissues (Gokhale & Satyanarayanajois 2014; Agyei *et al.*, 2016). Thus additional investigations are merited to identify the sequence of the fragment of κ-CAS responsible for the observed effects and elucidate the exact mechanisms by which these effects are exerted.

References

Abraham, C., & Cho, J.H. 2009. Inflammatory bowel disease. *N Engl J Med*, 361(21), pp. 2066-2078.

Abreu, M.T., Fukata, M., Arditi, M. 2005. TLR signaling in the gut in health and disease. J *Immunol*, 174(8), pp. 4453-4460.

Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M. 2014. Fasciola hepatica tegumental antigens indirectly induce an M2 macrophage-like phenotype in vivo. *Parasite Immunol*, 36, pp. 531-9. doi: 10.1111/pim.12127.

Agyei, D., Ongkudon, C.M., Wei, C.Y., Chan, A.S., Danquah, M.K. 2016. Bioprocess challenges to the isolation and purification of bioactive peptides. *Food and Bioproducts Processing*, 98, pp. 244–256.

Ahmed, C. M., Dabelic, R., Bedoya, S. K., Larkin, J., & Johnson, H. M. 2015. A SOCS1/3 Antagonist Peptide Protects Mice Against Lethal Infection with Influenza A Virus. *Frontiers in immunology*, 6, 574. doi:10.3389/fimmu.2015.00574

Aihara, K., Ishii, H. & Yoshida M. 2009. Casein-derived tripeptide, Val-Pro-Pro (VPP), modulates monocyte adhesion to vascular endothelium. *J Atheroscler Thromb*, 16(5), pp. 594-603.

Akira, S., Uematsu, S., Takeuchi, O. 2006. Pathogen recognition and innate immunity. *Cell*, 124(4), pp. 783-801.

Aldridge, A., O'Neill, S.M. 2016. Fasciola hepatica tegumental antigens induce anergic-like T cells via dendritic cells in a mannose receptor-dependent manner. *Eur J Immunol*, 46(5), pp.1180-1192. doi: 10.1002/eji.201545905.

Antoni, D., Burckel, H., Josset, E., & Noel, G. 2015. Three-dimensional cell culture: a breakthrough in vivo. *International journal of molecular sciences*, 16(3), pp. 5517–5527. doi:10.3390/ijms16035517

Anwar, M.A., Basith, S., & Choi, S. 2013. Negative regulatory approaches to the attenuation of Toll-like receptor signaling. Experimental & molecular medicine, 45(2), e11. doi:10.1038/emm.2013.28

Ashare, A., Powers, L.S., Butler, N.S., Doerschug, K.C., Monick, M.M., Hunninghake, G.W. 2005. Anti-inflammatory response is associated with mortality and severity of infection in sepsis. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 288, pp. 633–640.

Atreya, R., Zimmer, M., Bartsch, B., Waldner, M.J., Atreya, I., Neumann, H., Hildner, K., Hoffman, A., Kiesslich, R., Rink, A.D., Rau, T.T., Rose-John, S., Kessler, H., Schmidt, J., Neurath, M.F. 2011. Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal $CD14^{+}$ macrophages. *Gastroenterology*, 141(6), pp. 2026-2038. doi: 10.1053/j.gastro.2011.08.032.

Autissier, P., Soulas, C., Burdo, T.H., Williams, K.C. 2010. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. *Cytometry A*, 77(5), pp. 410-419.

Baba, N., Van, V.Q., Wakahara, K., Rubio, M., Fortin, G., Panzini, B., Soucy, G., Wassef, R., Richard, C., Tamaz, R., Lahaie, R., Bernard, E.J., Caussignac, Y., Leduc, R., Lougnarath, R., Bergeron, C., Racicot, M.A., Bergeron, F., Panzini, M.A., Demetter, P., Franchimont, D., Schäkel, K., Weckbecker, G., Kolbinger, F., Heusser, C., Huber, T., Welzenbach, K., Sarfati, M. 2013. CD47 fusion protein targets CD172a+ cells in Crohn's disease and dampens the production of IL-1β and TNF. *J Exp Med*, 210(6), pp. 1251-1263.

Bachmann, M. F., & Oxenius, A. 2007. Interleukin 2: from immunostimulation to immunoregulation and back again. *EMBO Reports*, 8(12), pp. 1142–1148. http://doi.org/10.1038/sj.embor.7401099

Baeuerle, P.A. 1998. Pro-inflammatory signaling: last pieces in the NF-κB puzzle? *Curr Biol*, 8, pp. 19–22.

Baldwin, A.S. 1995. The NF-κB and IκB proteins: new discoveries and insights. *Annu Rev Immunol*, 14, pp. 649–681.

Balsano, C., Alisi, A. 2009. Antioxidant effects of natural bioactive compounds. *Curr Pharm Des*, 15(26), pp. 3063-3073.

Bamdad, F., Shin, S.H., Suh, J.W., Nimalaratne, C., Sunwoo, H. 2017. Anti-Inflammatory and Antioxidant Properties of Casein Hydrolysate Produced Using High Hydrostatic Pressure Combined with Proteolytic Enzymes. *Molecules*, 22(4), pii: E609. doi: 10.3390/molecules22040609.

Banchereau, J., Briere, F., Caux, C., Daavoust, J., Lebecque, S., *et al.* 2000. Immunobiology of dendritic cells. *Annu Rev Immunol*, 18, pp. 767-811.

Banchereau, J. & Steinman, R.M. 1998. Dendritic cells and the control of immunity. *Nature*, 392, pp. 245–252. doi:10.1038/32588.

Banerjee, S., Biehl, A., Gadina, M., Hasni, S., Schwartz, D.M. 2017. JAK-STAT Signaling as a Target for Inflammatory and Autoimmune Diseases: Current and Future Prospects. *Drugs*, 77(5), pp. 521-546. doi: 10.1007/s40265-017-0701-9.

Bangchi, D., Zafra-Stone, S., & Bagchi, M. 2010. Recent advances in nutraceuticals and functional foods. In: Bangchi, D., Lau, F.C., & Bagchi, M. (eds.) Genomics, Proteomics and Metabolomics in Nutraceuticals and Functional Foods. Iowa, USA: Blackwell Publishing Inc.

Baumann, H., & Gauldie, J. 1994. The acute phase response. *Immunol Today*, 15, pp. 74–80.

Barton, G.M. & Kagan, J.C. 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol,* 9, pp.535-542.

Bates, J., & Diehl, L. 2014. Dendritic cells in IBD pathogenesis: an area of therapeutic opportunity? *J Pathol*, 232(2), pp. 112-120. doi: 10.1002/path.4277.

Baumgart, D.C., Thomas, S., Przesdzing, I., Metzke, D., Bielecki, C., Lehmann, S.M., Lehnardt, S., Dörffel, Y., Sturm, A., Scheffold, A., Schmitz, J., Radbruch, A. 2009. Exaggerated inflammatory response of primary human myeloid dendritic cells to lipopolysaccharide in patients with inflammatory bowel disease. *Clin Exp Immunol*, 157(3), pp. 423-436.

Begue, B., Wajant, H., Bambou, J.C., Dubuquoy, L., Siegmund, D., Beaulieu, J.F., Canioni, D., Berrebi, D., Brousse, N., Desreumaux, P., *et al*. 2006. Implication of TNF-related apoptosis-inducing ligand in inflammatory intestinal epithelial lesions. *Gastroenterology*, 130, pp. 1962–1974.

Bianchi, M.E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol*, 81, pp. 1–5. doi:10.1189/jlb.0306164.

Biswas, S.K., Chittezhath, M., Shalova, I.N., Lim, J.Y. 2012. Macrophage polarization and plasticity in health and disease. *Immunol Res*, 53, pp. 11–24.

Bouglé, D., & Bouhallab, S. 2015. Dietary bioactive peptides: Human studies. *Crit. Rev. Food Sci. Nutr*, 57, pp. 335–343.

Boyman, O., Kolios, A.G., & Raeber, M.E. 2015. Modulation of T cell responses by IL-2 and IL-2 complexes. *Clin Exp Rheumatol*, 33(4 Suppl 92), pp. S54-57.

Bradley, J.R. 2008. TNF-mediated inflammatory disease. *J Pathol*, 214(2), pp. 149-160.

Breese, E., Braegger, C.P., Corrigan, C.J., Walker-Smith, J.A., MacDonald, T.T. 1993. Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. *Immunology*, 78(1), pp. 127-131.

Brill, A., Baram, D., Sela, U., Salamon, P., Mekori, Y.A., Hershkoviz, R. 2004. Induction of mast cell interactions with blood vessel wall components by direct contact with intact T cells or T cell membranes in vitro. *Clin Exp Allergy.* 34(11), pp. 1725-1731.

Brown, K., Park, S., Kanno, T., Granzoso, G., & Siebenlist, U. 1993. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc. Natl. Acad. Sci. U.S.A*, 90, pp. 2532–2536.

Buchbinder, E. I., & Desai, A. 2016. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *American Journal of Clinical Oncology*, 39(1), pp. 98–106. http://doi.org/10.1097/COC.0000000000000239

Butte, M.J., Keir, M.E., Phamduy, T.B., Sharpe, A.H. and Freeman, G.J. 2007. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity*, 27(1), pp.111-122.

Cacalano, N., Yoshimura, A. and Mui, A.L. 2006. Divergent mechanisms utilized by SOCS3 to mediate interleukin-10 inhibition of tumor necrosis factor a and nitric oxide production by macrophages. *J. Biol. Chem*, 281, pp. 6316–6324.

Cao, Q., Wang, Y., Zheng, D., Sun, Y., Wang, Y., Lee, V.W., Zheng, G., Tan, T.K., Ince, J., Alexander, S.I., Harris, D.C. 2010. IL-10/TGF-beta-modified macrophages induce regulatory T cells and protect against adriamycin nephrosis. *J Am Soc Nephrol*, 21(6), pp. 933-942. doi: 10.1681/ASN.2009060592.

Carow, B., & Rottenberg, M.E. 2014. SOCS3, a Major Regulator of Infection and Inflammation. *Frontiers in Immunology*, 5(58), pp. 1-13.

Carr, R.I., Webster, D., Sadi, D., Williams, H. & Walsh, N. 1990. Immunomodulation by opioids from dietary casein. *Annals of the New York Academy of Sciences*, 574, pp. 374-376.

Cassetta, L., Cassol, E., & Poli, G. 2011. Macrophage Polarization in Health and Disease. *ScientificWorldJournal*, 11, pp. 2391–2402.

Cederlund, A., Gudmundsson, G.H., Agerberth, B. 2011. Antimicrobial peptides important in innate immunity. *FEBS J*, 278, pp. 3942-3951.

Cejas, P.J., Carlson, L.M., Zhang, J., Padmanabhan, S., Kolonias, D., Lindner, I., Haley, S., Boise, L.H., Lee, K.P. 2005. Protein kinase C betaII plays an essential role in dendritic cell differentiation and autoregulates its own expression. *J Biol Chem*, 280(31), pp. 28412-28423.

Chakrabarti, S., Jana, M., Roy, A., & Pahan, K. 2018. Upregulation of Suppressor of Cytokine Signaling 3 in Microglia by Cinnamic Acid. *Current Alzheimer research*, 15(10), pp. 894-904.

Chan, E.D., & Riches, D.W. 2001. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am. J. Physiol. Cell Physiol*, 280, pp. 441–450.

Chassaing, B., Aitken, J. D., Malleshappa, M., & Vijay-Kumar, M. 2014. Dextran sulfate sodium (DSS)-induced colitis in mice. *Current protocols in immunology*, 104, pp. 15-25. doi:10.1002/0471142735.im1525s104.

Chatterton, D.E.W., Nguyen, D.N., Bering, S.B., & Sangild, P.T. 2013 Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *The International Journal of Biochemistry and Cell Biology*, 45, pp. 1730–1747.

Chen, H.M., Muramoto, K., & Yamauchi, F. 1995. Structural Analysis of Antioxidative Peptides from Soybean beta.-Conglycinin. *J. Agric. Food Chem*, 43, pp. 574–578.

Cheng, X., Gao, D., Chen, B. and Mao, X. 2015. Endotoxin-Binding Peptides Derived from Casein Glycomacropeptide Inhibit Lipopolysaccharide-Stimulated Inflammatory Responses via Blockade of NF-κB activation in macrophages. *Nutrients*, 7, pp. 3119-3137.

Cobrin, G.M., & Abreu, M.T. 2005. Defects in mucosal immunity leading to Crohn's disease. *Immunol Rev*. 206, pp. 277-295.

Cohen, B.,L. 2017. Update on anti-tumor necrosis factor agents and other new drugs for inflammatory bowel disease. *BMJ*, 357, j2505. doi: https://doi.org/10.1136/bmj.j2505.

Cordeiro-da-Silva, A., Tavares, J., Araújo, N., Cerqueira, F., Tomás, A., Kong Thoo Lin, P., Ouaissi, A. 2004. Immunological alterations induced by polyamine derivatives on murine splenocytes and human mononuclear cells. *Int Immunopharmacol*, 4(4), pp. 547-556.

Craik, D.J., Fairlie, D.P., Liras, S., Price, D. 2013. The Future of Peptide-based Drugs. *Chemical discovery and drug design.* 81 (1), pp. 136-147.

Croft, M., So, T., Duan, W., & Soroosh, P. 2009. The significance of OX40 and OX40L to T-cell biology and immune disease. *Immunological reviews*, 229(1), pp. 173-191.

Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S.Y., Senechal, B., Puel, A., Biswas, S.K., Moshous, D., Picard, C., Jais, J.P., D'Cruz, D., Casanova, J.L., Trouillet, C., Geissmann, F. 2010. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*, 33(3), pp. 375-386.

Cross, M.L. & Gill, H.S. 2000. Immunomodulatory properties of milk. *Br J Nutr*, 84, Suppl 1, S81-89.

Curtsinger, J.M. & Mescher, M.F. 2010. Inflammatory cytokines as a third signal for T cell activation. Curr Opin Immunol, 22(3), pp. 333-340. doi: 10.1016/j.coi.2010.02.013.

