Regulation of Complement Receptor Immunoglobulin (CRIg) Expression by Cytokines

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DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due references is made in the text.

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Usma Munawara

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PUBLICATIONS

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ABBREVIATIONS

A - F

α	Alpha
β	Beta
BSA	Bovine serum albumin
bp	Base pair
C	Complement component
CA	Crude antigen
C. albicans	*Candida albicans*
C-terminus	Carboxyl-terminus
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
C1q	Complement component 1, q subcomponent
C1r	Complement component 1, r subcomponent
C1s	Complement component 1, s subcomponent
CO_2	Carbon dioxide
CR	Complement receptor
CRIg	Complement receptor immunoglobulin
$CRIg^+$	Complement receptor immunoglobulin positive
$CRIg^-$	Complement receptor immunoglobulin negative
CR1	Complement receptor 1
CR2	Complement receptor 2
CR3 /CD11b	Complement receptor 3
CR4/ CD11c	Complement receptor 4
Ctrl	Control

DC	Dendritic cells
Dex	Dexamethasone
dia	Diameter
Dr.	Doctor
DTT	Dithiothreitol
dxDC	Dexamethasone treated dendritic cell
°C	Degree centigrade
EAU	Experimental autoimmune uveoretinitis
ECL	Enhanced chemiluminescence kit
EDTA	Ethylenediaminetetraacetic acid
FACs	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
Fc	Fragment crystallisable
FITC	Fluorescein isothiocyanate
fI	Factor I
fS	Factor S

G - L

g	Gravitational (acceleration)
γ	Gamma
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Glucocorticoid
GM-CSF	Granulocyte macrophage colony-stimulating factor
hrs	Hours
HBV	Hepatitis B virus
HBSS	Hank’s balanced salt solution

HCL	Hydrochloric acid
HEPES	2-[4-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
HLA-DR	Major histocompatibility complex, class II, DQ beta 1
HSC	Heamatopoietic stem cells
HRP	Horse-radish peroxidase
huCRIg	Human CRIg
huCRIg(L)	Human CRIg long
huCRIg(S)	Human CRIg short
IBD	Inflammatory bowel disease
iDC	Immunogenic dendritic cells
IFN	Interferon
Ig	Immunoglobulin
IgC_2	Immunoglobulin domain C_2-type
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgV	Immunoglobulin domain V (variable)-type
IL	Interleukin
IR	Ischemia/reperfusion
KCs	Kupffer cells
kDa	Kilo dalton
KO	Knockout
L. monocytogenes	*Listeria monocytogenes*
LPS	Lipopolysaccharide
LT	Lymphotoxin
LT-α	Lymphotoxin alpha

M - S

mAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimole
µl	Microliter
µg	Microgram
min	Minute
muCRIg	Murine CRIg
MAC	Membrane attack complex
MBL	Mannose-binding lectin
MCP	Membrane-cofactor protein
MDM	Monocyte-derived macrophages
MDP	Heamatopoietic-derived stem cell progenitors
MHC	Major histocompatibility complex
M1	Classically activated macrophages
M2	Alternatively activated macrophages
nm	Nanometre
ng	Nanogram
NaCl	Sodium chloride
Na^2HPO_4	Sodium phosphate dibasic
NaN_3	Sodium azide
NaOH	Sodium hydroxide
Na/K	Sodium/Potassium

NO	Nitrogen oxide
NOD	Non-obese diabetic
NK	Natural killer
NP40	Nonly phenoxypolyethoxylethanol
P	Properdin
pH	Hydrogen ion concentration
%	Percentage
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
PKC	Protein kinase C
PKCα	Protein kinase C alpha
PKCζ	Protein kinase C zeta
PLA_2	Phospholipase A_2
PMA	Phorbol-12-myristate-13-acetate
qRT-PCR	Quantitative real-time PCR
RA	Rheumatoid arthritis
RPMI	Roswell park memorial institute
sec	Second
SCRs	Short consensus repeats
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SP	Signal peptide

shRNA	Short hairpin RNA

T - Z

TAMs	Tumour-associated macrophages
TCR	T cell receptor
tDCs	Tolerogenic dendritic cells
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
THP-1	Human acute monocytic leukaemia cell line
TLR	Toll like receptor
TNF	Tumour necrosis factor
Treg	T regulatory cells
UV	Ultraviolet
v/v	Volume/Volume
V	Volt
VSIG4	V-set and Ig domain-containing 4
VSIG4$^{-/-}$	VSIG4 knock out
VSIG4$^{+/+}$	VSIG4 wild type
WT	Wild type
w/v	Weight/Volume
H_2O	Water
Z39Ig	Z39 immunoglobulin

ABSTRACT

The product of a B7 family-related protein V-set and Ig domain-containing 4 was recently identified to have complement binding properties and termed Complement Receptor Immunoglobulin (CRIg). Although it shares the phagocytosis promoting activities of the classical complement receptors, CR3 and CR4, it differs structurally and in biological properties, from these receptors. CRIg is expressed selectively on macrophages and has been found to display anti-inflammatory and immunosuppressive properties. Despite the importance of CRIg in infection, immunity and inflammation there is little known on the regulation of its expression by inflammatory mediators. This was the subject of the present thesis. The research addressed the hypothesis that cytokines differentially regulate CRIg expression in human macrophages, in a manner distinct from their effects on CR3 and CR4 expression. Furthermore, that expression on human dendritic cells is also regulated by cytokines.

The effects of cytokines on the regulation of CRIg expression on macrophages were examined when cytokines were added either to the monocytes or monocyte-derived macrophages (MDM) per se. The effects of these mediators were also examined on monocyte-derived dendritic cells (DC). Expression of complement receptors was evaluated by qRT-PCR as well as protein expression using Western blotting and flow cytometry. Macrophage phagocytosis activity was assessed by measuring the uptake of complement opsonised heat-killed *Candida albicans* and cytokine production by cytometric bead assay. MDM deficient in PKCα or PKCζ were generated by nucleofection with isozyme specific shRNA. The levels of PKC isozymes in macrophages were determined by Western blots.

The findings show that CRIg expression on macrophages is regulated by several cytokines which can be grouped into Th1, Th2, pyrogenic, immunosuppressive,

regulatory and haematopoietic growth factor patterns. The data demonstrated that CRIg was absent in monocytes but the cells began to express the receptor during their culture as they differentiated into macrophages. While, LT-α, IL-1β, IL-6, IL-10, GM-CSF, M-CSF caused an increase, IFN-γ, TNF, TGF-β1, IL-4 and IL-13 induced a decrease in CRIg expression, showing that both Th1 and Th2 cytokines can similarly control expression of CRIg expression on macrophages. Cytokines acting directly on MDM expressing CRIg modulated this expression. However in this case, all of the cytokines apart from GM-CSF and LT-α caused a decrease in expression. Interestingly in both of the above scenarios the majority of cytokines had opposite effects on CR3 and CR4 expression. Cytokine induced modulation of complement receptor expression was not limited to MDM. The results showed that in monocyte-derived DC, CRIg, CR3 and CR4 expression could be either increased or decreased by cytokines but again the effects differed between CRIg and the classical complement receptors.

The mechanisms of regulation of CRIg expression in MDM were explored and the results revealed that PKCα is critical in the cytokine induced modulation of CRIg expression. MDM rendered PKCα deficient by nucleofection with shRNA showed increased expression of CRIg and showed significant reduction in the LPS-induced CRIg down regulation. The importance of TNF as a key regulatory cytokine was shown as the addition of TNF neutralisation antibody also increased CRIg expression. Although limited studies were conducted on functional aspects of CRIg expressing macrophages, the data revealed that increased and decreased CRIg expression correlated with the respective changes in phagocytosis of complement-opsonised *C. albicans* by macrophages and not with changes in CR3 and CR4. Furthermore MDM induced to express increased amounts of CRIg over CR3 while showing increased phagocytosis of complement opsonised fungi, released significantly less TNF. The effects of cytokines were evident when measuring CRIg protein e.g. by Western blotting as well as CRIg

mRNA, suggesting that regulation is likely to be at a pre-transcriptional level. MDM expressed two forms of CRIg the long and short forms but interestingly DC expressed these as well as an additional intermediatory form. The different forms were similarly regulated by cytokines.

The thesis represents the first comprehensive study on how inflammatory mediators regulate CRIg expression in cells relevant to both innate and adaptive immunity. In the evolution of the inflammatory reaction macrophages encountering microbial pathogens release cytokines which in the main down regulate CRIg and enable the generation of a cascade of mediator/cytokine release which cause monocyte infiltration to the site which differentiate into macrophages which express CRIg levels dictated by the cytokines. Our data identified TNF and PKCα as a key intercellular and intracellular signalling molecules in this expression and that these may function by differentially regulating CRIg versus CR3/CR4 in the inflammatory sites. Our findings have significance in the adaptive immune response by showing that cytokines alter the expression of CRIg on DC. Accordingly cytokines (IL-10, TGF-β1) and agents (dexamethasone, PKC inhibitors) which upregulate CRIg expression promote the development of tolerogenic DC and those which decrease expression (TNF, IFN-γ) break the tolerance function of DC.

The findings not only increase our knowledge of the immune-biology of CRIg but are likely to lead to better interpretation of action of anti-inflammatory drugs including anti-TNF therapy in diseases such as rheumatoid arthritis (RA).

CHAPTER ONE

Introduction

1.1 Introduction

The prime role of the immune system is to provide mechanisms for effective and efficient removal of foreign matter and self-altered tissues from our body. Microbial pathogens including viruses, bacteria, fungi, and parasites present a risk to human health by directly or indirectly damaging tissues and organs. The immune system is designed to engage these pathogens and respond to them in a manner which confines and eliminates them from tissues (Table 1.1). To achieve this goal components of the immune system are interactive, enhancing each other's activities. Lymphocytes, through their subpopulations generate cells (cytotoxic T cells) which can directly interact with infectious targets or promote antibody production (B cells) which has specificity for the antigens on the pathogen. T cell subpopulations may play a helper function (Th) to B cells to produce antibody and the class/sub class (isotype) of antibody. Regulatory T cells have a suppressive role and may prevent the development of autoimmunity (Ferrante, 2005). Innate lymphoid cells (ILCs) that mirror the phenotypes and functions of T cells are also involved in repair responses upon infection (Eberl et al., 2015) (Figure 1.1).

The phagocytic cells of the immune system comprise the polymorphonuclear cells and the mononuclear cells. The former essentially play the simple role of engulfing bacteria and eliminating them from tissues and the body. They mobilize both oxidative and non-oxidative microbicidal mechanisms in the phagocytic vacuole to kill the engulfed bacteria (Ferrante, 2005). The mononuclear phagocytic system, in comparison, is far more complex with these cells playing not only a phagocytic and microbial killing role but also a more intricate intercellular function to activate other leukocytes, including T cells to establish an immune response. The macrophages may be found strategically positioned in organs such as the liver to act as a microbial pathogen

filtering system. They serve a similar function in the lungs, and act as residents in compartments such as the peritoneum. The fixed tissue macrophages play a strategic role in sensing the invading bacteria or damaged tissues. They thus play key roles in innate immunity by directly killing microbial pathogens and in adaptive immunity by presenting antigens and signals to the T cells. Supplementing the macrophages localized in tissues are the blood monocytes which become mobilized and move from the vasculature to the tissues in response to microbial invasion and tissue damage. These differentiate into macrophages.

The literature review that follows has been organized to introduce components of the inflammatory reaction, whether in infection and immunity or chronic inflammatory settings, and a relatively newly described Complement Receptor Immunoglobulin (CRIg) which is found on macrophages. The present study used the *in vitro* culture and primarily based on the human monocytes developing into macropahges or dendritic cells. This chapter mainly focus on the immunobiology of mononuclear phagocytes including monocyte, macrophages and dendritic cells, their association with the complement system and complement receptors including CRIg, CR3 and CR4 and the effects of endogenous inflammatory mediators, cytokines.

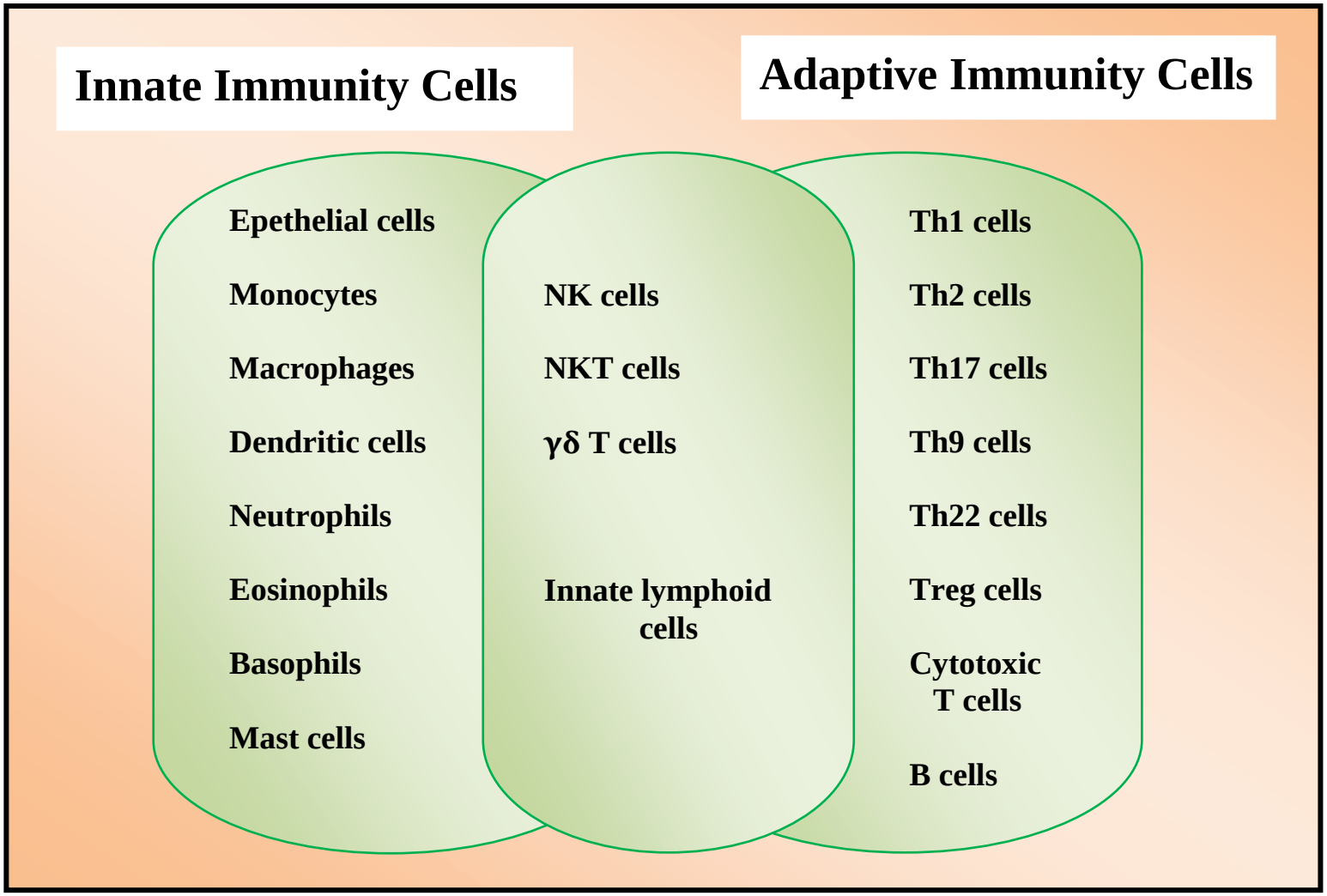

Figure 1.1: Innate and adaptive immune cells (Striz et al., 2014). Both systems collaborate with each other and some of the cells share characteristics of both innate and adaptive systems. Adapted and redrawn from (Striz et al., 2014).

Table 1.1: Receptors of the innate immune system used to detect infection.

Receptors of the innate immune system			
Receptor	**Location**	**Target**	**Effects**
Complement	Bloodstream, tissue fluids	Microbial cell wall components	Complement activation, opsonisation, lysis
Mannose-binding lectin (MBL)	Bloodstream, tissue fluids	Mannose-containing microbial carbohydrates (cell walls)	Complement activation, opsonisation
C-reactive protein (CRP)	Bloodstream, tissue fluids	Phosphatidylcholine, pneumococcal polysaccharide (microbial membranes)	Complement activation, opsonisation,
Lipopolysaccharide (LPS) receptor, LPS-binding protein (LBP)	Bloodstream, tissue fluids	Bacterial lipopolysaccharide (gram-negative cell walls)	Delivery to cell membrane
Toll-like receptors	Cell surface or internal compartments	Microbial components not found in hosts	Induces innate responses
NOD family receptors	Intracellular	Bacterial cell wall components	Induces innate responses
Scavenger receptors	Cell membrane	Many targets, gram-positive and gram-negative bacteria, apoptotic host cells	Induces phagocytosis or endocytosis

Adapted from Kuby Immunology (sixth edition), Innate immunity, Chapter 3 (Kindt et al., 2006).

1.2 Mononuclear phagocytes

Mononuclear phagocytes are diverse and primarily consist of monocytes circulating in the blood and macrophages (Mantovani et al., 2009) and dendritic cells in the tissues linking innate and adaptive immunity (Haniffa et al., 2015). Histiocytes or immature macrophages (monocytes) or myeloid cells of bone marrow origin circulate for about one to three days in the bloodstream during which time they may be attracted to an area of inflammation by chemokines (Imhof and Aurrand-Lions, 2004).

Monocytes are continuously entering and leaving the bloodstream, maintaining 1-6 % of the total normal adult white blood cell count. Migration of monocytes is a random phenomenon; it occurs in the presence of specific sets of transcription factors (Valledor et al., 1998, Nagamura-Inoue et al., 2001, Lehtonen et al., 2007) (Figure 1.2). A fraction of circulating monocytes undergoe a series of changes and modifications to become resident and tissue-specific macrophages (Gordon and Taylor, 2005, Lehtonen et al., 2007).

Originating from haematopoietic-stem cell progenitors in the bone marrow (Geissmann et al., 2010), circulating monocytes and tissue macrophages are the key modulators and effector cells in the immune response. Macrophages are involved in diverse activities (Gordon et al., 1992) including pro-inflammatory versus anti-inflammatory, tissue-destructive versus tissue-restorative and immunogenic versus tolerogenic activities (Stout and Suttles, 1997, Gordon, 2003). Haematopoietic stem cells (HSC) generate intermediate precursors including common myeloid progenitors, granulocyte and macrophage progenitors and MDP (Haematopoietic-derived stem cell progenitors (myeloid colony-forming units (M-CFU)) in the bone marrow of the adult or the yolk sac of the developing embryo develop into MDP). In the bone marrow,

MDPs give rise to two separate subpopulations of pro-monocyte and common DC progenitors (CDPs).

Monocytes and tissue macrophages develop from an MDP-derived pro-monocyte precursor. During homeostasis, most of the tissue macrophages are probably derived from lymphocyte antigen 6C negative ($LY6C^-$) blood monocytes, while under inflammatory conditions lymphocyte antigen 6C positive ($LY6C^+$) blood monocytes differentiate into inflammatory macrophages. The macrophages can be polarized into three phenotypes, classically activated macrophages (M1), alternatively activated macrophages (M2) and tumour associated macrophages (TAMs). In secondary lymphoid organs, pro-DC which have developed from CDP can give rise to lymphoid DC. The $LY6C^+$ monocytes can also develop into monocyte-derived DC (Auffray et al., 2009, Lawrence and Natoli, 2011) (Figure 1.3).

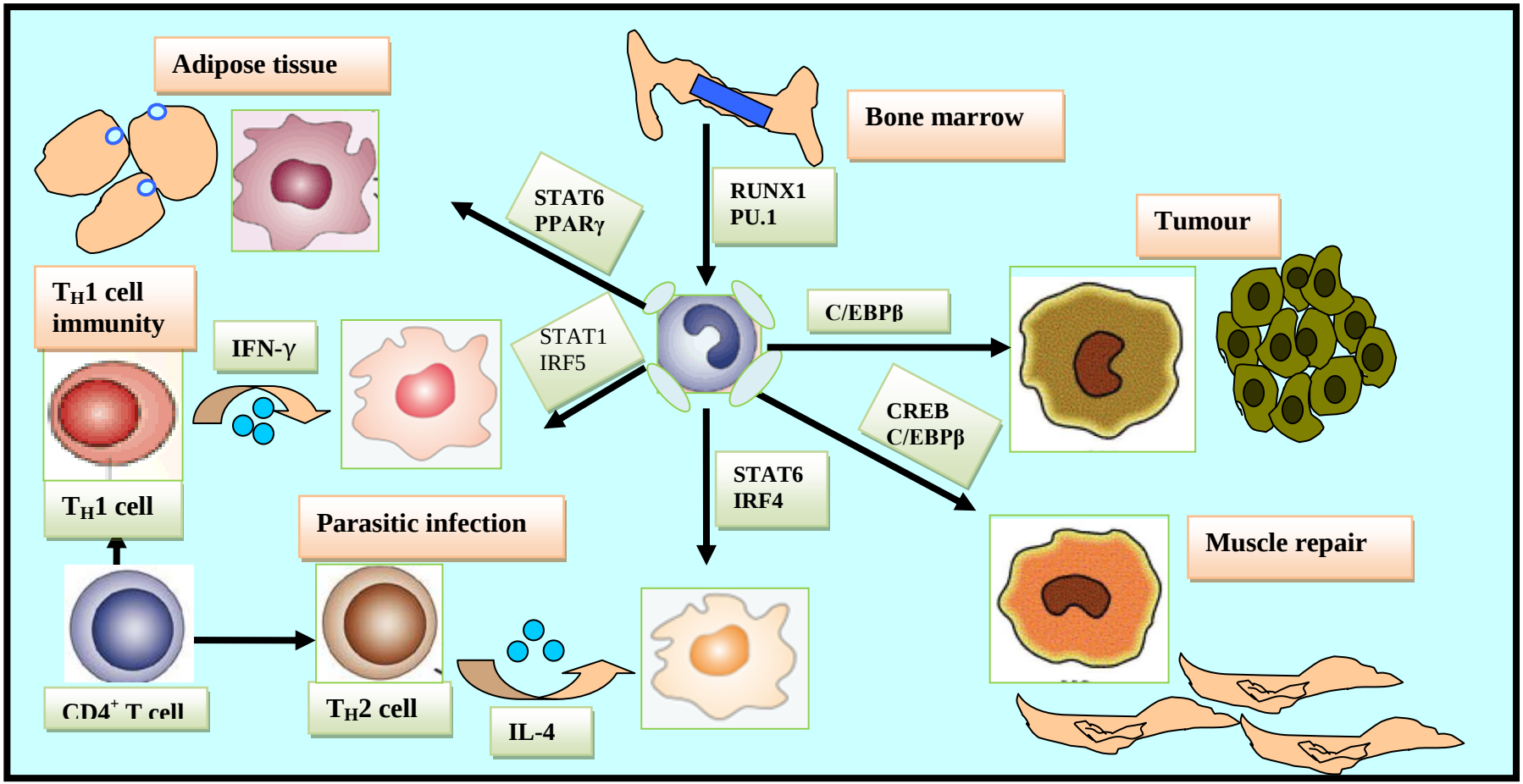

Figure 1.2: Transcription factors involved in macrophages polarization. The key transcription factors associated with the functional polarization of macrophages. C/EBPβ, CCAAT/enhancer-binding protein-β; CREB, cAMP-responsive element-binding protein; IFN-γ, interferon-γ; IL-4, interleukin-4; IRF, interferon-regulatory factor; PPARγ, peroxisome proliferator-activated receptor-γ; RUNX1, Runt-related transcription factor 1; STAT, signal transducer and activator of transcription; T_H, T helper. Adapted and redrawn from (Lawrence and Natoli, 2011).

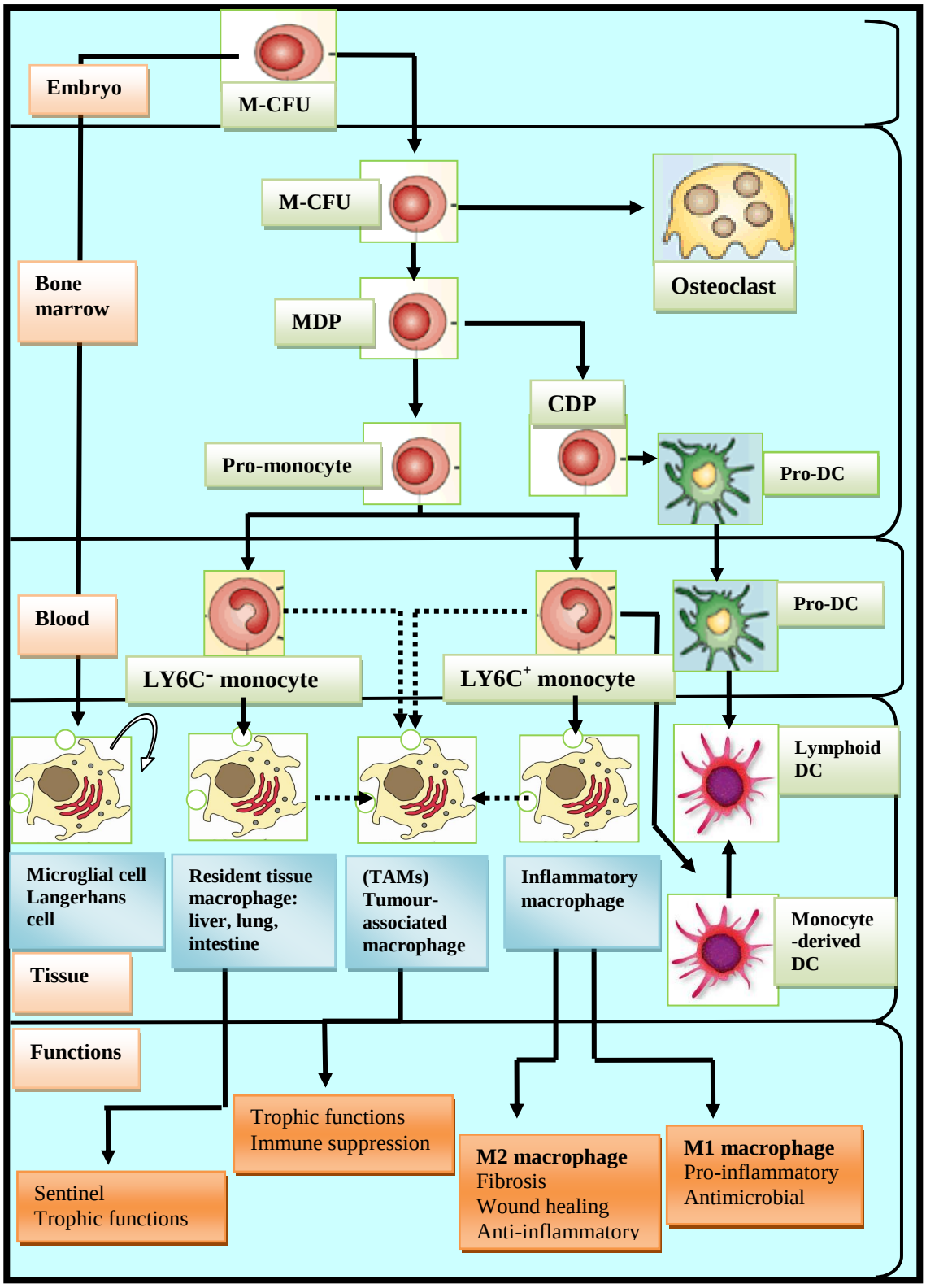

Figure 1.3: A schematic representation of the mononuclear phagocyte system. Tissue macrophages and dendritic cells are derived from haematopoietic stem cells (HSC) that resides in the bone marrow (see text for description)**.** M-CFU, myeloid colony-forming units; MDP, macrophage and dendritic cell progenitor; DC, dendritic cells; CSF1, macrophage colony-stimulating factor 1; CDPs, common DC progenitors; TAMs, tumour-associated macrophages. Adapted and redrawn from (Lawrence and Natoli, 2011).

1.2.1 Monocytes

The monocyte is destined to circulate and to play a role in eliminating dead and damaged tissues. Monocyte levels can be increased (monocytosis) in response to chronic infections, cancers, autoimmune disorders and in various blood disorders. Reduced monocyte numbers (monocytopenia) are seen in response to toxins in the blood (endotoxemia), as well as following the treatment of patients with chemotherapeutics. These phagocytose agents by either binding to the substances directly via pattern-recognition receptors or by binding to opsonins such as complement or antibodies that coat the pathogen surface. Monocytes possess flexible differentiation potential, and can differentiate into macrophages, dendritic cells, osteoclasts and microglia, depending on the stimuli (Stout and Suttles, 2004). The most relevant stimuli are cytokines, and the initial cytokines to which a monocyte is exposed determines its subsequent differentiation program (Erwig et al., 1998).

1.2.2 Macrophages

Macrophages reside in tissues (Naito, 2008) to perform phagocytic functions. These are long lived cells remaining biosynthetically active up to several months. Besides their activity *per se* the cells regulate the activity of other cell types systemically, thus have a dominating role in tissue homeostasis. Their role in innate immunity is through the cell's ability to recognize molecular patterns on pathogens (Lehtonen et al., 2007).

Macrophages play a central role in immunity by providing a first line of defence against invading microbial pathogens (Stout and Suttles, 2004, Mantovani et al., 2009). They are strategically placed in many tissues of the body to play this role (Mantovani et al., 2009) (Table 1.2). They identify and phagocytose invading pathogens and shape

inflammatory processes by secreting cytokines and other mediators into tissues (Aderem and Underhill, 1999, Janeway and Medzhitov, 2002, Shi and Pamer, 2011).

When the innate immune system is unable to cope with the invading microorganisms, macrophages, under the influence of appropriate activation signals, function as effector cells of the adaptive immune system. Other important functions of macrophages include wound healing, tissue repair and resolution of inflammation, and the maintenance of tissue homeostasis. To accomplish this array of tasks, macrophages are in a state of plasticity with gene expression programs adapting to specific phenotypes depending on the stimuli and surrounding microenvironment. While macrophages are functionally heterogeneous under normal conditions, exposure to microbial pathogens and to endogenous factors increases their functional heterogeneity (Gordon and Taylor, 2005).

Besides anti-microbial functions macrophages may contribute to the progression of chronic inflammatory conditions and to tissue damage under the influence of chemokines, growth factors and cytokines (Hansson and Libby, 2006, Qian and Pollard, Gordon and Martinez, 2010). For example in the presence of IFN-γ and LPS, macrophages exhibit a potent pro-inflammatory phenotype (Gordon and Taylor, 2005, Gordon, 2007, Meghari et al., 2007, Mosser and Edwards, 2008, Martinez et al., 2009, Gordon and Martinez, 2010), under the influence of Th2 cytokines macrophages adopt an alternative or different phenotype (Stein et al., 1992), while in the presence of immune complexes macrophages becomes regulatory cells, secreting high levels of IL-10. During inflammatory responses macrophages express mixed phenotypes (Gordon, 2003, Gordon and Taylor, 2005, Mosser and Edwards, 2008, Martinez et al., 2008). The heterogeneity of macrophages is based on the complex environment stimuli and type and state of infection (Mantovani et al., 2004, Gordon and Taylor, 2005).

Macrophage activation has been linked to a variety of inflammatory diseases involving the secretion of growth factors, cytokines and enzymes that may participate either in tissue damage or repair at the site of inflammation. For example, mouse models have shown that inhaled allergens activate macrophages and dendritic cells that produce Th2 (IL-4 and IL-13) cytokines to initiate and propagate the symptoms of asthma (Dasgupta and Keegan, 2012). In another study, human macrophages have been recognized as the main contributor of inflammatory responses in lungs subjected to chronic cigarette smoke exposure leading to chronic obstructive pulmonary disease (Mannino and Buist, 2007, Lee, 2012).

Table 1.2: Macrophages in various tissues. Information sourced from (Weidenbusch and Anders, 2012).

Resident macrophages in various tissues		
Macrophages	**Organs / Cell types**	**Reference (s)**
Dermal macrophages	Skin	(Gordon and Taylor, 2005)
Microglia	Brain	(Gordon and Taylor, 2005)
Alveolar macrophages	Lung	(Gordon and Taylor, 2005)
Intestinal macrophages	Stomach	(Mowat and Bain, 2011)
Intestinal macrophages	Ileum	(Mowat and Bain, 2011)
Intestinal macrophages	Colon	(Mowat and Bain, 2011)
Kupffer cells	Liver	(Gordon and Taylor, 2005)
Marginal zone macrophages, red pulp macrophages	Spleen	(Gordon and Taylor, 2005)
Ovarian macrophages	Ovary / Testis	(Yang et al., 2011)
Bone marrow macrophages	Bone marrow	(Ehninger and Trumpp, 2011)
Osteoclasts	Bone	(Gordon and Taylor, 2005)

1.2.3 Classification of macrophages depending on their activation state

It is evident now that macrophages demonstrate a range of activation profiles. This concept of phenotypic alteration of immune cells was first noted by Mosmann and his colleagues (Mosmann and Sad, 1996).

1.2.3.1 Classically activated macrophages (M1)

Macrophage activation or classical activation was introduced by Mackaness in the 1960s (Mackaness, 1962), and defined as the first pathway of macrophage activation (M1) (Gordon and Martinez, 2010). Classical activation is also regarded as the pro-inflammatory activation state of macrophages (Mackaness, 1964, van Furth et al., 1972), depending on the secreted molecules of activated T helper 1 (Th1) $CD4^+$ lymphocytes or natural killer cells including, IFN-γ, and TNF and bacterial LPS (Gordon and Taylor, 2005, Gordon, 2007). Classically activated macrophages (M1) undergo cell activation in response to Th1 cytokines such as IFN-γ alone or in concert with microbial stimuli (e.g. LPS) or other cytokines (e.g. TNF and GM-CSF) (Gordon and Taylor, 2005, Gordon, 2007, Meghari et al., 2007, Mosser and Edwards, 2008, Gordon and Martinez, 2010). M1 macrophages have an IL-12 ^{high}, IL-23^{high} and IL-10^{low} phenotype (Mantovani et al., 2007). Classically activated macrophages show enhanced microbicidal activity and up regulated pro-inflammatory cytokines to strengthen cell-mediated adaptive immunity (Gordon and Taylor, 2005, Gordon, 2007) (Table 1.3, 1.4) (Figure 1.4).

1.2.3.2 Alternatively activated macrophages (M2)

An alternatively activated macrophage state has been designated as “M2” macrophages by analogy to Th2 (CD4 helper T cells) (Mills et al., 2000). M2 macrophages are known to be induced by Th2 cytokines such as IL-4 or IL-13, IL-10, and glucocorticoid and immune complexes (Gordon, 2003, Gordon and Taylor, 2005, Martinez et al., 2008). In addition, M2 macrophages are activated by IL-21 and TGF-β (activin A) (Gallina et al., 2006, Kzhyshkowska et al., 2006, Ogawa et al., 2006, Pesce et al., 2006, Martinez et al., 2008). The M2 macrophage activation states are further subdivided into M2a, M2b and M2c. M2a depends on IL-4 and IL-13, M2b follows stimulation by immune complexes in the presence of Toll-like receptor ligand, M2c macrophages form in the presence of anti-inflammatory stimuli including IL-10 or TGF-β and glucocorticoid hormones (Martinez et al., 2008). M2 macrophages have an IL-12^{low}, IL-23^{low} and IL-10^{high} phenotype (Mantovani et al., 2007). M2 macrophages are not only involved in the immune response against parasites, but also in allergy, wound healing and tissue remodelling (Gordon, 2003, Martinez et al., 2009) (Table 1.3, 1.4) (Figure 1.4).

Table 1.3: Tissue distribution of macrophages. Information sourced from (Mantovani et al., 2007).

Macrophage polarization	
M1 **Classically activated macrophages**	**M2** **Alternatively activated macrophages**
LPS + IFN-γ	**IL-4 / IL-13, Glucocorticoids, TGF-β**
• IL-12 ^{high} • IL-23 ^{high} • TNF ^{high} • IL-1 ^{high} • IL-10 ^{low} • M1 Chemokines (e.g. CXCL10) • ROI • RNI	• IL-12 ^{low} • IL-23 ^{low} • TNF ^{low} • IL-1ra ^{high} • IL-10 ^{high} • M2 Chemokines (e.g. CCL22) • Scavenger, mannose, galactose receptors ^{high}
• Type I inflammation • Tissue destruction • Killing of intracellular parasites • Tumour resistance	• Type II inflammation • Tissue remodelling and angiogenesis • Parasite encapsulation • Tumour promotion

Table 1.4: Effects of M1 (IFN-γ) and M2 (IL-4/IL-13) on macrophages. Information sourced from (Martinez and Gordon, 2014).

Effects on macrophages			
M1 (IFN-γ)	**Reference (s)**	**M2 (IL-4/IL-13)**	**Reference (s)**
Inhibits replication of HIV at early pre-integration steps	(Cassol et al., 2009)	Decreases phagocytosis of particles while increasing inflammatory cytokine production	(Varin et al., 2010)
Decreases Fc-mediated phagocytosis	(Frausto-Del-Rio et al., 2012)	Decreases autophagy in TB infection	(Harris et al., 2007)
Decreases complement-mediated phagocytosis	(Schlesinger and Horwitz, 1991)	Induces fusion	(Helming and Gordon, 2007)
Induces autophagy in TB infection	(Matsuzawa et al., 2012)	Inhibits IFN-γ-induced fusion	(Takashima et al., 1993)
Increases fusion in combination with concanavalin A	(Takashima et al., 1993)	Favours Arginase-I vs. i-NOS, ArgI + macrophages suppress Th2 inflammation and fibrosis	(Pesce et al., 2009)
Induces fusion in alveolar macrophages	(Nagasawa et al., 1987)	Inhibits HIV replication at post-integration level	(Cassol et al., 2009)
Induces mycobacteria killing vía NO	(Herbst et al., 2011)		
Mediates parasite killing via NO	(Thomas et al., 1997, Piedrafita et al., 2001)		

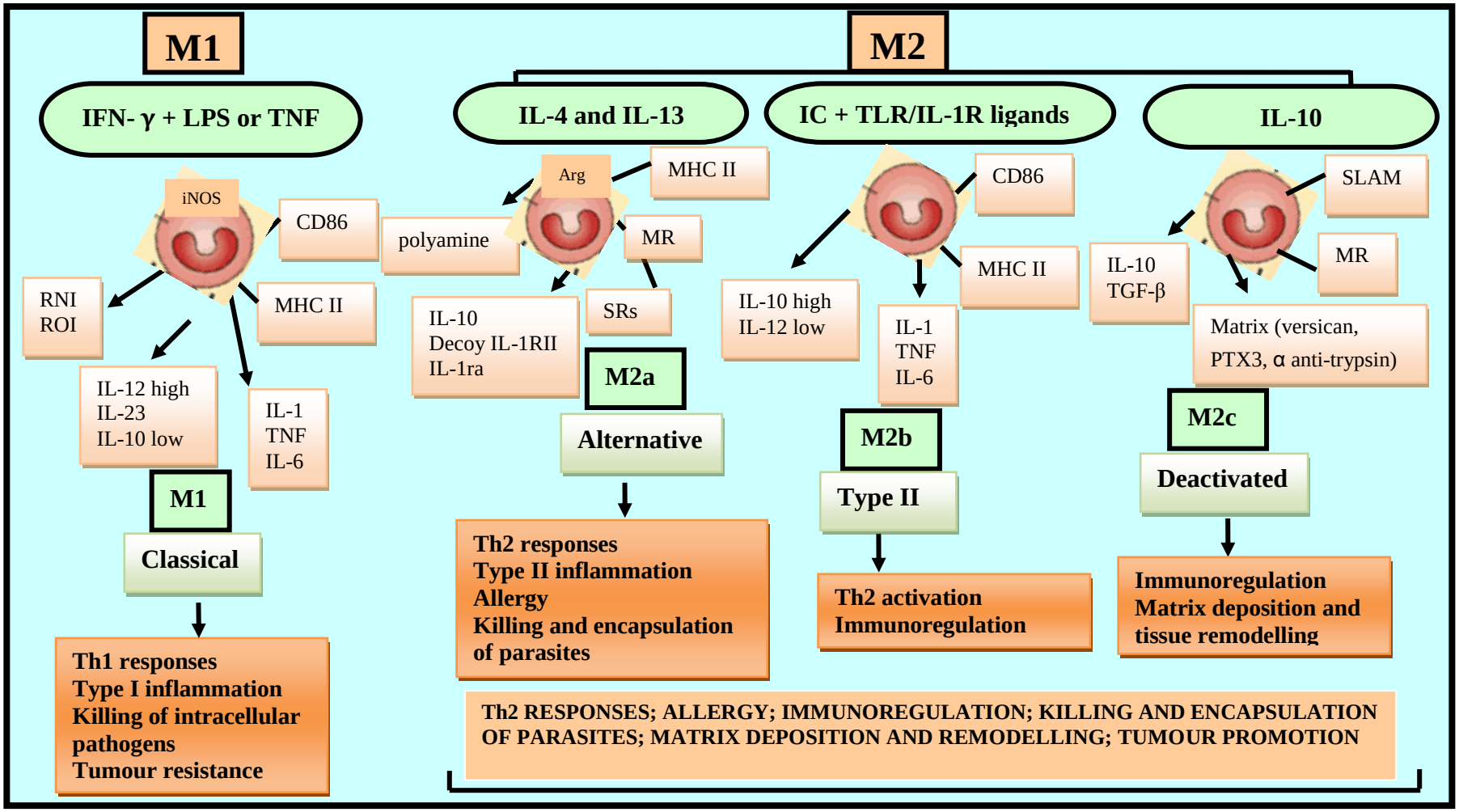

Figure 1.4: M1 and M2 model of macrophage polarization. M1 activation state occurs after exposure to IFN-γ, LPS or TNF, while the M2 state is functionally subdivided into M2a, M2b and M2c depending on exposure. M2a occurs after IL-4 and IL-13 exposure; M2b, exposure to immune complex + Toll like receptor (TLR) ligand; M2c, exposure to IL-10 and glucocorticoids. Adapted and redrawn from (Martinez and Gordon, 2014).

1.3 Dendritic cells

Monocytes can also differentiate into dendritic cells (DC), known as antigen presenting cells which are present in almost all tissues in an immature state (Banchereau et al., 2000, Lehtonen et al., 2007, Steinman, 2008) (Table 1.5), and have a unique ability to migrate out of tissues. Different subsets of DC exist (Schlitzer et al., 2015), including dermal DCs (dDC) ($CD1a^{+}DC\text{-}SIGN^{+}$), Langerhans cells (LDC) ($CD1a^{+}Langerin^{+}$), myeloid DCs (mDC) ($CD1c^{+}CD19^{-}$), monocyte-derived DC (moDC) ($CD1a^{+}CD14^{-}$) and plasmacytoid-derived DC (pDC) ($CD45RA^{+}CD123^{+}$) (Alvarez et al., 2008, Shortman and Heath, 2010, Cheong et al., 2010, Li et al., 2011). These different subsets originate from HSC by different pathways (van Furth and Cohn, 1968, Geissmann et al., 2010).

DC populations are distributed throughout the body at strategic sites including skin and mucosal surfaces where pathogens are entering into the system and secondary lymphoid organs where adaptive immune responses are initiated. DC perform the distinct function of antigen capture in one location and antigen presentation in another. Immature forms of these cells monitor the body and can capture intruding pathogens. They then leave the tissues and migrate into secondary lymphoid organs where they present the antigen to prime naïve T cells (Gordon and Martinez, 2010, Biswas and Mantovani, 2010, Murray and Wynn, 2011, Lawrence and Natoli, 2011). In lymphoid organs, they are able to activate antigen specific T and B cells and induce adaptive immune responses (Lehtonen et al., 2007). Macrophages and DC are the important antigen presenting cells of the immune system and share many similar functions in regulating innate and adaptive immune responses during infections (Banchereau et al., 2000, Thery and Amigorena, 2001, Foti et al., 2004, Stout and Suttles, 2004, Hume, 2006). DC also serve as a major link between innate and adaptive immune responses

because of their unique ability to stimulate naïve T cells (Banchereau et al., 2000, Steinman, 2008). DC have the ability to detect microbes and ingest pathogenic material (Banchereau et al., 2000, Thery and Amigorena, 2001, Banchereau et al., 2003, Foti et al., 2004), playing a key role in the recruitment of other immune cells such as macrophages, immature DC, neutrophils and natural killer (NK) cells to sites of infection (Lambrecht et al., 1998, Banchereau et al., 2000). For a more detailed explanation of how dendritic cells can control the adaptive immune system see a recent review by (Iwasaki and Medzhitov, 2015).

Table 1.5: Different dendritic cells in various tissues. Information sourced from (Weidenbusch and Anders, 2012).

Dendritic cells in various tissues		
Dendritic cells (DC)	**Organs / Cell types**	**Reference (s)**
Dermal DC, Langerhans cells	Skin	(Gordon and Taylor, 2005)
Lamina propia DC	Stomach	(Mowat and Bain, 2011)
Lamina propia DC	Ileum	(Mowat and Bain, 2011)
Lamina propia DC	Colon	(Mowat and Bain, 2011)
Plasmacytoid DC, cDC	Liver	(Crispe, 2011)
iDC, follicular DC	Spleen	(Sathe and Shortman, 2008)
Dendritic cell precursors	Pancreas	(Welzen-Coppens et al., 2012)
Interstitial DC	Kidney	(John and Nelson, 2007, Nelson et al., 2012)

1.4 Mediators of inflammation

Mediators of inflammation play a crucial role in shaping the nature, intensity, chronicity and resolution of the inflammatory response. These may be of exogenous origin e.g. as a result of tissue being invaded by bacteria and the release of cell active microbial substances such as endotoxin. These exogenous mediators are interactive with endogenously generated mediators of inflammation. Amongst the different groups of mediators are the cytokines (Table 1.6) (Figure 1.5). The importance of these can be seen from the fact that TNF and other inflammatory mediators have been targeted for their therapeutic benefits in diseases such as rheumatoid arthritis (Scott et al., 2010).

Mediators of inflammation include humoral factors such as complement (see complement section 1.5) which is activated following contact with microbial pathogens and altered or damaged tissue of the body. The complement fragments generated in this system promote inflammation by a combination of anaphylatoxic activity, making the vascular wall permeable, chemotaxis, cell activation and direct tissue damage.

An understanding has been reached over the last two to three decades of a set of cytokines which have highly overlapping biological properties, produced primarily by macrophages in human, termed pyrogenic cytokines, TNF, IL-1β and IL-6 because of their ability to induce fever (Hanson and Murphy, 1984, Dinarello et al., 1986, Nijsten et al., 1987, Saper and Breder, 1994, Dinarello et al., 1999). These three cytokines while important in controlling immunity against infections are at the centre of causing cell activation and tissue damage in chronic inflammatory diseases. There is evidence to suggest that these cytokines may be induced in a sequential manner i.e. TNF then IL-1β and then IL-6 thereby not only maintaining the actions of the previous cytokine but also promoting new actions to the inflammatory response (Zetterstrom et al., 1998, Netea et al., 2000). IL-10 is considered as an immunosuppressive cytokine and its production

may protect against chronic inflammation (de Waal Malefyt et al., 1991, Fiorentino et al., 1991a, Fiorentino et al., 1991b, Asadullah et al., 2003).

Other cytokines which are known primarily for their property of being haematopoietic growth factors are the M-CSF and GM-CSF. While their role is mostly restricted to the development of macrophages from precursor cells, there is also compelling evidence that they act on mature mononuclear phagocytes.

To gain an understanding of the regulation of development of $CRIg^+$ macrophages and its expression this thesis will focus on a set of cytokines which are known to act on macrophages and which allow us to determine the role of the various cytokine patterns generated during inflammation. The properties of some of these cytokines are introduced below (Table 1.7).

Table 1.6: Cytokines involved in immune responses (Striz et al., 2014).

Cytokines	Reference (s)
TNF	(Aggarwal et al., 2012)
IL-1 (IL-1α, IL-1β)	(Dinarello et al., 1983, Kobayashi et al., 1990, Kostura et al., 1989)
IL-18	(Piper et al., 2013)
IL-33	(Dinarello, 2005)
IL-5	(Salmond et al., 2012)
IL-13	(Wynn, 2003)
IL-4	(Velazquez et al., 2000)
IL-36 (IL-36α, IL-36β, IL-36γ, IL-36Ra)	(Carrier et al., 2011)
IL-37	(Boraschi et al., 2011)
IL-38	(van de Veerdonk et al., 2012)
IL-6	(Horii et al., 1988)
IL-11, LIF (leukaemia inhibitory factor), oncostatin M, CNTF (ciliary neurotrophic factor), cardiotrophin-1.	(Taga, 1997)
IL-31	(Kasraie et al., 2010)
Type I IFNs (IFN-α, IFN-β)	(Adachi et al., 1997)
Type II IFNs (IFN-γ)	(Frasca et al., 2008)
Type III IFNs (IFN-λ1 (Il-28A), IFN-λ2 (IL-28B), IFN-λ3 (IL-29))	(Ioannidis et al., 2013)
IL-26	(Donnelly et al., 2010)
TGF-β	(Doerner and Zuraw, 2009)
M-CSF	(Chitu and Stanley, 2006)
IL-8	(Reape and Groot, 1999)
LT-α	(Schneider et al., 2004)
GM-CSF	(Richardson et al., 1992)
G-CSF	(Tsuji et al., 1994)
Chemokines (approximately 50 different chemokines identified)	(Nomiyama et al., 2013)
IL-12	(Lyakh et al., 2008)
IL-23	(Hannas et al., 2011)
IL-27 (p28/EBi3 complex)	(Hall et al., 2012)
IL-35 (p35/EBi3 complex)	(Collison et al., 2007)
IL-17/IL-17A (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F	(Miao et al., 2013)
IL-7	(Shalapour et al., 2012)
IL-15	(Perera et al., 2012)
IL-10	(Enk et al., 1995)
IL-19	(Azuma et al., 2011)
IL-20	(Lebre et al., 2012)
IL-22	(Chung et al., 2006)
IL-24	(Wang and Liang, 2005)

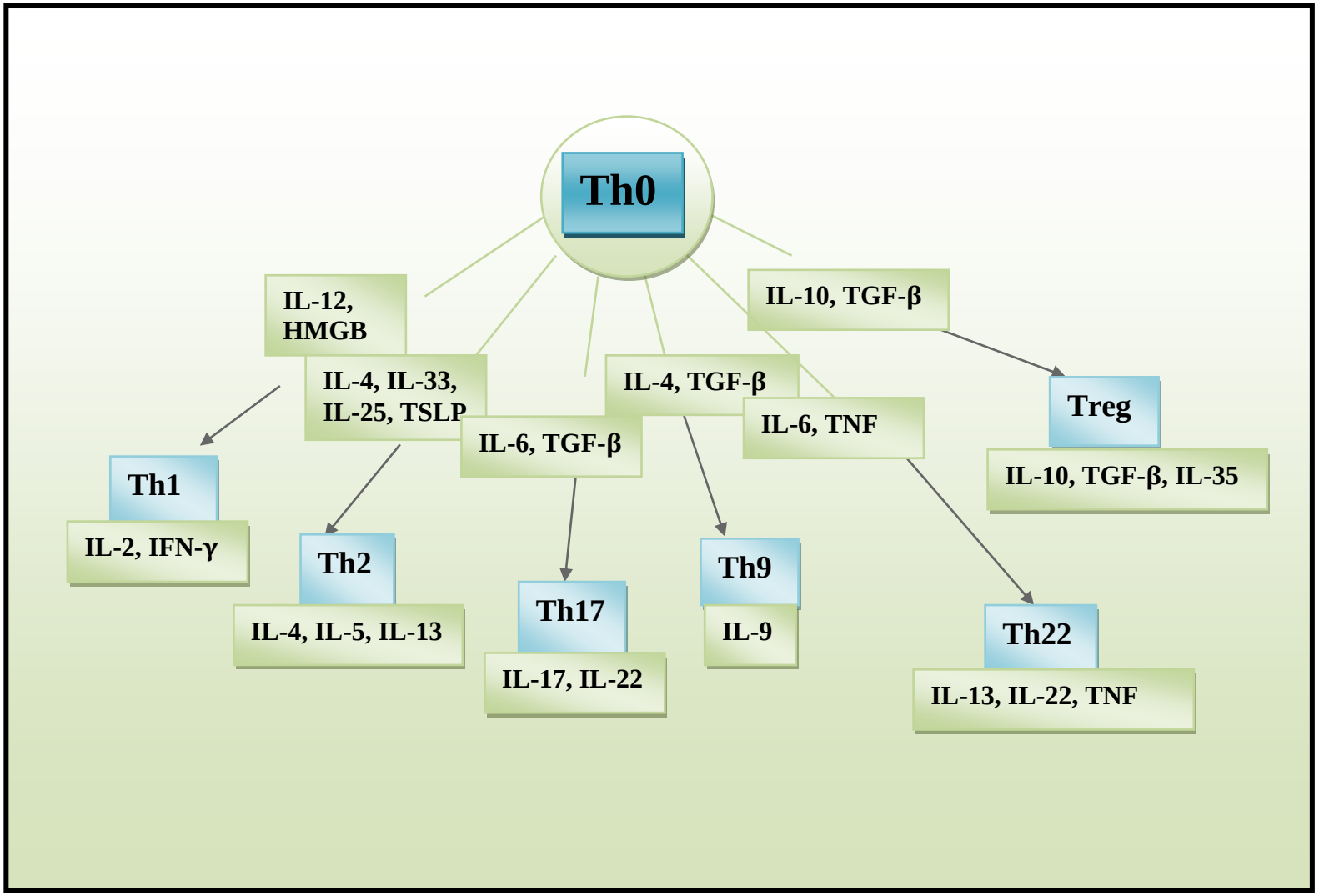

Figure 1.5: Polarization of Th cell responses based on cytokine patterns (Striz et al., 2014). Th cells can be divided into six functional subpopulations: Th1, Th2, Th17, Th9, Th22 and Treg, characterized by cytokine network. Adapted and redrawn from (Striz et al., 2014).

Table1.7: Effects of cytokines on macrophage function.

Cytokine	Effect on macrophages	Reference(s)
IL-4 and IL-13	IL-4$^{-/-}$ mice show increased bacterial clearance from both joints and kidneys in experimental *Staphylococcus aureus*-induced sepsis and septic arthritis Human macrophages treated with rIL-4 show depressed killing of asexual erythrocytic forms of *Plasmodium falciparum.* *In vitro* exposure of macrophages to IL-4 and IL-13 inhibited autophagy-mediated killing of mycobacteria Autophagy of *Citrobacter* was inhibited by treating macrophages with IL-4 and IL-13 IL-13$^{-/-}$ mice showed delayed clearance of bacteria	(Hultgren et al., 1998) (Kumaratilake and Ferrante, 1992) (Harris et al., 2007) (Su et al., 2012) (Olszewski et al., 2007)
IL-1β	Involved in clearing infections in models utilizing *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* Was required for the clearance of *Bordetella pertussis* in the murine lung	(Place et al., 2014, Mayer-Barber et al., 2010, Karmakar et al., 2012) (McElvania Tekippe et al., 2010)
IL-10	Is a potent inhibitor of IFN-γ-induced macrophage microbicidal activity against intracellular and extracellular parasites Down regulating various macrophages function, such as respiratory burst, Ia expression and cytokine production (TNF, IL-1β and IL-6) Plays a critical role in phagocytic functions of macrophages	(Gazzinelli et al., 1992, Oswald et al., 1992) (Fiorentino et al., 1991b, de Waal Malefyt et al., 1991, Bogdan et al., 1991) (Li et al., 2012)
TNF and LT-α	TNF $^{-/-}$ LT-α $^{-/-}$ mice were highly susceptible to infection with viable *Salmonella Typhimurium* as compared to wild type	(Dharmana et al., 2002)

IFN-γ	Priming human MDM with IFN-γ, increases the non-oxidative early intracellular killing of *Salmonella enterica Serovar Typhimurium.*	(Gordon et al., 2005)
	Enhances internalization and non-oxidative killing following infection with *Salmonella Typhimurium* Generally increases the bacterial killing effect of MDM in the presence of antibiotics Enhances the respiratory burst, the NO secretion and anti-tumour cytotoxic activity Increases killing of group B *Streptococcus* Type III in adult macrophages	(Gordon et al., 2005) (Smith et al., 2010) (Boehm et al., 1997) (Lin et al., 1995) (Arenzana-Seisdedos et al., 1985) (Cavaillon, 1994) (Marodi et al., 2000)
GM-CSF	Effects tumoricidal activity Increases the production of antibody-dependent cellular cytotoxicity (ADCC) Increases the expression of adhesion molecules, respiratory burst and phagocytic capacity Increases the killing of group B *Streptococcus* Type III in adult macrophages Increases killing capacity of cord blood macrophages with recombinant human GM-CSF Deficiencies in GM-CSF increase susceptibility to gram-negative pathogen *Pseudomonas aeruginosa* infection *in vivo*.	(Pojda et al., 1989) (Inamura et al., 1990) (Selgas et al., 1996) (Williams et al., 1995) (Nagler et al., 1996) (Marodi et al., 2000)

M-CSF	Effects tumoricidal activity Increases the production of antibody-dependent cellular cytotoxicity (ADCC)	(Munn and Cheung, 1989) (Munn and Cheung, 1990) (Qi et al., 1995) (Adachi et al., 1993) (Sakurai et al., 1997)
TGF-β	Using a murine bone marrow transplant (BMT) model, TGF-β signalling induces microRNA (miR)-29b and promoting impaired alveolar macrophages (AM) responses (impaired bacterial killing) TGF-β-activated kinase (TAK1) activators, TAK1-binding protein 1 (TAB1) and TAK1-binding protein 2 (TAB2), play important role in the survival of activated macrophages	(Ballinger et al., 2006) (Domingo-Gonzalez et al., 2014) (Mihaly et al., 2014)

1.5 The complement system

1.5.1 Introduction

The complement system plays a key role in innate immunity and is involved in providing defence against infections. Additionally, it maintains cell homeostasis by recognizing and eliminating apoptotic and necrotic cells. Although the complement system has been primarily recognized in performing a role in the innate immune system, its role in the adaptive immune response has also been recognized (Walport, 2001a, Walport, 2001b, Mastellos and Lambris, 2002, Morgan et al., 2005) (Figure 1.6).

Complement serves as a dominant opsonin in the absence of antibodies for the effective removal of apoptotic cells, immune complexes and pathogenic waste from the circulation by promoting phagocytosis (Mevorach et al., 1998, Taylor et al., 2000, Walport, 2001a, Walport, 2001b, Rus et al., 2005, Trouw et al., 2008). The complement system consists of some thirty five or more humoral and cell-associated proteins which when activated interact to initiate a cascade of proteolytic reactions leading to cleavage products with several important biological activities. This includes anaphylatoxins, chemotactic factors, opsonins and lytic complexes. These mediate tissue permeability, cell migration, phagocytosis of foreign matter and lysis of bacteria, respectively. Complement activation is usually triggered by the interaction of pattern-recognition receptors with foreign surface structures and follows one of the three pathways, the classical, alternative and the lectin pathways (Gros et al., 2008) (Figure 1.7). Some bacteria activate complement directly as in the alternative pathway, whereas others require antibody binding to their surface (Frank, 2010).