Curtsinger, J.M., Schmidt, C.S., Mondino, A., Lins, D.C., Kedl, R.M., Jenkins, M.K., Mescher, M.F. 1999. Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. J Immunol, 162(6), pp. 3256-3262.

Daddaoua, A., Puerta, V., Zarzuelo, A., Suarez, M.D., Sanchez de Medina, F., Martinez-Augustin, O. 2005. Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *Journal of Nutrition*, 135, pp. 1164–1170.

Dalgleish, D. G., & Law, A. J. R. 1988. Sodium caseinates ? composition and properties of different preparations. *International Journal of Dairy Technology*, 41(1), pp. 1–4.

Dalpke, A.H., Opper, S., Zimmermann, S., Heeg, K. 2001. Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. *J Immunol*, 166(12): pp. 7082-7089.

De Kruif, C.G., Holt, C., 2003. Casein micelle structure, functions and interactions. In: Fox, P.F., McSweeney, P.L.H. (Eds.), Advanced Dairy Chemistry, third ed. Proteins, vol. 1. Kluwer Academic/Plenum Publishers, New York, pp. 233–276.

Dhaval, A., Yadav, N., & Purwar, S. 2016. Potential Applications of Food Derived Bioactive Peptides in Management of Health. *Int. J. Pept. Res. Ther*, 22, pp. 377–398.

Dillon, S.M., Rogers, L.M., Howe, R., Hostetler, L.A., Buhrman, J., McCarter, M.D., Wilson, C.C. 2010. Human intestinal lamina propria CD1c+ dendritic cells display an activated phenotype at steady state and produce IL-23 in response to TLR7/8 stimulation. *J Immunol*, 184(12), pp. 6612-6621.

Dominguez-Melendez, V., Silvestre-Santana, O., Moreno-Fierros, L., Aguiniga-Sanchez, I., Martinez, L., Marroquin-Segura, R., *et al.* 2012. Sodium caseinate induces mouse granulopoiesis. *Inflammation Research*, 61, pp. 367–373.

Donnelly, S. *et al.,* 2005. Thioredoxin peroxidase secreted by Fasciola hepatica induces the alternative activation of macrophages. *Infection and immunity*, 73(1), pp.166–73.

Donnelly, R. P., S. L. Freeman, and M. P. Hayes. 1995. Inhibition of IL-10 expression by IFN-gamma up-regulates transcription of TNF-alpha in human monocytes. *J. Immunol*, 155, pp. 1420–1427.

Dowling, D., Hamilton, C.M., & O'Neill, S.M. 2008. A comparative analysis of cytokine responses, cell surface marker expression and MAPKs in DCs matured with LPS compared with a panel of TLR ligands. *Cytokine,* 41(3), pp. 254-62. doi: 10.1016/j.cyto.2007.11.020.

Driessler, F., K. Venstrom, R. Sabat, K. Asadullah, and A. J. Schottelius. 2004. Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. *Clin. Exp. Immunol,* 135, pp. 64–73.

Dudek, A.M., Martin, S., Garg, A.D., Agostinis, P. 2013. Immature, semi-mature, and fully mature dendritic cells: toward a DC-cancer cells interface that augments anticancer immunity. *Front Immunol*, 4, pp. 438. doi:10.3389/fimmu.2013.00438.

Duffield, J.S. 2003. The inflammatory macrophage: a story of Jekyll and Hyde. *Clinical Science (London)*. 104, pp. 27–38.

Dunay, I.R., Fuchs, A., Sibley, L.D. 2010. Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice. *Infect Immun,* 78(4), pp. 1564–1570. doi: 10.1128/IAI.00472-09.

Duncan, S. A., Baganizi, D. R., Sahu, R., Singh, S. R., & Dennis, V. A. 2017. SOCS Proteins as Regulators of Inflammatory Responses Induced by Bacterial Infections: A Review. *Frontiers in Microbiology*, 8, pp. 2431. http://doi.org/10.3389/fmicb.2017.02431

Dziuba, M., Dziuba, B., & Iwaniak, A. 2009. A milk proteins as precursors of bioactive peptides. *Acta Scientiarum Polonorum. Technologia Alimentaria*, 8, pp. 71–90.

Ebrahimpour, S., Shahbazi, M., Khalili, A., Tahoori, M.T., Zavaran Hosseini, A., Amari, A., Aghili, B., Abediankenari, S., Mohammadizad, H., Mohammadnia-Afrouzi, M. 2017. Elevated levels of IL-2 and IL-21 produced by CD4+ T cells in inflammatory bowel disease. *J Biol Regul Homeost Agents*, 31(2), pp. 279-287.

Ehrenstein, M.R., Evans, J.G., Singh, A., Moore, S., Warnes, G., Isenberg, D.A., Mauri, C. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med*, 200(3), pp. 277-285.

El-Gabalawy, H., Guenther, Lyn C., and Bernstein, Charles, N. 2010. Epidemiology of Immune-Mediated Inflammatory Diseases: Incidence, Prevalence, Natural History, and Comorbidities. *J Rheumatol Suppl*, 85, pp. 2-10. doi: 10.3899/jrheum.091461.

Elitsur, Y. & Luk, G.V. 1991. Beta-casomorphin (BCM) and human colonic lamina propria lymphocyte proliferation. *Clinical and Experimental Immunology.* 85, pp. 493–497.

Eming, S. A., Krieg, T., & Davidson, J.M. 2007. Inflammation in wound repair: molecular and cellular mechanisms. J. *Invest. Dermatol*, 127, pp. 514–525.

Espagnolle, N., Balguerie, A., Arnaud, E., Sensebé, L., & Varin, A. 2017. CD54-Mediated Interaction with Pro-inflammatory Macrophages Increases the Immunosuppressive Function of Human Mesenchymal Stromal Cells. Stem cell reports, 8(4), pp. 961-976.

Evans, H. G., Gullick, N. J., Kelly, S., Pitzalis, C., Lord, G. M., Kirkham, B. W., & Taams, L. S. 2009. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proceedings of the National Academy of Sciences of the United States of America*, 106(15), pp. 6232-6237.

Evans, H.G., Suddason, T., Jackson, I., Taams, L.S., Lord, G.M. 2007. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor activated monocytes. *Proc Natl Acad Sci USA*, 104, pp. 17034 –17039.

Evel-Kabler, K., Song, X. T., Aldrich, M., Huang, X. F., & Chen, S. Y. 2005. SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. The Journal of clinical investigation, 116(1), pp. 90-100.

Farrell, H.M., Malin, E.L., Brown, E.M., & Mora-Gutierrez, A. 2009. Review of the chemistry of αS2-casein and the generation of a homologous molecular model to explain its properties. 92(4), pp. 1338–1353. doi: https://doi.org/10.3168/jds.2008-1711.

Farrell, H.M., Brown, E.M., Hoagland, P.D., Malin, E.L., 2003. Higher order structures of the caseins: a paradox? In: Fox, P.F., McSweeney, P.L.H. (Eds.), Advanced Dairy Chemistry. Proteins, vol. 1. Springer, New York, pp. 203–231.

Feghali, C. A., & Wright, T.M. 1997. Cytokines in acute and chronic inflammation. Front. Biosci. 2: d12–26.

Feng, Y., & Chao, W. 2012. Toll-like receptors and myocardial inflammation. *Int J Inflam*, 2011, 170352. doi: 10.4061/2011/170352.

Fernandez-Fernandez, A.M., Lopez-Pedemonte, T., Medrano-Fernandez, A. 2017. Evaluation of antioxidant, antiglycant and ACE-inhibitory activity in enzymatic hydrolysates of α-lactalbumin. *Food Nutr. Sci*, 8, pp. 84–98. doi: 10.4236/fns.2017.81006.

Ferreiro, D. U., & Komives, E. A. 2010. Molecular Mechanisms of System Control of NF-κB Signaling by IκBα. *Biochemistry*, 49(8), pp. 1560–1567.

Figueroa-Santiago, O., & Espino, A.M. 2014. Fasciola hepatica fatty acid binding protein induces the alternative activation of human macrophages. *Infection and immunity*, 82(12), pp. 5005-5012.

Food and Agriculture Organization of the United Nations (FAO), authors Report on Functional Foods, Food Quality and Standards Service (AGNS) 2007. Available online: http://www.fao.org/ag/agn/agns/files/Functional_Foods_Report_Nov2007.pdf.

Fox, P.F. 2009. Milk: an overview. In: Thompson, A., Boland, M., & Singh, H. (eds.) Milk Proteins from expression to food. New York: Elsevier, Inc., pp. 1-54.

Fukui, S., Iwamoto, N., Takatani, A., Igawa, T., Shimizu, T., Umeda, M., Nishino, A., Horai, Y., Hirai, Y., Koga, T., Kawashiri, S. Y., Tamai, M., Ichinose, K., Nakamura, H., Origuchi, T., Masuyama, R., Kosai, K., Yanagihara, K., Kawakami, A. 2018. M1 and M2 Monocytes in Rheumatoid Arthritis: A Contribution of Imbalance of M1/M2 Monocytes to Osteoclastogenesis. *Frontiers in immunology*, 8, 1958. doi:10.3389/fimmu.2017.01958.

Garrett, W.S., Lord, G.M., Punit, S., Lugo-Villarino, G., Mazmanian, S.K., Ito, S., Glickman, J.N., Glimcher, L.H. 2007. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell*, 131(1), pp. 33-45.

Geijtenbeek, T.B., Engering, A. and Van Kooyk, Y. 2002. DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology. J. Leukocyte Biol, 71, pp. 921.

Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y., Figdor, C.G. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*, 100(5), pp. 575-585.

Geissmann, F., Auffray, C., Palframan, R., Wirrig, C., Ciocca, A, Campisi, L., Narni-Mancinelli, E., Lauvau, G. 2008. Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses. *Immunol Cell Biol*, 86(5), pp. 398-408. doi: 10.1038/icb.2008.19.

Geissmann, F., Jung, S., Littman, D.R. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*, 19(1), pp. 71-82.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., Ley, K. 2010. Development of monocytes, macrophages and dendritic cells. *Science*, 327, pp. 656–661.

Ghashghaeinia, M., Toulany, M., Saki, M., Bobbala, D., Fehrenbacher, B., Rupec, R., Rodemann, H.P., Ghoreschi, K., Röcken, M., Schaller, M., Lang, F., Wieder, T. The NFκB pathway inhibitors Bay 11-7082 and parthenolide induce programmed cell death in anucleated Erythrocytes. Cell Physiol. Biochem. 2011, 27, 45-54. doi: 10.1159/000325204.

Gill, H.S., Doull, F., Rutherfurd, K.J., Cross, M.L. 2000. Immunoregulatory peptides in bovine milk. *British Journal of Nutrition*. 84, pp.S111-S117.

Gill, H.S., Rutherfurd, K.J. & Cross, M.L. 2000. Bovine milk: A unique source of immumodulatory ingredients for functional foods. *In Functional Foods II - Claims and Evidence*, pp. 82- 90. [J Buttriss and M Saltmarsh, editors]. Cambridge: Royal Society of Chemistry Press.

Givens, D., Livingstone, K., Pickering, J., Fekete, Á., Dougkas, A., Elwood, P. 2014. Milk: White elixir or white poison? An examination of the associations between dairy consumption and disease in human subjects. *Anim Front*, 4, pp. 8-15.

Gokhale, A.S., & Satyanarayanajois, S. 2014. Peptides and peptidomimetics as immunomodulators. *Immunotherapy,* 6, pp. 755–774.

Gordon, S. & Taylor. P.R. *2005.* Monocyte and macrophage heterogeneity. *Nat Rev Immunol.* 5(12), pp. 953-964.

Green, D.R., Ferguson, T., Zitvogel, L., Kroemer, G. 2009. Immunogenic and tolerogenic cell death. *Nat Rev Immunol*, 9, pp. 353–363. doi:10.1038/nri2545.

Greeneltch, K.M., Haudenschild, C.C., Keegan, A.D., Shi, Y. 2004. The opioid antagonist naltrexone blocks acute endotoxic shock by inhibiting tumor necrosis factor-alpha production. *Brain Behav Immun*, 18(5), pp. 476-484.

Gren, S.T., & Grip, O. 2016. Role of Monocytes and Intestinal Macrophages in Crohn's Disease and Ulcerative Colitis. *Inflamm Bowel Dis*, 22(8), pp. 1992-1998. doi: 10.1097/MIB.0000000000000824.

Grewal, I. S., and Flavell R.A. 1996. The role of CD40 ligand in costimulation and T cell activation. *Immunol Rev*, 153, pp. 85–106.

Grip, O., Bredberg, A., Lindgren, S., Henriksson, G. 2007. Increased subpopulations of CD16(+) and CD56(+) blood monocytes in patients with active Crohn's disease. *Inflamm Bowel Dis*, 13(5), pp. 566-572.

Gu, W., Yao, L., Li, L., Zhang, J., Place, A. T., Minshall, R. D., & Liu, G. 2017. ICAM-1 regulates macrophage polarization by suppressing MCP-1 expression via miR-124 upregulation. *Oncotarget*, 8(67), pp. 111882–111901.

Guha, M., O'Connell, M.A., Pawlinski, R., Hollis, A., McGovern, P., Yan, S.F., Stern, D., & Mackman, N. 2001. Lipopolysaccharide activation of the MEK-ERK1/2 pathway in human monocytic cells mediates tissue factor and tumor necrosis factor alpha expression by inducing Elk-1 phosphorylation and Egr-1 expression. *Blood,* 98, pp. 1429–1439.

Hanada, T., Tanaka, K., Matsumura, Y., Yamauchi, M., Nishinakamura, H., Aburatani, H., Mashima, R., Kubo, M., Kobayashi, T., Yoshimura, A. 2005. Induction of hyper Th1 cell-type immune responses by dendritic cells lacking the suppressor of cytokine signaling-1 gene. J Immunol, 174(7), pp. 4325-4332.

Hancock, R.E.W., & Sahl, H.G. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol*, 24, pp. 1551-1557.

Harden, J.L., & Egilmez, N.K. 2012. Indoleamine 2,3-dioxygenase and dendritic cell tolerogenicity. *Immunol Invest*, 41:738–64. doi:10.3109/08820139.2012.676122.