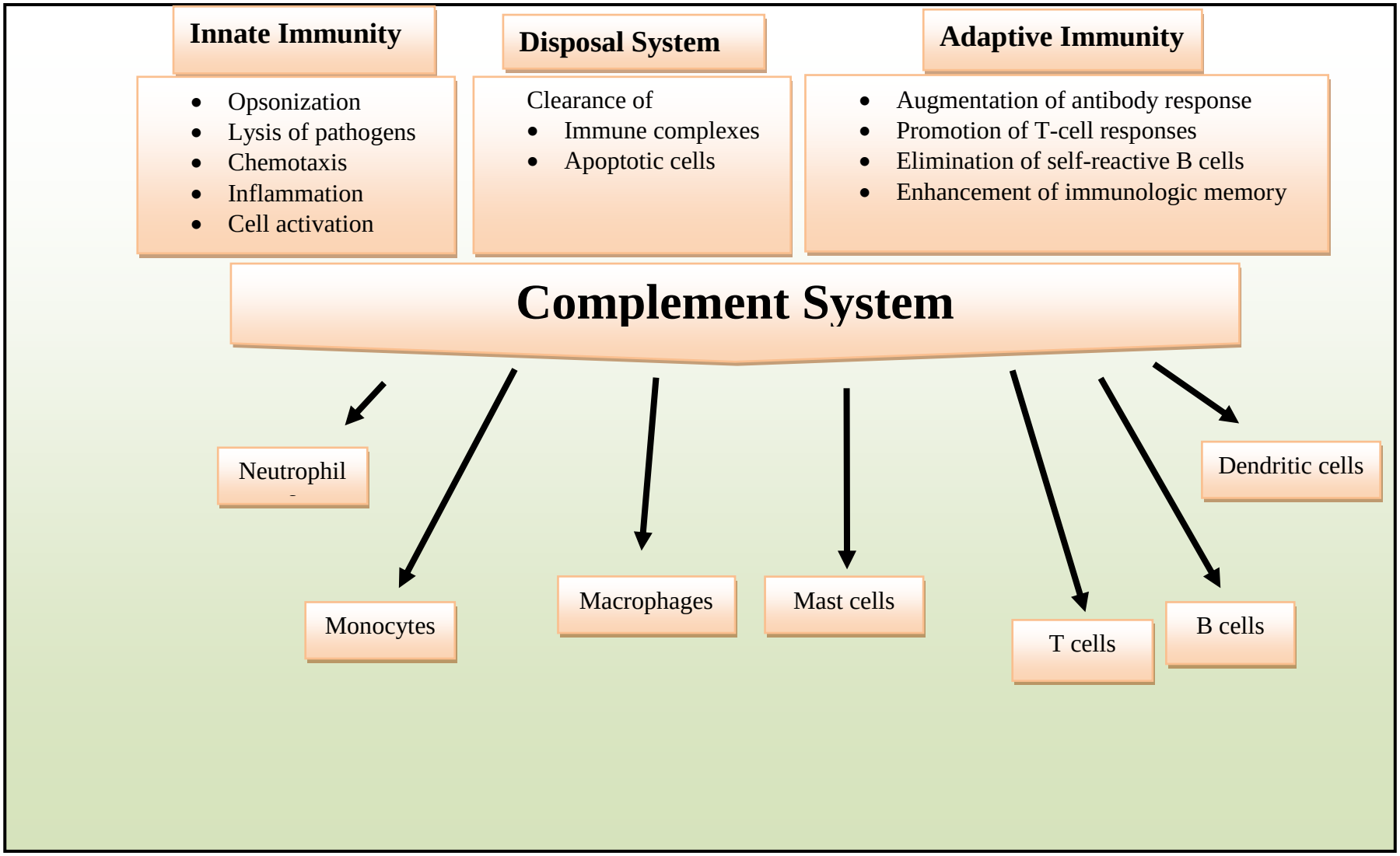

Figure 1.6: The complement system maintains cell homeostasis by eliminating immune complexes and act as a bridge between innate and adaptive immunity. Information sourced from (Walport, 2001a, Walport, 2001b, Mastellos and Lambris, 2002, Morgan et al., 2005)

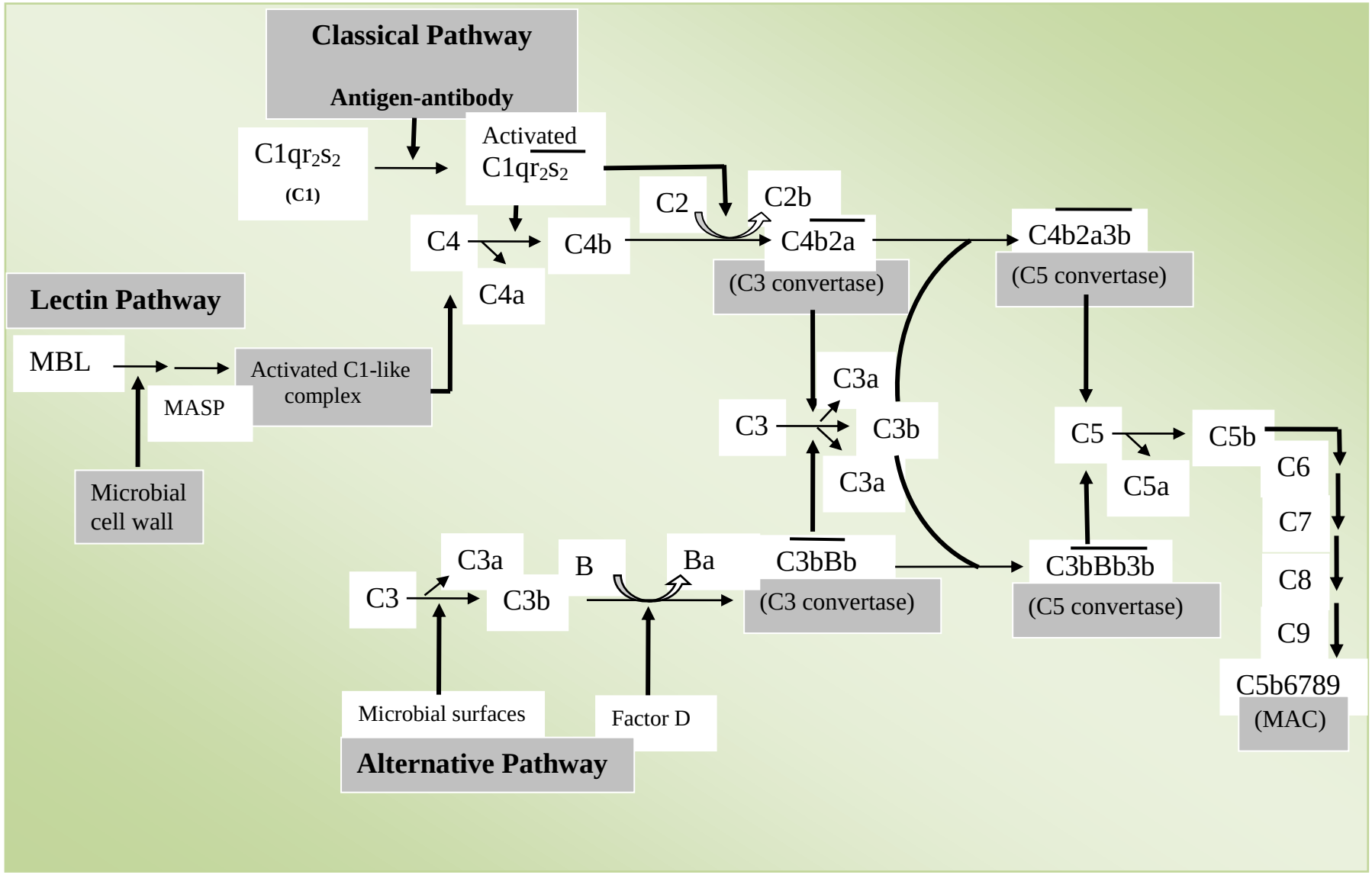

Figure 1.7: Schematic diagram of complement activation by three pathways: the classical, lectin and alternative pathways. Adapted and redrawn from Kuby Immunology (sixth edition) 2006; The complement system, Chapter 7 (Kindt et al., 2006).

1.5.2 Complement activation

Activation of complement is initiated via at least three distinct pathways. The pathways are distinguished by the factors required for the initiation and the activation but all three pathways lead to the proteolytic cleavage of component C3 (Walport, 2001a, Walport, 2001b, Frank, 2010). The classical pathway is operative following an immune response and the production of antibodies which promote the activation of complement once they have bound to antigen. The alternative pathway and lectin pathway represent the action of the innate immune system to defend the body against invading microbial pathogens (Walport, 2001a, Walport, 2001b, Frank, 2010).

1.5.2.1 Complement component C3

Complement component C3 is a complex protein of molecular weight 185kDa, the structure is very flexible and consists of two chains that originate from a single chain precursor with a total of 13 distinct domains (Janssen et al., 2005). The basic part of the C3 molecule is formed by eight macroglobulin (MG) domains, which specifically consist of an alpha (α) and beta (β) chain. C3 is activated by the proteolytic enzyme "C3 convertase" (Pangburn et al., 1981, Sahu and Lambris, 2001). There are specific structural patterns of interest called "CUB and TED" domains in the centre of the alpha chain of C3, which as a result of cleavage of C3 generate the small C3a peptide (9kDa) called anaphylatoxin, and the large C3b fragment (177kDa) (Muller-Eberhard and Gotze, 1972, Makrides, 1998, Walport, 2001a, Thai and Ogata, 2003). Formation of C3b fragment results in conformational changes and exposes a buried thioester moiety (Tack et al., 1980) that allows covalent attachment of C3b with pathogen surfaces. The C3b fragment, once attached to hydroxyl and amino groups on a particle surface,

degrades into iC3b, C3c and C3dg fragments, which are recognized by different complement receptors (Ross and Medof, 1985, Carroll, 2000, Walport, 2001a, Helmy et al., 2006) and preferentially activate the formation of membrane attack complex (MAC) which is capable of directly damaging cellular membranes (Guo and Ward, 2005).

1.5.2.2 Classical complement pathway

The classical pathway is activated by antibodies bound to antigens on the pathogen surface. The binding antibody to the antigen leads to conformational changes in the antibody molecule and exposes the binding site for the C1 (C1q) component (Walport, 2001a). Binding of C1q induces changes in C1r to become an active serine protease enzyme C1r and C1s to be the active enzyme C1s (Bally et al., 2009). Active C1s has two substrates C4 and C2. The C4 is a glycoprotein and is activated when C1s hydrolyses a small fragment (C4a) and exposes a binding site on the larger fragment (C4b) (Dodds et al., 1996). C4a, is an anaphylatoxin (Hugli, 1986) and mediator of inflammation. The C4b fragment attaches on the target surface of C1 and then C2 attaches on the exposed binding site of C4b. C2 is then cleaved by the C1s into a smaller fragment (C2b), whereas the resulting C4b2a complex (C3 convertase) cleaves C3 into its active form, C3b. Some of the C3b binds to C4b2a to form a complex C4b2a3b (C5 convertase). This complex cleaves C5 to generate C5a and C5b. C5b binds C6 and initiates the formation of the MAC (Walport, 2001a, Walport, 2001b, Guo and Ward, 2005, Frank, 2010).

1.5.2.3 Alternative complement pathway

The alternative pathway activation can occur in the absence of antibody (Thurman and Holers, 2006). In the alternative pathway, the internal C3 ester is activated by direct reaction with the pathogen surface, leading to a rapid amplification of C3b deposition through the action of pro-enzyme factor B (fB) and properdin (P) (Muller-Eberhard and Gotze, 1972) and factor D releasing the enzyme C3 convertase (C3bBb) complex (Lesavre et al., 1979, Fishelson et al., 1984). The C3 convertase, a proteolytic enzyme, activates the C3 and C5 components, generating the small C3a peptide (9kDa) (anaphylatoxin) and the large C3b fragment (177kDa) or C5a and C5b, respectively. Cleavage of C3 into its active peptides exposes the buried thioester moiety (Tack et al., 1980) that can now covalently attach to hydroxyl and amino groups on a particle surface (Muller-Eberhard and Gotze, 1972). Several different molecules can initiate C3 conversion, such as LPS and other bacterial products (Wu et al., 2009, Lachmann, 2009, Frank, 2010, Forneris et al., 2010).

1.5.2.4 Lectin complement pathway

The activation of the lectin pathway is initiated through the interaction of serum lectins mannose-binding lectin (MBL), L-Ficolin and H-Ficolin with specific carbohydrate groups on the surface of different microorganisms including bacteria leading to complement activation via the mannose-binding-lectin-associated serine proteases (MASPs), MASP-1 and MASP-2 (Fujita, 2002, Teillet et al., 2005, Hansen et al., 2010, Megyeri et al., 2013). MBL is structurally similar to C1q, while MASP-1 and MASP-2 are similar to C1r and C1s, respectively. The lectin pathway does not depend on antibody for its activation (Jensenius, 2005), but the mechanism is more like that of the

classical pathway. After MBL binds to mannose residues or carbohydrates on the surface of the pathogen or cell, this association then causes cleavage and activation of C4 and C2 for the generation of the lectin C3 convertase (C4b2a). As a result, serine proteases activating component C4 leads to the key C3 step of activation without antibody binding and represents an important innate defence mechanism (Thiel et al., 1997, Thiel, 2007, Degn et al., 2009, Dunkelberger and Song, 2010, Frank, 2010).

1.5.2.5 Membrane attack complex

All three complement pathways converge in the generation of an active C5 convertase that cleaves C5 into the small C5a fragment and the large C5b fragment (DiScipio et al., 1983). C5b binds to the surface of the target cell and provides a binding site for the subsequent complement components and initiates the formation of the MAC (Guo and Ward, 2005). The formation of the MAC involves C5b, C6, C7, C8 and C9, which interact sequentially and mediate the lysis of invading pathogens (Podack et al., 1976, Tschopp, 1984, Nauta et al., 2002).

1.5.3 Regulation of the complement system

Complement has evolved as a first line of defence against pathogens and as indicated plays a central role in immune reactions, so even small disruptions in its activation and regulation can lead to excessive complement activity and may trigger autoimmune, neurodegenerative and microbial pathogenesis (Sahu and Lambris, 2000, Walport, 2001b). To prevent unwanted complement activation there are numerous inhibitory mechanisms and complement regulatory proteins that inactivate various complement components (Hourcade et al., 1989, Morgan and Gasque, 1996, Kim et al., 2008),

including decay accelerating factor (DAF, CD55) (Lublin and Atkinson, 1989) and complement receptor 1-related gene y (Crry) (Paul et al., 1989), membrane cofactor protein (MCP) (Liszewski et al., 1991) and CD59 (Rollins and Sims, 1990) (Table 1.8).

In the absence of these regulators via mutations or blockage, complement activation can cause certain chronic inflammatory diseases such as age-related macular degeneration, ischemia and arthritis (Walport, 2001a, Morgan and Harris, 2003, Banda et al., 2003, Thurman and Holers, 2006, Jha et al., 2007, Katschke et al., 2007, Holers, 2008). The C1 inhibitor (C1Inh) regulates the classical pathway by preventing excessive activation of C4 and C2 by C1 (Frank, 2006, Zuraw, 2008, Walford and Zuraw, 2014).

Table 1.8: Regulatory proteins of the human complement system. Information sourced from (Sacks, 2010, Zipfel et al., 2006, Klaska and Nowak, 2007, Morgan et al., 2005, Zipfel and Skerka, 2009, Tedesco, 2008).

Regulatory protein	Name	Immunologic function
Serum	C1Inh (C1-inhibitor, serpin)	Inhibitor of C1r and C1s, removal of MASP-1 and MASP-2 from active MASP / MBL complexes
	C4bBP (C4b-binding protein)	Blocks formation of C3 convertase by binding C4b, fI co-activator (participation in the activation of the classical pathway convertase C3)
	Factor H (fH)	Blocks formation of C3 convertase by binding C3b, fI co-activator (participation in the activation of the alternative pathway convertase C3)
	Factor I (fI)	Control of C3 and C5 convertases activity (breakdown of the α chains in C3b and C4b molecules-in cooperation with appropriate co-activators
	C3INA (C3-inactivator)	Inactivation of C3a
	α2-macroglobulin	Regulation of MASP / MBL complexes activity
	Factor S (fS, vitronectin)	Preventing the incorporation of C5b-7 complexes in the membrane
	Clusterin	Preventing the incorporation of C5b-7 complexes in the membrane
Cellular	CR1 (CD35)	Control of C3 and C4b convertases activity through the distribution of C3b and C4b
	CR2 (CD21)	Regulation of antibody synthesis by B cells
	CR3/CR4 (CD11b/CD18)/(CD11c/CD18)	Phagocytosis supporting
	MCP (Membrane-cofactor protein, CD46)	Supporting C3b and C4b degradation by fI
	DAF (Decay-accelerating factor, CD55)	Accelerates dissociation of C4b2a and C3bBb (classical and alternative C3 convertases) or shortening the half-life of the classical and alternative pathways
	S protein	Binds soluble C5b67 and prevents its insertion into cell membrane
	HRF (Homologous restriction factor, CD59)	Bind to C5b678 on autologous cells , blocking of MAC formation by binding C8 and C9
	MIRL (Membrane inhibitor of reactive lysis)	Blocking C9 attachment and MAC creation
	Anaphylatoxin in activator	Inactivates anaphylatoxin activity of C3a and C5a by carboxypeptidase N-catalysed removal of C-terminal Arg

1.5.4 Complement deficiencies

The importance of complement in immunity to infection and other disease conditions is evidenced by the observations made in patients with complement deficiencies. Deficiencies of complement may lead to increased susceptibility to bacterial infections (Sjoholm et al., 2006). This is seen particularly with deficiencies of the late components C3 to C9 (Fijen et al., 1989, Pettigrew et al., 2009, Keiser and Broderick, 2012). Deficiencies in any of the early components of the classical pathway (C1q, C1r, C1s, C4 and C2) result in complications of immune-complex diseases such as system lupus erythematosus (SLE) (Sturfelt, 2002, Pettigrew et al., 2009, Frank, 2010, Bryan and Wu, 2014), glomerulonephritis, and vasculitis (Sjoholm et al., 2006, Pettigrew et al., 2009).

Depletion of the early components of the alternative pathway (factor D and properdin) appears to be associated with *Neisseria* infections (Figueroa and Densen, 1991, Platonov et al., 1993, Pettigrew et al., 2009). MBL deficiency is associated with increased respiratory tract infections (Ip et al., 2005, Sorensen et al., 2005, Jensenius, 2005, Valles et al., 2010). C3 deficiency leads to recurrent bacterial infections and may play a role in immune-complex diseases (Pickering and Walport, 2000, Sjoholm et al., 2006, Pettigrew et al., 2009). The importance of regulatory proteins of complement are also seen when individuals have deficiencies of these e.g. a deficiency in the C1q esterase inhibitor leads to hereditary angioedema (Frank, 2006, Zuraw, 2008, Walford and Zuraw, 2014).

1.5.5 Complement receptors

1.5.5.1 Complement receptor (CR1)

CR1 is found on chromosome 1 at the locus 1q32 (Weis et al., 1987). CRI (CD35) is a ~ 200 kDa glycoprotein which is comprised of 30 short consensus repeats (SCRs) organized in four long homologous repeats (LHRs) (Krych et al., 1992, Krych-Goldberg and Atkinson, 2001). There are four major structural domains of the CR1 protein; the signal peptide, extracellular domain, transmembrane sections and cytoplasmic domain (Wong, 1990). The extracellular domain is made of the SCR (Klickstein et al., 1988). SCRs are modular structures of about 60 amino acids, each with two pairs of disulphide bonds that provide structural rigidity (Krych et al., 1992). SCR 15-17 of CR1 has been determined to be the binding site of both C3b and C4b (Krych-Goldberg and Atkinson, 2001, Smith et al., 2002), module 15 is identified for C4b binding and module 16 is critical for C3b binding. CR1 also binds to C1q and MBL and plays a role in the phagocytosis of particles coated by these proteins (Klickstein et al., 1997, Ghiran et al., 2000).

CR1 is expressed on the extracellular membrane of blood cells and is also found in soluble form in the plasma (Wong, 1990, Vik and Wong, 1993). CR1 is expressed on monocytes, macrophages, neutrophils, eosinophils, erythrocytes, B-lymphocytes, $CD4^+$ T cells, follicular dendritic cells, and Langerhans cells in the skin (Klickstein et al., 1988, Liu and Niu, 2009). CR1 performs a number of different functions in the immune system. Erythrocyte-associated CR1 binds to immune complexes in a process of immune adherence (Rothman et al., 1975, Fearon, 1980, Taylor et al., 1997, Birmingham et al., 2003) (Table 1.9).

1.5.5.2 Complement receptor (CR2)

CR2 (CD21) consist of 15 SCR, like those observed in CR1 (Krych et al., 1992). CR2 plays a immuno-regulatory role (Carroll, 2000), enhancing humoral immunity (Carroll, 2000, Fleming et al., 2002, Haas et al., 2002) and regulating T-cell immunity to self and non-self-antigens (Kaya et al., 2001, Fairweather et al., 2006). In addition, the expression of CR2 modules on follicular dendritic cells and B cells is critical for the generation of normal immune responses (Molina et al., 1996, Qin et al., 1998, Fang et al., 1998, Boackle, 2003). The CR2 receptor has been considered to be the binding site of both iC3b and C3dg and promotes B cell responses (Weis et al., 1984, Carter and Fearon, 1992, Carroll, 2004) (Table 1.9).

1.5.5.3 Complement receptor CR3 (CD11b / CD18)

CR3 is a heterodimeric complex of two transmembrane proteins; consisting of an alpha subunit (CD11b or α_M) of 150kda and a common beta chain (CD18 or β_2) of 95kda (Ross, 2000). CR3 is abundantly expressed on monocytes, macrophages, neutrophils, dendritic cells and lymphoid NK cells (Ross, 2000). CR3 ligates with the inactivated fragment of C3, iC3b (Rosen and Law, 1990, Ross and Vetvicka, 1993). In addition to iC3b, CR3 has a binding affinity for other host molecules such as fibrinogen and intercellular adhesion molecule – 1 (ICAM–1) (Diamond et al., 1993) and can interact with diverse microbial molecules including enterobacterial LPS, *Bordetella pertussis* filamentous hemagglutinin, *leishmania gp 63* and *P. gingivalis* fimbriae (Russell and Wright, 1988, Diamond et al., 1993, Ingalls et al., 1997, McGuirk and Mills, 2000, Hajishengallis et al., 2005).

CR3 co-associates with pattern-recognition receptors including CD14 and toll like receptors (TLRS) (TLR2 and TLR4) in activated phagocytes (Triantafilou et al., 2004, Hajishengallis et al., 2006). These ligands are bound by the Van Willebrand factor A domain in the α-chain named as αI domain. The CR3 αI domain is well established as the primary binding site for iC3b (Diamond et al., 1993, Ueda et al., 1994). Binding of ligands to CR3 leads to conformational changes in its ectodomain transmitting an outside-in signal through the cell membrane (Diamond et al., 1993, Springer and Dustin, 2012).

CR3 plays diverse roles in immunity and inflammation, including phagocytosis of iC3b-opsonized particles or complexes (Bajtay et al., 2004), promotion of leukocyte migration to sites of extravascular inflammation and induction of cytokine responses (Myones et al., 1988, Ehlers, 2000, Barreiro et al., 2007, Dupuy and Caron, 2008) (Table 1.9).

1.5.5.4 Complement receptor CR4 (CD11c / CD18)

CR4 is a transmembrane heterodimer, consisting of an alpha subunit (CD11c or α_X) of 150kda and the common beta chain (CD18 or β_2) of 95kda. CR4 belong to a β_2-integrin family and is abundantly present on monocytes, macrophages and dendritic cells. CR4 is able to bind the proteolytically inactive form of C3b, iC3b on a pathogen, facilitating the phagocytosis of iC3b-opsonized particles, which assists the clearance of microbial pathogens. In addition, it also performs important functions during leukocyte extravasation across the endothelium and interaction with extracellular matrix (Carroll, 2004, Barreiro et al., 2007, Dupuy and Caron, 2008) (Table 1.9).

1.5.5.5 Complement receptor immunoglobulin (CRIg)

CRIg is a recently identified novel member of the complement receptor immunoglobulin superfamily (Helmy et al., 2006), also described as VSIG4, V-set and Ig domain-containing 4 and a B7 family-related molecule (Vogt et al., 2006) and as Z39Ig (Langnaese et al., 2000) (Table 1.9). As this receptor is the subject of this thesis, the structure and function of this protein will be dealt with in detail below.

Table 1.9: Receptors that regulate the complement system, their molecular structure, binding ligands, cellular distribution and different biological activities. Information sourced from (He et al., 2008).

Complement receptors	Structure	Major ligands	Cellular distribution	Biological activities
CR1 (CD35)	30 SCRs of about 60 amino acids	• C3b • C4b	Monocytes Macrophages Neutrophils Follicular dendritic cells Eosinophils B-cells Erythrocytes	Blocks formation of C3 convertase Binds immune complexes to cells
CR2 (CD21)	15 SCRs of about 60 amino acids	• iC3b • C3d • C3d(g)	B-cells Follicular dendritic cells	Part of B-cell co-receptor Binds Epstein-Barr virus
CR3 (CD11b / CD18)	Belong to integrin family is transmembrane heterodimers (α_M / β_2)	• iC3b	Monocytes Macrophages Neutrophils NK cells	Binds cells adhesion molecules on neutrophils Facilitating their extravasation Bind immune complexes Enhancing their phagocytosis
CR4 (CD11c / CD18)	Belong to integrin family is transmembrane heterodimers (αx / β_2)	• iC3b	Monocytes Macrophages Neutrophils Dendritic cells NK cells	Binds cells adhesion molecules on neutrophils Facilitating their extravasation Bind immune complexes Enhancing their phagocytosis
CRIg	Extracellular IgV and IgC domain	• C3b • iC3b • C3c	Macrophages Dendritic cells	Promotes rapid phagocytosis Anti-inflammatory and immuno-suppressive

1.6 Complement receptor immunoglobulin (CRIg)

1.6.1 Introduction

CRIg was first identified as Z39Ig by Langnaese et al. (2000), as a novel gene expressed highly in lungs, placenta and synovium (Walker, 2002, Lee et al., 2006). Further studies showed that CRIg was a novel complement receptor of the Ig superfamily, expressed selectively on a sub-population of macrophages including tissue resident macrophages and liver Kupffer cells (KCs) (Helmy et al., 2006). CRIg binds to C3b and iC3b and plays an important host defence role by promoting rapid phagocytosis of microbial pathogens (Helmy et al., 2006, Gorgani et al., 2008) (Figure 1.8).

Interestingly, in the same year this receptor which has also been termed as (VSIG4), V-set and Ig domain-containing 4 (VSIG4), was reported to be a potent inhibitor of T cell responses and negative regulator of human and mouse T cells proliferation (Vogt et al., 2006). This coupled with recent findings that CRIg has anti-inflammatory activity in experimental chronic inflammatory diseases makes the receptor functionally unique compared to the classical complement receptors, CR3 and CR4 described above (Chen et al., 2009, Guo et al., 2010, Gorgani et al., 2011, Jung et al., 2012). To respect those who named this molecule, the three terminologies are used interchangeably throughout the thesis.

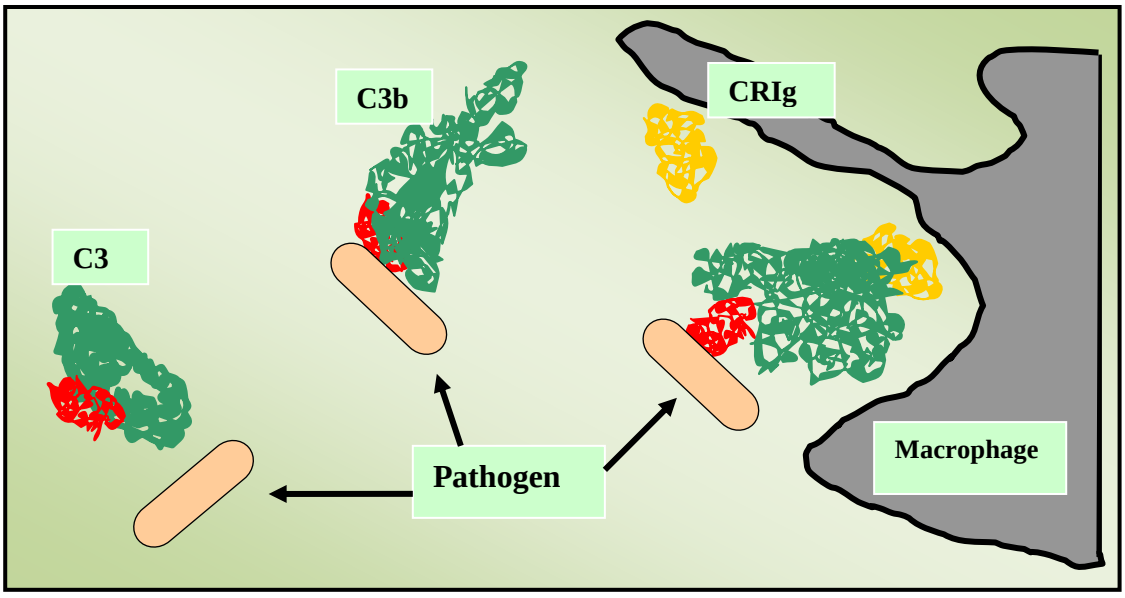

Figure 1.8: Recognition of C3b fragment by CRIg receptor. Cleavage of C3 generates the large C3b fragment and exposes a buried thioester moiety that allows covalent attachment of C3b with pathogen surface and recognition of C3b by CRIg expressed on a macrophage. Adapted and redrawn from (He et al., 2008).

1.6.2 Z39Ig is located on human chromosome X

Z39Ig belongs to the immunoglobulin superfamily member and the gene is localized in the pericentromeric region of human X chromosome and has been considered a gene involved in mental retardation (Langnaese et al., 2000). However, when its expression was examined in 1432 human cDNA libraries, it was found that this gene was linked to the complement system as a cell-surface receptor in activated macrophages and most likely involved in phagocytosis. The gene is abundantly expressed on the synovium, placenta, and lungs tissue (Walker, 2002). Lower levels of Z39Ig expression were detected in endocrine and exocrine tissues, and occasionally in cardiovascular tissue (Walker, 2002).

Further analysis of its expression, showed Z39Ig protein to be expressed on immune cells such as the human monocytic cell line THP-1 cells, mature $CD14^+$ DC differentiated from umbilical-cord blood $CD34^+$ haematopoietic progenitor cells (Kim

et al., 2005). CRIg is not expressed by B and T cells (Kim et al., 2005). The selective expression of CRIg on mononuclear phagocytes but not lymphocytes suggested that this receptor filled a unique role in infection and immunity and inflammation (Kim et al., 2005). In addition, the expression of CRIg on human mature $CD14^+$ DCs confirmed the observation of increasing CRIg expression during the process of $CD34^+$ haematopoietic progenitor cells differentiating to $CD14^+$ DCs by Ahn et al. in 2002 (Ahn et al., 2002).

Co-expression studies examined by the Guilt-by-Association (GBA) method indicated that the CRIg expression pattern is similar to early genes in the classical complement system (C1qA, C1qB, C1qC, C1r and C1 inhibitor), major histocompatibility complex (MHC) class II genes (HLA-DR alpha, HLA-DR alpha 1 and HLA-DR beta1), Fc receptors (Fc gamma RIIa and Fc epsilon R1), lysosomal protein (LAPTm5) and macrophage receptors (MACRO and CD163/M130) (Walker, 2002).

1.6.3 CRIg structure

CRIg / VSIG4 / Z391g is a type -1 transmembrane protein 399 amino acids in length. Analysis of the protein amino acid sequence identifies an extracellular region which consists of two immunoglobulin (Ig) like domains, a signal peptide and transmembrane region, and an intracellular domain (Langnaese et al., 2000, Kim et al., 2005, Lee et al., 2006). The intracellular domain of CRIg contains a cAMP/cGMP-dependent protein kinase phosphorylation site and a protein kinase C phosphorylation site (Langnaese et al., 2000). The presence of two phosphorylation sites in its intracellular domain might indicate their role in signal transduction (Kim et al., 2005). CRIg is localized at position Xq12 between hephaestin and moesin on the X chromosome (Langnaese et al., 2000).

CRIg exists as at least two alternatively spliced forms huCRIg long (L) (Helmy et al., 2006) and huCRIg short (S) (Helmy et al., 2006). The longer form huCRIg(L) consists of both a V-type and C_2-type terminal Ig domains (Smith and Xue, 1997) and the huCRIg(S) spliced version encodes only a V-type Ig domain (Figure 1.9, 1.10). Murine CRIg (muCRIg) encodes only the spliced version V-type Ig domain (Helmy et al., 2006). The muCRIg and huCRIg share 67% overall amino acid sequence identity with 83% amino acid sequence identity residing in the IgV domain, and the AP-2 internalization motif, YARL and DSQALI are consensus sequences shared by human and mouse CRIg (Helmy et al., 2006).

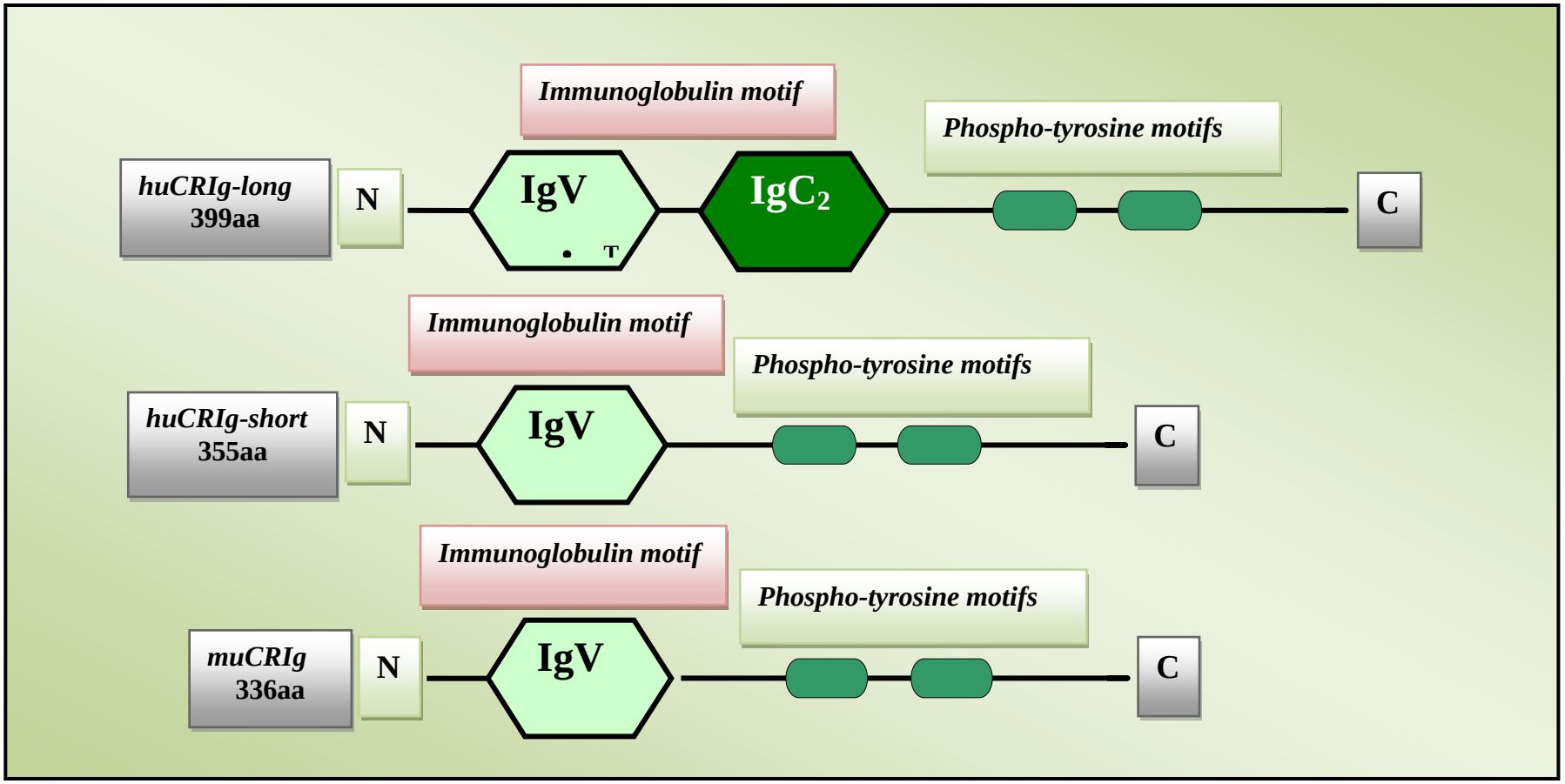

Figure 1.9: Structure of human and murine CRIg. CRIg is a type 1 transmembrane protein consisting of either an IgV and IgC_2-type domain huCRIg(L) or IgV-type immunoglobulin domain huCRIg(S) and muCRIg. Adapted and redrawn from (Helmy et al., 2006).

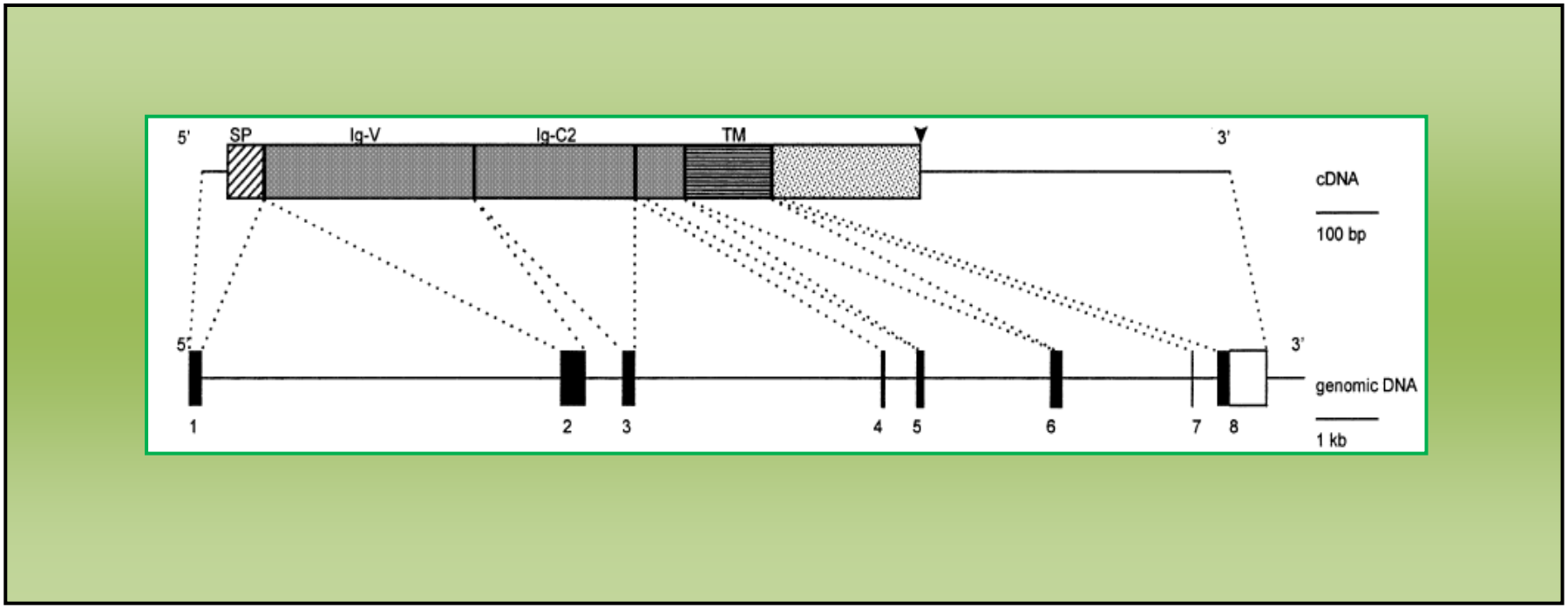

Figure 1.10: Genomic structure of CRIg cDNA. Deduced exon/intron organization of the human CRIg (Z39Ig) gene. SP, signal peptide; Ig-V, immunoglobulin domain V-type; IgC_2, immunoglobulin C_2-type; TM, transmembrane region. Adapted and copied from (Langnaese et al., 2000).

1.6.4 CRIg expression in cells and tissues

CRIg is highly expressed on liver KCs, on interstitial macrophages in the heart, synovial lining macrophages in the joint, foam cells in atherosclerotic plaques and resident peritoneal macrophages (Helmy et al., 2006, Lee et al., 2006, Vogt et al., 2006). In humans it is additionally expressed on alveolar macrophages, on adrenal gland macrophages and on Hofbauer cells in the placenta (Helmy et al., 2006).

Expression of CRIg is absent on human and mouse splenic macrophages, langerhans cells, microglial cells, bone-marrow derived macrophages and many monocytes/macrophage cell lines such as RAW264.7, PU5-1.8, P388D1, WEHI-3, J774 and IC-21 (Helmy et al., 2006). Other leukocytes T and B cells and neutrophils do not express CRIg (Vogt et al., 2006).

1.6.5 CRIg in infection and immunity

KCs constitute 90% of the tissue macrophages present in the reticulo-endothelial system (Bilzer et al., 2006). KCs are stationed at strategic positions where they are constantly exposed to bacteria and microbial debris (Fox et al., 1987), KCs clear bacterial endotoxins, immune complexes and microbial pathogens from the circulation (He et al., 2008). Mice deficient in KCs show increased susceptibility to bacterial infections and increased mortality (Pinto et al., 1991). Human and mouse KCs express the complement receptors, CR3 and CRIg (Gregory et al., 2002, Helmy et al., 2006). Human KCs also express CR1, CR2 (Hinglais et al., 1989). CRIg and CR3 are co-expressed on KCs and facilitate the rapid phagocytosis of opsonized bacteria (Gregory et al., 2002, Helmy et al., 2006, Wiesmann et al., 2006, Gorgani et al., 2008). CRIg-deficient KCs internalized fewer bacteria and mice show increased mortality, associated with overwhelming

infection and generation of a cytokine storm. Studies by Gorgani et al 2008 demonstrated that CRIg was more efficient than CR3 in promoting phagocytosis of bacteria in a subpopulation of tissue resident macrophages (Gorgani et al., 2008).

CRIg binds to C3b and iC3b and C3c, but not to the parent molecule C3 (Helmy et al., 2006, Wiesmann et al., 2006). On a structural level, C3b is composed of two chains an α and a β-chain (Wiesmann et al., 2006), and most of the binding partners of C3b are linked to the α-chain (Taniguchi-Sidle and Isenman, 1994, Oran and Isenman, 1999, Sahu and Lambris, 2001, Janssen and Gros, 2007), while CRIg selectively binds to the β-chain of C3b (Wiesmann et al., 2006) (Figure 1.11).

Binding of C3 proteins to CRIg on macrophages resulted in clearance of microbial pathogens (Helmy et al., 2006). $CRIg^{+}$ macrophages showed increased and rapid binding of complement C3-opsonized particles compared to $CRIg^{-}$ macrophages. The IgV domain of CRIg in humans and mouse is considered important for binding of complement fragments such as C3b, iC3b and C3c (Wiesmann et al., 2006).

In 2006, CRIg was identified on a subset of tissue resident macrophages with distinct properties to other known complement receptors such as CR3 and CR4. It is regarded as an important component of the innate immune system because of its high expression on KCs and on other tissue resident macrophages. CRIg plays an important role in host defense by promoting the rapid phagocytosis of circulating pathogens, for example when expressed on KCs lining the sinusoids of the liver. C3b and iC3b opsonized bacteria bind to CRIg and are effectively phagocytosed to control bacterial dissemination. CRIg is present on a constitutively recycling pool of membrane vesicles (Helmy et al., 2006).

Macrophages provide a first line defence against bacterial infections, but the intracellular bacteria *Listeria monocytogenes* escapes the mechanism of protection and mostly survives. Fusion of phagosomes and lysosomes is enhanced when the

phagosomal lumen becomes acidic and this phagosomal acidic environment is essential for the killing of bacteria and requires H^+-ATPase activity to provide microbial defence by macrophages (Hackam et al., 1997). Chloride has an important role in the acidification of intracellular organelles (Di et al., 2006) and CRIg signalling activates the chloride intracellular channel 3 (CLIC3) to increase [Cl^-] in the phagosomal lumen and induces phagosomal acidification through the coactivation of Na^+/K^+-ATPase and Na^+/H^+ exchangers. Kim et al showed that the chloride intracellular channel 3 (CLIC3), an intracellular chloride channel protein binds to the cytoplasmic domain of CRIg receptor and triggers the killing of *L. monocytogenes* (Kim et al., 2013).

CRIg was also identified on a sub-population of resident peritoneal macrophages with significantly increased binding and internalization of C3-opsonized particles (Gorgani et al., 2008). CRIg binds readily to C3-opsonized pathogens in the absence of $Ca2^+$ and $Mg2^+$, which differs from the CR3 receptor that requires divalent cations for activation, thus these receptors contribute independently to complement-mediated phagocytosis of iC3b-opsonized particles (Gorgani et al., 2008).

1.6.6 Role and dynamics of CRIg expression in inflammation

1.6.6.1 Expression of CRIg in inflammatory tissues

Rheumatoid arthritis (RA) is an inflammatory disorder in which elements of the immune system attack the synovial membrane and joints. There is compelling evidence that macrophages play a key role in the inflammatory reaction and tissue damage in RA (Smeets et al., 2003, Szekanecz and Koch, 2007). The intimal macrophages in the synovium consist of at least two sub-populations; tissue resident macrophages and inflammatory macrophages (Revell, 1989, Athanasou and Quinn, 1991, Edwards et al., 1997).

In the RA synovial lining layer, mononuclear cells were stained and analysed to be $Z39Ig^+$ cells, $Z39Ig^+CD11c^+$cells and $Z39Ig^+CD11c^-$ cells, i.e. CRIg positive cells. The expression of Z39Ig/CRIg protein was observed only in intimal macrophages of normal and also RA, osteoarthritis (OA) and psoriatic arthritis (PsA) patients (Tanaka et al., 2008). Increased numbers and ratio of $Z391g^+CD11c^+$cells to $Z391g^+$cells was observed in the synovial lining layer of RA compared to those of OA and PsA, $CRIg/Z39Ig^+$ expression was used as a marker of tissue resident macrophages in normal synovium and the corresponding macrophages in the synovial lining layer of inflammatory arthritis. The $Z391^+CD11c^+$cells were shown to be a useful tool for identification of the RA synovial lining layer (Tanaka et al., 2008).

In humans and mice, the tissue macrophages are abundantly present in the mucosa of the small and large intestine (Weber et al., 2009). Intestinal macrophages are primarily involved in the clearance of apoptotic cells and microbial pathogens (Nagashima et al., 1996, Weber et al., 2009). In humans, these intestinal macrophages lack pattern recognition receptors such as the Fc receptors of IgA and IgG, CD14, TLR2, TLR4, complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Weber et al., 2009). However, CRIg expression has been observed in the major subset of human and murine macrophages in the large intestine. $CRIg^+$ macrophages in the intestine showed significant level of C3b-dependent phagocytosis, to eliminate foreign particles in the large intestine (Tanaka et al., 2012).

1.6.6.2 VSIG4, is a negative regulator of T cell activation

VSIG4 has been reported to be an inhibitor of T cell activation (Vogt et al., 2006). By using human VSIG4-Ig fusion molecules *in vitro,* it was demonstrated that VSIG4 is a strong negative regulator of murine and human T cell proliferation and IL-2 production.

In mice, VSIG4 was shown to inhibit the production of Th cell-dependent IgG responses *in vivo* (Vogt et al., 2006).

T cell induction is a complex process and regulated by a network of activating and inhibitory signals. Presentation of peptides by MHC molecules is insufficient to complete T cell activation; it requires additional signals from co-stimulatory molecules (Langman and Cohn, 1993, Lenschow et al., 1996, Chambers and Allison, 1999, Schwartz, 2003). CD28 is an important co-stimulatory molecule expressed on T cells, interacting with the B7 family members CD80 and CD86 and regulates T cell activation, proliferation and differentiation (Coyle and Gutierrez-Ramos, 2001, Sharpe and Freeman, 2002, Leibson, 2004, Keir and Sharpe, 2005, Collins et al., 2005, Flies and Chen, 2006). These co-stimulatory molecules are important in activation and inhibition of T cell responses. CD28 and inducible co-stimulatory (ICOS) usually promote the T cell response, while cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (a close homolog of CD28) and programmed death ligand 1 (PD-L1) inhibits T cell responses and VSIG4 is as potent at inhibiting T cell responses as PD-L1. Hence, VSIG4 acts as a potent inhibitor of mouse and human $CD4^+$ and $CD8^+$ T cells activation and proliferation *in vivo* and *in vitro* (Vogt et al., 2006), however the molecular mechanism by VSIG4 inhibit T cell responses remains to be established (Vogt et al., 2006).

1.6.6.3 Dendritic cells (DC) transfected with VSIG4 and T cells suppression

DC are distributed throughout the body at strategic points of pathogen entry or tissue injury (Banchereau et al., 2000, Steinman et al., 2003), and play a critical role in priming the immune responses in adaptive immunity (Steinman, 2006). The complement system is not only important in the innate immune system but it can also

influence the adaptive immune responses by modulating DC function and regulating antigen specific T cell responses. Complement activation is an integral part of the development of powerful effector functions in immune responses (Peng et al., 2006, Li et al., 2008, Strainic et al., 2008, Baruah et al., 2009). Most work has been conducted with murine DC and these studies highlight the importance of local production of complement by antigen presenting cells in modulating DC activation and functional development (Peng et al., 2006, Li et al., 2008, Strainic et al., 2008, Peng et al., 2008, Peng et al., 2009).

Studies on human DC showed the expression of many of the complement components of classical, alternative and terminal pathways (Nauta et al., 2004, Castellano et al., 2004, Reis et al., 2006, Reis et al., 2007, Reis et al., 2008, Castellano et al., 2007). In addition, human DC expressed complement receptors including CR3, CR4 and CRIg which are known to bind the active fragments C3b, iC3b and C3d which serve in immune adhesion (Li et al., 2011). The CRIg receptor was detected in monocyte-derived (mo)DC after 6 days of culture in the absence of any stimulation (Li et al., 2011).

T cells responses are tightly regulated by the activity of co-stimulatory molecules which are required to mount an appropriate immune response (Zhu et al., 2001, Cassell, 2001, Lim et al., 2005) and T cells are important in the pathogenesis of immune disorders, such as autoimmune diseases and graft rejection (Cobbold, 2002, Simpson, 2003, Wang et al., 2008). DC are involved in the maintenance of immunological self-tolerance and responsible for the priming of naïve T cell during the initiation of the immune response (Villadangos and Schnorrer, 2007, Tokita et al., 2008, Ohnmacht et al., 2009). DC transfected with VSIG4 (hVSIG4) recombinant adenovirus were co-cultured with allogenic T cells. Treatment with hVSIG4 recombinant

adenovirus-transfected DC caused allogenic T cell suppression and decreased cytokine production including IL-2 and IFN-γ (Xu et al., 2009).

1.6.6.4 Immune-mediated liver injury and protective role of CRIg on Kupffer cells

In a concanavalin A (ConA)-induced hepatitis (CIH) model VSIG4 knockout (KO) mice showed severe liver pathology and poor survival rates. Administration of CRIg-Ig fusion protein to wild type mice prevents CIH development, suggesting that regulation of this disease is dependent on CRIg expression. Furthermore, liver KCs lacking CRIg failed to induce liver T- and NKT-cell tolerance toward their cognate antigens. Hence, CRIg contributes to the induction and maintenance of KC-mediated liver T- and NKT-cell tolerance (Jung et al., 2012).

1.6.6.5 VSIG4 alleviates renal tubulointerstitial injury in VSIG4 KO mice

Renal tubulointerstitial injury is characterized by activation and infiltration of T cells and macrophages and is considered an indicator of chronic nephrosis (Kuroiwa and Lee, 1998, Tipping and Holdsworth, 2003). Studies showed that nephritic tissue injury can be lessened by T cells suppression through its co-stimulatory signalling molecules (Biancone et al., 1995, Bertram et al., 2004). VSIG4 KO ($VSIG4^{-/-)}$ and wild type ($VSIG4^{+/+}$) phenotypes were assessed using the unilateral ureteric obstruction mouse model of renal inflammation and tubulointerstitial fibrosis. The results from this study determined that macrophage expressing VSIG4 reduces T cell infiltration and protects the renal interstitium from damage. Interestingly, the ($VSIG4^{+/+}$) mice showed lower levels of IL-2, IFN-γ and TNF, while ($VSIG4^{-/-}$) mice showed decreased production of IL-10 (Li et al., 2014).

1.6.6.6 CRIg and the alternative pathway of complement system

In the alternative complement pathway, the enzyme C3 convertase splits C3 component into the small C3a peptide and the large C3b fragment that exposes the thioester "war-head" for the covalent attachment of C3b to particle surface before degradation to iC3b and C3c (Pangburn et al., 1981, Makrides, 1998, Walport, 2001a, Sahu and Lambris, 2001, Liddington and Bankston, 2005). C3b in the alternative pathway then binds with the serine protease factor Bb and is responsible for subsequent cleavage of the substrates C3 and C5 (Muller-Eberhard and Gotze, 1972). CRIg (human CRIg), binds to the β-chain of the C3b fragment, resulting in the inhibition of complement activation *in vitro* and selectively inhibits the C3 and C5 convertases of the alternative pathway by blocking their interaction with the substrates C3 and C5, respectively (Wiesmann et al., 2006). However, binding of CRIg to C3b and not C4b, does not inhibit C5 binding to the classical pathway C3bC4b heterodimer (Wiesmann et al., 2006), thus the interaction of CRIg, selectivity with the β-chain of C3b subunit blocks the cleavage of substrates C3 and C5 in the alternative pathway but not classical pathway convertases. Interaction of CRIg to C3b and not C4b selectively inhibits the alternative pathway of complement and blocks unwanted complement activation.

Advantage has been taken of this property to develop potential therapeutic anti-inflammatory agent in diseases such as the kidney disorder membrano-proliferative glomerulonephritis (Pickering et al., 2002, Cook and Botto, 2006) and age-related macular degeneration (Edwards et al., 2005, Hageman et al., 2005, Haines et al., 2005, Klein et al., 2005, Mollnes et al., 2007). Excessive complement activation plays a role in the pathogenesis of chronic inflammatory diseases, including asthma, ischemia-reperfusion injury and cardiopulmonary bypass (Walport, 2001a, Pickering et al., 2002, Thurman and Holers, 2006).

Intestinal ischemia / reperfusion (IR) is a transient loss of blood flow followed by reperfusion which causes a strong local and systemic inflammatory response. IR injury is observed in most of the clinical situations including transplantation, shock and surgery (Fleming and Tsokos, 2006). Complement activation contributes significantly to the initiation and amplification of the inflammatory response induced by IR (Tsokos and Fleming, 2004). Inhibition of complement activity protects tissues from developing IR injury (Fleming et al., 2003b, Fleming et al., 2003a, Atkinson et al., 2005). Complement activity by ischemic tissues involves the three complement pathways, the lectin, classical and alternative (Williams et al., 1999, Fleming et al., 2002, Stahl et al., 2003). It has been demonstrated that murine CRIg-Fc (soluble form of CRIg) can be used in clinical settings to potentially protect from IR-induced injury by inhibiting the activation of the alternative pathway of complement (Katschke et al., 2007). Treatment with CRIg-Fc prior to mesenteric IR prevents local (intestinal) and remote (lung) injury by reducing complement deposition to the sites of injury (Chen et al., 2009).

Experimental autoimmune uveoretinitis (EAU) is a Th1 and Th17 CD4 T cell mediated disease (Amadi-Obi et al., 2007, Luger et al., 2008) and represents a T-cell driven autoimmune response (Liversidge and Forrester, 1988, Caspi et al., 1990). Complement is an important part of the innate immune system and its activation is essential in resolving infection and regulating tissue homeostasis, although uncontrolled complement activity may lead to inflammation and pathology in autoimmune diseases. The role of alternative pathway of complement in retinal inflammation and pathology was investigated using an EAU as a model. In mice with EAU, increased levels of C3d and complement factor B (CFB) expression were observed in the retina. Treatment with exogenous CRIg-Fc suppressed retinal inflammation. Accordingly C3d deposition and CFB expression was decreased as was the severity of EAU. Treatment with CRIg-Fc

inhibited T cell proliferation and production of cytokines such as TNF, IFN-γ, IL-17 and IL-6 in BM-derived macrophages (Chen et al., 2010).

The over activation of the alternative pathway of complement plays a role in disease induction and progression in inflammatory and tissue damaging diseases (Makrides, 1998, Taylor et al., 1998, Walport, 2001a, Katschke et al., 2007). The absence of a functional alternative pathway of complement through genetic deletion of factor B protects against experimental arthritis (Banda et al., 2006). To prevent excessive complement activation, cells are equipped with regulators (Hourcade et al., 1989). Uncontrolled complement activation due to the absence of regulators results in complement-mediated diseases such as nephrotoxic nephritis and arthritis (Morgan and Harris, 2003, Thurman and Holers, 2006).

The soluble form of CRIg (not naturally soluble) (Vogt et al., 2006), in two experimental models of arthritis, reversed inflammation and bone loss by inhibiting the alternative pathway of complement in the joint (Katschke et al., 2007). In addition it has been shown that platelets deficient in complement regulatory proteins could be rescued from complement attack by soluble CRIg (Kim et al., 2008).

1.6.6.7 Diabetes and CRIg

Type 1 diabetes is an organ-specific autoimmune disease driven mainly by T lymphocytes, but other cells of the immune system such as B lymphocytes, NK cells, macrophages and DC also have important roles in the onset of this disease (Andre-Schmutz et al., 1999, Bluestone et al., 2010). Fu et al used a new method, magnetic resonance imaging of magnetic nano-particles as a prediction tool in the NOD mouse model of Type 1 diabetes (Fu et al., 2012), which is sensitive to such changes, permitting monitoring of disease progression and signalling of the imminent onset of

diabetes (Denis et al., 2004, Turvey et al., 2005). Administration of a CRIg-Fc had a protective effect on the pathogenesis of Type 1 diabetes in nonobese diabetic (NOD) mice (Fu et al., 2012). These findings indicated a potential therapeutic benefit in treating this Type 1 diabetes with soluble CRIg.

1.6.6.8 VSIG4 gene as a potential blood biomarker in severe preeclampsia

Preeclampsia (PE) is a placental disorder and characterized by high blood pressure and proteinuria (protein in the urine) (Bolte et al., 2001). PE can occur in the 2nd half of pregnancy and is associated with multiple adverse effects, and is a leading cause of maternal and neonatal morbidity and mortality worldwide (Bolte et al., 2001, Redman and Sargent, 2005). For effective treatment of PE, circulating factors associated with the coagulation pathway (fibrinogen, fibronectin, factor VIII, antithrombin, protein S and protein C) and, endothelial activation (endoglin and CD146) have been measured (Carty et al., 2008, Romero et al., 2008, Grill et al., 2009, Kar, 2014); however these markers differentiate normal pregnancy from PE disease but do not differ between severe and non-severe PE conditions (Carty et al., 2008, Romero et al., 2008).

A microarray approach identified the CRIg/VSIG4 gene as a potential diagnostic marker of severe PE (Textoris et al., 2013). Of the eight genes identified via the microarray experiment, CRIg was the gene most upregulated in severe PE upon validation with real time PCR (Textoris et al., 2013). These results are also consistent with the findings that the VSIG4 gene was slightly overexpressed in PBMC from patients with PE (Sun et al., 2009).

1.6.7 Regulation of CRIg expression in macrophages

1.6.7.1 Arachidonate and LPS regulated CRIg expression in macrophages

Gorgani et al (Gorgani et al., 2011) demonstrated that inflammatory mediators significantly alter the expression of CRIg in human macrophages. Human monocytes matured to $CRIg^+$ macrophages cultured in the presence of arachidonate show depressed CRIg expression and similar results were observed with mature macrophages. In addition they found that this effect was independent of P13 Kinase, P38 and ERK1/ERK2, but dependent on protein kinase C (PKC) (Gorgani et al., 2011).

CRIg expression is also absent upon activation by LPS or in autoimmune inflammatory foci (Vogt et al., 2006).

1.6.7.2 Regulation of CRIg expression by anti-inflammatory agents in macrophages

Previous work in our laboratory has shown that the steroidal anti-inflammatory agent dexamethasone positively modulates CRIg expression, while nonsteroidal anti-inflammatory agents such as indomethacin and nordihydroguaiaretic acid had no effect on CRIg expression in macrophages (Gorgani et al., 2011). The increase in CRIg expression by dexamethasone correlated with an increase in phagocytosis by the macrophage.

1.6.7.3 Cytokines IFN-γ, TGF-β1, IL-4 and IL-10 regulate CRIg expression in macrophages

T cell activation requires TCR-mediated signalling and additional signals from co-stimulatory molecules. If co-stimulation is insufficient, T cells become tolerant (Langman and Cohn, 1993, Lenschow et al., 1996, Chambers and Allison, 1999, Schwartz, 2003, Dong et al., 2003). CRIg can inhibit T cell proliferation and IL-2

production (Vogt et al., 2006). Hepatitis B virus (HBV) infection is a serious health concern, and often strong cytotoxic T lymphocyte responses (CTL) and Th1 polarization develop in patients who are cured from acute HBV infection. These HBV-specific CTLs are also present in high frequencies in chronic hepatitis B patients, and are dysfunctional in producing anti-viral inflammatory cytokines such as TNF and IFN-γ and leading to an inability to control HBV infection (Rehermann et al., 1996, Wu et al., 2004, Blackburn et al., 2009).

Z39Ig is highly expressed on liver KCs (Helmy et al., 2006), but in liver tissues of chronic hepatitis B patients it is down regulated in comparison to healthy controls. In addition, *in vivo* and *in vitro* studies indicated that decreasing Z39Ig expression was caused by IFN-γ, suggesting that T cells can protect themselves from inactivation and possibly maintain their anti-viral function by down-regulating Z39Ig expression (Guo et al., 2010). Furthermore, our laboratory has also shown that IL-10 increased CRIg expression while IFN-γ, TGF-β1 and IL-4 decreased its expression (Gorgani et al., 2011). Thus CRIg expression seems to be regulated differently by different cytokines and other inflammatory mediators.

1.7 Signalling mechanisms involved in regulating CRIg expression

Up until 2011, there was virtually nothing known about the molecular mechanisms that regulate the expression of CRIg. Our group then reported (Gorgani et al., 2011) that arachidonic acid caused a down regulation in CRIg expression in monocyte-derived macrophages. In contrast, dexamethasone causes an up regulation in CRIg expression. An investigation into the mechanism by which arachidonic acid caused the down regulation revealed that protein kinase C (PKC) but not the mitogen-activated protein kinases or phosphatidylinositol 3-kinase was involved. These are some of the signalling

molecules that arachidonic acid stimulates in phagocytic cells (Hii et al., 1998, Hii et al., 2001). A role for PKC in causing the down regulation of CRIg expression was supported by the observation that the direct PKC activator, phorbol 12-myristate 13-acetate (PMA) (Aihara et al., 1991), mimicked arachidonic acid in reducing CRIg expression.

PKC is a family of serine/threonine protein kinases which consists of at least 10 isozymes. These are grouped into classical/conventional (α, βI, βII, and γ), novel (δ, ε, η, θ), and atypical (ζ, λ) isozymes depending on their cofactor requirements. Whereas all isozymes require a phospholipid e.g. phosphatidylserine, for activation, the classical isozymes also require calcium and diacylglycerol for activation; the novel isozymes do not require calcium but require diacylglycerol; neither calcium nor diacylglycerol are needed for the activation of the atypical isozymes (Aihara et al., 1991, Tsai et al., 2015). The differential requirement for the co-factors is dictated by the structural elements present on each class of PKC (Tsai et al., 2015). Owing to these properties, the classical and novel PKC isozymes can be directly activated by phorbol esters such as PMA. Another level of regulation is at the post translation stage, during which PKC isozymes are phosphorylated at specific residues in the activation loop by kinases such as phosphoinositide-dependent kinase-1 (Storz and Toker, 2002), an example of which is shown for PKCα (Figure 1.11). Each PKC isozyme is activated at specific locations within the cell and this is regulated by scaffold proteins (Antal et al., 2014). It has been proposed that this dictates substrate specificity for each isozyme, thus enabling different isozymes to perform different functions within the cell (Antal et al., 2014).

Human monocytes express α, βI, βII, δ, ε, and ζ isozymes (Huang et al., 1997). Thus, any of these could be involved in down regulating CRIg. However, our group has previously reported that arachidonic causes the activation of PKCα, βI, βII and ε in human macrophages (Huang et al., 1997), thereby narrowing the possibility to four PKC

isozymes. To select the most likely isozyme for investigation, it is necessary to have some knowledge of what the downstream targets are for the PKC isozymes. One of these targets is the type IV cytosolic phospholipase A_2 ($cPLA_2$), a known target of PKCα (Li et al., 2007).

Our group had previously reported in neutrophils that arachidonic acid causes the activation of $cPLA_2$ (Robinson et al., 1998) that releases arachidonate from membrane phospholipids (Robinson et al., 1998), leaving behind a lysophospholipid. This could be caused by the phosphorylation of $cPLA_2$ by PKCα in response to arachidonic stimulation. The regulation of CRIg expression is therefore likely to involve $cPLA_2$ because this phospholipase is not only activated by agents which down regulate CRIg expression (arachidonic acid and PMA), but $cPLA_2$ expression and activity in monocytes and macrophages are inhibited by glucocorticoids such as dexamethasone (Stone et al., 1990, Gewert and Sundler, 1995) that cause an upregulation of CRIg expression (Gorgani et al., 2011). Furthermore, LPS, which also causes a down-regulation of CRIg expression (Vogt et al., 2006), is known to promote the expression and activation of $cPLA_2$ in macrophages and Kupffer cells (Dieter et al., 2002, Miller et al., 2007). There is also evidence that LPS can act via PKCα in murine RAW264.7 macrophages to stimulate cytokine production (St-Denis et al., 1998). Considering that arachidonic acid/PMA/LPS and dexamethasone have opposite effects on CRIg expression, we hypothesise that the PKCα-activating agents and dexamethasone exert opposite actions on a common intracellular target, $cPLA_2$, to regulate CRIg expression (Figure 1.12). Indeed preliminary results have shown that $cPLA_2$ inhibitors cause upregulation of CRIg mRNA, (data not presented). Given these data, it is possible that PKCα is the isozyme that promotes the down regulation of CRIg expression.