Hart, A.L., Al-Hassi, H.O., Rigby, R.J., Bell, S.J., Emmanuel, A.V., Knight, S.C., Kamm, M.A., Stagg, A.J. 2005. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology*, 129(1), pp. 50-65.

Heufler, C., Koch, F., Stanzl, U., Topar, G., Wysocka, M., Trinchieri, G., Enk, A., Steinman, R.M., Romani, N., Schuler, G. 1996. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. *Eur J Immunol*, 26(3), pp. 659-668.

Hoffman, K.M., Ho, D.G. & Sampson, H.A. 1997. Evaluation of the usefulness of lymphocyte proliferation assays in the diagnosis of allergy to cow's milk. J *Allergy Clin Immunol*, 99, pp. 360–366.

Holden, N.S., Squires, P.E., Kaur, M., Bland, R., Jones, C.E., Newton, R. 2008. Phorbol ester-stimulated NF-kB-dependent transcription: roles for isoforms of novel protein kinase C, Cell Signal, 20(7), pp. 1338–1348.

Holmer-Jensen, J., Karhu, T., Mortensen, L.S., Pedersen, S.B., Herzig, K.H., Hermansen, K. 2011. Differential effects of dietary protein sources on postprandial low-grade inflammation after a single high fat meal in obese non-diabetic subjects. *Nutr J*, 10, pp. 115.

Hoving, J.C., Wilson, G.J., & Brown, G.D. 2014. Signalling C-Type lectin receptors, microbial recognition and immunity. *Cell Microbiol*. 16(2), pp. 185–194.

Hovsepian, E., F. Penas, S. Siffo, G. A. Mirkin, and N. B. Goren. 2013. IL-10 inhibits the NF-κB and ERK/MAPK-mediated production of pro-inflammatory mediators by up-regulation of SOCS-3 in Trypanosoma cruzi-infected cardiomyocytes. *PLoS One* 8, e79445.

Hubbard, A.K. & Giardina, C. 2000. Regulation of ICAM-1 expression in mouse macrophages. *Inflammation.* 24(2), pp. 115-125.

Hume, D. A. 2008. Macrophages as APC and the dendritic cell myth. *J. Immunol*, 181, pp. 5829–5835.

Huppertz, T., Fox, P.F., & Kelly, A.L. 2018. The caseins: Structure, stability, and functionality. In R. Y. Yada 298 (Ed.), Proteins in Food Processing (Second Edition), pp. 49-92.

Hvas, C.L., Dige, A., Bendix, M., Wernlund, P.G., Christensen, L.A., Dahlerup, J.F., Agnholt, J. 2016. Casein glycomacropeptide for active distal ulcerative colitis: a randomized pilot study. Eur *J Clin Invest*, 46(6), pp. 555-563. doi: 10.1111/eci.12634.

Iliev, I.D., Spadoni, I., Mileti, E., Matteoli, G., Sonzogni, A., Sampietro, G.M., Foschi, D., Caprioli, F., Viale, G., Rescigno, M. 2009. Human intestinal epithelial cells promote the differentiation of tolerogenic dendritic cells. Gut, 58(11), pp. 1481-1489.

Italiani, P., & Boraschi, D. 2014. From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation. *Front. Immunol.* 5.

Iwasaki, A. & Medzhitov, R. 2010. Regulation of adaptive immunity by the innate immune system. *Science*, 327, pp. 291–295. doi:10.1126/science.1183021.

Jani, P., Manseta, P., Patel, S. 2012. Pharmaceutical Approaches related to Systemic Delivery of Protein and Peptide Drugs: An Overview, *Int J Pharm Sci Rev Res*, pp. 42-52.

Jersmann, H. P., Hii, C.S., Ferrante, J.V., & Ferrante, A. 2001. Bacterial lipopolysaccharide and tumor necrosis factor alpha synergistically increase expression of human endothelial adhesion molecules through activation of NF-kappaB and p38 mitogen-activated protein kinase signaling pathways. *Infect. Immun*, 69, 1273–1279.

Jiang, Z. & Zhu, L. 2016. Update on the role of alternatively activated macrophages in asthma. *J Asthma Allergy*. 9, pp. 101-107.

Jin, M., Opalek, J.M., Marsh, C.B., Wu, H.M. 2004. Proteome comparison of alveolar macrophages with monocytes reveals distinct protein characteristics. *Am J Respir Cell Mol Biol*, 31(3), pp. 322-329.

Jo, D., Liu, D., Yao, S., Collins, R.D., Hawiger, J. 2005. Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis. *Nat Med*, 11, pp. 892–810.

Jollès, P. & Fiat, A.M. 1979. The carbohydrate portions of milk glycoproteins. *J Dairy Res*. 46(2), pp. 187-191.

Jones, G.E. 2000. Cellular signaling in macrophage migration and chemotaxis. *Journal of Leukocyte Biology.* 68, pp. 593–602.

Juillerat-Jeanneret, L., Robert, M.C., & Juillerat, M.A. 2011. Peptides from Lactobacillus hydrolysates of bovine milk caseins inhibit prolylpeptidases of human colon cells. *Journal of Agricultural and Food Chemistry*, 59, pp. 370–377.

Kalra, E.,K. 2003. Nutraceutical--definition and introduction. *AAPS PharmSci*, 5(3), E25.

Kamada, N., Hisamatsu, T., Okamoto, S., Chinen, H., Kobayashi, T., Sato, T., Sakuraba, A., Kitazume, M.T., Sugita, A., Koganei, K., Akagawa, K.S., Hibi, T. 2008. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *J Clin Invest*, 118(6), pp. 2269-2280. doi: 10.1172/JCI34610.

Karin, M., & Ben-Neriah, Y. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol*, 18, pp. 621–663.

Kaspar, A.A., & Reichert, J.M. 2013. Future directions for peptide therapeutics development, Vorabveröffentlichung, *Drug Discovery Today*, 18 (17-18), pp. 807-817.

Kawai, T., Akira, S. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol*, 21(4), pp. 317-337.

Kawanaka, N., Yamamura, M., Aita, T., Morita, Y., Okamoto, A., Kawashima, M., Iwahashi, M., Ueno, A., Ohmoto, Y., Makino, H. 2002. CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis Rheum,* 46(10), pp. 2578-2586.

Kiesler, P., Fuss, I.J., Strober, W. 2015. Experimental Models of Inflammatory Bowel Diseases. *Cell Mol Gastroenterol Hepatol*, 2015 1(2), pp. 154-170.

Kiewiet, M.B.G., Dekkers, R., Gros, M., van Neerven, R.J.J., Groeneveld, A., de Vos, P., Faas, M.M. 2017. Toll-like receptor mediated activation is possibly involved in

immunoregulating properties of cow's milk hydrolysates. *PLoS One*, 12(6): e0178191. doi: 10.1371/journal.pone.0178191.ment. 85, pp.2-10.

Kim, Y.G., Kamada, N., Shaw, M.H., Warner, N., Chen, G.Y., Franchi, L., Núñez, G. 2011. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. *Immunity*, 34(5), pp. 769-780.

Kimura, A., Naka, T., Muta, T., Takeuchi, O., Akira, S., Kawase, I., & Kishimoto, T. 2005. Suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production by regulating JAK–STAT. *Proceedings of the National Academy of Sciences of the United States of America*, 102(47), pp. 17089–17094. http://doi.org/10.1073/pnas.0508517102.

Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M., Yoshimura, A. 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity*, 17(5), pp. 583-91.

Kitazawa, H., Yonezawa, K., Tohno, M., Shimosato, T., Kawai, Y., Saito, T., & Wang, J. M. 2007. Enzymatic digestion of the milk protein beta-casein releases potent chemotactic peptide(s) for monocytes and macrophages. *International immunopharmacology*, 7(9), pp. 1150-1159.

Kitts, D.D., & Weiler, K. 2003. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design*, 9 (16), pp. 1309-1323.

Klatzmann, D., & Abbas, A.K. 2015. The promise of low-dose interleukin-2 therapy for autoimmune and inflammatory diseases. *Nat Rev Immunol*, 15, pp. 283-294.

Koh, T.J., & DiPietro, L.A. 2011. Inflammation and wound healing: the role of the macrophage. *Expert Rev. Mol. Med.* 13, e23.

Kohchi, C., Inagawa, H., Nishizawa, T., Soma, G. 2009. ROS and innate immunity. *Anticancer Res*, 29(3), pp. 817-821.

Kreider, T., Anthony, R.M., Urban, J.F., & Gause, W.C. 2007. Alternatively activated macrophages in helminth infections. *Current opinion in immunology*, *19*(4), pp. 448-453.

Kubo, M., Hanada, T., Yoshimura, A. 2003. Suppressors of cytokine signaling and immunity. Nat Immunol, 4(12), pp. 1169-1176.

Kumar, S., Dwivedi, P.D., Das, M., Tripathi, A. 2013. Macrophages in food allergy: An enigma. *Molecular Immunology*. 56, pp. 612–618.

Kumosinski, T.F., King, G., Farrell Jr., H.M., 1994a. An energy-minimized casein submicelle working model. *J. Protein Chem*, 13, pp. 681–700.

Kwiatkowski, D., Hill, A.V., Sambou, I., Twumasi, P., Castracane, J., Manogue, K.R., Cerami, A., Brewster, D.R., Greenwood, B.M. 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria. *Lancet*, 336(8725), pp. 1201-1204.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, pp.680-685.

Laird, M. H. W., S. H. Rhee, D. J. Perkins, A. E. Medvedev, W. Piao, M. J. Fenton, and S. N. Vogel. 2009. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. *J. Leukoc. Biol*, 85, pp. 966–977.

Lawrence, T. 2009. The Nuclear Factor NF-κB Pathway in Inflammation. *Cold Spring Harbor Perspectives in Biology*, 1(6), a001651. http://doi.org/10.1101/cshperspect.a001651

Lemieux, L., Piot, J.M., Guillochon, D., Amiot, J. 1991. Study of the efficiency of a mobile phase used in size-exclusion HPLC for the separation of peptides from a casein hydrolysate according to their hydrodynamic volume. *Chromatographia*, 32, pp. 499–504.

Lenschow, D.J., Walunas, T.L. and Bluestone, J.A. 1996. CD28/B7 system of T cell costimulation. Annual Review of Immunology, 14(1), pp.233-258.

León, B., López-Bravo, M., Ardavín, C. 2005. Monocyte-derived dendritic cells. *Semin Immunol*, 17(4), pp. 313-318.

Li, S.N., *et al.* 2013. IL-21 modulates release of proinflammatory cytokines in LPS-stimulated macrophages through distinct signaling pathways. *Mediators Inflamm 2013*, e548073.

Li, T., Cheng, X., Du, M., Chen, B., Mao, X. 2017. Upregulation of heme oxygenase-1 mediates the anti-inflammatory activity of casein glycomacropeptide (GMP) hydrolysates in LPS-stimulated macrophages. *Food Funct*, 8(7). Pp. 2475-2484.

Li, Y., Chu, N., Rostami, A. and Zhang, G. 2006. Dendritic Cells Transduced with SOCS-3 Exhibit a Tolerogenic/DC2 Phenotype That Directs Type 2 Th Cell Differentiation In Vitro and In Vivo. *J Immunol*, 177(3), pp. 1679-1688.

Li, Y., de Haar, C., Peppelenbosch, M.P., van der Woude, C.J. 2012. SOCS3 in immune regulation of inflammatory bowel disease and inflammatory bowel disease-related cancer. *Cytokine Growth Factor Rev*, 23(3), pp. 127-138. doi: 10.1016/j.cytogfr.2012.04.005.

Li, J., Hsu, H. C., & Mountz, J. D. 2012. Managing macrophages in rheumatoid arthritis by reform or removal. *Current rheumatology reports*, 14(5), pp. 445-454.

Li, J., Hsu, H.C., Yang, P., *et al.* 2012. Treatment of arthritis by macrophage depletion and immunomodulation: testing an apoptosis-mediated therapy in a humanized death receptor mouse model. *Arthritis Rheum*, 64, pp. 1098–1109.

Li, E.W.Y., & Mine, Y. 2004 Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response andphagocytic activities of human macrophage like cells U937. *Journal of Agricultural and Food Chemistry*, 52, pp. 2704–2708.

Lichtenegger, F. S., Mueller, K., Otte, B., Beck, B., Hiddemann, W., Schendel, D. J., & Subklewe, M. 2012. CD86 and IL-12p70 Are Key Players for T Helper 1 Polarization and Natural Killer Cell Activation by Toll-Like Receptor-Induced Dendritic Cells. *PLoS ONE*, 7(9), e44266. http://doi.org/10.1371/journal.pone.0044266.

Lindner, I., Cejas, P.J., Carlson, L.M., Torruellas, J., Plano, G.V., Lee, K.P. 2007. Signal transduction in DC differentiation: winged messengers and Achilles' heel. *Adv Exp Med Biol*, 590, pp. 1-29.

Lissner, D., Schumann, M., Batra, A., Kredel, L.I., Kühl, A.A., Erben, U., May, C., Schulzke, J.D., Siegmund, B. 2015. Monocyte and M1 Macrophage-induced Barrier Defect Contributes to Chronic Intestinal Inflammation in IBD. *Inflamm Bowel Dis*, 21(6), pp. 1297-1305. doi: 10.1097/MIB.0000000000000384.

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. 2017. NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, 2, 17023. http://doi.org/10.1038/sigtrans.2017.23

Liu, X., Zhang, Y., Yu, Y., Yang, X., Cao, X. 2008. SOCS3 promotes TLR4 response in macrophages by feedback inhibiting TGF-beta1/Smad3 signaling. *Mol Immunol,* 45(5), pp. 1405-1413.

Lodes, M. J., Cong, Y., Elson, C. O., Mohamath, R., Landers, C. J., Targan, S. R., Fort, M., Hershberg, R. M. 2004. Bacterial flagellin is a dominant antigen in Crohn disease. *The Journal of clinical investigation*, 113(9), pp. 1296-1306.

Lombardo, E., Alvarez-Barrientos, A., Maroto, B., Boscá, L., & Knaus, U.G. 2007. TLR4-mediated survival of macrophages is MyD88 dependent and requires TNF-alpha autocrine signalling. *J. Immunol*, 178, pp. 3731–3739.