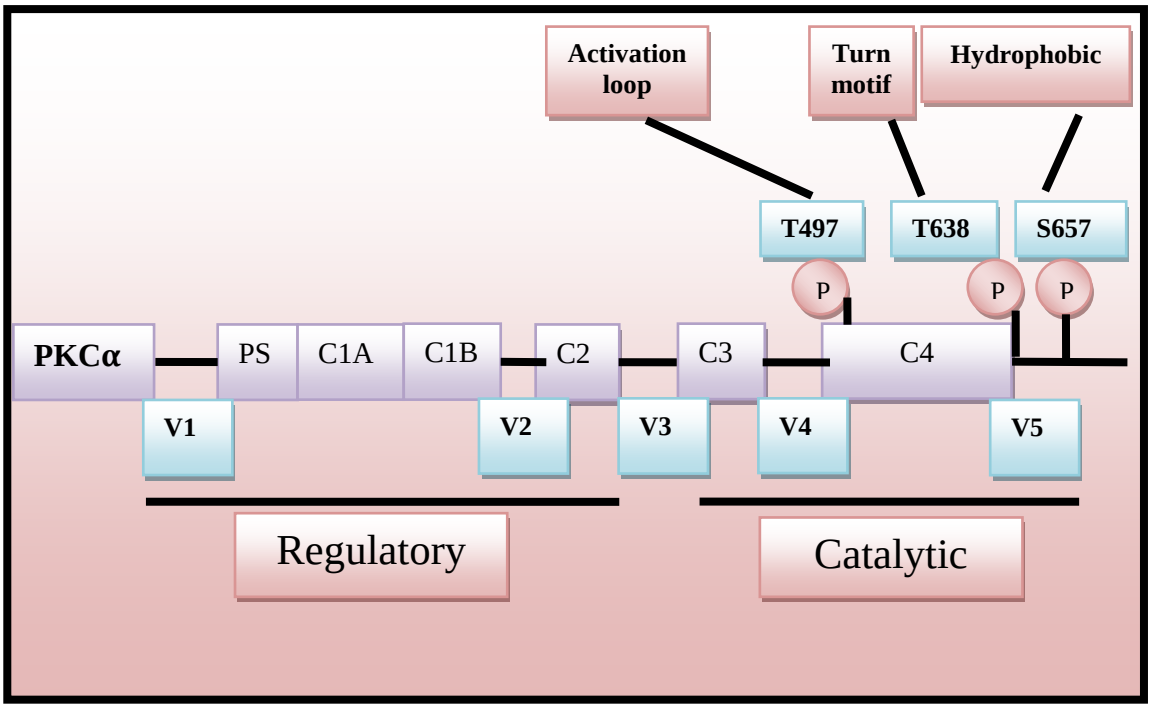

Figure 1.11: Structure of PKCα. PKCα is a 671 amino acid protein. C1A, C1B and C2 are regulatory domains. The C1 domains bind diacylglycerol and phosphatidylserine whereas the C2 domain binds anionic phospholipids, including phosphatidylinositol 4, 5-biphosphate, in a Ca^{2+}-dependent manner. C3 is ATP-binding domain and C4 domain is the substrate-binding kinase core. Adapted and redrawn from (Newton, 2001).

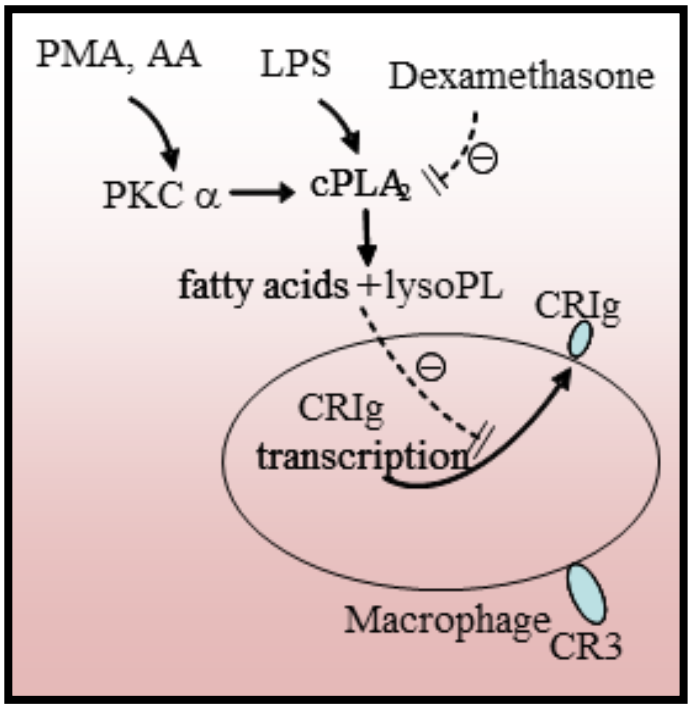

Figure 1.12. A model for the intracellular signaling molecules/pathways involved in the regulation of CRIg expression. LysoPL= lysophospholipids. Supportive evidence is provided in the text.

1.8 Hypotheses, aims and significance of this thesis

The macrophage is vital to protection against infection on the one hand and on the other can be detrimental leading to the tissue damage associated with inflammatory disorders. Cytokines in various patterns contribute significantly to this macrophage behaviour. Since CRIg, a macrophage specific complement receptor has been shown to play a role in immunity to infection and also mediate anti-inflammatory/immunosuppressive activity; it is likely that cytokines precipitate their effects via this receptor. However at present very little work has been done on the regulation of CRIg expression on macrophages by cytokines let alone inflammatory mediators in general and this question is the subject of the present thesis.

Hypotheses

1. CRIg expression on macrophages is amenable to modulation by cytokines which are known to alter macrophage function and which play roles in infection and immunity and inflammation.
2. CRIg is a control point in inflammation. Cytokines act through this control point by modulating the development of $CRIg^{+}$ macrophages and by acting on mature macrophages *per se*.
3. The modulatory effects of cytokines are not restricted to innate immunity but extend to adaptive immunity by regulating CRIg expression on DC.
4. The action of some anti-inflammatory drugs and anti-cytokine therapy acts through alleviating the effects of cytokines on CRIg expression.
5. Cytokines differentially regulate CRIg versus CR3/CR4 in macrophages in a PKCα-dependent manner.

Aims

The aims of this thesis are outlined below and the relationships between these aims are illustrated in Figure 1.13.

1. To examine the effects of a subset of cytokines known to act on macrophages for their effects on the development of $CRIg^+$ macrophages from human monocytes in culture: LT-α, IFN-γ, IL-4, IL-13, TNF, IL-1β, IL-6, TGF-β1, IL-10, M-CSF and GM-CSF (Chapter 3).
2. To study the effects of these cytokines on mature macrophages *per se* with respect to CRIg expression (Chapter 3).
3. To characterize the effects of these cytokines on CRIg expression in human DC (Chapter 4).
4. To determine the relationship between CRIg and CR3/CR4 expression in the above models (Chapter 3, Chapter 4, Chapter 5).
5. To study the intracellular signalling mechanisms which regulate CRIg expression in macrophages (Chapter 5).

Significance

Cytokines are known to alter cellular function during inflammation, arising from infection or autoimmune processes. In order to gain insights into the role that different cytokines play in autoimmune disease and infection it is important to examine the effects of different cytokines on the cells involved in these diseases i.e macrophages and dendritic cells.

This research plans to define the relationship between a new and important receptor (CRIg) in macrophages and cytokine-induced modulation, relevant to innate

and adaptive immunity. This should lead to a better understanding of how present therapies work and possibly provide avenues for their optimisation. It will also define CRIg as a new target for altering anti-microbial or inflammatory reactions in the body.

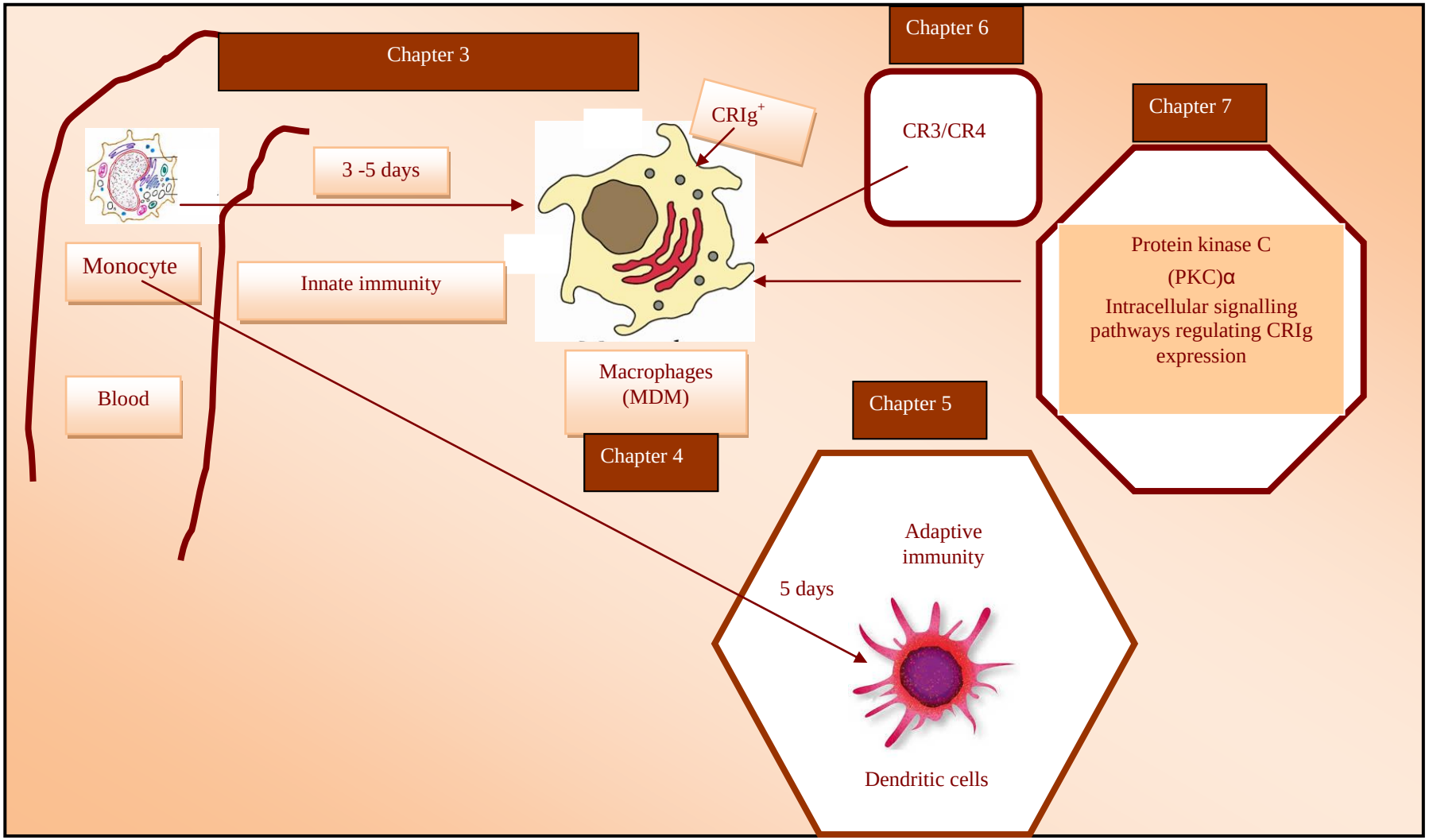

Figure 1.13: Schematic flow chart of the research presented in this thesis in each chapter.

CHAPTER TWO

Material and Methods

2.1 Ethical considerations

This study was approved by the Human Ethics Research Committee of the Children, Youth and Women's Health Service at the Women's and Children's Hospital, under the approval number REC 2165/4/2011.

2.2 Materials

2.2.1 Tissue culture media

Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium, foetal calf serum (FCS) and L-glutamine were purchased from SAFC Biosciences (Lenexa, Kansas, USA). Penicillin, streptomycin and Hank's balanced salt solution (HBSS) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Gradients / Cell separation medium

Ficoll-Paque Plus and Percoll gradient were purchased from (GE Health-care, Uppsala, Sweden; Little Chalfont, UK) sodium diatrizoate was from Sigma-Aldrich and angiografin was obtained from Schering AG (Berlin, Germany).

2.2.3 Cytokines

Recombinant cytokines, (LT)-α (TNF-β), GM-CSF, M-CSF, IL-1β, IL-6, IL-4, TNF, IL-13, IFN-γ and IL-10 were purchased from ProSpec-Tany Technogene (Rehovot, Israel) and TGF-β1 from R&D Systems (Minneapolis, Minnesota, USA), dexamethasone and LPS were purchased from Sigma-Aldrich.

2.2.4 Protease and phosphatase inhibitors

Benzamidine, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, and Sigma 104 (Phosphotase substrate) were purchased from Sigma-Aldrich and aprotinin from Calbiochem (Merck, Darmstadt, Germany).

2.2.5 Antibodies

A mouse monoclonal antibody that recognizes the IgV domain of human CRIg (clone 3C9) was kindly provided by Dr. van Lookeren Campagne (Genentech, San Francisco, CA, USA). Mouse monoclonal antibodies against PKCα, PKCζ and CRIg/Z39Ig (clone 6H8) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-CD11b/CD11c monoclonal antibodies and mouse IgG were purchased from Abcam (Cambridge, UK). Mouse IgG_1 isotype phycoerythrin-conjugated antibodies were purchased from eBioscience (San Diego, CA, USA) and IgG_{2b} isotype control phycoerythrin-conjugated antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti hDc-SIGN FITC (DC) was purchased from R&D Systems. Horse-radish peroxidase (HRP)-conjugated rabbit anti-mouse IgG was purchased from Dako, Agilent Technologies (Denmark). The anti-TNF mAb (2TNF-H34A) was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

2.2.6 General chemicals / Bio-chemicals

Sodium hydroxide, chloroform, and isopropanol were purchased from Ajax Chemicals (Auburn, NSW, Australia). Absolute ethanol, Giemsa stain and formaldehyde were obtained from Merck (Kilsyth, Vic, Australia). Bovine Serum Albumin (BSA) was

purchased from Bovogen Biologicals (Essendon, Vic, Australia). Trizma base, trypan blue, β-mercaptoethanol, and ethylenediaminetetraacetic acid (EDTA) were from Sigma-Aldrich. HEPES (2-[4-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), Ponceau S, TEMED (Tetramethylethylenediamine), DTT (Dithiothreitol), Glycine, Folin and Ciocalteaus phenol reagent were purchased from Sigma-Aldrich. Polyacrylamide was purchased from Bio-Rad (Hercules. CA, USA).

2.2.7 Markers for monocytes, macrophages and dendritic cells

These cell types are standard and well established in our laboratory i.e monocytes, MDM (Gorgani et al., 2011) and DCs. Morphologically these cells have distinct appearances but in the past the laboratory has used various markers (CD14, CD36, CD68, CD163, CD11b, CD11c, CD209) to check the phenotype of the cell populations after each protocol. The methods described in this thesis are well established in the literature (Seager Danciger et al., 2004, Mohana et al., 2015, Yamamichi et al., 2015, Sugimura et al., 2015, Larsen et al., 2015, Wang et al., 2015).

2.3 Purification of human monocytes

Human peripheral blood mononuclear cells (PBMC) were purified according to the method of Ferrante and Thong (1978). Venous blood collected from healthy volunteers was centrifuged over a medium of hypaque-ficoll at 600g /35 min. After centrifugation, the leukocytes resolve into two discrete bands with red blood cells at the bottom of the tube. PBMCs consisting of monocytes, T and B lymphocytes were obtained from the top band, while neutrophils were present in the lower band. The upper band was gently aspirated and washed with RPMI -1640 medium by repeated centrifugation (3 x 5 min,

600 x *g*) and re-suspension of the cells. Viability of the leukocytes as judged by their ability to exclude trypan blue being >99%. The PBMC (purity >98%) were used for monocyte purification. In addition PBMC were purified from "buffy coats" obtained from Australian Red Cross Blood Service (Adelaide, South Australia).

Monocytes were purified from PBMC by density gradient centrifugation, as described previously (Seager Danciger et al., 2004, Marantos et al., 2008). Briefly, cells were layered on a 46% iso-osmotic Percoll gradient and spun at 600 x *g* for 30 min at room temperature. The monocyte-containing layer was washed three times with ice-cold RPMI-FCS (RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin) containing 1 mmol/EDTA and centrifuging at 600 x *g* for 5 min at 4ºC. Monocytes were >90% pure (95 - 98%) as judged by standard Giemsa staining and $CD14^+$ staining by flow cytometry. Initially this method was used for phenotyping of purified cells, but once the method was well established the protocol was routinely followed with no phenotyping.

2.4 Preparation of monocyte-derived macrophages (MDM)

MDM were prepared as described previously (Gorgani et al., 2011). Monocytes were isolated from blood buffy coats of healthy donors (Australian Red Cross Blood Service, Adelaide, South Australia) by centrifugation on Hypaque-Ficoll medium and then adherence was carried out according to Human Macrophage Nucleofection instructions (Amaxa, Lonza Walkersville, MD, USA), except that culture dishes (150 mm diameter, Sarstedt (Postfach, Nümbrecht, Germany) were coated with neat autologous plasma in lieu of poly-D-Lysine. After plating for 1 h, the adherent cells were cultured for varying days depending on the nature of the experiment and allowed to differentiate into MDM in an incubator at 37°C, 5% CO_2 air mixture at a density of 1 x 10^6 cells/ml. Cells were

cultured in RPMI-10% FCS and the medium was replaced every second day during incubation. Macrophages were harvested by gentle scrapping (using rubber policeman) or by carefully dislodging the cells by 15 min incubation in detachment buffer (lidocaine/EDTA/PBS) (Robinson and Nau, 2008) at 37°C and followed by repeated pipetting over the monolayer. Macrophages were then washed three times and either used for further assays or pelleted and stored at -70ºC for later use. The preparation consisted of approximately 99% MDM.

In various experiments the monocytes were differentiated (differentiated/developed were used interchangeably throughout the thesis) into mature monocyte-derived macrophages (MDM) for three or five or seven days (Gorgani et al., 2011). Monocytes were isolated from PBMC (as described above in 2.3). The cells were cultured in (RPMI-1640 + P/S + L-Glut + 10% FCS) and incubated at 37ºC under an atmosphere of 5% CO_2 / air and high humidity over a period of a few days in the presence or absence of cytokines depending on the experimental plan of each results chapter. Cytokines were added at the concentration indicated in the results. After incubation, MDM were harvested and mRNA levels, intracellular protein and surface protein levels were measured by qRT-PCR, Western blotting and flow cytometry, respectively (see section 2.8, 2.9 and 2.10).

2.5 Knockdown of PKCα in MDM

Knockdown of PKCα was achieved using an Amaxa Nucleofector and a Human Macrophage Nucleofection Kit (Lonza Walkersville, MD, USA). For ~ 10^6 MDM, 4 µg non-targeting control shRNA or PKCα-specific shRNA (predesigned shRNA specific for PKCα (GenBank accession no. NM_002744), Sigma-Aldrich) (see chapter 5) was added to each cuvette and the cells were transfected using program Y-010 according to

the manufacturer's instructions. After transfection, MDM were cultured for 24 h before harvesting to assay functional responses. An aliquot of the cultures was used to confirm the knockdown of PKCα by Western blot analysis. Cell viability was also monitored by the trypan blue dye exclusion (add equal volumes of 0.1% trypan blue and cell suspension (1 x 10^6), mix well and load into the haemocytometer). Cell viability was retained at ~ 90%, which is consistent with the statement made by the Nucleofection Kit information document (Amaxa).

2.6 Macrophage stimulation with LPS

Transfected MDM were harvested using detachment buffer and plated in a 96-well plate at 1 x 10^5 cells per well. LPS (dilutions in culture medium) was added to cells at 10 ng/ml final concentration (Marantos et al., 2008). The plate was incubated in 37°C for 24 h, and the supernatants were harvested for cytokine measurements. To examine the effects of LPS on CRIg expression in PKCα-deficient MDM, cells were plated in 40 x 10 mm dishes with 2 ml of medium. After incubation with 10 ng/ml LPS at 37°C for 24 h, cells were harvested, and CRIg protein levels were determined by Western blot analysis (see section 2.10).

2.7 Preparation of dendritic cells

Monocyte-derived dendritic cells were generated from human peripheral blood monocytes by culturing these with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). Monocytes were isolated from PBMC (as described above in 2.3). The cells were cultured in RPMI-10%FCS, in the presence of GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) and incubated at 37ºC under an atmosphere of 5% CO_2 / air and high humidity at a

starting density of 1 x 10^6 cells/ml over a period of five days. After incubation, dendritic cells were harvested and mRNA levels, intracellular protein and surface protein levels were measured by qRT-PCR, Western blotting and flow cytometry, respectively (see section 2.8, 2.9 and 2.10).

2.8 Measurement of CRIg expression by quantitative real-time PCR for CRIg mRNA expression

2.8.1 Total RNA isolation and quantitation of RNA

Total RNA was isolated using an RNeasy Plus mini kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions (Gorgani et al., 2011). Briefly, the pelleted cells were lysed in RLT buffer and washed in RW1, RPE buffer and then treated with DNase I (DNA-free Kit, Ambion, Life Technologies, Mulgrave, Vic, Australia) to remove any genomic DNA contamination. The quantity of RNA was assessed on a NanoDrop (Thermo Fisher Scientific, MA, USA).

2.8.2 Synthesis of cDNA

Single-strand (first-strand) cDNA was synthesised from total RNA using iScript™ cDNA synthesis kit following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, to a tube was added 200 ng of total RNA, 4 µl of 5x Reaction Mix, 1 µl of iScript, and water up to 20 µl total volume. The mixture was heated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min and then stored at -20°C, until used for PCR.

2.8.3 Primer design

Primers for CRIg (Gorgani et al., 2011), CD11b and CD11c were manually designed for use in quantitative real-time PCR. Using the NCBI Reference Sequence of the CRIg transcript (VSIG4, NM_007268.2), the forward primer (5' ACACTTATGGCCGTCCCAT 3') was designed to anneal across exons 1 and 2 and reverse primer to anneal within exon 2 (5' TGTACCAGCCACTTCACCAA 3') to generate an expected 130 base pair (bp) PCR product. CD11b and CD11c transcripts were detected with the following primer pairs designed with Oligo Perfect Designer (Invitrogen, Carlsbad, CA, USA) (Gorgani et al., 2011) (F: 5' CCTGGTGTTCTTGGTGCCC 3'; R: 5' TCCTTGGTGTGGCACGTACTC 3'; 102 bp expected PCR product size, *ITGAM*, NM_001145808.1) and (F: 5' CCGATTGTTCCATGCCTCAT 3'; R: 5' AACCCCAATTGCATAGCGG 3'; 154 bp expected PCR product size, ITGAX, NM_001286375.1), respectively. Data were normalised against the expression of the house-keeping gene, GAPDH, amplified with F: 5' GAGTCAACGGATTTGGTCGT 3' and R' 5' GACAAGCTTCCCGTTCTCAGCCT 3', to generate an expected 185 bp PCR product.

2.8.4 Quantitative SYBR-Green-based Real Time PCR

This was conducted essentially as described previously (Gorgani et al., 2011). The synthesised cDNA was amplified in 20 µl triplicate reactions with 1 x iQ SYBR Green supermix (Bio-Rad Laboratories), 1 µl of cDNA (50 ng/µl) and 100 nM of each forward and reverse primer for CRIg, CD11b, CD11c or GAPDH, using the iQ5 Real Time Detection System with v3.1 software (Bio-Rad Laboratories). The thermal cycling cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Expression data were

analysed using the supplied software (BioRad iQ5 optical system version 2.1), with gene expression data for CRIg normalised against GAPDH expression data.

2.9 Flow cytometry for CRIg expression

2.9.1 Analysis of CRIg cell-surface expression

The expression of CRIg on the surface of macrophages and dendritic cells was measured by flow cytometry using the monoclonal anti-CRIg 6H8 antibody. After harvest from cell culture, Fc receptors on the cell surface were blocked by incubation with ice-cold PBS supplemented with 0.5% (w/v) BSA, 10 mg/ml Intragam P, and 5% (v/v) human AB serum (prepared from volunteer blood donors with human AB blood type), for 30 min. PE-conjugated anti-CRIg (Z39Ig, 6H8 clone) or isotype control (IgG1) antibodies, along with CD14-FITC or CD209-FITC / anti hDc-SIGN FITC antibodies were added to 1.5 x 10^5 cells in a total staining volume of 50 µl. After 30 min, the cells were washed with 2 ml of PBS containing 0.5% (w/v) BSA by centrifugation (600 x *g* for 5 min). After discarding the supernatant, the cells were then fixed in PBS containing 1% (v/v) formaldehyde and analysed on a Becton Dickinson FACSCanto flow cytometer (BD Biosciences, CA, USA). A minimum of 20,000 - 50,000 events were acquired. Using WinMDI 2.9 (http//en.bio.soft.net), cells were gated by forward and side scatter, followed by anti-CD14 (monocytes/macrophages) or anti-CD209 / anti hDc-SIGN FITC (dendritic cells) fluorescence, before CRIg expression was determined.

2.10 Western blotting for CRIg, PKCα and PKCζ expression

2.10.1 Sample preparation

Cells were harvested by gentle scrapping and centrifuged at 4ºC for 5 min at 1200 x *g* and then resuspended in 100 µl of lysis buffer (20 mM HEPES, pH 7.8, 0.5% (v/v) Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml Sigma 104, and 10 µg/ml leupeptin, aprotinin, pepstatin A, and benzamidine) and placed on a rocking platform for 2 hrs at room temperature. The samples were centrifuged at 4ºC for 5 min at 1200 x *g* and soluble fractions were collected. A protein assay was then performed (as described below in 2.10.2), prior to the addition of 3 x Laemmli buffer (20 mM Tris-HCl, pH 6.8, 40% (w/v) sucrose, 6% (w/v) SDS (Sodium dodecyl sulfate), 0.5% (w/v) bromophenol blue and 10% (v/v) β-mercaptoethanol). Samples were boiled at 100ºC for 5 min and then stored at -20ºC until analysis.

2.10.2 Protein quantitation by Lowry's protein assay

The protein content of the cell lysates was quantitated by the method described by Lowry (Lowry et al., 1951). BSA protein standards (0, 3.125, 6.25, 12.5, 25 and 50 µg) were prepared for each assay by serially diluting 1% BSA (1 mg/ml in PBS) with H_2O; whilst samples of cell lysates were diluted 1:10 for quantitation. One-hundred and fifty µl of Lowry's solution (2% Na_2CO_3, 1% SDS, 0.4% NaOH, 0.16% Na/K tartrate, and 1% 0.1 M $CuSO_4$) was added to 50 µl of standards and cell lysate samples diluted 1:10 in water to be quantitated. After 20 min of incubation at room temperature, 15 µl of 50% Folin and Ciocalteau's Phenol Reagent (diluted in water) was added. Following 20 min of incubation at room temperature, then 180 µl of each standard and sample was transferred into a 96 well flat-bottomed plate (Nunc, Roskilde, Denmark) and the optical density at 540 nm was measured using a Dynatech MR 5000 plate reader (Dynatech

Laboratories, Alexandria, VA). A standard curve was generated from the protein standards, enabling the concentration of protein in each sample to be determined.

2.10.3 Western blot

Twenty to 60 µg (equally loaded in each experiment) of each protein sample were separated by 12% SDS-Polyacrylamide (1.585ml MilliQwater (Merck Millipore, Australia), 50 µl 10% SDS, 1.25 ml Buffer A (1.5 M Tris (Base), 500 mls MilliQwater, pH 8.8), 2.085 ml Acrylamide, 2.5 µl TEMED, 25 µl 10% APS (Ammonium persulfate) gel electrophoresis (SDS-PAGE) by subjecting the gel to 175 V for approximately 1 h using the Bio-Rad Mini-PROTEAN 3 system (BioRad) (Costabile et al., 2001). The samples were electrophoretically transferred to a nitrocellulose membrane (Pierce, Illinois, USA) at 100 V for 1 hr (Transfer buffer: 25 mM TrisBase, 20% (v/v) methanol, 152mM Glycine, MilliQ water; 5 x Running buffer: 384 mM Glycine, 50 mM Tris Base, 1 litre MilliQ water, 0.1% SDS). To monitor the extent of protein transfer, the membrane was stained with Ponceau S (0.1% in 5% acetic acid). The membrane was immersed in blocking solution (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% (v/v) Tween-20, 5% (w/v) skim milk powder (Diploma instant, Fonterra) for 1 h at room temperature or overnight at 4ºC. The membrane was then incubated with primary antibody (mouse anti-CRIg, PKCα, PKCζ, GAPDH; all at 1:1000 dilutions in blocking solution) overnight at 4°C. Following washing (3 x 10 min, 10 ml blocking solution), the membrane was treated with secondary antibody, HRP-conjugated rabbit anti-mouse IgG (1:2000 dilutions in blocking solution) for 1 hr at room temperature on a rocking platform. Immunoreactive material was detected using Western Lightning® Plus-Enhanced Chemiluminescence Substrate from PerkinElmer (Waltham, Massachusetts, USA), according to the manufacturer's instru

ctions. Blot analysis was performed on a Chemi-Doc XRS^{+} system (Bio-Rad Laboratories, Hercules, CA) using the Bio-Rad Image Lab^{TM} software version 3.0.

2.11 Cytokine assays

IL-6, TNF and IL-1β levels were determined using Cytometric Beads Array Flex Sets obtained from BD Biosciences (San Jose, CA, USA), according to the manufacturer's instructions and as described previously (Melino et al., 2008). In brief, 50 µl of capture bead suspension and 50 µl of PE detection reagent were added to an equal amount of sample or standard dilution and incubated for 2 hr at room temperature in the dark. Subsequently, samples were washed and centrifuged at 200 x *g* at room temperature for 5 min. The supernatant was discarded and 150 µl of wash buffer was added. Samples were analysed on a BD FACSCanto System (BD Biosciences).

2.12 Phagocytosis assay

The phagocytosis assay was performed essentially as described previously (Gorgani et al., 2011). At 24 hr post transfection with shRNA (Chapter 5) or cytokine treatment (Chapter 3), MDM were washed and detached with detachment buffer. Then 1 x 10^5 *C. albicans* yeast was added to 5 x 10^4 MDM in a final volume of 0.5 ml HBSS. Complement-containing human AB serum was added to a final concentration of 10%. The cells were incubated for 15 min at 37°C on a rocking platform. Unphagocytosed yeast particles were removed by differential centrifugation at 175 x *g* for 5 min and then the MDM in the pellet were resuspended and cytocentrifuged onto a microscope slide and stained with Giemsa. The number of particles in phagocytic vacuoles was then determined (Gorgani et al., 2011). Phagocytosis was scored as both the number of

macrophages that had engulfed >4 fungi (line graph) as well as the number of fungi engulfed per cell (bar graph).

2.13 Statistical analysis

Statistical significance was calculated using GraphPad Prism 6.1 (GraphPad Software, Inc., La Jolla, CA, USA). To compare the control response to multiple groups, a two-way ANOVA or one-way ANOVA followed by Dunnett's Multiple Comparison test was performed. A paired or unpaired two-tailed Student's *t*-test was used to compare the means of two groups with matched or unmatched responses, respectively. A value of $p < 0.05$ was considered significant.

Experimental designs involved in Chapter 3 and 4 are as (Figure 2.1, 2.2, 2.3, 2.4, 2.5).

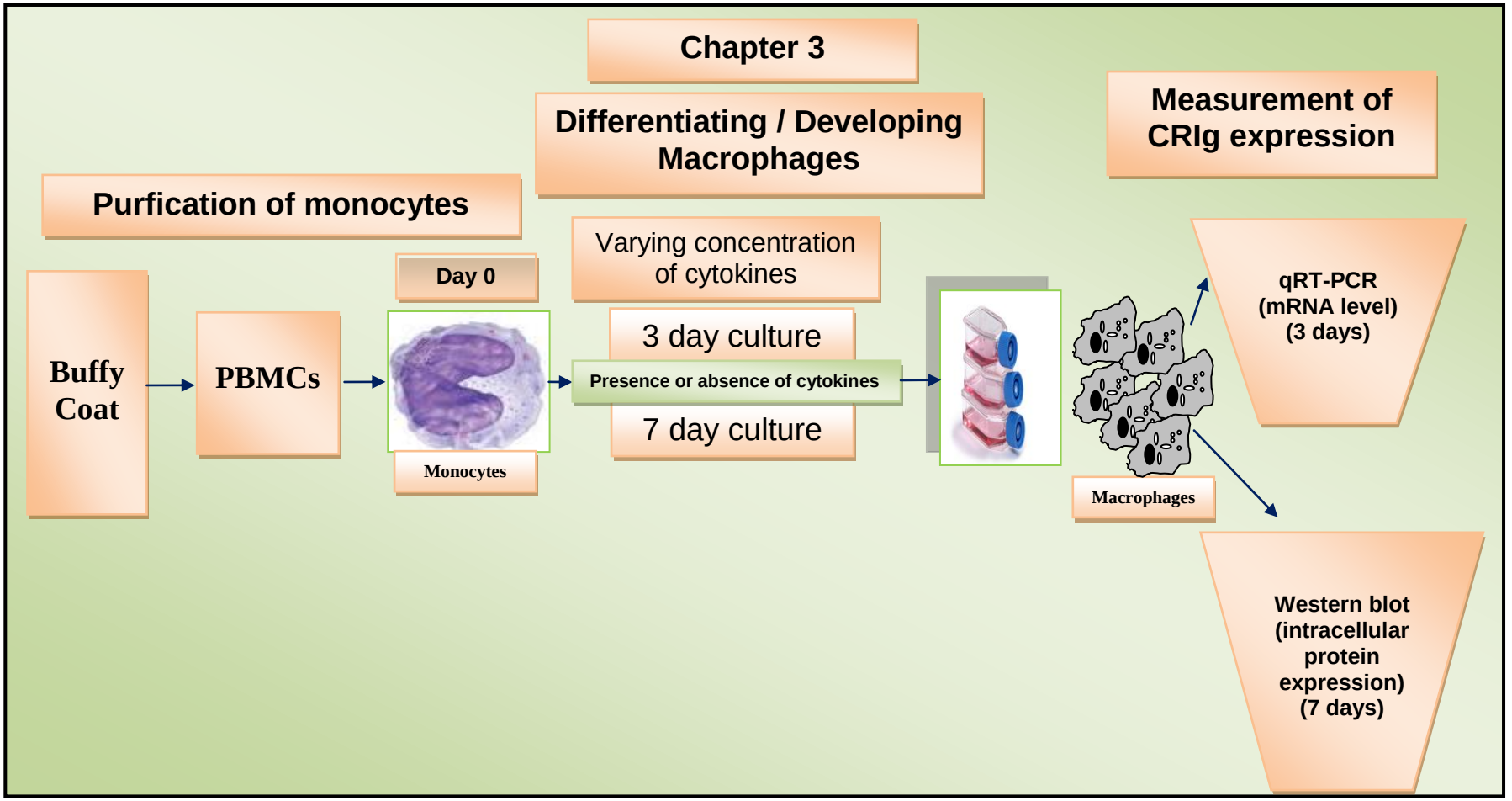

Figure 2.1: Schematic flow chart of the experimental procedures involved in the measurement of CRIg expression during the development of human monocyte into macrophages in Chapter 3. Monocytes were cultured in RPMI-1640+P/S+L-Glut+10% FCS for 3 or 7 days with or without cytokines. CRIg mRNA were measured by qRT-PCR at 3 days and CRIg protein by Western blot at 7 days of culture.

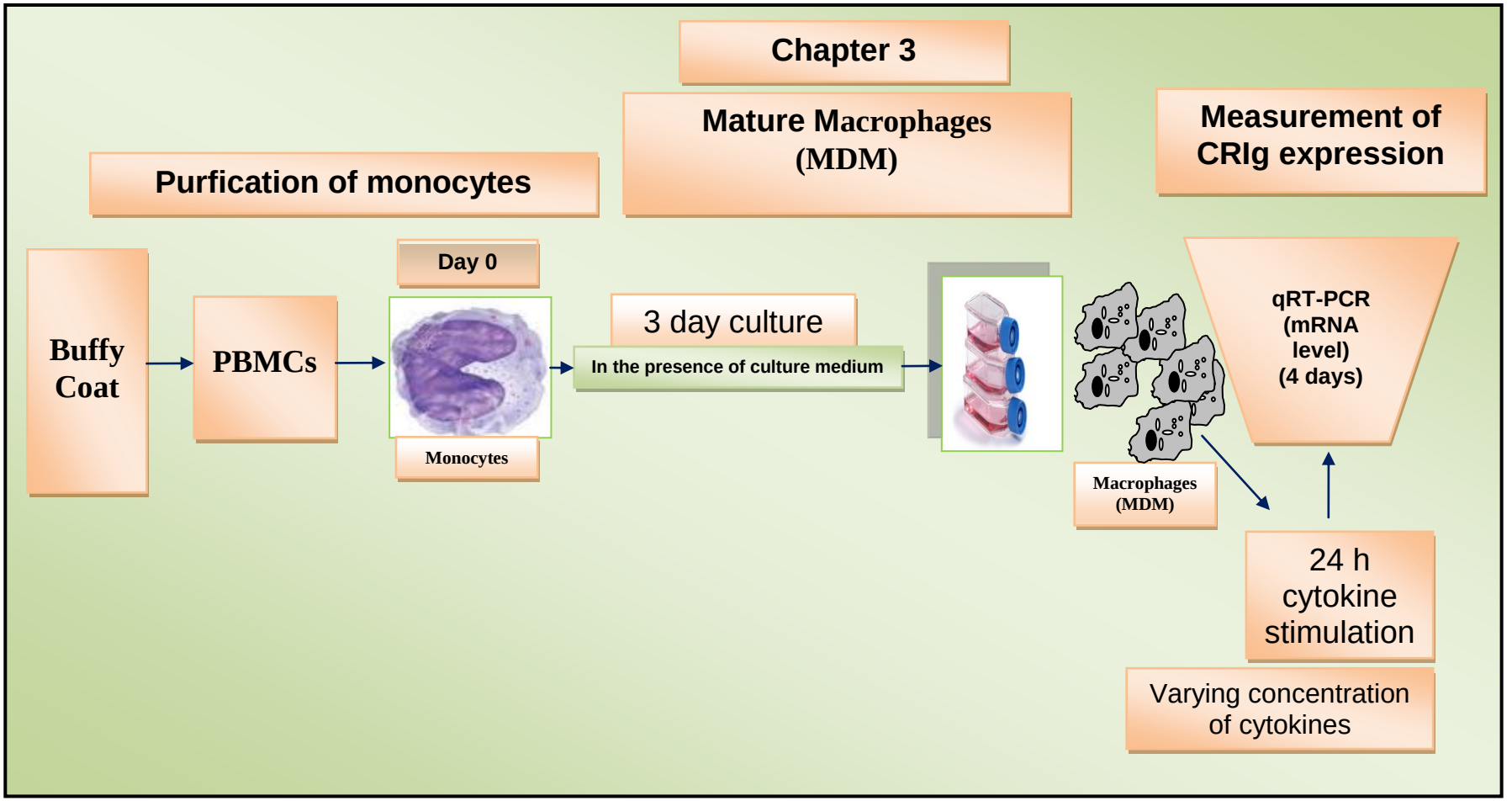

Figure 2.2: Schematic flow chart of the experimental procedures involved in the measurement of CRIg expression in human monocyte matured into MDM in Chapter 3. Monocytes were cultured in RPMI-1640+P/S+L-Glut+10% FCS for 3 days and then MDM were treated with cytokines for 24 hrs. CRIg mRNA were measured by qRT-PCR at 4 days of culture.

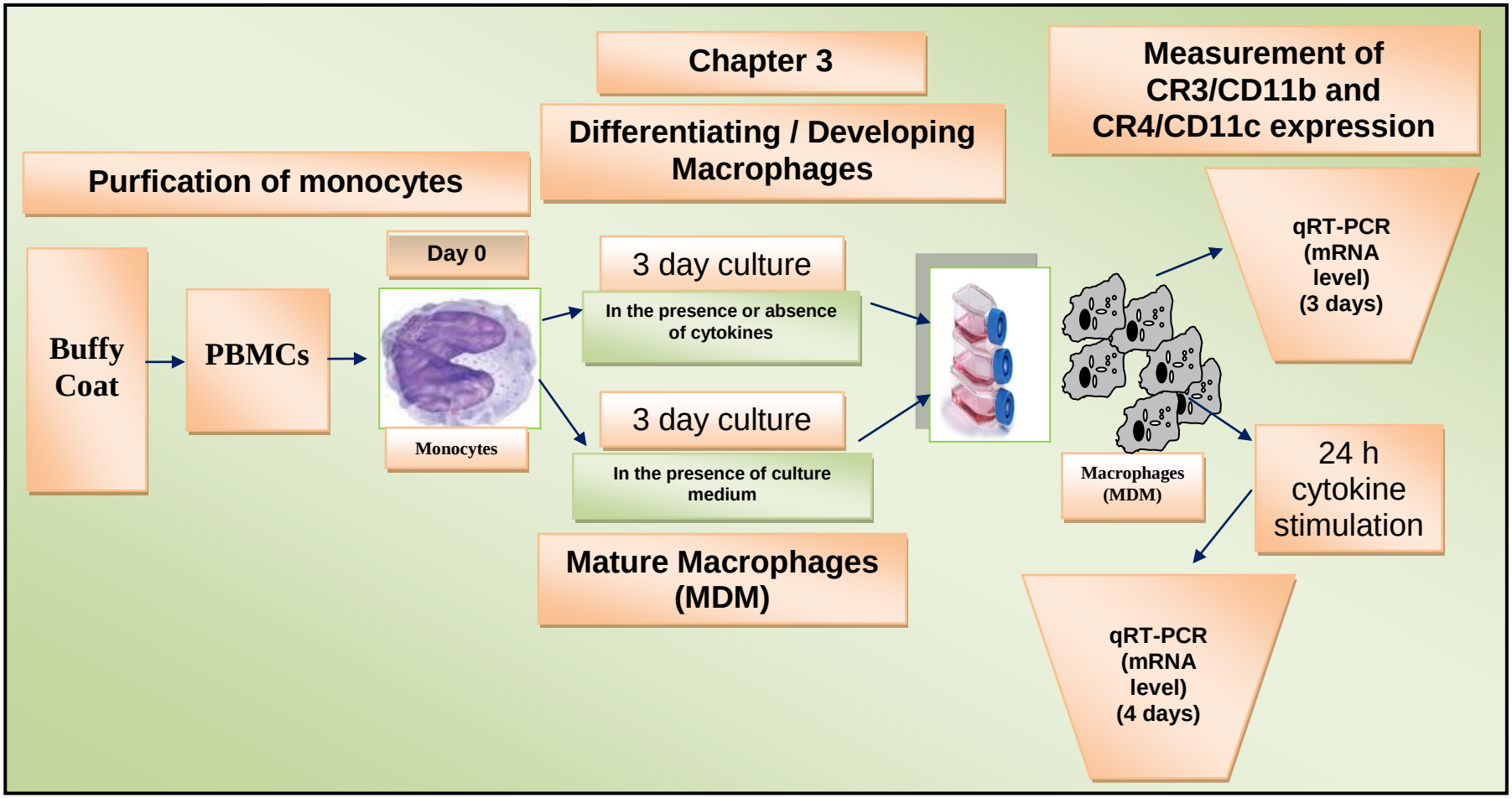

Figure 2.3: Schematic flow chart of the experimental procedures involved in the measurement of CR3/CD11b and CR4/CD11c expression during the development of human monocyte into macrophages and MDM in Chapter 3. In human monocyte differentiating into macrophages, monocytes were cultured in RPMI-1640+P/S+L-Glut+10% FCS for 3 days with or without cytokines. In human MDM, monocytes were cultured in RPMI-1640+P/S+L-Glut+10% FCS for 3 days and then MDM were treated with cytokines for 24 hrs. CR3/CD11b and CR4/CD11c mRNA were measured by qRT-PCR at 3 days and 4 days of culture.

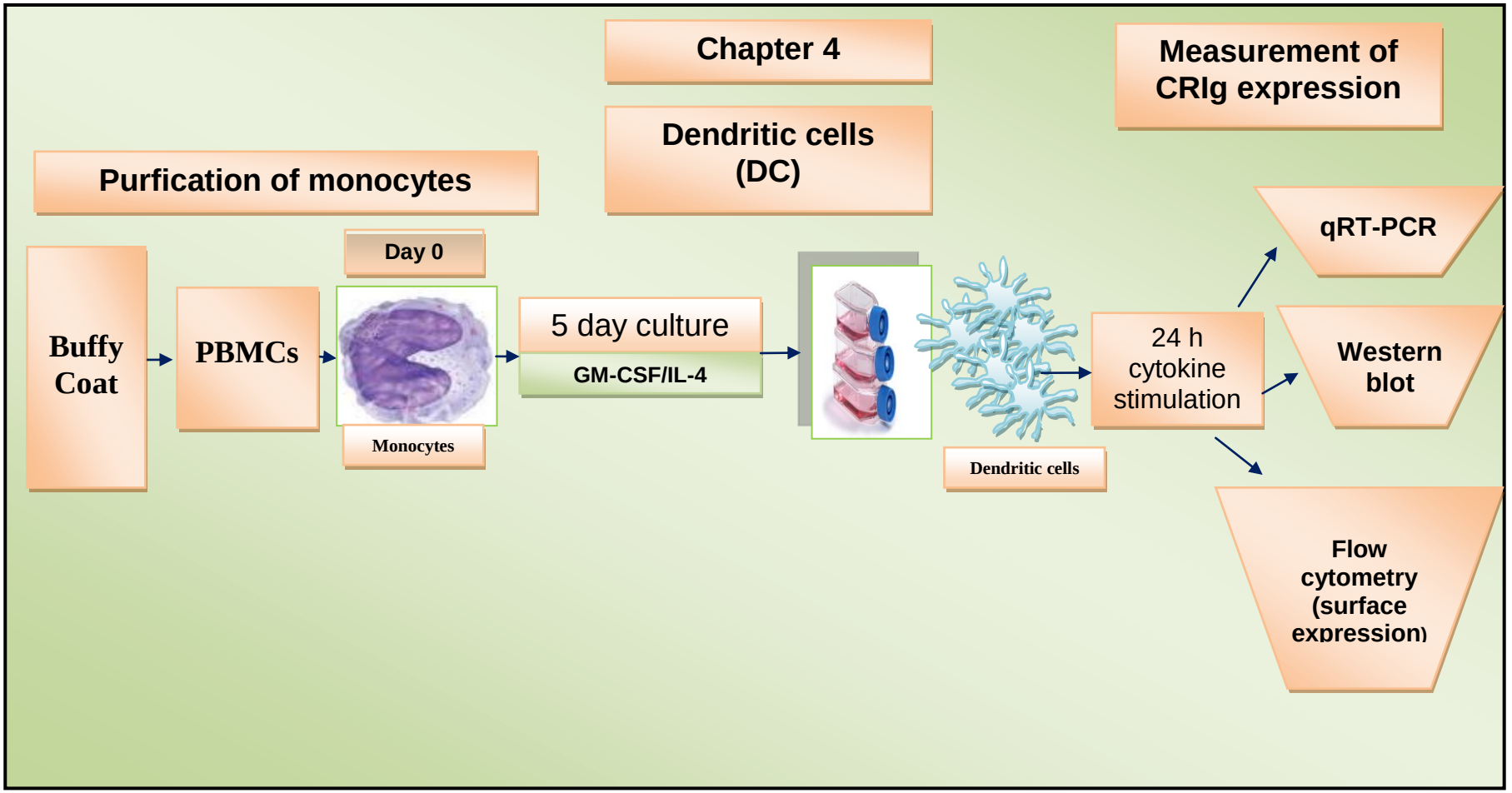

Figure 2.4: Schematic flow chart of the experimental procedures involved in the measurement of CRIg expression in monocyte-derived human DC in Chapter 4. Monocytes were cultured in the presence of (RPMI-1640+P/S+L-Glut+10% FCS and cytokines (GM-CSF/IL-4) over a period of five days, these cells are then termed as DC. The DC were then treated for 24 hrs with cytokines. DC were harvested and mRNA levels, intracellular protein and surface protein levels were measured by qRT-PCR, Western blotting and flow cytometry, respectively.

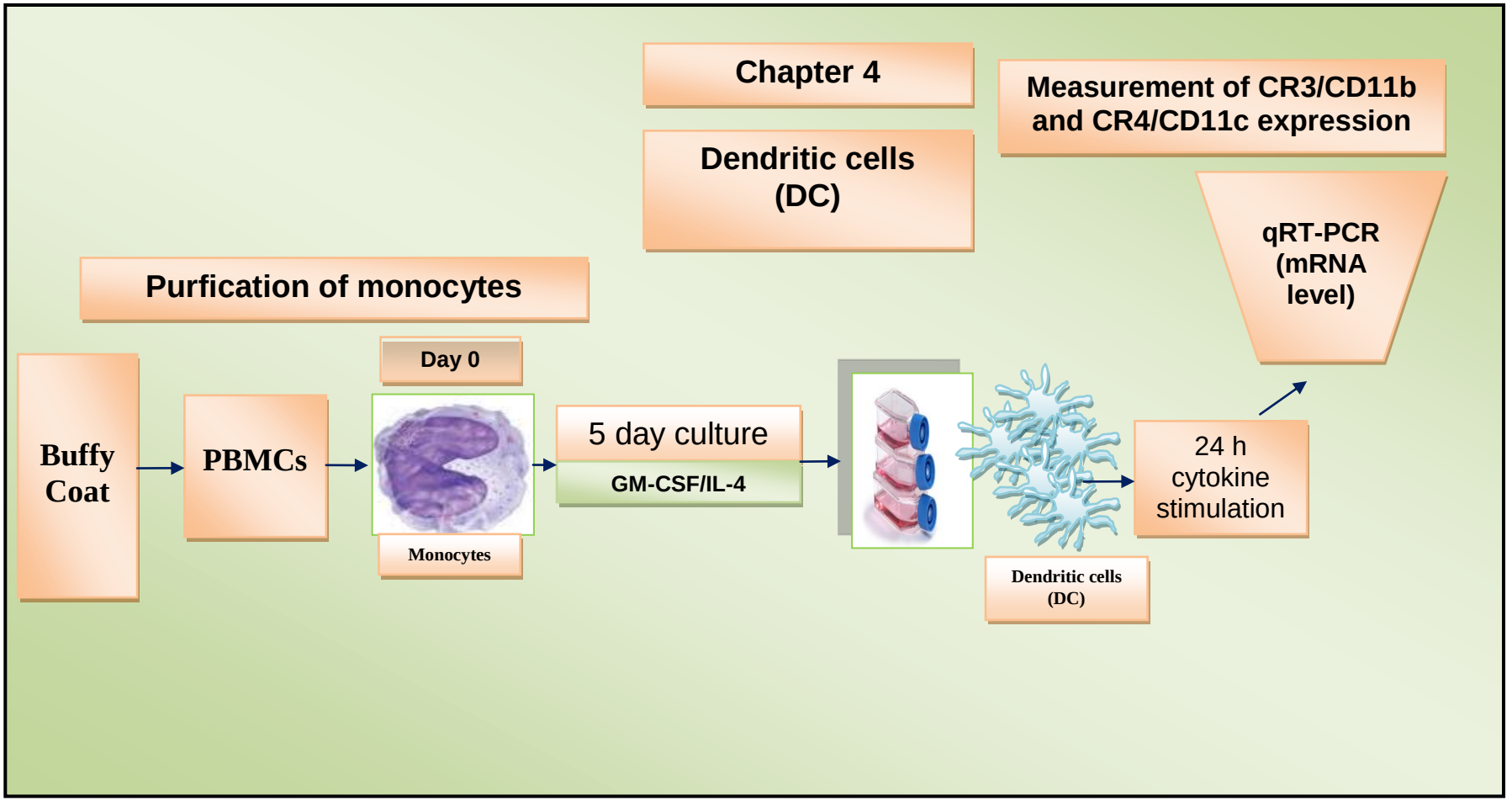

Figure 2.5: Schematic flow chart of the experimental procedures involved in the measurement of CR3/CD11b and CR4/CD11c expression in monocyte-derived human DC in Chapter 4. Monocytes were cultured in the presence of (RPMI-1640+P/S+L-Glut+10% FCS) and cytokines (GM-CSF/IL-4) over a period of five days, these cells are then termed as DC. The DC were then treated for 24 hrs with cytokines. CR3/CD11b and CR4/CD11c mRNA were measured by qRT-PCR at 6 days of culture.

CHAPTER THREE

Cytokines Modulate CRIg, CR3, CR4 Expression and Phagocytosis During the Development of Human Macrophages and in Human Mature Macrophages

3.1 Introduction

Tissue resident macrophages are the first line of contact with microbial pathogens and altered tissues. The type of genes expressed by these macrophages may dictate the mode of immune responses elicited. Members of complement, Toll-like and scavenger receptors as well as C-type lectins are amongst the groups of receptors that initially recognize opsonised-pathogen or pathogen-associated molecular patterns. In the last decade, the B7 family-related protein V-set and Ig domain-containing 4 (VSIG4) (Z39Ig) (Langnaese et al., 2000, Walker, 2002, Kim et al., 2005), was found to be an important complement (CRIg) receptor (Helmy et al., 2006). This receptor differs structurally and functionally from the classical complement receptors, CR3 and CR4. CRIg is expressed selectively by macrophages and is involved in the rapid phagocytosis of complement (C3b/iC3b)-opsonised pathogens/IgM-coated erythrocytes (Gorgani et al., 2008)**.** The presence of CRIg on KC surfaces results in the rapid uptake of circulating *Listeria monocytogenes* and *Staphylococcus aureus*, thereby limiting bacterial dissemination and pathogenesis (Helmy et al., 2006), $CRIg^{-/-}$ mice infected with these bacteria exhibited exaggerated levels of inflammatory cytokines, and died earlier than wild type mice. The absence of CRIg, therefore, resulted in excessive innate and adaptive immune system activation. In line with these findings, murine CRIg-Fc fusion protein depressed complement activation (Katschke et al., 2007), inhibited T cell function and ameliorated humoral and cellular immune responses (Vogt et al., 2006). Thus, CRIg represents a novel complement receptor with properties distinct from those of the classically known complement receptors.

Monocytes attracted to sites of inflammation differentiate into macrophages, where by possessing certain genes they are positioned strategically to recognise modified-self, non-self and opsonised particles and thereby play a critical defence

role(s), particularly during the initial encounter of infiltrating microbial pathogens and altered tissues. Macrophages are heterogeneous (Mantovani et al., 2009) and cytokines can direct monocyte differentiation to different macrophage subtypes. Since these subpopulations can have distinct properties of critical importance to protection against infection or promoting pathogenesis, the role of the various endogenous mediators in influencing the recruitment, maturation and development of macrophage subtypes while important, still remains poorly understood.

Our laboratory previously reported that monocytes in culture begin to express CRIg mRNA as they develop into macrophages (Gorgani et al., 2011). The development of $CRIg^{+}$ macrophages was found to be up (IL-10) or down (IFN-γ, IL-4 and TGF-β1) regulated by cytokines based on CRIg mRNA levels. The aim of this research was to examine the effects of these cytokines on CRIg protein expression using Western blot analysis to reveal the effects on both of the spliced forms of CRIg. In addition this research aimed to characterize better the cytokine network's influence on CRIg expression by examining the effects of other cytokines which play an important role in macrophages during inflammation. The cytokines chosen, TNF, IL-13, IL-1β, IL-6, LT-α, M-CSF and GM-CSF are known to have direct action on developing and mature macrophages (monocyte-derived macrophages). This research also aimed to compare effects of these cytokines on the CRIg receptor versus the CR3 and CR4 receptors.

3.2 Methods

For details of methods see chapter 2 (section 2.3; 2.4; 2.8; 2.10). The *in vitro* model involved the culturing of monocytes to allow their development into macrophages. CRIg, CR3/CD11b and CR4/CD11c mRNA were measured by qRT-PCR at 3 days of

culture and CRIg protein by Western blot at 7 days (Figure 2.1, 2.3). During these periods of time the monocyte cultures were treated by the addition of varying concentrations of cytokines or dexamethasone.

The effects of cytokines on the expression of CRIg, CR3/CD11b and CR4/CD11c were also examined in MDM. Purified monocytes were cultured for 3 days and used as mature monocyte-derived macrophages (MDM) (see section 2.3; 2.4 in chapter 2). MDM were then treated for 24 hr in the presence of varying concentrations of cytokines (Figure 2.2). The macrophages were harvested after 4 days and then CRIg, CR3/CD11b and CR4/CD11c mRNA levels were measured by qRT-PCR (see section 2.8 in chapter 2) (Figure 2.3). For phagocytosis, monocytes were cultured for 5 days into MDM and then treated with cytokines and dexamethasone for 24 hr. The macrophages were harvested after 6 days and then challenged with complement opsonised heat killed *C. albicans* (see section 2.12 in Chapter 2).

3.3 Results

Previously it was shown that cultured human monocytes displayed maximal increase in CRIg mRNA expression on day 3 of culture and protein on day 7 (Gorgani et al., 2011). Thus experiments in this chapter were designed around these time points in order to examine the effects of cytokines on macrophage CRIg expression. Cytokines known to regulate macrophage function and produced in inflammatory sites were evaluated for their effects on CRIg expression. Monocytes were treated with or without cytokines and the expression of CRIg mRNA and protein were examined in human monocytes developing into macrophages and MDM.

3.3.1 Effect of cytokines on monocytes developing into CRIg$^+$ macrophages

Monocytes were cultured in the presence of either the Th1 cytokines, LT-α and IFN-γ or the Th2 cytokines IL-4 and IL-13. In the presence of LT-α there was an increase in CRIg mRNA and marked increase in CRIg protein (Figure 3.1 A and B). In contrast, IFN-γ caused a marked decrease in CRIg mRNA and protein expression (Figure 3.1 C and D). These effects were seen over a concentration range of 5-40 ng/ml for LT-α and 10-40 ng/ml for IFN-γ. The Th2 cytokines, IL-4 and IL-13 both markedly inhibited the expression of CRIg at the mRNA and protein level (Figure 3.2 A and B). These cytokines reduced CRIg expression in a concentration dependent manner over a range of 1-40 ng/ml for IL-4 and 5-40 ng/ml for IL-13.

Western blot analysis enabled us to distinguish between the two different forms of CRIg, the long (L) and short (S) forms. The data in Figures 3.1 (B, D, E) and 3.2 (B, D, E) showed that the effects of each cytokines on the two forms were essentially similar. It is also evident that CRIg(L) is the more abundant form in these macrophages, even after treatment with cytokines.

TNF, IL-1β and IL-6 are referred to as pyrogenic and pro-inflammatory cytokines which predominate during infection and inflammation associated with chronic inflammatory diseases. Because of the importance of CRIg in phagocytosis and regulation of inflammation, their effects on CRIg expression in cultured macrophages were examined. Treatment of monocytes with TNF caused a marked reduction of CRIg mRNA and protein in the mature macrophages (Figure 3.3 A and B). This reduced expression occurred in a concentration dependent manner. In particular, TNF caused approximately 80% reduction in CRIg protein expression. IL-1β, and in particular IL-6 substantially increased CRIg expression in macrophages (Figure 3.3 C, D, E, and F). CRIg protein expression analysed by Western blotting demonstrated that both forms of

CRIg, L and S, were similarly altered in expression when monocytes were cultured in the presence of these cytokines (Figure 3.3 B, D, and F).

TGF-β1 and IL-10 share a number of properties and have been shown to depress inflammation. Their effects on macrophage function have been reported. Culturing monocytes with TGF-β1 led to a concentration (2-15 ng/ml) dependent decrease in CRIg mRNA expression with almost complete suppression of CRIg protein expression (Figure 3.4 A and B). In contrast IL-10 caused a marked increase in CRIg expression in human monocytes developing into macrophages (Figure 3.4 C and D). When these effects were compared to those of dexamethasone it was evident that IL-10 was as effective as dexamethasone in inducing CRIg expression (Figure 3.4 E and F). This was seen at both the mRNA and protein level. Although the effects of dexamethasone on total CRIg cellular protein was not previously studied it is evident from the Western blot analysis that the steroid increased the cellular expression of both CRIg(L) and CRIg(S) forms (Figure 3.4 F). The effects of both TGF-β1 and IL-10 were similar on both forms of CRIg (Figure 3.4 B and D).

The studies were extended to another set of cytokines which are involved in controlling macrophage function, GM-CSF and M-CSF. When monocytes were cultured in the presence of these cytokines, both caused an increase in CRIg mRNA and protein expression in macrophages (Figure 3.5). Both of these cytokines caused a marked increase in CRIg expression, comparable to that seen with IL-10. As per other cytokines, both GM-CSF and M-CSF caused an increase of both CRIg(L) and CRIg(S) (Figure 3.5 B and D).

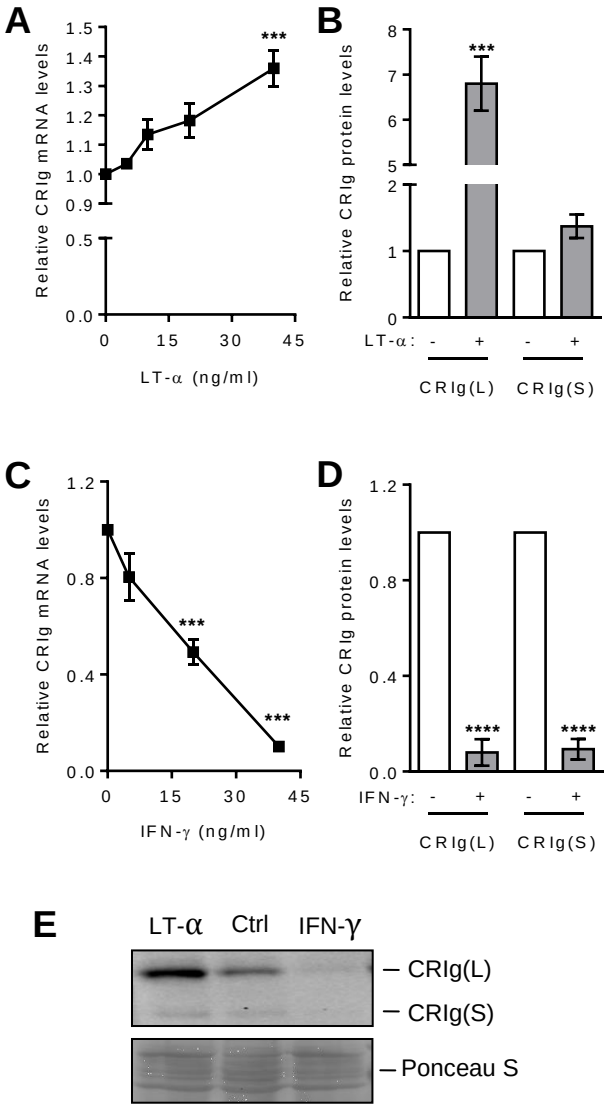

Figure 3.1: The Th1 cytokines LT-α and IFN-γ regulate the development of CRIg$^+$ macrophages from monocytes (see Figure 2.1 for protocol). Monocytes were cultured in the presence of either LT-α **(A)** (0, 5, 10, 20, 40 ng/ml) or IFN-γ **(C)** (0, 10, 20, 40 ng/ml) for 3 days and then CRIg mRNA expression measured by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. For CRIg protein expression monocytes were cultured in the absence (Ctrl) or presence of 40 ng/ml LT-α **(B, E)** or IFN-γ **(D, E)** for 7 days and then the CRIg protein levels measured by Western blot (clone 3C9). Note both the long and short forms of CRIg are expressed. **(E)** A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. Data are presented as means ± SEM of three experiments each conducted with monocytes from different individuals, ***p<0.001, ****p<0.0001.