Lopez-Posadas, R., Requena, P., Gonzalez, R., Suarez, M.D., Zarzuelo, A., Sanchez de Medina, F. *et al.* 2010. Bovine glycomacropeptide has intestinal anti-inflammatory effects in rats with dextran sulfate-induced colitis. *Journal of Nutrition*, 140, pp. 2014–2019.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rössner, S., Koch, F., Romani, N., Schuler, G. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods, 223(1), pp.77-92.

Lutz, M.B., & Schuler, G. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol*, 23, pp. 445–449. doi:10.1016/S1471-4906(02)02281-0.

Ma, J., Wang, J.H., Guo, Y.J., Sy, M.S., Bigby, M. 1994. In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes. *Cell Immunol,* 158(2), pp. 389-399.

Maloy, K.J., & Powrie, F. 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*, 474, pp. 298–306.

Mansell, A., Smith, R., Doyle, S.L., Gray, P., Fenner, J.E., Crack, P.J., Nicholson, S.E., Hilton, D.J., O'Neill, L.A., Hertzog, P.J. 2006. Suppressor of cytokine signaling 1

negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat Immunol*, 7(2), pp. 148-155.

Mantovani, A., Biswas, S.K., Galdiero, M.R., Sica, A., Locati, M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*, 229(2), pp. 176-185. doi: 10.1002/path.4133.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., Locati, M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*, 25(12), pp. 677-86.

Marble, D.J., Gordon, K.B., Nickoloff, B.J. 2007. Targeting TNFalpha rapidly reduces density of dendritic cells and macrophages in psoriatic plaques with restoration of epidermal keratinocyte differentiation. *J Dermatol Sci*, 48(2), pp. 87-101.

Marine, J.C., Topham, D.J., McKay, C., Wang, D., Parganas, E., Stravopodis, D, *et al*. 1999. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell*, 98, pp. 609–616.

Martinez, F., & Gordon, S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep*, pp. 6-13. doi: 10.12703/P6-13.

Martinez, F., Gordon, S., Locati, M., & Mantovani, A. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol*, 177, pp. 7303–7311.

Martinez, F.O., Helming, L. & Gordon, S. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol,* 27, pp. 451-483.

Martinez, F.O., Sica, A., Mantovani, A., Locati, M. 2008. Macrophage activation and polarization. *Front Biosci,* 13, pp. 453-461.

Martinez-Pomares, L. 2012. The mannose receptor. *Jouornal of Leukocyte Biology*, 92, pp. 1177-1186.

Marx, V. 2005. Watching Peptides Grow Up. *Chemical and Engineering News,* 83, pp. 17-24.

Mason, J.M. 2010. Design and development of peptides and peptide mimetics as antagonists for therapeutic intervention. *Future medicinal chemistry*, 2, pp. 1813-1822.

McCarthy, D.A., Macey, M.G., Bedford, P.A., Knight, S.C., Dumonde, D.C., Brown, K.A. 1997. Adhesion molecules are upregulated on dendritic cells isolated from human blood. *Immunology*, 92(2), pp. 244-251.

McGuckin, M.A., Eri, R., Simms, L.A., *et al.* 2009. Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis*, 15, pp. 100–113.

McWhitney, R.L. 1988. Proteins of milk. In: Wing NP, editor. *Fundamentals of dairy chemistry. New York: Van Nostrand Reinhold*, pp. 81-169.

Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature*, 454, pp. 428–435.

Meisel, H. 1997. Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers.* 43, pp. 119–128.

Menard, R., D. K. Nagler, R. Zhang, W. Tam, T. Sulea, and E. O. Purisima. 2000. Human cathepsin X. A novel cysteine protease with unique specificity. *Adv. Exp. Med. Biol*, 477, pp. 317–322.

Meuret, G., Bammert, J., Hoffmann, G. 1974. Kinetics of human monocytopoiesis. *Blood*, 44(6), pp. 801-816.

Migliore-Samour, D. & Jolles, P. 1988. Casein, a prohormone with an immunomodulating role for the newborn? *Experientia*. 44, pp. 188–193.

Mikkelsen, T.L., Bakman, S., Sørensen, E.S., Barkholt, V., Frøkiaer, H. 2005. Sialic acid-containing milk proteins show differential immunomodulatory activities independent of sialic acid. *J Agric Food Chem*, 53(20), pp. 7673-7680.

Mills, C.D., K. Kincaid, J. M. Alt, M. J. Heilman, & A. M. Hill. 2000. M-1/M-2 Macrophages and the Th1/Th2 paradigm. *J. Immunol*, 164, pp. 6166–6173.

Ming, Z., Jia, Y., Yan, Y., Pang, G., & Chen, Q. 2015. Amelioration effect of bovine casein glycomacropeptide on ulcerative colitis in mice. Food and Agricultural Immunology, 26(5), pp. 717-728.

Mitra, N., Liu, Y., Liu, J., Serebryany, E., Mooney, V., DeVree, B. T., Sunahara, R. K., Yan, E. C. 2013. Calcium-dependent ligand binding and G-protein signaling of family B GPCR parathyroid hormone 1 receptor purified in nanodiscs. *ACS chemical biology*, 8(3), pp. 617-25.

Mohanty, D.P., Mohapatra, S., Misra, S., & Sahu, P.S. 2015. Milk derived bioactive peptides and their impact on human health - A review. *Saudi journal of biological sciences*, 23(5), pp. 577-583.

Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., Kaplan, G.G. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology*, 142(1), pp. 46-54.

Monje, P., Hernández-Losa, J., Lyons, R.J., Castellone, M.D., Gutkind, J.S.. 2005. Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. *J. Biol. Chem*, 280, pp. 35081–35084.

Cheng, M. & Otani, H. 1997. Effect of bovine kappa-caseinoglycopeptide on secretion of interleukin-1 family cytokines by P388D1 cells, a line derived from mouse monocyte/macrophage. *Milchwissenshaft*, 52, pp. 192-196.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., O'Garra, A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 19, pp. 683-765.

Mosser, D.M. 2003. The many faces of macrophage activation. *J Leukoc Biol*. 73(2), pp. 209-212.

Mosser D.M. & Edwards, J.P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 8(12), pp. 958–969.

Mukhopadhya, A., Noronha, N., Bahar, B., Ryan, M. T., Murray, B. A., Kelly, P. M., Sweeney, T. 2014. Anti-inflammatory effects of a casein hydrolysate and its peptide-

enriched fractions on TNFα-challenged Caco-2 cells and LPS-challenged porcine colonic explants. *Food Science & Nutrition*, 2(6), pp.712–723.

Muraille, E., Leo, O., Moser, M. 2014. Th1/Th2 Paradigm Extended: Macrophage Polarization as an Unappreciated Pathogen-Driven Escape Mechanism? *Front Immunol.* 5, pp. 603.

Murata, K., Ishii, N., Takano, H. *et al*. 2000. Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *J Exp Med*, 191, pp. 365–374.

Murch, S.H., Braegger, C.P., Walker-Smith, J.A., MacDonald, T.T. 1993. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut,* 34, pp. 1705–1709.

Murray, P.J., Allen, J.E., Biswas, S.K., Fisher, E.A., Gilroy, D.W., Goerdt, S., Gordon, S., Hamilton, J.A., Ivashkiv, L.B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F.O., Mege, J.L., Mosser, D.M., Natoli, G., Saeij, J.P., Schultze, J.L., Shirey, K.A., Sica, A., Suttles, J., Udalova, I., van Ginderachter, J.A., Vogel, S.N., Wynn, T.A. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*, 41(1), pp. 14-20. doi: 10.1016/j.immuni.2014.06.008.

Naka, T., Fujimoto, M., Tsutsui, H., Yoshimura, A. 2005. Negative regulation of cytokine and TLR signalings by SOCS and others. *Adv Immunol*, 87, pp. 61-122.

Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., Akira, S., Yamanishi, K., Kawase, I., Nakanishi, K., Kishimoto, T. 2002. SOCS-1 participates in negative regulation of LPS responses. *Immunity*, 17(5), pp. 677–687.

Nakahara, T., Moroi, Y., Uchi, H., Furue, M. 2006. Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. *J Dermatol Sci*, 42, pp. 1-11.

Nair, S., Pandey, A.D. and Mukhopadhyay, S. 2011. The PPE18 protein of Mycobacterium tuberculosis inhibits NF-κB/rel-mediated proinflammatory cytokine production by upregulating and phosphorylating suppressor of cytokine signaling 3 protein. *J Immunol*, 186(9), pp. 5413-5424. doi: 10.4049/jimmunol.1000773.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., Paul, W.E. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol.* 17, pp. 701-738.

Nongonierma, A.B., & FitzGerald, R.J. 2015. The scientific evidence for the role of milk protein-derived bioactive peptides in humans. *Journal of Functional Foods,* 17, pp. 640–656.

O'Connor, D. The World Dairy Situation. 2009. 2008 - by International Dairy Federation. *International journal of dairy technology*, 62, pp. 286-287. doi: 10.1111/j.1471-0307.2009.00486.x

O'Farrell, A.M., Liu, Y., Moore, K.W., Mui, A.L. 1998. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. *EMBO J.* 17(4), pp.1006-1018.

Ogle, M. E., Segar, C. E., Sridhar, S., & Botchwey, E. A. 2016. Monocytes and macrophages in tissue repair: Implications for immunoregenerative biomaterial design. *Experimental biology and medicine,* 241(10), pp. 1084-1097.

Okazaki, T., & Honjo, T. 2006. The PD-1–PD-L pathway in immunological tolerance. *Trends in Immunology*, 27(4), pp.195-201.

O'Neill, L.A., Golenbock, D., & Bowie, A.G. 2013. The history of Toll-like receptors - redefining innate immunity. *Nat Rev Immunol,* 13(6), pp. 453-460. doi: 10.1038/nri3446.

Ortega-Gómez, A., M. Perretti, and O. Soehnlein. 2013. Resolution of inflammation: an integrated view. *EMBO Mol. Med*, 5, pp. 661–674.

Otani, H., & Futakami, M. 1994. Effects of bovine milk proteins on the phagocytic property and formation of nitrite by mouse peritoneal macrophages. *Animal Science and Technology,* 65, pp. 423-431.

Otani, H., & Futakami, M. 1996. Modification of nitrite production and phagocytosis of thioglycollate-elicited mouse peritoneal macrophages by bovine casein digests. *Food and Agricultural Immunology,* 8, pp. 59-69.

Otani, H., & Hata, I. 1995. Inhibition of proliferative responses of mouse spleen lymphocytes and rabbit Peyer's patch cells by bovine milk caseins and their digests. *J Dairy Res*. 62(2), pp. 339-348.

Otani, H. & Monnai, M. 1993. Inhibition of proliferative responses of mouse spleen lymphocytes by bovine milk k-casein digests. Food Agric. I*mmunol,* 5, pp. 219-229.

Otani, H., Monnai, M., Hosono, A. 1992. Bovine k-casein as inhibitor of the proliferation of mouse splenocytes induced by lipopolysaccharide stimulation. *Milchwissenschaft,* 47, pp. 512-515.

Otani, H., Monnai, M., Kawasaki, Y., Kawakami, H. & Tanimoto, M. 1995. Inhibition of mitogen-induced proliferative responses of lymphocytes by bovine kappa-caseinoglycopeptides having different carbohydrate chains. *Journal of Dairy Research*, 62, pp. 349-357.

Ozaki, M.E., Coren, B.A., Huynh, T.N., Redondo, D.J., Kikutani, H., Webb, S.R. 1999. CD4+ T cell responses to CD40-deficient APCs: defects in proliferation and negative selection apply only with B cells as APCs. *J Immunol*, 163(10), pp. 5250-5256.

Ozer, K. & Siemionow, M. 2001. Combination of anti-ICAM-1 and anti-LFA-1 monoclonal antibody therapy prolongs allograft survival in rat hind-limb transplants. *J Reconstr Microsurg*, 17(7), pp. 511-518.

Parameswaran, N., and Patial, S. 2010. Tumor necrosis factor-α signaling in macrophages. *Crit. Rev. Eukaryot. Gene Expr*, 20, pp. 87–103.

Pardoll DM. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer,* 12, pp. 252–264. doi:10.1038/nrc3239

Pasare, C., & Medzhitov, R. 2004. Toll-like receptors and acquired immunity. *Semin Immunol*, 16, pp. 23-26.

Pasqual, G., Chudnovskiy, A., Tas, J.M., Agudelo, M., Schweitzer, L.D., Cui, A., Hacohen, N., Victora, G.D. 2018. Monitoring T cell–dendritic cell interactions in vivo by intercellular enzymatic labeling. *Nature*. 553(7689), pp. 496–500. doi: 10.1038/nature25442.

Patil, S., Pincas, H., Seto, J., Nudelman, G., Nudelman, I., & Sealfon, S. C. 2010. Signaling network of dendritic cells in response to pathogens: a community-input supported knowledgebase. *BMC Systems Biology*, 4, 137. http://doi.org/10.1186/1752-0509-4-137.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research, 29(9), pp.e45-e45.

Plevy, S.E., Landers, C.J., Prehn, J., et al. 1997. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. *J Immunol*, 159, pp. 6276–6282.

Popov, A., Driesen, J., Abdullah, Z., Wickenhauser, C., Beyer, M., Debey-Pascher, S., Saric, T., Kummer, S., Takikawa, O., Domann, E., Chakraborty, T., Kronke, M., Utermohlen, O., and Schultze, J. L. 2008. Infection of myeloid dendritic cells with Listeria monocytogenes leads to the suppression of T cell function by multiple inhibitory mechanisms. *J. Immunol,* 181, pp. 4976–4988.

Posselt, G., Schwarz, H., Duschl, A., & Horejs-Hoeck, J. 2011. Suppressor of cytokine signaling
2 is a feedback inhibitor of TLR-induced activation in human monocyte-derived dendritic cells. *J Immunol,* 187(6):2875-2884.

Pulendran, B. & Maddur, M.S. 2015. Innate immune sensing and response to influenza. *Curr Top Microbiol Immunol*, 386, pp. 23–71. doi:10.1007/82_2014_405.

Qualls, J.E., Kaplan. A.M., van Rooijen, N., Cohen. D.A. 2008. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol*, 80, pp. 802–815.