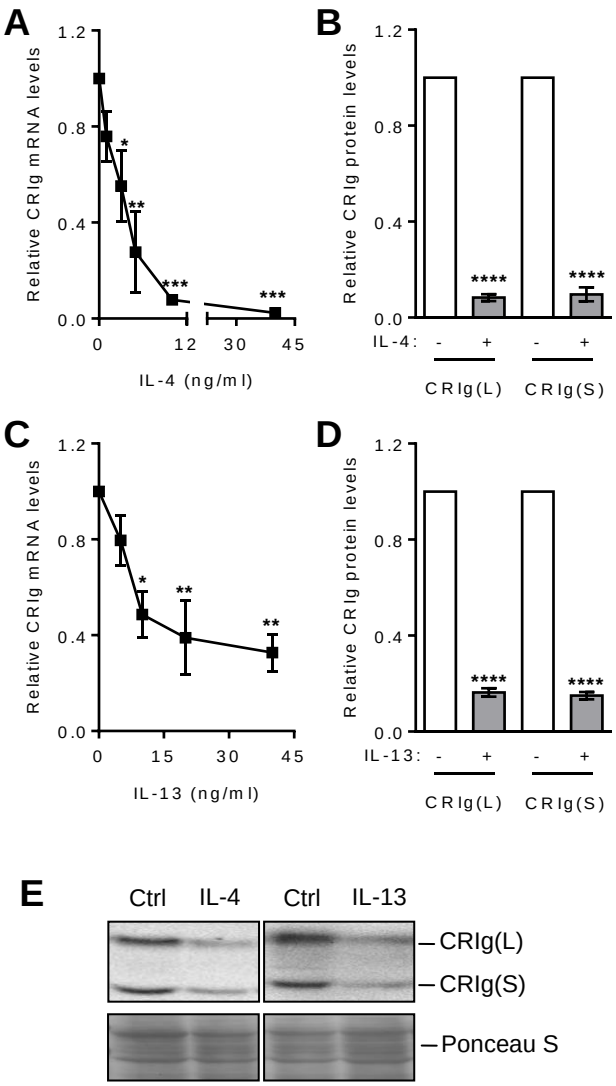

Figure 3.2: The Th2 cytokines IL-4 and IL-13 down-regulate CRIg expression during the development of human monocytes into macrophages (see Figure 2.1 for protocol). Monocytes were cultured in the presence of either IL-4 **(A)** (0, 1, 3, 5, 10, 40 ng/ml) or IL-13 or **(C)** (0, 5, 10, 20, 40 ng/ml) for 3 days and then CRIg mRNA expression measured by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA expression. For CRIg protein expression the monocytes were cultured in the absence (Ctrl) or presence of 40 ng/ml **(B)** IL-4 or IL-13 **(D)** for 7 days and then CRIg levels measured by Western blot (clone 3C9). Note that macrophages express the long and short forms of CRIg. **(E)** A representative Western blot of CRIg levels and total protein by Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. Data are presented as means ± SEM of three experiments, each conducted with monocytes from different individuals, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

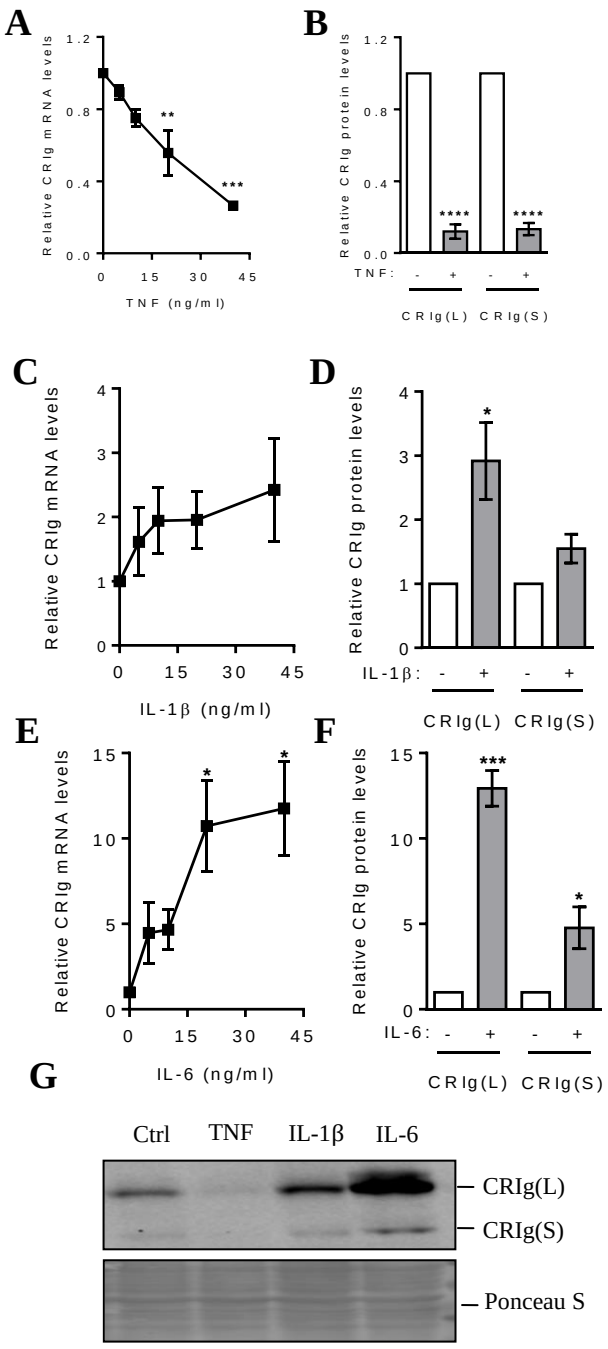

Figure 3.3: The pyrogenic cytokines, TNF, IL-1β and IL-6 differentially regulate CRIg expression during the development of human monocytes into macrophages (see Figure 2.1 for protocol). Monocytes cultured in the presence of either TNF **(A)** (0, 5, 10, 20, 40 ng/ml), or IL-1β **(C)** (0, 5, 10, 20, 40 ng/ml) or IL-6 **(E)** (0, 5, 10, 20, 40 ng/ml) for 3 days and then CRIg mRNA expression measured by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA expression. For CRIg protein expression monocytes were cultured in the absence (Ctrl) or presence of 40 ng/ml TNF **(B)**, IL-1β **(D)** or IL-6 **(F)** for 7 days and then CRIg expression analysed by Western blotting (clone 3C9). Both forms of CRIg were present. **(G)** A representative Western blot of CRIg levels and total protein in lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. Data are presented as means ± SEM of experiments, each conducted with monocytes from different individuals, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

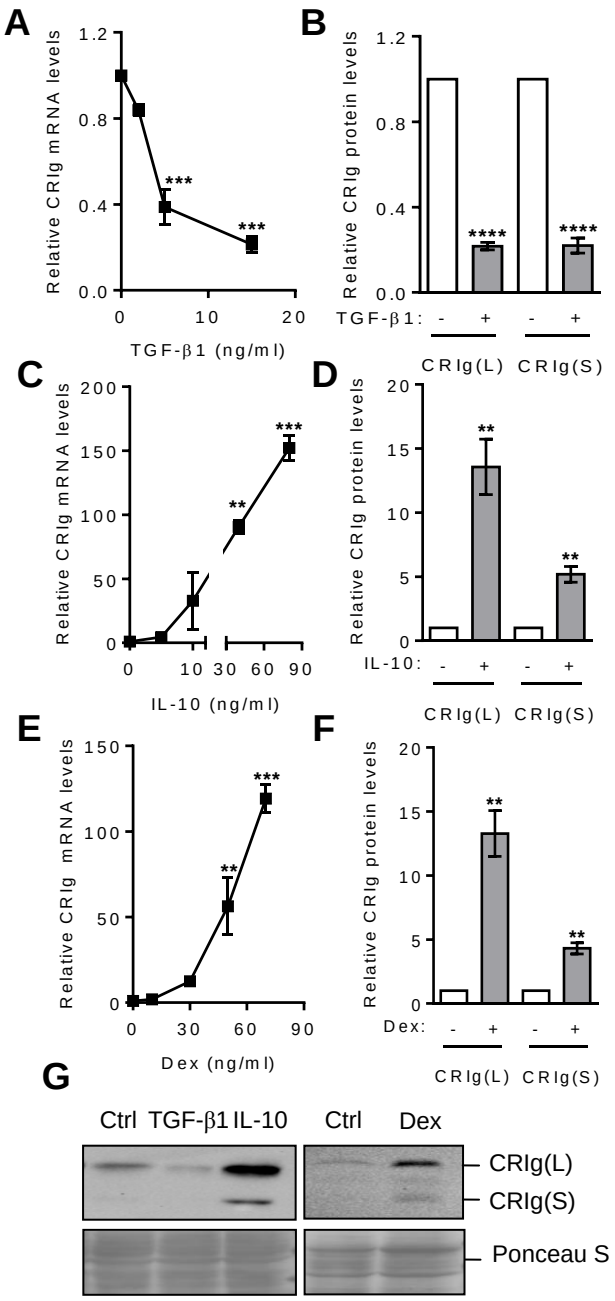

Figure 3.4: TGF-β1 has different effects to IL-10 and dexamethasone on CRIg expression during the development of human monocytes into macrophages (see Figure 2.1 for protocol). Human monocytes were cultured in the presence of either TGF-β1 **(A)** (0, 2, 5, 15 ng/ml), or IL-10 **(C)** (0, 5, 10, 40, 80 ng/ml) or dexamethasone **(E)** (0, 10, 30, 50, 70ng/ml) for 3 days and then CRIg mRNA expression measured by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA expression. For CRIg protein expression monocytes were cultured in the absence (Ctrl) or the presence of 15 ng/ml TGF-β1 **(B)**, 40 ng/ml IL-10 **(D)** or 30 ng/ml dexamethasone **(F)** for 7 days and then examined by Western blotting (clone 3C9) and data expressed as fold-difference in CRIg band intensity with CRIg expression in the absence of cytokine set as 1. **(G)** A representative blot is shown. Data are expressed as fold-difference in band intensity as determined by densitometry. Data are presented as means ± SEM of experiments, each conducted with monocytes from different individuals, **p<0.01, ***p<0.001, ****p<0.0001.

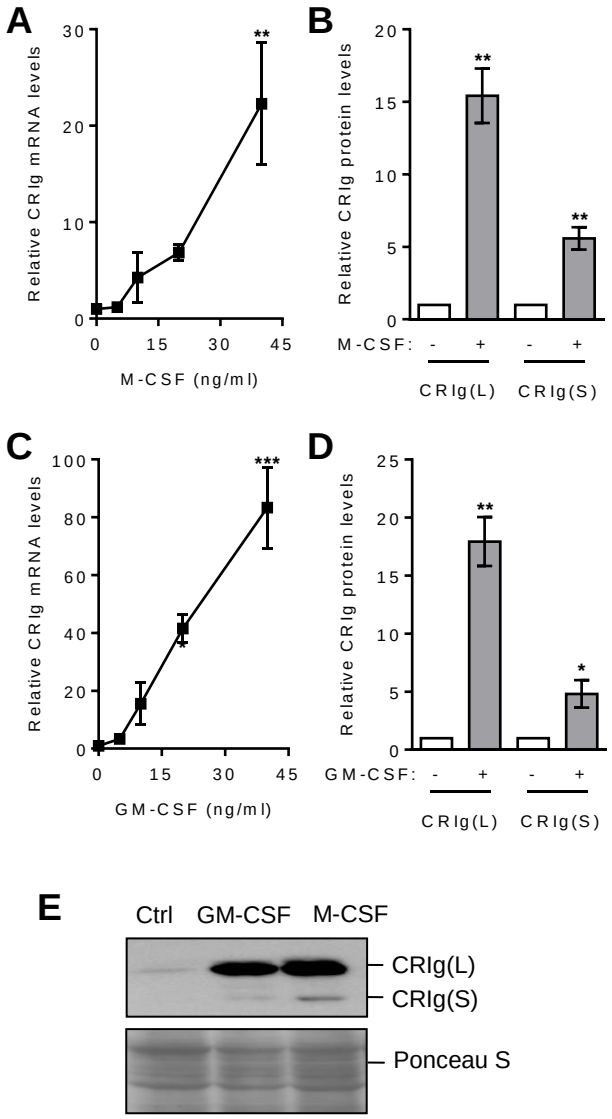

Figure 3.5: The haematopoietic growth factors, M-CSF and GM-CSF promote CRIg expression during the development of human monocytes into macrophages (see Figure 2.1 for protocol). Human monocytes were cultured in the presence of either M-CSF **(A)** (0, 5, 10, 20, 40 ng/ml) or GM-CSF **(C)** (0, 5, 10, 20, 40 ng/ml) for 3 days and then CRIg mRNA expression measured by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. For CRIg protein expression monocytes were cultured in the absence (Ctrl) or presence of 40 ng/ml M-CSF **(B)** or GM-CSF **(D)** for 7 days and CRIg expression measured by Western blotting (clone 3C9). (**E)** A representative Western blot of total protein load is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. Data are presented as means ± SEM of experiments, each conducted with monocytes from different individuals, *p<0.05, **p<0.01, ***p<0.001.

3.3.2 The effect of cytokines on CRIg expression in macrophages (MDM)

In the previous section the effects of cytokines on the development of $CRIg^+$ macrophages were examined. While this forms one stage of understanding the mediators that control CRIg expression in macrophages in particular during inflammation, it does not reveal whether macrophages present in tissues and already expressing CRIg can be modulated in the presence of cytokines. Thus a second stage for regulating inflammation is for cytokines to act on these resident tissue macrophages.

MDM expressing CRIg were generated from monocytes in culture. The MDM were treated with either cytokines or dexamethasone for 24 h and then examined for levels of CRIg mRNA. The results showed that treatment with dexamethasone increased CRIg expression (Figure 3.6 J). Treatment with 5-40 ng/ml of LT-α caused a small but non-significant increase in CRIg mRNA (Figure 3.6 A). In comparison, another Th1 cytokine IFN-γ caused a marked decrease in CRIg mRNA expression over a concentration range of 5-40 ng/ml reaching a decrease of approximately 60% (Figure 3.6 B) at 40 ng/ml. IL-4 down regulated CRIg mRNA expression in MDM over a concentration range of 1-40 ng/ml, with a 60% reduction at 10 ng/ml (Figure 3.6 C). Decreased CRIg mRNA expression could be detected at low 1-3 ng/ml concentrations of IL-4. IL-13 caused a significant reduction in expression of CRIg mRNA over a concentration range of 5-40 ng/ml (Figure 3.6 D). A decrease in CRIg mRNA expression was observed at 5 ng/ml and significance reached at 20 ng/ml.

TNF caused a substantial decrease in CRIg mRNA over a concentration range of 1-20 ng/ml (Figure 3.6 E). The effect was evident at 1 ng/ml to almost complete reduction of CRIg mRNA at 20 ng/ml (Figure 3.6 E). In contrast to TNF, IL-1β was not as effective at depressing CRIg levels in MDM over a concentration range of 5-40

ng/ml (Figure 3.6 F), however, IL-1β caused a small and significant decrease at the highest concentration tested 40 ng/ml (Figure 3.6 F). Similarly IL-6 tested at concentration of 5-40 ng/ml had little effect on CRIg mRNA expression at low concentrations but a significant effect was observed at 40 ng/ml (Figure 3.6 G).

The regulatory cytokine TGF-β1 caused a substantial decrease in CRIg mRNA over a concentration range of 2-15 ng/ml (Figure 3.6 H). But IL-10 caused a marked decrease in CRIg mRNA expression in MDM at a concentration range of 5-40 ng/ml (Figure 3.6 I). Dexamethasone up regulated CRIg mRNA over a concentration range of 10-50 ng/ml (Figure 3.6 J) in MDM. M-CSF caused a significant increase in CRIg mRNA expression in MDM over a concentration range of 5-40 ng/ml (Figure 3.6 K). In contrast to the effects of M-CSF, GM-CSF down regulated CRIg mRNA expression at 5-40 ng/ml (Figure 3.6 L).

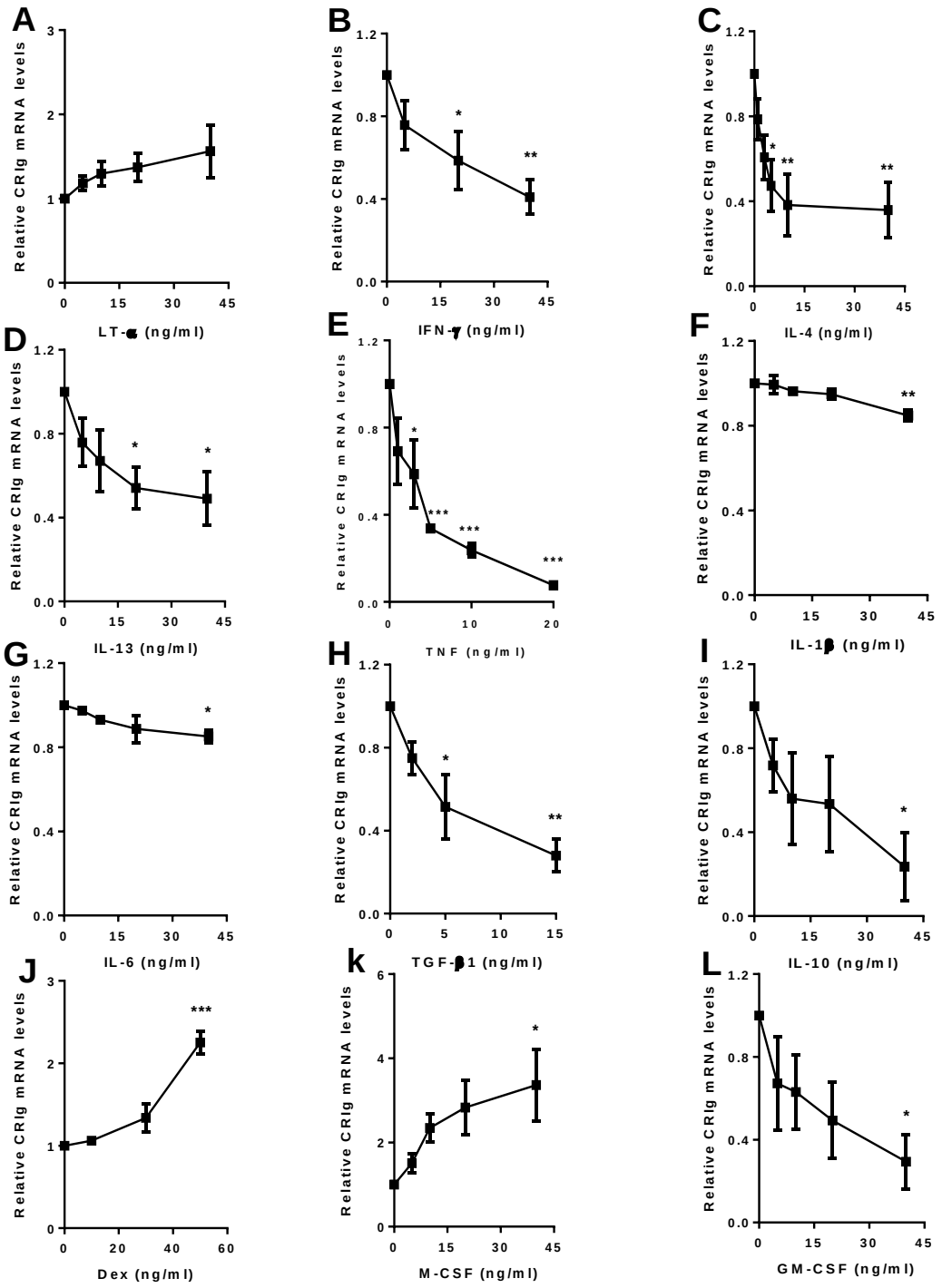

Figure 3.6: Effects of cytokines on CRIg expression in human MDM (see Figure 2.2 for protocol). In these studies MDM were prepared by culturing human monocytes for 3 days (for CRIg mRNA). MDM from 3 day cultures were treated with LT-α **(A)** (0, 5, 10, 20 and 40 ng/ml) or IFN-γ **(B)** (0, 5, 20 and 40 ng/ml) or IL-4 **(C)** (0, 1, 3, 5, 10 and 40 ng/ml) or IL-13 **(D)** (0, 5, 10, 20 and 40 ng/ml) or TNF **(E)** (0, 1, 3, 5, 10 and 20 ng/ml) or IL-1β **(F)** (0, 5, 10, 20 and 40 ng/ml) or IL-6 **(G)** (0, 5, 10, 20 and 40 ng/ml) or TGF-β1 **(H)** (0, 2, 5 and 15 ng/ml) or **(I)** IL-10 (0, 5, 10, 20 and 40 ng/ml) or dexamethasone **(J)** (0, 10, 30 and 50 ng/ml) or M-CSF **(K)** (0, 5, 10, 20 and 40 ng/ml) or GM-CSF **(L)** (0, 5, 10, 20 and 40 ng/ml) for 24 h and then CRIg mRNA levels relative to GAPDH mRNA were assessed by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ***p<0.001.

3.3.3 Effects of cytokines on human MDM phagocytosis

To examine whether the effects of cytokines on CRIg expression in human MDM corresponded to functional changes, phagocytosis was examined. In these experiments the MDM (5 day) were treated with the cytokines for 24h and were then challenged with *C. albicans* which had been preopsonised with complement-containing human AB group serum. Figure 3.7 shows that cytokine treatment of MDM altered their capacity to phagocytose *C. albicans.* While LT-α and M-CSF caused an increase in phagocytosis, all the other cytokines caused a decrease in fungal phagocytosis by the macrophages. The effect of cytokines on phagocytosis correlated with changes in CRIg expression but not in CR3/CR4 expression.

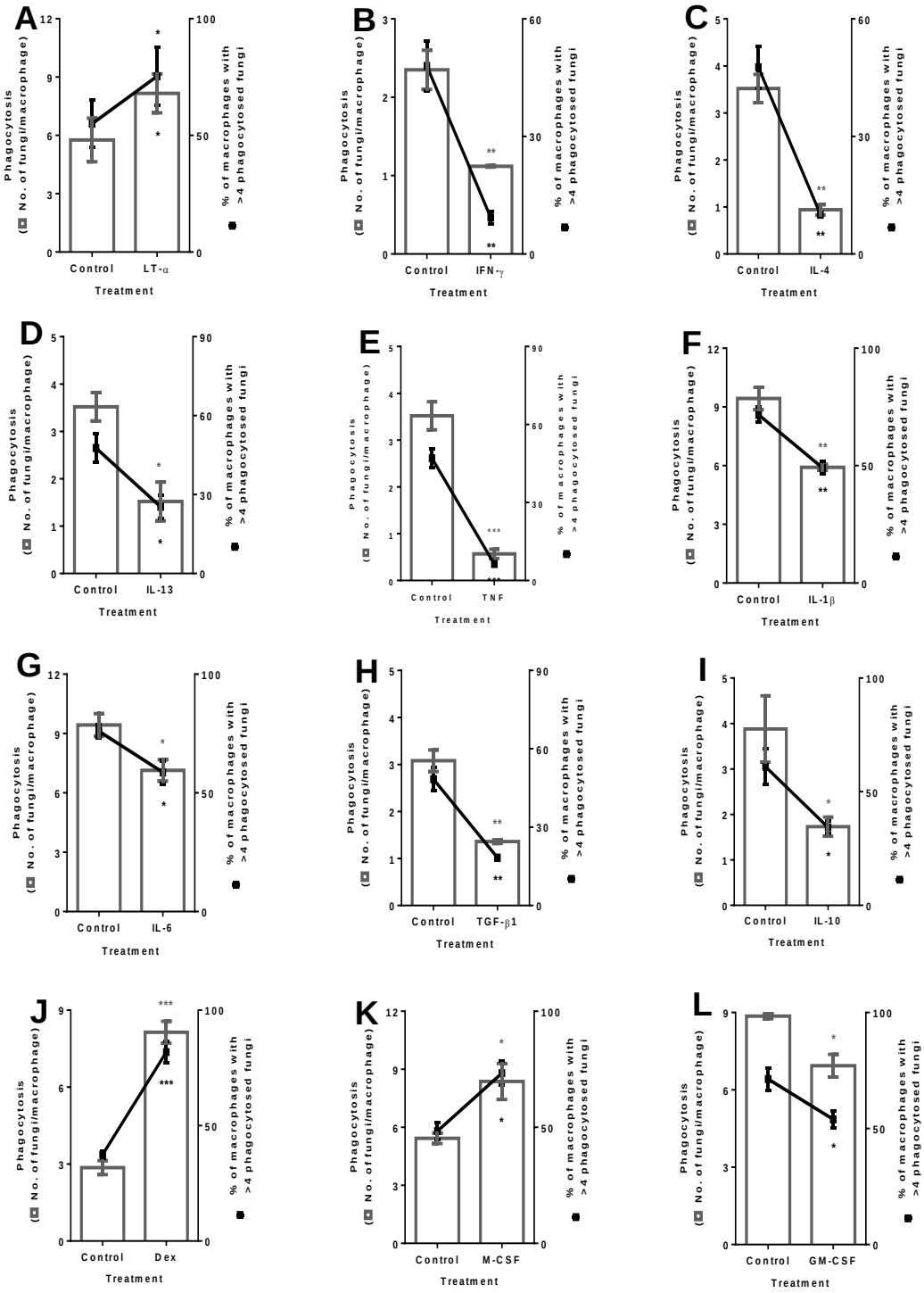

Figure 3.7: Effects of CRIg expression on human MDM phagocytosis (see Figure 2.2 for protocol). In these studies MDM were prepared by culturing human monocytes for 5 days (for phagocytosis). MDM were treated with LT-α **(A)** (40 ng/ml) or IFN-γ **(B)** (40 ng/ml) or IL-4 **(C)** (40 ng/ml) or IL-13 **(D)** (40 ng/ml) or TNF **(E)** (20 ng/ml) or IL-1β **(F)** (40 ng/ml) or IL-6 **(G) (**40 ng/ml) or TGF-β1 **(H)** (15 ng/ml) or **(I)** IL-10 (40 ng/ml) or dexamethasone **(J)** (50 ng/ml) or M-CSF **(K)** (40 ng/ml) or GM-CSF **(L)** (40 ng/ml) for 24 h and examined for their ability to phagocytose complement opsonised *C. albicans*. Phagocytosis was scored as both the number of macrophages that had engulfed more than >4 fungi (line graph) and the number of fungi engulfed per cell (bar graph). Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ***p<0.001.

3.3.4 Effect of cytokines on CR3/CD11b and CR4/CD11c expression during the development of human monocytes into macrophages and in human MDM

To gain a more effective understanding of the consequences of cytokine-induced modulation of CRIg expression (studied in previous sections) it is important to assess these changes in comparison to the induction of other functional receptors. Particularly important in this context is the expression of the classical complement receptors, CR3 and CR4, which also promote the phagocytosis of iC3b-opsonized particles (Myones et al., 1988, Barreiro et al., 2007, Dupuy and Caron, 2008, Gorgani et al., 2008). However CRIg and CR3/CR4 differ in that while the former has anti-inflammatory and immunosuppressive activity, the latter promotes inflammation (Helmy et al., 2006, Vogt et al., 2006, Gorgani et al., 2011). Thus the relative expression of these receptors may be a critical determinant of the severity of the inflammatory reaction. It was therefore considered important to understand whether CR3 and CR4 were also regulated by these cytokines and the type of changes cytokines induced.

The effects of the same cytokines outlined in the previous sections on the expression of CR3/CD11b and CR4/CD11c were examined during the human monocytes developing into macrophages and on human MDM.

Cytokines caused changes in CR3 and CR4 expression during human monocytes developed into macrophages (Figure 3.8). While TNF, IL-6, IL-10, M-CSF and GM-CSF caused a decrease in expression of CD11b and CD11c mRNA (Figure 3.8 E, G, I, K, L). The other cytokines induced very little effect or increase in these receptors. Dexamethasone caused no effect in expression of CD11b and CD11c mRNA (Figure 3.8 J).

To examine the direct effects of cytokines on human MDM, the MDM (3 day) were treated with cytokines for 24hrs and the expression of CD11b and CD11c mRNA

were examined. Several of the cytokines had very little effect or decreased expression of these receptors (Figure 3.9 A-C, F-H, K, L) on MDM. However IL-13, TNF, IL-10 and dexamethasone caused an increase in CR3 and CR4 expression (Figure 3.9 D, E, I, J).