Raes, G., Van den Bergh, R., De Baetselier, P., Ghassabeh, G.H., Scotton, C., Locati, M., Mantovani, A., Sozzani, S. 2005. Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells. *J Immunol.* 174(11), pp. 6561-6562.

Reis e Sousa C. 2006. Dendritic cells in a mature age. *Nat Rev Immunol*, 6, pp. 476–483. doi:10.1038/nri1845.

Rescigno, M., Martino, M., Sutherland, C. L., Gold, M. R., & Ricciardi-Castagnoli, P. 1998. Dendritic Cell Survival and Maturation Are Regulated by Different Signaling Pathways . The *Journal of Experimental Medicine*, 188(11), pp. 2175–2180.

Requena, P., Daddaoua, A., Guadix, E., Zarzuelo, A., Suárez, M. D., de Medina, F.S., Martínez-Augustin, O. 2009. Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-kB signal transduction pathways. British *Journal of Pharmacology*, 157, pp. 1232–1240.

Requena, P., Daddaoua, A., Martinez-Plata, E., Gonzalez, M., Zarzuelo, A., Suarez, M.D. *et al.* 2008. Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation ofinterleukin 17. *Br J Pharmacol*, 154, pp. 825–832.

Riss, T.L., Moravec, R.A., Niles, A.L. *et al.* 2013. Cell Viability Assays. [Updated 2016 Jul 1]. In: Sittampalam, G.S., Coussens, N.P., Brimacombe, K. *et al.*, editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004.

Robak T, Gladalska A, Stepien H. 1998. The tumour necrosis factor family of receptors/ligands in the serum of patients with rheumatoid arthritis. *Eur Cytokine Netw*, 9(2), pp. 145–154.

Roberts, A.W., Robb, L., Rakar, S., Hartley, L., Cluse, L., Nicola, N.A., *et al*. 2001. Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. Proc Natl *Acad Sci U S A*, 98, pp. 9324–9329.

Rocher, C., *et al.* 2013. SMAD-PI3K-Akt-mTOR pathway mediates BMP-7 polarization of monocytes into M2 macrophages. *Plos One*, 8, e84009.

Rogacev, K.S., Ulrich, C., Blömer, L., Hornof, F., Oster, K., Ziegelin, M., Cremers, B., Grenner, Y., Geisel, J., Schlitt, A., Köhler, H., Fliser, D., Girndt, M., Heine, G.H. 2010. Monocyte heterogeneity in obesity and subclinical atherosclerosis. *Eur Heart J*, 31(3), pp. 369-376. doi: 10.1093/eurheartj/ehp308.

Roseberg, I.M. 1996. Protein Analysis and Purification: Benchtop techniques, Boston: Birkhauser.

Rosengart, M.R., Arbabi, S., Garcia, I., Maier, R.V. 2000. Interactions of calcium/calmodulin-dependent protein kinases (CaMK) and extracellular-regulated kinase (ERK) in monocyte adherence and TNFalpha production. *Shock*, 13, pp. 183–189.

Roskoski, R. 2012. ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol. Res*, 66, pp. 105–143.

Rossman, M. D., B. T. Maida, and S. D. Douglas. 1990. Monocyte-derived macrophage and alveolar macrophage fibronectin production and cathepsin D activity. *Cell. Immunol*, 126, pp. 268–277.

Rossol, M., Kraus, S., Pierer, M., Baerwald, C., Wagner, U. 2012. The CD14(bright) CD16+ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 cell population. *Arthritis Rheum*, 64(3), pp. 671-677. doi: 10.1002/art.33418.

Rőszer, T. 2015. Understanding the mysterious M2 macrophage through activation markers and effector mechanisms. *Mediators Inflamm*, 2015, e816460.

Rugtveit, J., Nilsen, E.M., Bakka, A., Carlsen, H., Brandtzaeg, P., Scott, H. 1997. Cytokine profiles differ in newly recruited and resident subsets of mucosal macrophages from inflammatory bowel disease. *Gastroenterology*, 112(5), pp. 1493-1505.

Rutella, S., Danese, S., & Leone, G. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood*, 108, pp. 1435–1440. doi:10.1182/blood-2006-03-006403.

Rutgeerts, P., Sandborn, W.J., Feagan, B.G., Reinisch, W., Olson, A., Johanns, J., Travers, S., Rachmilewitz, D., Hanauer, S.B., Lichtenstein, G.R., de Villiers, W.J., Present, D., Sands, B.E., Colombel, J.F. 2005. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med*, 353(23), pp. 2462-2476.

Rutherfurd-Markwick, K.J., & Moughan P.J. 2005. Bioactive peptides derived from food. *Journal of AOAC International*, 88 (3), pp. 955-966.

Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y.C., Wulf, G., Rottapel, R., Yamaoka, S., Lu, K.P. 2003. Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol Cell*. 12(6), pp. 1413-1426.

Sabapathy, K., Hochedlinger, K., Nam, S.Y., Bauer, A., Karin, M., Wagner, E.F. 2004. Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. *Mol. Cell*, 15, pp. 713–725.

Saghaeian-Jazi,M., Mohammadi, S. and Sedighi, S. 2016. Culture and Differentiation of Monocyte Derived Macrophages Using Human Serum: An Optimized Method. *Res Med Sci*, 18(6), e7362.

Sakai, J., Cammarota, E., Wright, J. A., Cicuta, P., Gottschalk, R.A., Li, N., Fraser, I., Bryant, C.E. 2017. Lipopolysaccharide-induced NF-κB nuclear translocation is primarily dependent on MyD88, but TNFα expression requires TRIF and MyD88. *Scientific reports*, 7(1), pp. 1428. doi:10.1038/s41598-017-01600-y.

Sakuraba, A., Sato, T., Kamada, N., Kitazume, M., Sugita, A., Hibi, T. 2009. Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. *Gastroenterology*, 137(5), pp. 1736-1745.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M.L., Stammati, A., Zucco, F. 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol.* 21(1), pp. 1-26.

Sanchez-Munoz, F., Dominguez-Lopez, A., Yamamoto-Furusho, J.K. 2008. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol*, 14, pp. 4280–4288.

Santiago-Schwarz, F., Anand, P., Liu, S., Carsons, S.E. 2001. Dendritic cells (DCs) in rheumatoid arthritis (RA): progenitor cells and soluble factors contained in RA synovial fluid yield a subset of myeloid DCs that preferentially activate Th1 inflammatory-type responses. J *Immunol*, 167(3), pp. 1758-1768.

Satoh, T., *et al.* 2010. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol*, 11, pp. 936-944.

Savijoki, K., Ingmer, H., & Varmanen, P. 2006. Proteolytic systems of lactic acid bacteria. *Applied Microbiology and Biotechnology*, 71 (4), pp. 394-406.

Scarpa, M., Castagliuolo, I., Castoro, C., Pozza, A., Scarpa, M., Kotsafti, A., Angriman, I. 2014. Inflammatory colonic carcinogenesis: a review on pathogenesis and immunosurveillance mechanisms in ulcerative colitis. *World J. Gastroenterol*, 20, pp. 6774–6785.

Scheeren, R.A., Koopman, G., Van der Baan, S., Meijer, C.J., Pals, S.T. 1991. Adhesion receptors involved in clustering of blood dendritic cells and T lymphocytes. *Eur J Immunol*, 21(5), pp. 1101-1105.

Scheller, E.L., Hankenson, K.D., Reuben, J.S. & Krebsbach, P.H. 2011. Zoledronic acid inhibits macrophage SOCS3 expression and enhances cytokine production. *J Cell Biochem*, 112(11), pp. 3364-3372.

Schlitt, A., Heine, G.H., Blankenberg, S., Espinola-Klein, C., Dopheide, J.F., Bickel, C., Lackner, K.J., Iz, M., Meyer, J., Darius, H., Rupprecht, H.J. 2004. CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. *Thromb Haemost*, 92(2), pp. 419-424.

Schwartz, R.H. 2003. T cell anergy. *Annu Rev Immunol*, 21, pp. 305-334.

Schwarzmaier, D., Foell, D., Weinhage, T., Varga, G., Däbritz, J. 2013. Peripheral monocyte functions and activation in patients with quiescent Crohn's disease. PLoS One, 8(4), e62761. doi: 10.1371/journal.pone.0062761.

Scott, C.L., Aumeunier, A.M. & Mowat, A.M. 2011. Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends Immunol*, 32(9), pp. 412-419.

Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., Kuziel, W.A., Pamer, E.G. 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity*, 19(1), pp. 59-70.

Shaji, J., & Patole, V. 2008. Protein and Peptide drug delivery: oral approaches. *Indian journal of pharmaceutical sciences*, *70*(3), pp. 269–277. doi:10.4103/0250-474X.42967

Shakibaei, M., Csaki, C., Nebrich, S., Mobasheri, A. 2008. Resveratrol suppresses interleukin-1beta-induced inflammatory signaling and apoptosis in human articular chondrocytes: potential for use as a novel nutraceutical for the treatment of osteoarthritis. *Biochem Pharmacol*, 76(11), pp. 1426-1439. doi: 10.1016/j.bcp.2008.05.029.

Sharif, O., Bolshakov, V.N., Raines, S., Newham, P., Perkins, N.D. 2007. Transcriptional profiling of the LPS induced NF-kappaB response in macrophages. *BMC Immunol*, 8(1).

Sheikh, N.A. & Jones, L.A. 2008. CD54 is a surrogate marker of antigen presenting cell activation. *Cancer Immunol Immunother*, 57(9), pp. 1381-1390. doi: 10.1007/s00262-008-0474-9.

Sheikholeslami, V.D., & Ahmadi, K.G.F. 2012. Changes in antioxidant status and cardiovascular risk factors of overweight young men after six weeks supplementation of whey protein isolate and resistance training. *Appetite*, 59, pp. 673-678.

Shen, L., Evel-Kabler, K., Strube, R., Chen, S.Y. 2004. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. Nat Biotechnol, 22(12), pp. 1546-1553.

Shouda, T., Yoshida, T., Hanada, T., Wakioka, T., Oishi, M., Miyoshi, K., Komiya, S., Kosai, K., Hanakawa, Y., Hashimoto, K., Nagata, K., Yoshimura, A. 2001. Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. *J Clin Invest*, 108(12), pp. 1781-1788.

Shreffler, W. G., R. R. Castro, Z. Y. Kucuk, Z. Charlop-Powers, G. Grishina, S. Yoo, A. W. Burks, H. A. Sampson. 2006. The major glycoprotein allergen from Arachis hypogaea, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant in vitro. *J. Immunol.* 177, pp. 3677-3685.

Silva, M.A. 2009. Intestinal dendritic cells and epithelial barrier dysfunction in Crohn's disease. *Inflamm Bowel Dis*, 15, pp. 436–453.

Slavik, J., Hutchcroft, J. and Bierer, B. 1999. CD28/CTLA-4 and CD80/CD86 families. *Immunologic Research*, 19(1), pp.1-24.

Sletten, G.B., Halvorsen, R., Egaas, E., Halstensen, T.S. 2007. Memory T cell proliferation in cow's milk allergy after CD25+ regulatory T cell removal suggests a role for casein-specific cellular immunity in IgE-mediated but not in non-IgE-mediated cow's milk allergy. *Int Arch Allergy Immunol*, 142(3), pp. 190-198.

Smith, P. *et al.*, 2004. Schistosoma mansoni worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. *Journal of immunology*, 173(2), pp.1240–1248.

Snoeren, T.H., van Markwijk, B., van Montfort, R. 1980. Some physical chemical properties of bovine αS2-casein. *Biochim. Biophys. Acta*, 622, pp. 268–276.

Soldano, S., Pizzorni, C., Paolino, S., Trombetta, A.C., Montagna, P., Brizzolara, R., *et al.* 2016. Alternatively Activated (M2) Macrophage Phenotype Is Inducible by Endothelin-1 in Cultured Human Macrophages. *PLoS ONE*, 11(11): e0166433.

Song, J., Wang, Q., Du, M., Chen, B., Mao., X. 2017. Peptide IPPKKNQDKTE ameliorates insulin resistance in HepG2 cells via blocking ROS-mediated MAPK signaling. *Journal of Functional Foods*, 31, pp. 287-294.

Song, J.S., Kim, Y.J., Han, K.U., Yoon, B.D., & Kim, J.W. 2015. Zymosan and PMA activate the immune responses of Mutz3-derived dendritic cells synergistically. Immunology Letters, 167(1), pp. 41–46. doi:10.1016/j.imlet.2015.07.002.

Sorvillo, N., Pos, W., van den Berg, L.M., Fijnheer, R., Martinez-Pomares, L., Geijtenbeek, T.B., Herczenik, E., Voorberg, J. 2012. The macrophage mannose receptor promotes uptake of ADAMTS13 by dendritic cells. *Blood.* 119, pp. 3828-3835.

Srivastava, V., Manchanda, M., Gupta, S., Singla, R., Behera, D., Das, G., Natarajan, K. 2009. Toll-like receptor 2 and DC-SIGNR1 differentially regulate suppressors of cytokine signaling 1 in dendritic cells during Mycobacterium tuberculosis infection. *J Biol Chem*, 284(38), pp. 25532-41. doi: 10.1074/jbc.M109.006221.

Steinbach, E.C., Plevy, S.E. 2014. The role of macrophages and dendritic cells in the initiation of inflammation in IBD. *Inflamm Bowel Dis*, 20(1), pp. 166-175. doi: 10.1097/MIB.0b013e3182a69dca.

Steinman, R.M., & Idoyaga, J. 2010. Features of the dendritic cell lineage. *Immunol Rev*, 234, pp. 5–17. doi:10.1111/j.0105-2896.2009.00888.x

Steinman, R.M., Inaba, K., Turley, S., Pierre, P., Mellman, I. 1999. Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies. *Hum Immunol*, 60, pp. 562–567. doi:10.1016/S0198-8859(99)00030-0.

Sun, W. & Yang, J. 2010. Molecular basis of lysophosphatidic acid-induced NF-B activation, Cell Signal, 22(12), pp. 1799–1803.

Syme, C.D., Blanch, E.W., Holt, C., Jakes, R., Goedert, M., Hecht, L., Barron, L.D. 2002. A Raman optical activity study of rheomorphism in casein, synucleins and tau. New insight into the structure and behavior of natively unfolded proteins. *Eur. J. Biochem*, 269, pp. 148–156.

Strober, W., & Fuss, I. J. 2011. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology*, 140(6), pp. 1756-1767.

Takeda, N., *et al.* 2010. Differential activation and antagonistic function of HIF-α isoforms in macrophages are essential for NO homeostasis. *Genes Dev*, 24, pp. 491-501.

Takeda, K., & Akira, S. 2004. TLR signaling pathways. *Semin Immunol*, 16(1), pp. 3-9.

Tamang, J.P. 2010. Diversity of fermented foods. In: Tamang, J.P., & Kailasapathy, K. (eds.) Fermented foods and beverages of the world. Boca Raton: CRC Press, pp. 41-84.

Tamiya, T., Kashiwagi, I., Takahashi, R., Yasukawa, H., and Yoshimura, A. 2011. Suppressors of Cytokine Signaling (SOCS) Proteins and JAK/STAT Pathways: Regulation of T-cell Inflammation by SOCS1 and SOCS3. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31(5), pp. 980-985.

Tamoutounour, S., Henri, S., Lelouard, H., de Bovis, B., de Haar, C., van der Woude, C.J., Woltman, A.M., Reyal, Y., Bonnet, D., Sichien, D., Bain, C.C., Mowat, A.M., Reis e Sousa, C., Poulin, L.F., Malissen, B., Guilliams, M. 2012. CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol*, 42(12), pp. 3150-3166. doi: 10.1002/eji.201242847.

Takeda, A.K., Kaisho, T., & Akira, S. 2003. Toll-like receptors. *Annu Rev Immunol*, 21**,** pp. 335-376.

Takeuchi, H., Trang, V.T., Morimoto, N., Nishida, Y., Matsumura, Y., Sugiura, T. 2014. Natural products and food components with anti-Helicobacter pylori activities. *World J Gastroenterol*, 20, pp. 8971.

Targan, S.R., Hanauer, S.B., van Deventer, S.J., Mayer, L., Present, D.H., Braakman, T., DeWoody, K.L., Schaible, T.F., Rutgeerts, P.J. 1997. A short-term study of chimeric

monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med*, 337(15), pp. 1029-1035.

Targan, S.R., & Karp, L.C. 2005. Defects in mucosal immunity leading to ulcerative colitis. *Immunol Rev*. 206, pp. 296-305.

Taylor, P.C., Peters, A.M., Paleolog, E., Chapman, P.T., Elliott, M.J., McCloskey, R., Feldmann, M., Maini, R.N. 2000. Reduction of chemokine levels and leukocyte traffic to joints by tumor necrosis factor alpha blockade in patients with rheumatoid arthritis. *Arthritis Rheum*, 43(1), pp. 38-47.

Tesmer, L.A., Lundy, S.K., Sarkar, S., & Fox, D.A. 2008. Th17 cells in human disease. *Immunological reviews*, 223, pp. 87-113.

Thaiss, C. A., Semmling, V., Franken, L., Wagner, H., & Kurts, C. 2011. Chemokines: A New Dendritic Cell Signal for T Cell Activation. *Frontiers in Immunology*, 2, pp. 31. http://doi.org/10.3389/fimmu.2011.00031.

Toma, S.J., Nakai, S. 1973. Calcium sensitivity and molecular weight of αS2-casein. *J. Dairy Sci*, 56, pp. 1559–1562.

Tseng, W.P., Su, C.M., & Tang, C.H. 2010. FAK activation is required for TNF-alpha-induced IL-6 production in myoblasts. *J. Cell. Physiol*, 223, pp. 389–396.

Tsuiji, M., Fujimori, M., Ohashi, Y., Higashi, N., Onami, T.M., Hedrick, S.M., Irimura, T. 2002. Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1. *J Biol Chem.* 277(32), pp. 28892-28901.

Ueno, A., Jeffery, L., Kobayashi, T., Hibi, T., Ghosh, S., Jijon, H. 2018. Th17 plasticity and its relevance to inflammatory bowel disease. *J Autoimmun*, 87, pp. 38-49. doi: 10.1016/j.jaut.2017.12.004.

Van Seventer, G.A., Shimizu, Y., Horgan, K.J. & Shaw, S. 1990. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *Journal of Immunology*, 144(12), pp. 4579-4586.

Verstrepen, L., Carpentier, I., Beyaert, R. 2014. The biology of A20-binding inhibitors of NF-kappaB activation (ABINs). *Adv Exp Med Biol*, 809, pp. 13-31.

Vidya, M. K., Kumar, V. G., Sejian, V., Bagath, M., Krishnan, G., & Bhatta, R. 2017. Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals. *International Reviews of Immunology*, 37(1), pp. 20–36.

Vogel, D.Y., Vereyken, E.J., Glim, J.E., Heijnen, P.D., Moeton, M., van der Valk, P., Amor, S., Teunissen, C.E., van Horssen, J., Dijkstra, C.D. 2013. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *J Neuroinflammation*. 10, pp. 35.

Von Bergwelt-Baildon, M. S., Popov, A., Saric, T., Chemnitz, J., Classen, S., Stoffel, M. S., Fiore, F., Roth, U., Beyer, M., Debey, S., Wickenhauser, C., Hanisch, F. G., and Schultze, J. L. 2006. CD25 and indoleamine 2, 3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo: additional mechanisms of T-cell inhibition. *Blood*, 108, pp. 228–237.

Vordenbäumen, S., Braukmann, A., Altendorfer, I., Bleck, E., Jose, J., & Schneider, M. 2013. Human casein alpha s1 (CSN1S1) skews in vitro differentiation of monocytes towards macrophages. *BMC immunology*, 14, 46. doi:10.1186/1471-2172-14-46.

Vordenbaumen, S., Braukmann, A., Petermann, K., Scharf, A., Bleck, E., von Mikecz, A., *et al*. 2011. Casein alpha s1 is expressed by human monocytes and upregulates the production of GM-CSF via p38 MAPK. *Journal of Immunology*, 186, pp. 592–601.

Vukman, K.V., Adams, P.N., Metz, M., Maurer, M., O'Neill, S.M. 2013. Fasciola hepatica tegumental coat impairs mast cells' ability to drive Th1 immune responses. *J Immunol*, 190(6), pp. 2873-2879. doi: 10.4049/jimmunol.1203011

Waiboci, L.W., Ahmed, C.M., Mujtaba, M.G., Flowers, L.O., Martin, J.P., Haider, M.I., Johnson, H.M. 2007. Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: implications for the development of a SOCS-1 antagonist. *J Immunol*, 178(8), pp. 5058-5068.

Walstra, P., Geurts, T.J., Noomen, A., Jellema, A., Van Boekel, M.A.J.S. 1999. Dairy Technology: principles of milk properties and processes, Basel, Switzerland, Markel Dekker, Inc.

Wautier, J.L., Setiadi, H., Vilette, D., Weill, D., Wautier, M.P. 1990. Leukocyte adhesion to endothelial cells. *Biorheology*, 27 (3-4), pp. 425–432.

Weaver, C. T., Elson, C. O., Fouser, L. A., & Kolls, J. K. 2012. The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annual review of pathology*, 8, pp. 477-512.

Weimann, C., Meisel, H. & Erhardt, G. 2009. Short communication: bovine kappa-casein variants result in different angiotensin I converting enzyme (ACE) inhibitory peptides. *J Dairy Sci*, 92(5), pp. 1885-1888.

Weischenfeldt, J., Porse, B. 2008. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protoc, 2008, pdb.prot5080.

Wells, A.D., Walsh, M.C., Bluestone, J.A. and Turka, L.A. 2001. Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *The Journal of Clinical Investigation*, 108(6), pp.895-904.

Whiting, C.C., Su, L.L., Lin, J.T., & Fathman, C.G. 2010. GRAIL: a unique mediator of CD4 T-lymphocyte unresponsiveness. *The FEBS journal*, 278(1), pp. 47-58.

Whyte, C.S., Bishop, E.T., Ruckerl, D., Gaspar-Pereira, S., Barker, R.N., Allen, J.E., Rees, A.J., Wilson, H.M. 2011. Suppressor of cytokine signaling (SOCS)1 is a key determinant of differential macrophage activation and function. *J Leukoc Biol*, 90(5), pp. 845-854.

Wilson, H. M. 2014. SOCS Proteins in Macrophage Polarization and Function. Frontiers in *Immunology*, 5, pp. 357. http://doi.org/10.3389/fimmu.2014.00357.

Wong, C.W., Seow, H.F., Liu, A.H., Husband, A.J., Smithers, G.W., & Watson, D.L. 1996. Modulation of immune responses by bovine beta-casein. *Immunology and Cell Biology*, 74, pp. 323-329.

Wong, K.L., Tai, J.J., Wong, W.C., Han, H., Sem, X., Yeap, W.H., Kourilsky, P., Wong, S.C. 2011. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood,* 118(5), pp. 16-31.

Wu, Y.J., Wang, H., Liang, J.H., Miao, Y., Liu, L., Qiu, H. Li, J. Y. 2016. Using the geometric mean fluorescence intensity index method to measure ZAP-70 expression in patients with chronic lymphocytic leukemia. *OncoTargets and therapy*, *9*, pp. 797–805. doi:10.2147/OTT.S94613

Wu, Z.Q., Han, X.D., Wang, Y., Yuan, K.L., Jin, Z.M., Di, J.Z., *et al.* 2011. Interleukin-1 recep-tor antagonist reduced apoptosis and attenuated intestinal mucositis in a5-fluorouracil chemotherapy model in mice. *Cancer Chemotherapy and Pharmacology*, 68, pp. 87–96.

Wynn, T.A., Chawla, A. & Pollard, J.W. 2013. Macrophage biology in development, homeostasis and disease. *Nature*, 496(7446), pp.445-455.

Wynn, T.A., & Vannella, K.M. 2016. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*, *44*(3), pp. 450-462.

Xu, H., Friedrichs, U., Gieseler, R.K., Ruppert, J., Ocklind, G., Peters, J.H. 1992. Human blood dendritic cells exhibit a distinct T-cell-stimulating mechanism and differentiation pattern. *J Immunol*, 36(5), pp. 689-696.

Yamaguchi, N., Kawaguchi, K. & Yamamoto, N. 2009. Study of the mechanism of antihyperten-sive peptides VPP and IPP in spontaneously hypertensive rats by DNA microarrayanalysis. *European Journal of Pharmacology*, 620, pp. 71–77.

Yang, J., Zhang, L., Yu, C., Yang, X.F., & Wang, H. 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark. Res*. 2: 1.

Yang, J., Zhang, L., Yu, C., Yang, X. F., & Wang, H. 2014. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomarker research*, 2(1), 1. doi:10.1186/2050-7771-2-1

Yasaka, T., Mantich, N.M., Boxer, L.A., Baehner, R.L. 1981. Functions of human monocyte and lymphocyte subsets obtained by countercurrent centrifugal elutriation:

differing functional capacities of human monocyte subsets. *J Immunol*, 127(4), pp. 1515-1518.

Yawalkar, N., Tscharner G.G., Hunger R.E., Hassan A.S. 2009. Increased expression of IL-12p70 and IL-23 by multiple dendritic cell and macrophage subsets in plaque psoriasis. *J Dermatol Sci*, 54, pp. 99–105.

Yoshimura, A., Nake, T., and Kubo, M. 2007. SOCS Proteins, Cytokine Signalling and Immune Regulation. *Nature Reviews: Immunology*, 7, pp. 45-465.

Yoshimura, A., Suzuki, M., Sakagushi, R., Hanada, T., and Yasukawa, H. 2012. SOCS, Inflammation, and Autoimmunity. *Frontiers in Immunology*, 3, pp. 1-9.

Yu, A., Zhu, L., Altman, N.H., Malek, T.R. 2009. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. *Immunity*, 30, pp. 204-217.

Yun, S.S., Sugita-Konishi, Y., Kumagai, S. & Yamauchi, K. 1996. Isolation of mitogenic glycophosphopeptides from cheese whey protein concentrate. *BioScience, Biotechnology and Biochemistry*, 60, pp. 429-433.

Zaba, L.C., Fuentes-Duculan, J., Eungdamrong, N.J., Abello, M.V., Novitskaya, I., Pierson, K.C., Gonzalez, J., Krueger, J.G., Lowes, M.A. 2009. Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. *J Invest Dermatol*, 129(1), pp. 79-88.

Zarif, J. C., Hernandez, J. R., Verdone, J. E., Campbell, S. P., Drake, C. G., & Pienta, K. J. 2016. A phased strategy to differentiate human CD14+monocytes into classically and alternatively activated macrophages and dendritic cells. *BioTechniques*, 61(1), pp. 33-41. doi:10.2144/000114435.

Zarubin, T., & Han J. 2005. Activation and signaling of the p38 MAP kinase pathway. *Cell Res*, 15, pp11–18.

Zayas, J.F. 1997. Functionality of proteins in food, Berlin, Springer-Verlag.

Zhang, Y., Li, X., Ciric, B., Ma, C.G., Gran, B., Rostami, A., Zhang, G.X. 2015. Therapeutic effect of baicalin on experimental autoimmune encephalomyelitis is mediated by SOCS3 regulatory pathway. *Sci Rep*, 5, pp. 17407. doi: 10.1038/srep17407.

Zhao, J. 2007. Nutraceuticals, nutritional therapy, phytonutrients, and phytotherapy for improvement of human health: a perspective on plant biotechnology application. *Recent Pat Biotechnol*, 1(1), pp. 75-97.

Zheng, Y., Delgoffe, G.M., Meyer, C.F., Chan, W., Powell, J.D. 2009. Anergic T cells are metabolically anergic. *J Immunol*. 2009, 183(10), pp. 6095-6101.

Zhou, D., Chen, L., Yang, K., Jiang, H., Xu, W., & Luan, J. 2017. SOCS molecules: the growing players in macrophage polarization and function. *Oncotarget*, 8(36), 60710–60722. http://doi.org/10.18632/oncotarget.19940.

Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J., Liu, Y.J., MacPherson, G., Randolph, G.J., Scherberich, J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J.M., Lutz, M.B. 2010. Nomenclature of monocytes and dendritic cells in blood. *Blood*, 2, pp. 74-80.

Zigmond, E., Varol, C., Farache, J., Elmaliah, E., Satpathy, A.T., Friedlander, G., Mack, M., Shpigel, N., Boneca, I.G., Murphy, K.M., Shakhar, G., Halpern, Z., Jung, S. 2012. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity*, 37(6), pp. 1076-1090. doi: 10.1016/j.immuni.2012.08.026.

Appendix

Appendix A - No cytotoxic effects exhibited by κ-CAS treatment.

To ensure that the observed immuno-modulatory effects displayed by κ-CAS treatment were not due cytotoxic effects, cells were pre-treated for 2.5 hr with κ-CAS (1 mg/mL) prior to stimulation with and without LPS (100 ng/ml) for 24 hr. PBS and LPS alone were used as positive controls. 4 % PFA were used as negative controls. The results demonstrated that the doses used for κ-CAS (Figure 9.1) did not exhibit any significant cytotoxic effect on cells *in-vitro* compared to controls and as such were used at the same concentrations or lower for all subsequent experiments.

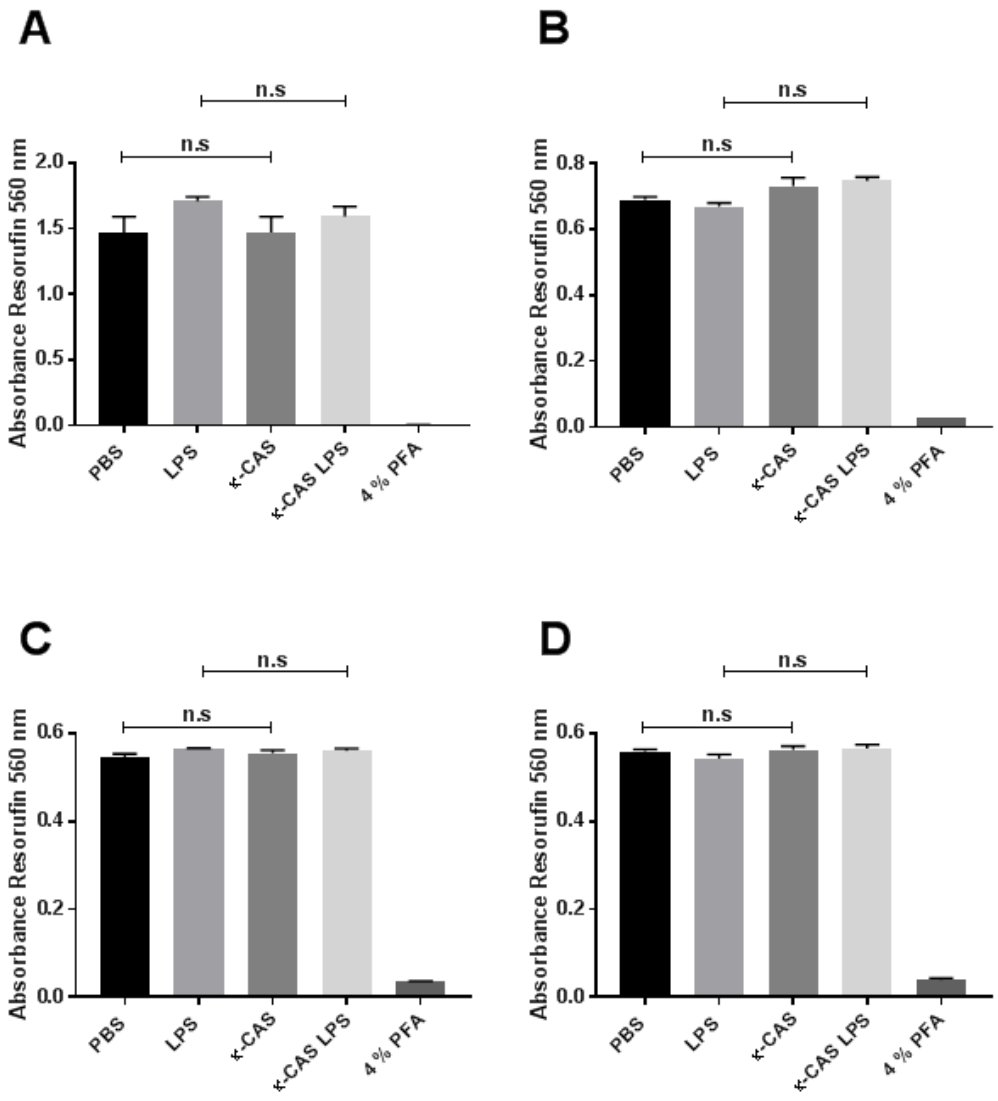

Figure 9.1 κ-CAS does not affect cell viability. Resazurin assays were performed on murine BMMφ (A), murine BMDC (B), human PBMCs (C) or human $CD14^+$ monocytes (D). 1 x 10^5 cells were pre-treated for 2.5 hr with κ-CAS (1 mg/mL) prior to stimulation with and without LPS (100 ng/ml) for 24 hr. PBS and LPS alone were used as positive controls while 4 % PFA was used as negative controls. Results are expressed as mean ±SD of at least 3 independent experiments in triplicate. P-values were calculated using multiple student's t-test. Ns; no significant difference compared to positive control groups.

Appendix B - The Suppressive mechanism exerted by κ-CAS on LPS induced cytokines is independent of heme oxygenase-1

More recently, GMP and its derivatives were found to supress TLR4 meditated responses in macrophages by attenuating the activation of NFκB signalling (Cheng *et al.,* 2015). The mechanism by which this occurred was found to be due to the upregulation of heme oxygenase-1, which when inhibited resulted in the restoration of inflammatory cytokine release and NFκB activity (Li *et al.,* 2017). Given that we attained similar results with κ-CAS, which was also shown to abrogate LPS mediated inflammatory cytokine release and NFκB activation, the effect of heme oxygenase-1 inhibitors was examined on the suppressive capacity of κ-CAS on LPS induced cytokine release (Figure 9.2). BMMφ derived from C57BL/6J mice were pre-treated with PBS or the heme oxygenase-1 inhibitor; ZnPPIX (10 or 20 μM/mL) for 1 hr and then treated with or without κ-CAS (1 mg/mL) for 2.5 hr, followed by stimulation with LPS (100 ng/mL) for 18 hr. While κ-CAS significantly supressed LPS induced TNF-α (Figure 9.2 ** , $p \leq 0.01$), the inhibition of heme oxygenase-1 by ZnPPIX did not restore inflammatory cytokine release. These results would suggest that the suppressive mechanism exerted by κ-CAS on LPS induced cytokine production is independent of heme oxygenase-1.

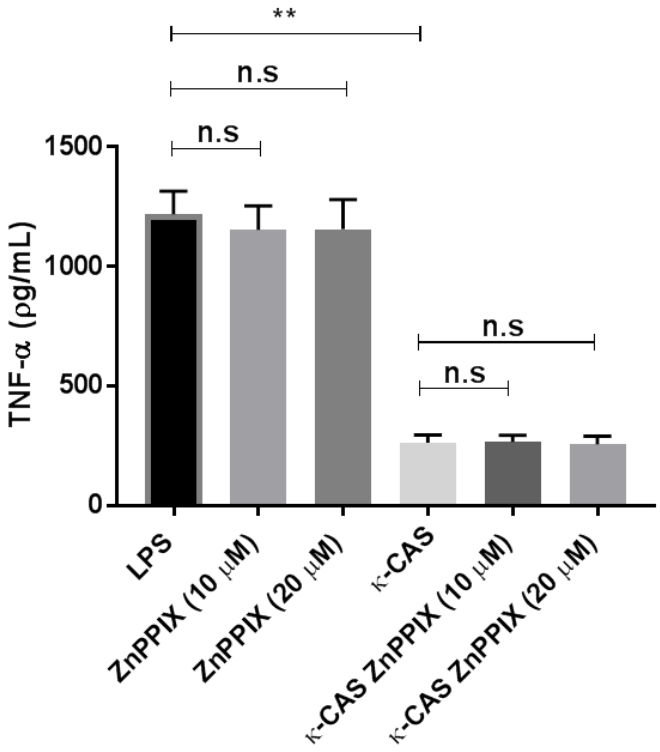

Figure 9.2 Effect of selective inhibitor ZnPPIX on LPS induced TNF-α suppression by κ-CAS in BMMφ. BMMφ derived from C57BL/6J mice werepre-treated with ZnPPIX (10 μM or 20 μM) for 1 hr and subsequently treated with κ-CAS (1 mg/mL) for 2.5 hr followed by stimulation with LPS (100 ng/mL) for 18 hr. . Supernatants were analysed for the secretion of TNF-α by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were calculated using multiple two-tailed student's t-test. ** , $p \leq 0.01$, compared to κ-CAS treated control. n.s; non-significant, compared to non-ZnPPIX treated controls.

Appendix C - The suppressive effects exerted by κ-CAS on LPS induced cytokines is reversed following trypsin treatment.

GMP and its derivatives have been found to supress TLR4 meditated responses in macrophages and other cells types (Cheng *et al.*, 2015). However, after GMP digestion with trypsin, Otani and Monnai demonstrated that their inhibitory effects were increased (Otani and Monnai 1993). Given that we attained similar results with the whole κ-CAS subunit, which was also shown to abrogate LPS mediated inflammatory cytokine release; the effect of trypsin treatment on the suppressive capacity of κ-CAS was exaimed (Figure 9.3). κ-CAS (0.5 mg) was treated with trypsin (150 μg/mL) for 1 hr at 37^0C. After incubation, the trypsin was heat inactivated at 90^oC for 5 min. κ-CAS without trypsin was used as a control. BMMφ derived from C57BL/6J mice were treated with or without trypsenised κ-CAS (0.5 mg/mL) for 2.5 hr, followed by stimulation with LPS (100 ng/mL) for 18 hr. While κ-CAS significantly supressed LPS induced TNF-α (Figure 9.3 * , $p \leq 0.05$), trypsin treatment reversed the suppressive effects exerted by κ-CAS on TNF-α secreation.

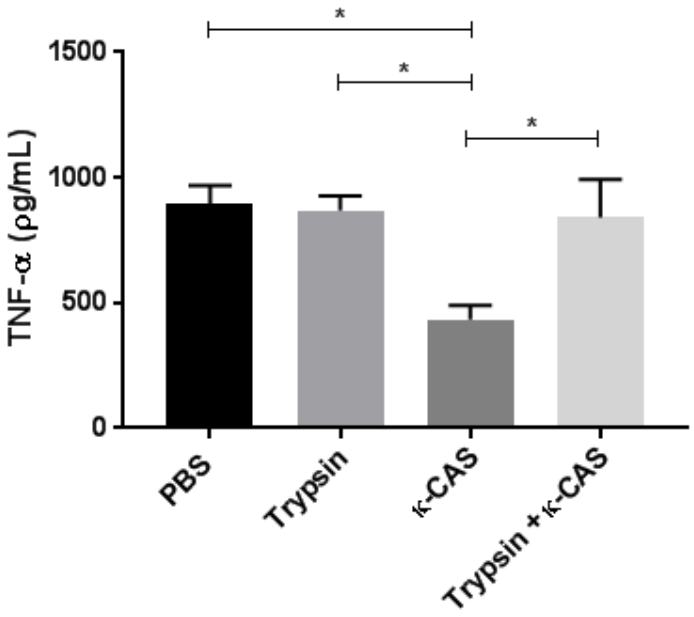

Figure 9.3 Effect of trypsin on κ-CAS suppressive activity in BMMφ. BMMφ derived from C57BL/6J mice were seeded at 1 x 10^6 cells/mL, rested for 2 hr, pre-treated with and without trypsin treated κ-CAS (0.5 mg/mL) for 2.5 hr followed by stimulation with LPS (100 ng/mL) for 18 hr. Supernatants were analysed for the secretion of TNF-α by ELISA. Results are expressed as mean ±SD of 3 independent experiments in triplicate. P-values were analysed using ANOVA. * , $p \leq 0.05$

Appendix D – Representative scatter plots for flow cytometry data

In order to confirm the presence, absence, increase or reduction of cellular markers, cells were stained with panels of marker specific fluorochrome-labelled monoclonal antibodies and analysed by flow cytometry. Results were presented as the geometric mean fluorescence intensity of multiple experiemnts, with a representative histogram. Representative scatter plot of all data is also presented below.

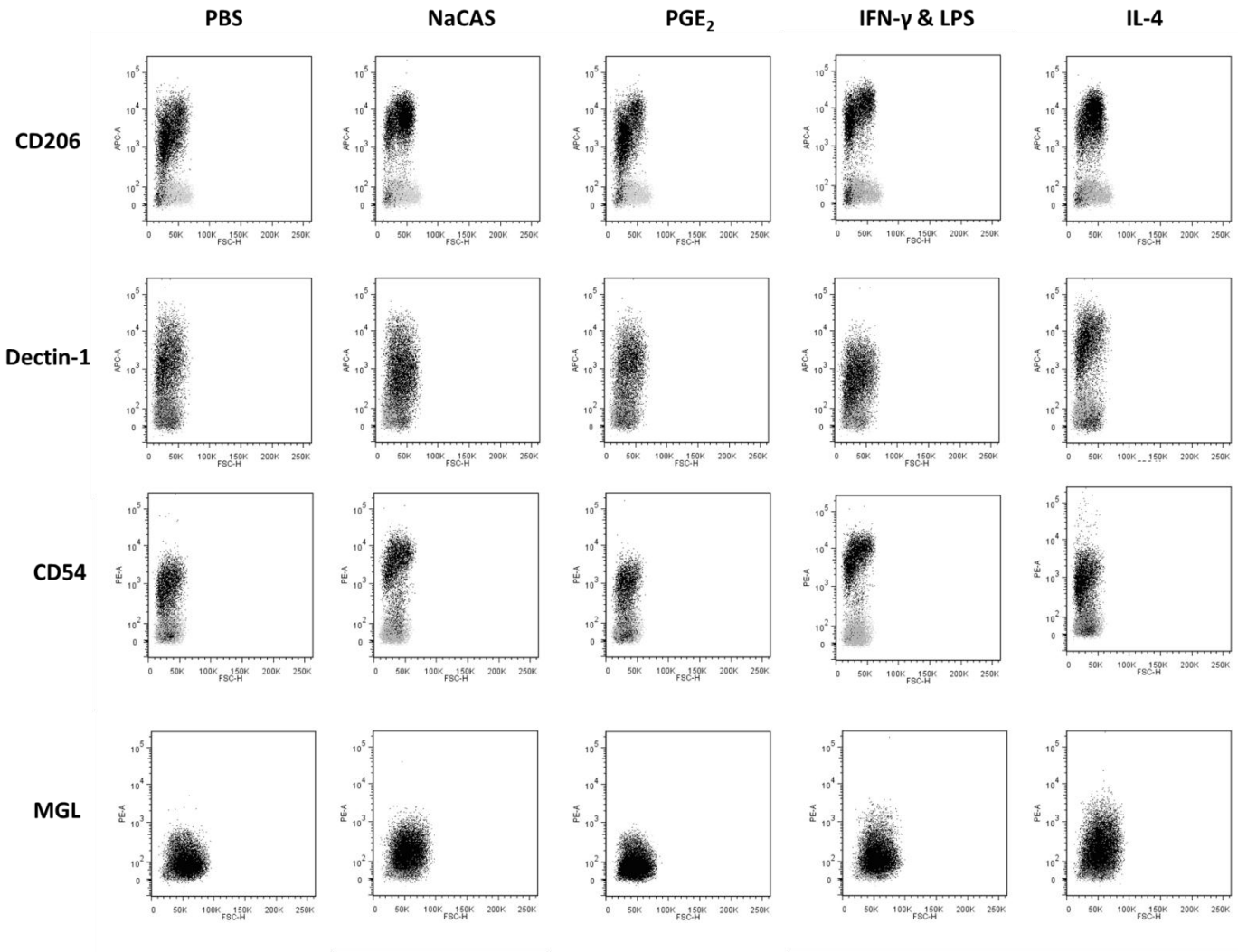
PBS
NaCAS
PGE2
IFN-γ & LPS
IL-4
CD206
Dectin-1
CD54
MGL
APC-A
PE-A
FSC-H

Figure 9.4 NaCAS induced receptors associated with both M1 & M2 macrophages. BMMφ derived from C57BL/6 mice were pre-treated with NaCAS (1 mg/mL) for for 24 hr. M2 stimulants IL-4 (20 ng/mL) or PGE_2 (5μM) and M1 stimulants IFNγ (20 ng/mL) & LPS (100ng.mL) were used as macrophage differentiating controls. PBS was used as a control. Cells were analysed by flow cytometry and representative scatter plots show the surface expression of CD206, MGL, Dectin-1 and CD54; isotype (gray), treated (black).

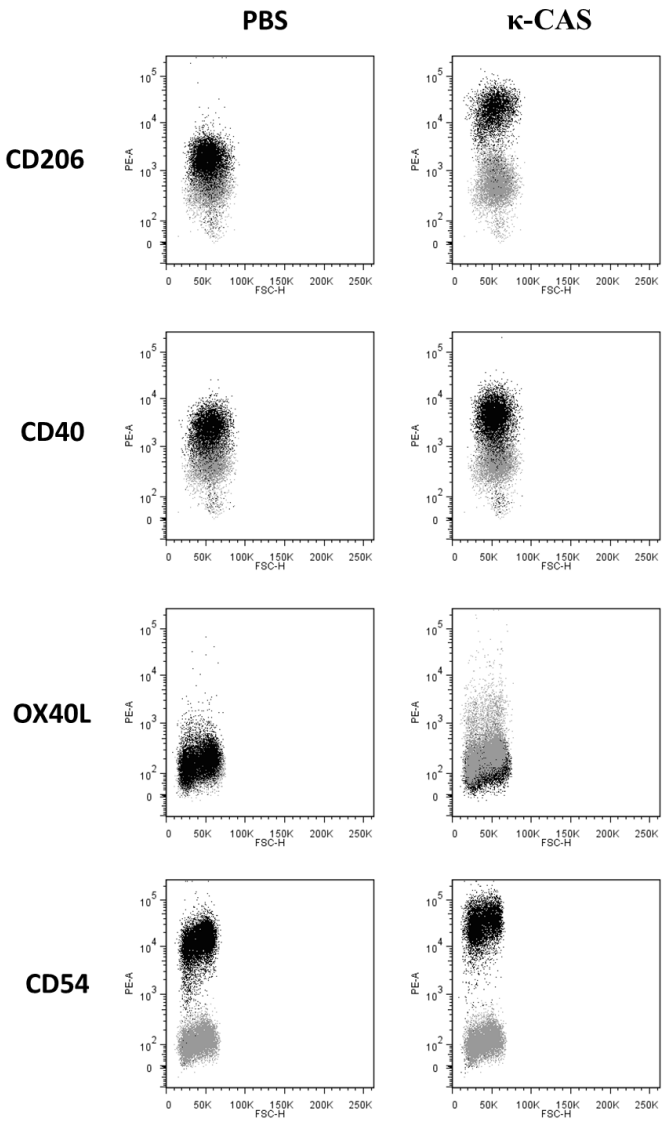

Figure 9.5 κ-CAS induced a mixed M1 & M2 receptor repertoire and selectively upregulated co-stimulatory molecules. BMMφ derived from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for for 24 hr. PBS was used as a negative control. Cells were analysed by flow cytometry and representative scatter plots show the surface expression of of CD206, CD40, OX40L and CD54; isotype (gray), treated (black).

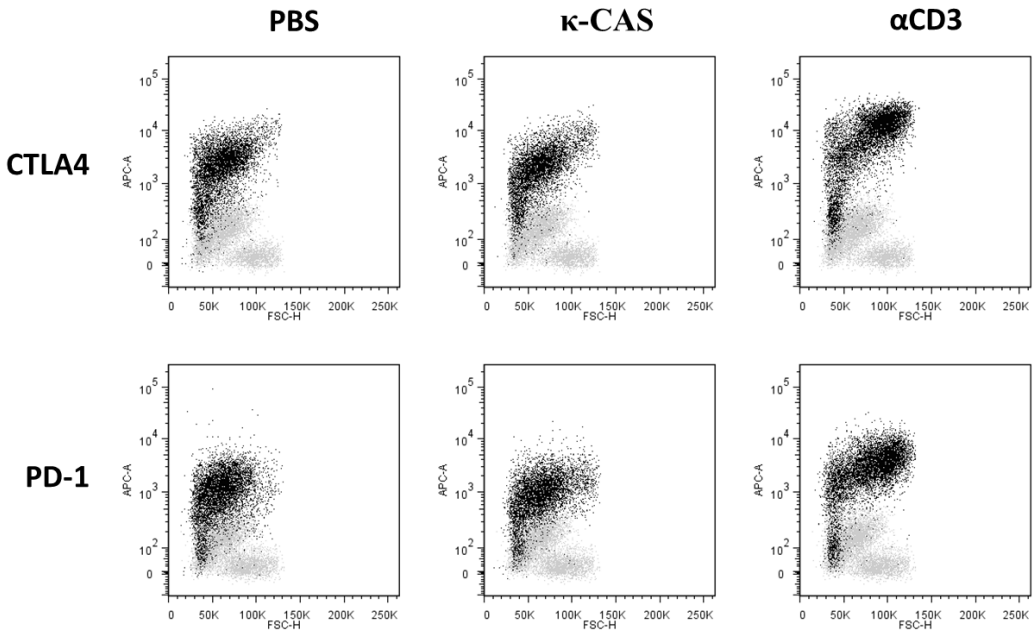

Figure 9.6 CD4$^+$ T-cells cultured with κ-CAS treated BMMφ do not display anergic markers. BMMφs from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for 18hr. Control BMMφ were treated with PBS. PBS and κ-CAS treated macrophages were subsequently co-cultured with CD4$^+$ T-cells at a ratio of 1:4 on plates pre-coated with anti-CD3 (1 μg/well). CD4$^+$ T-cells cultured with anti-CD3 alone were used an anergic control. Cells were analysed by flow cytometry and representative scatter plots show the surface expression of CTLA4 and PD-1; isotype (gray), treated (black).

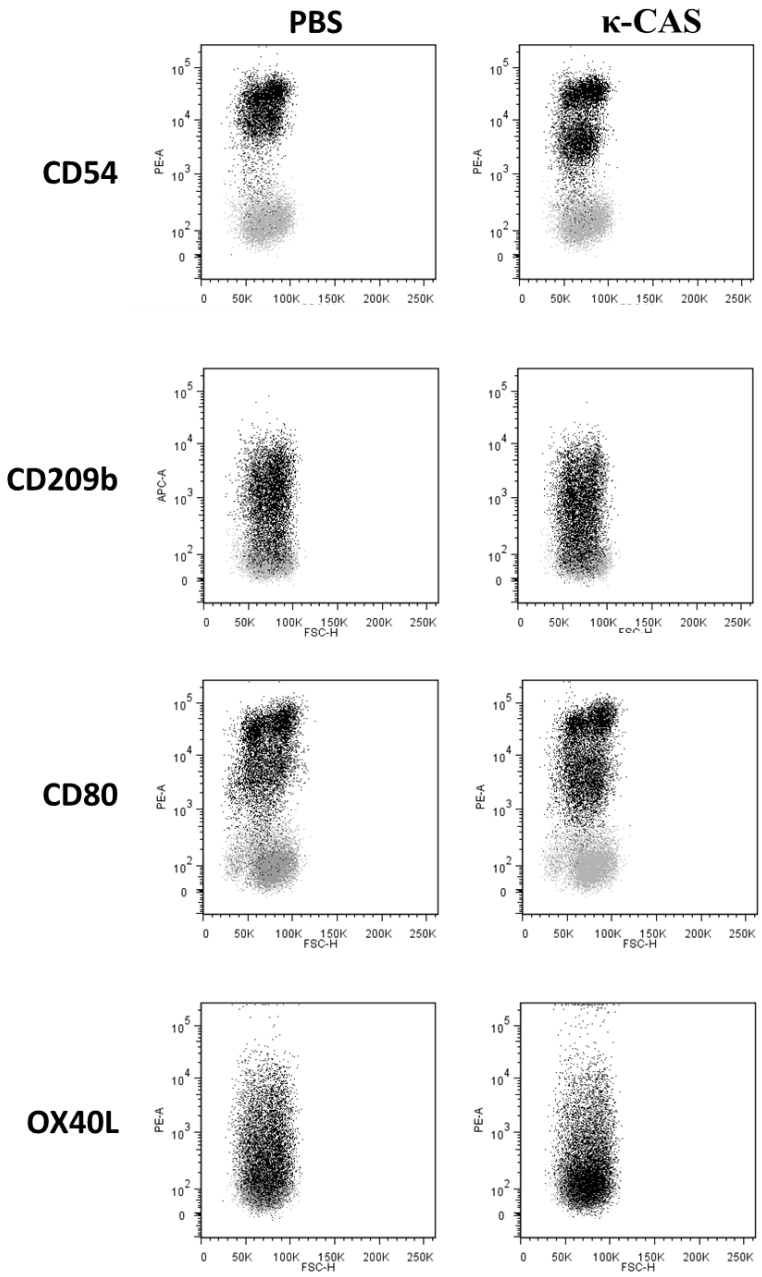

Figure 9.7 κ-CAS downregulates co-stimulatory & adhesion receptors. BMDCs derived from C57BL/6 mice were pre-treated with κ-CAS (1 mg/mL) for 18 hr. PBS was used as a negative control. Cells were analysed by flow cytometry and representative scatter plots show the surface expression of CD54 (E), CD209 (F), CD80 (G) and OX40L; isotype (gray), treated (black).

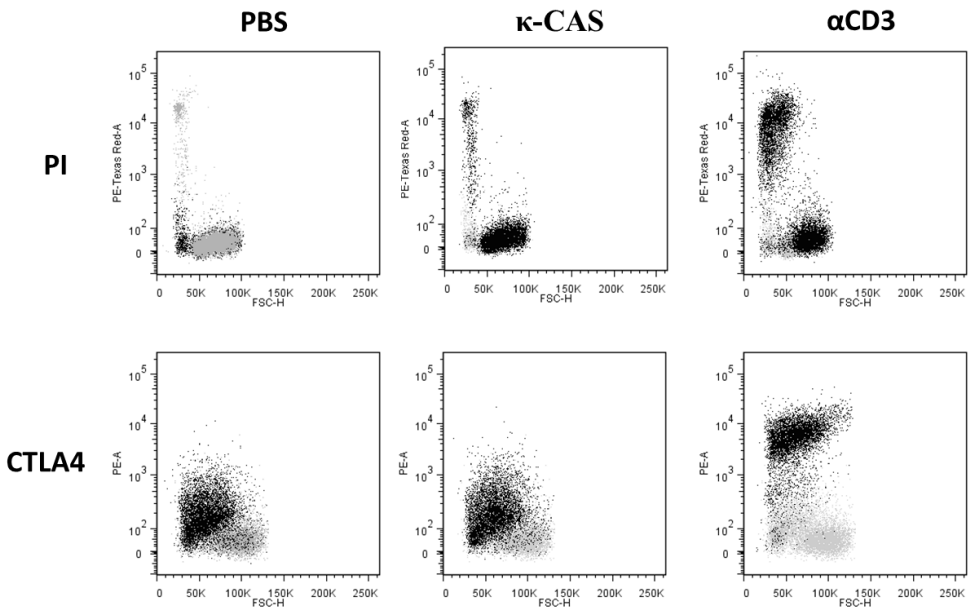

Figure 9.8 κ-CAS treated CD14$^+$ monocytes do not induce anergy markers in CD4$^+$ T-cells. After 72 hrs, non-adherent human CD4 cells were harvested following allogenic co-culture at a ratio of 1:10 with CD14$^+$ monocytes pre-treated with PBS or κ-CAS (1 mg/mL) for 18 hrs. CD4 cells cultured with anti-CD3 alone were used as an anergic control. Cells were analysed by flow cytometry and representative scatter plots show the uptake of the apoptotic marker PI or the surface expression of CTLA4; isotype (gray), treated (black).