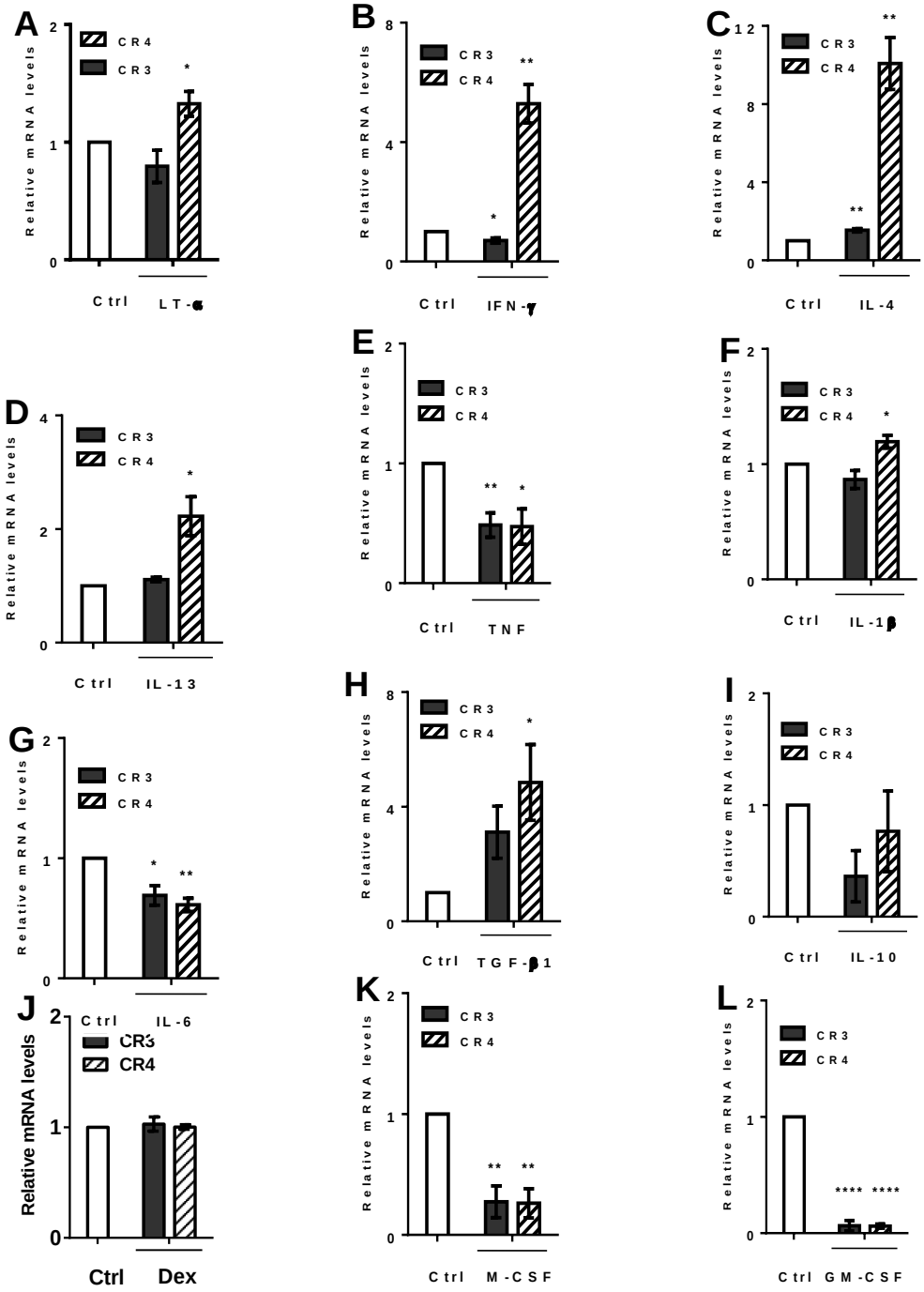

Figure 3.8: The effect of cytokines on the expression of CR3/CD11b and CR4/CD11c during the development of human monocytes into macrophages (see Figure 2.3 for protocol). Monocytes were treated with LT-α **(A)** (40 ng/ml) or IFN-γ **(B)** (40 ng/ml) or IL-4 **(C)** (40 ng/ml) or IL-13 **(D)** (40 ng/ml) or TNF **(E)** (20 ng/ml) or IL-1β **(F)** (40 ng/ml) or IL-6 **(G)** (40 ng/ml) or TGF-β1 **(H)** (15 ng/ml) or **(I)** IL-10 (40 ng/ml) or dexamethasone **(J)** (50 ng/ml) or M-CSF **(K)** (40 ng/ml) or GM-CSF **(L)** (40 ng/ml). The level of CD11b and CD11c mRNA was measured on day 3 using qRT-PCR. Data are expressed as fold-change over GAPDH-normalized CD11b and CD11c mRNA. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ****p<0.0001.

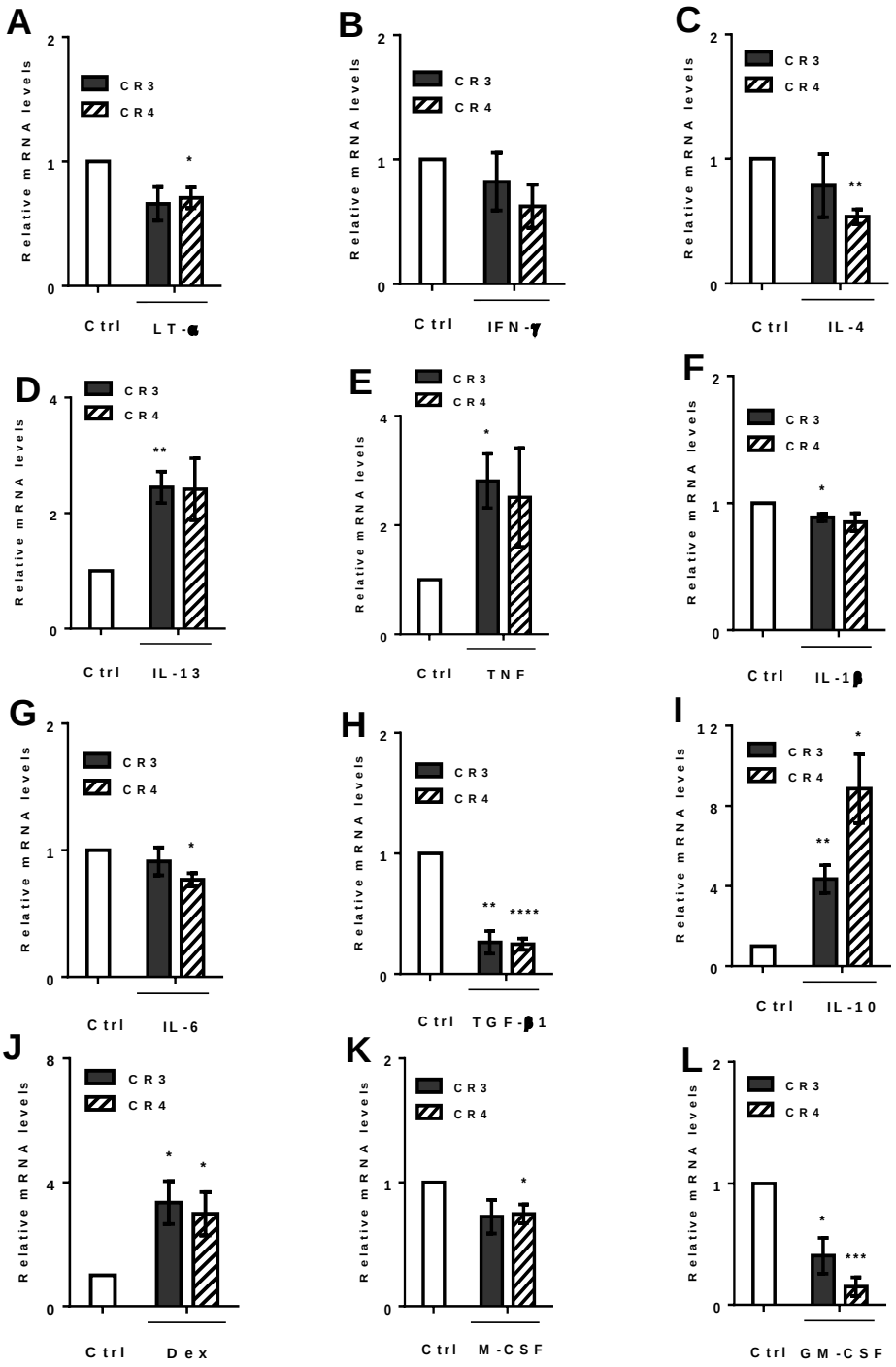

Figure 3.9: The effect of cytokines on CR3/CD11b and CR4/CD11c expression in human MDM (see Figure 2.3 for protocol). Monocytes were cultured for 3 days to develop into human MDM. Human MDM were then incubated for 24 h with LT-α **(A)** (40 ng/ml) or IFN-γ **(B)** (40 ng/ml) or IL-4 **(C)** (40 ng/ml) or IL-13 **(D)** (40 ng/ml) or TNF **(E)** (20 ng/ml) or IL-1β **(F)** (40 ng/ml) or IL-6 **(G)** (40 ng/ml) or TGF-β1 **(H)** (15 ng/ml) or **(I)** IL-10 (40 ng/ml) or dexamethasone **(J)** (50 ng/ml) or M-CSF **(K)** (40 ng/ml) or GM-CSF **(L)** (40 ng/ml). The level of CD11b and CD11c mRNA was measured using qRT-PCR. Data are expressed as fold-change over GAPDH-normalized CD11b and CD11c mRNA. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4 Summary

This work demonstrates that cytokines regulate the development of $CRIg^{+}$ macrophages from monocytes, supporting and extending the previous observations from our laboratory (Gorgani et al., 2011). This work suggests that CRIg expression may be a control point in infection and immunity, through which cytokines control macrophage function. The cytokines tested could be divided into those which caused an increase in CRIg expression, LT-α, IL-1β, IL-6, IL-10, GM-CSF, M-CSF and those which caused a decrease, IFN-γ, TNF, TGF-β1, IL-4 and IL-13. This work identifies for the first time the cytokine patterns which regulate CRIg expression on macrophages but also reveals new and unexpected properties for some of these cytokines, which may have implications in understanding the ability of the immune system to resist microbial pathogens. Surprisingly both the Th1 (IFN-γ) and Th2 (IL-4, IL13) cytokines decreased expression of CRIg. In contrast LT-α, caused an increase in the expression of this receptor. The findings imply that the role of these cytokine patterns in infection and immunity and inflammation may need re-evaluation.

The immuno-suppressive cytokine IL-10 caused a substantial increase in CRIg expression. Interestingly, this cytokine was as effective as the anti-inflammatory agent, dexamethasone in increasing the levels of CRIg in human macrophages. Another regulatory cytokine, TGF-β1 shares properties with IL-10, profoundly decreasing CRIg expression in macrophages. While TNF, IL-1β and IL-6 share many biological activities, the effects on macrophage CRIg expression differed. TNF caused a decrease and IL-1β and IL-6 increased expression.

CRIg expression is further compromised by TNF as the cytokine also acts on mature macrophages compared to IL-1β and IL-6 which had no effects on the mature macrophages (Dorn and Force, 2005). Since TNF has been shown to be a therapeutic

target for rheumatoid arthritis (Schett et al., 2013) and CRIg is protective in this disease (Katschke et al., 2007, Tanaka et al., 2008), the finding that the cytokine inhibits the development of $CRIg^+$ macrophages and induces a decrease in the expression on mature macrophages (Dorn and Force, 2005) suggests that the cytokine may be causing a significant part of its effects through the modulation of CRIg expression. Later in Chapter 5 it is reported that TNF causes these effects via activation of PKCα and that macrophages treated with anti-TNF antibody showed increased expression of CRIg (Dorn and Force, 2005).

GM-CSF and M-CSF regulate monocytes-macrophage differentiation and function. In this work macrophages developing under the influence of these growth factors show increased expression of CRIg. Thus the effects of GM-CSF and M-CSF on macrophage function may be precipitated by the ability to alter CRIg expression. The findings suggest that an important role for CSF in defence against infection may occur via increasing the phagocytosis of bacteria and may be of relevance to their application in therapy.

To appreciate the control point of inflammation via macrophages which are either fixed in tissues or that infiltrate sites of inflammation, the action of cytokines on the differentiated monocyte-derived macrophage (MDM) was examined. Interestingly most cytokines caused a decrease in CRIg expression on MDM, except for LT-α and M-CSF which caused an increase (Table 3.1).

There is little known about the effects of cytokines on the expression of CR3 and CR4 on macrophages. It is important to consider the changes in CRIg expression caused by cytokines in relation to changes in CR3 and CR4 expression caused by cytokines since inflammation may be a function of the relative expression of these receptors. Cytokines were found to alter both CR3 and CR4 expression in macrophages, both during monocyte development into a MDM and directly on the MDM. It was evident

that several of the cytokines had different effects on CRIg versus CR3/CR4 expression. Furthermore the cytokines were found to also cause different effects on CR3 compared to CR4 expression.

It is evident from the results that while cytokines regulate the development of monocytes to macrophages, these mediators were able to alter expression of these complement receptors by acting directly on the human MDM. This then becomes the second major control point in the inflammatory reaction, whereby cytokines can regulate inflammation by altering CRIg expression on a human MDM (Zang and Allison, 2006).

The most striking cytokines which can be highlighted in terms of their down regulation of the inflammatory reaction in terms of the concept of alteration in CRIg versus CR3/CR4 were LT-α and M-CSF which upregulated CRIg and down regulated CR3/CR4 expression. Further studies to elucidate their role via effects on these receptors may yield interesting results.

In addition, Chapter 3 demonstrates that the changes in CRIg protein caused by the cytokines are also observed at the CRIg mRNA level, suggesting that cytokines regulate CRIg at a pre-transcriptional level (see Chapter 5). It was also evident from our results that both the L and S spliced forms of CRIg were similarly regulated by the cytokines. More importantly changes in CRIg expression correlated with the changes in phagocytic activity of the macrophages.

Table 3.1: Effect of cytokines on the development of $CRIg^+/CR3^+/CR4^+$ macrophages and on MDM.

Cytokine	Development			MDM		
	CRIg	CR3	CR4	CRIg	CR3	CR4
LT-α	↑	↓	↑	↑	↓	**↓a/r**
IFN-γ	↓	↓	↑	↓	↓	↓
IL-4	↓	↑	**↑p**	↓	↓	↓
IL-13	↓	-	↑	↓	↑	**↑p**
IL-10	↑	↓	**↓a**	↓	↑	**↑p**
TGF-β1	↓	↑	**↑p**	↓	↓	↓
TNF	↓	↓	↓	↓	↑	**↑p**
IL-1β	↑	↓	↑	↓	-	-
IL-6	↑	↓	**↓a**	↓	-	↓
M-CSF	↑	↓	**↓a**	↑	↓	**↓a/r**
GM-CSF	↑	↓	**↓a**	↓	↓	↓
Dexamethasone	↑	-	-	↑	↑	**↑a**

a = anti-inflammatory; r = resolving; p = pro-inflammatory

The ↑ and ↓ arrows represent an increase and a decrease in receptor mRNA expression respectively. The – represent no change in mRNA levels.

CHAPTER FOUR

Expression of CRIg in Human Dendritic Cells is Modulated by Cytokines

4.1 Introduction

In Chapter 3 the effects of cytokines on the development of $CRIg^+$ macrophages and directly on human MDM, cells important in killing of microbial pathogens was examined. This has relevance primarily for innate immunity. However the importance of CRIg extends to adaptive immunity since it has been demonstrated that CRIg is expressed on DC.

DC play an essential role in the induction and regulation of immune responses due to their unique ability to stimulate naïve T cells and serve as a major link between innate and adaptive immunity (Banchereau et al., 2000, Steinman, 2008). In their immature state, DC harbour tolerogenic properties with an ability to induce regulatory T cells (T reg) and suppress naïve T cells activation (Steinman et al., 2003). During infection and inflammation, DC migrate to lymphoid organs and differentiates into mature cells to regulate immunity against pathogens, through the induction of effector cells such as Th1 and Th2 (Banchereau and Steinman, 1998, Lanzavecchia and Sallusto, 2001, Scott et al., 2011).

The complement receptors CR3/CD11b and CR4/CD11c are abundantly expressed on the surface of DC (Eberl et al., 2015). It has been reported that DC expressing CR3 interact with T cell surface bound iC3b and this leads to significantly enhanced T-cell proliferation (Schlitzer et al., 2015).

In contrast CRIg acts as a co-stimulatory molecule and negatively regulates adaptive immunity (Vogt et al., 2006). For example; human DC transfected with VSIG4 inhibit T cell proliferation and the production of pro-inflammatory cytokines (Xu et al., 2009). Thus modulation of CRIg expression in DC is likely to have an impact on immune responses. Therefore the aim of this research was to examine the effects of the cytokines, LT-α, IFN-γ, TNF, IL-1β, IL-6, IL-4, IL-13, TGF-β1, IL-10, M-CSF and

GM-CSF and also dexamethasone (since this has been shown to induce tolerogenic DC) on the expression of CRIg, CR3/CD11b and CR4/CD11c in DC.

4.2 Methods

The human DC were developed from monocytes cultured over a period of 5 days under the influence of GM-CSF (50 ng/ml) and IL-4 (20 ng/ml) (see section 2.3; 2.7 in Chapter 2). The DC were treated with either cytokines or dexamethasone at the indicated concentrations for 24 h. After 24 h incubation, cells were harvested and CRIg, CD11b and CD11c mRNA levels were measured by qRT-PCR (see section 2.8 in Chapter 2). Total CRIg protein was measured by Western blotting (see section 2.10 in Chapter 2) and cell-surface CRIg expression was examined by flow cytometry (see section 2.9 in Chapter 2).

4.3 Results

4.3.1 Development of CRIg$^+$ dendritic cells from human monocytes

The DC expressed CRIg mRNA (Figure 4.1 A) and CRIg protein on their surface (Figure 4.1 B, C). The DC showed several fold more CRIg expression than the corresponding MDM developed without addition of GM-CSF and IL-4.

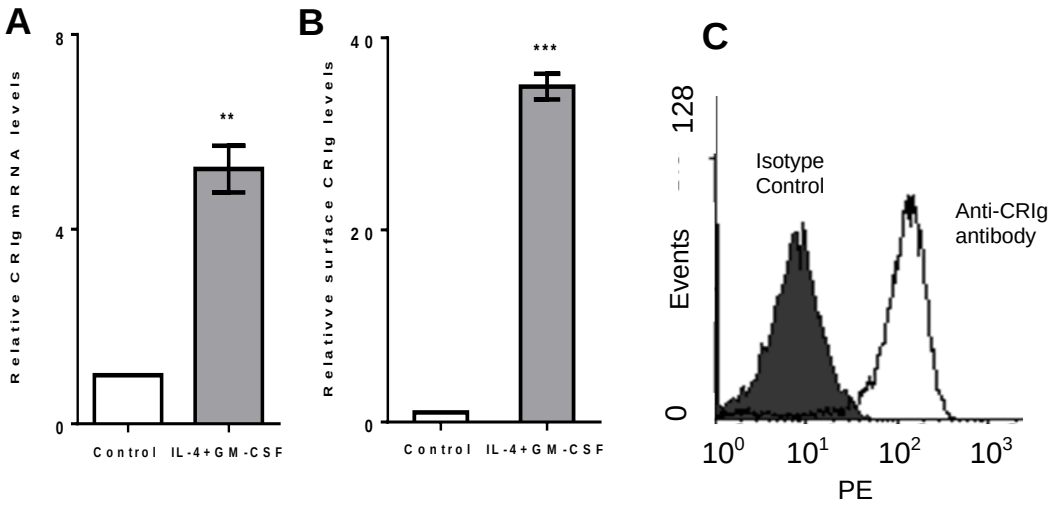

Figure 4.1: CRIg expression on DC (see Figure 2.4 for protocol). DC were generated by culturing monocytes at 37ºC in a 5% CO_2 humidified atmosphere for 5 days in the presence of GM-CSF (50 ng/ml) and IL-4 (20 ng/ml).
(**A)** CRIg mRNA measured by qRT-PCR relative to housekeeping gene GAPDH. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. (**B)** CRIg cell-surface expression on the DC was analysed using flow cytometry, after staining with anti-CRIg antibody (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control. **(C)** A representative experimental run displaying CRIg expression on DC for the data in B. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, **p<0.01, ***p<0.001.

4.3.2 Dexamethasone alters CRIg expression in DC

Previous work in our laboratory demonstrated that dexamethasone increased CRIg expression in MDM. In addition, it has been reported that DC develop under the influence of dexamethasone to have a tolerogenic phenotype (Spiering et al., 2014). It was therefore investigated whether dexamethasone altered the expression of CRIg on DC. The human DC were treated with dexamethasone at a concentration of 50 ng/ml for 24 h. Dexamethasone caused an increase (2-folds) in CRIg mRNA levels (Figure 4.2 A). This was reflected in an increase in CRIg protein measured by Western blotting (Figure 4.2 B, C). Western blotting showed the presence of the two isoforms of CRIg in DC as previously described in MDM and in Chapter 3 (Helmy et al., 2006).

Interestingly, the anti-CRIg antibody detected an additional protein migrating between the L (50 kDa) and S (45 kDa) forms. Details of these results will be presented at the end of this chapter. Examination of the Western blots also revealed that dexamethasone caused an increase in the levels of both the L and S forms of CRIg on DC (Figure 4.2 C). The changes induced by dexamethasone were also evident in expression of CRIg on the surface of DC which has implications for the function of DC as antigen presenting cells.

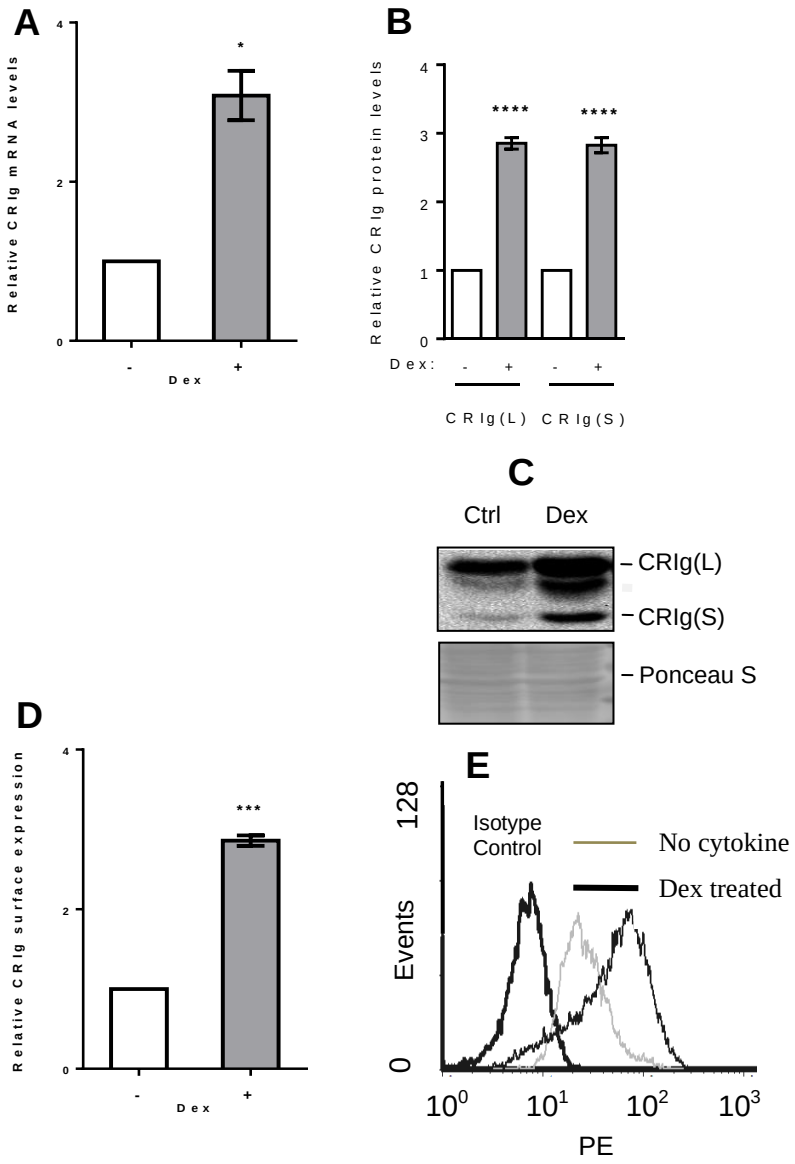

Figure 4.2: Dexamethasone increases CRIg expression in DC (see Figure 2.4 for protocol). DC were incubated in the presence or absence of 50 ng/ml dexamethasone over a period of 24 h. **(A)** CRIg mRNA expression relative to housekeeping gene GAPDH was examined using quantitative RT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Dexamethasone treated and untreated cells were examined by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** CRIg cell-surface expression were analysed by flow cytometry using an anti-CRIg monoclonal antibody (Z39Ig, 6H8-PE). Cells treated for 24 h with dexamethasone were stained for CRIg. Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, ***p<0.001, ****p<0.0001.

4.3.3 Effect of cytokines on CRIg$^+$ expression in DC

When CRIg$^+$ DC were treated with 40 ng/ml of LT-α for 24 h the cells showed a decrease in CRIg mRNA, although this did not reach significance (Figure 4.3 A). LT-α caused a significant decrease in total CRIg protein measured by Western blotting (Figure 4.3 B, C). Examination of effects on cell surface CRIg showed that LT-α decreased expression (Figure 4.3 D, E), both for the L and S forms (Figure 4.3 C).

When DC were treated with 40 ng/ml of IFN-γ, there was a similar decrease in both CRIg mRNA and total CRIg protein (Figure 4.4 A, B, C). Furthermore when cell-surface CRIg expression was examined by flow cytometry, IFN-γ caused a significant drop in CRIg expression (Figure 4.4 D, E). By Western blotting analysis, both the L and S forms were reduced by IFN-γ treatment (Figure 4.4 C).

The cytokines TNF, IL-1β and IL-6 are pyrogenic cytokines which share several properties (Netea et al., 2000, Dinarello, 2007) including stimulation of macrophages (Hanson and Murphy, 1984, Dinarello et al., 1986, Nijsten et al., 1987, Saper and Breder, 1994, Dinarello et al., 1999). It was therefore of interest to examine this group of cytokines on CRIg expression in DC. DC treated with 20 ng/ml of TNF for 24 h showed a decrease in CRIg mRNA expression (Figure 4.5 A), and a significant decrease in total CRIg protein assessed by Western blot (Figure 4.5 B, C). Both forms of CRIg were affected. TNF caused a significant reduction in expression of cell-surface CRIg (Figure 4.5 D, E). The effects of IL-1β and IL-6 were comparable to those of TNF, showing a decrease in CRIg mRNA, total CRIg protein and cell surface CRIg expression (Figure 4.6, 4.7). Both the L and S forms were down regulated by the cytokines (Figure 4.5 C; Figure 4.6 C; Figure 4.7 C).

The Th2 cytokines IL-4 and IL-13 provide another regulatory pathway for DC. Treating DC with 40 ng/ml IL-4 for 24 h, caused a decrease in the level of CRIg mRNA

(Figure 4.8 A). This reflected in a decrease in the level of total CRIg protein expressed by the DC treated with IL-4 (Figure 4.8 B, C) as well as in cell-surface CRIg expression (Figure 4.8 D, E). DC treated with 40 ng/ml of IL-13 displayed decreased expression of CRIg mRNA and protein (Figure 4.9 A - E). IL-13 also caused a decrease in cell surface CRIg expression (Figure 4.9 D, E). Examination of Western blots revealed that IL-4 and IL-13 caused a decrease in expression of both the L and S forms of CRIg (Figure 4.8 C and 4.9 C).

TGF-β1 regulates inflammation and IL-10 has immunosuppressive activity (de Waal Malefyt et al., 1991, Fiorentino et al., 1991a, Fiorentino et al., 1991b, Asadullah et al., 2003). Their action could in part be through the regulation of CRIg on DC. Cells treated with 15 ng/ml of TGF-β1 showed an increase in CRIg mRNA and total CRIg protein by Western blot, although this effect did not reach significance at mRNA level (Figure 4.10 A - C). The cell-surface expression of CRIg on DC was significantly increased by treating them with TGF-β1 (Figure 4.10 D, E).

DC treated with 40 ng/ml of IL-10 showed a significant increase in CRIg mRNA expression (Figure 4.11 A). Examination by Western blot of total CRIg protein showed a corresponding increase in CRIg protein expression (Figure 4.11 B, C). The increase caused by IL-10 treatment was evident also by increased cell-surface CRIg expression (Figure 4.11 D, E). Examination of the Western blots showed that TGF-β1 and IL-10 caused an increase in the levels of the L and S forms of CRIg on DC (Figure 4.10 C and 4.11 C).

Both M-CSF and GM-CSF have been reported to alter macrophage function. These results show that both cytokines regulate CRIg expression in human macrophages. Examination of the effects of M-CSF and GM-CSF on the expression of CRIg in DC showed that cells treated with 40 ng/ml of M-CSF display a marked increase in CRIg mRNA expression (Figure 4.12 A). This was confirmed when total

CRIg protein was assayed by Western blot. The cytokine caused several fold increase in the levels of CRIg protein expression (Figure 4.12 B, C). M-CSF treated DC also displayed an increase in cell-surface CRIg expression (Figure 4.12 D, E).

DC treated for 24 h with 40 ng/ml of GM-CSF showed an increase in CRIg mRNA, although this did not reach significance (Figure 4.13 A). When total CRIg protein expression was measured by Western blots the results showed that GM-CSF significantly increases CRIg expression (Figure 4.13 B, C). The surface expression of CRIg was also significantly increased by GM-CSF (Figure 4.13 D, E). Examination of the Western blots revealed that M-CSF and GM-CSF caused an increase in the levels of both the L and S forms of CRIg on DC (Figure 4.12 C and 4.13 C).

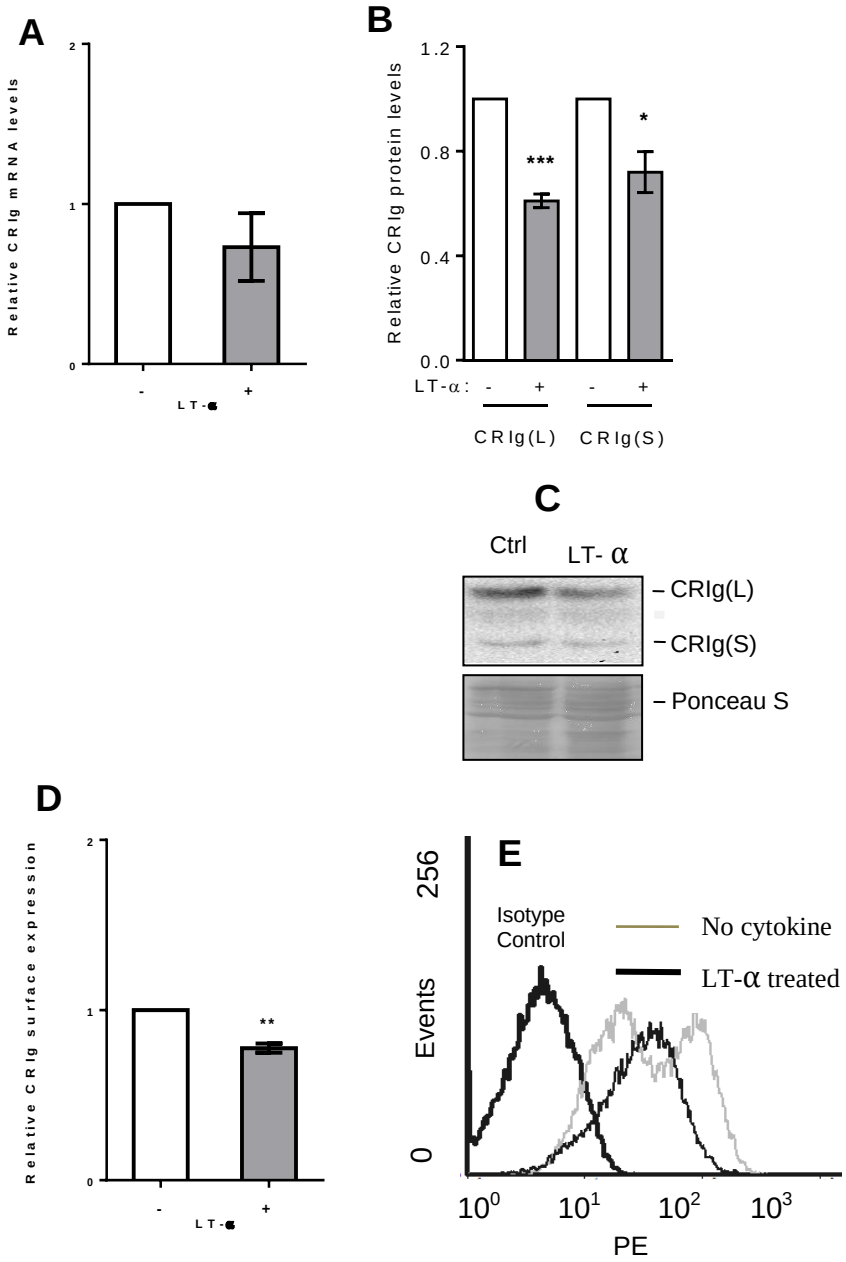

Figure 4.3: LT-α decreases CRIg expression in DC (see Figure 2.4 for protocol). (A) DC were treated with 40 ng/ml of LT-α and after 24 h the cells were examined for CRIg mRNA expression by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Western blot analysis for CRIg protein expression using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** CRIg expression on the surface of DC as determined by flow cytometry (Z39Ig, 6H8-PE). Cells were stained for CRIg or the IgG1 isotype control. Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ***p<0.001.

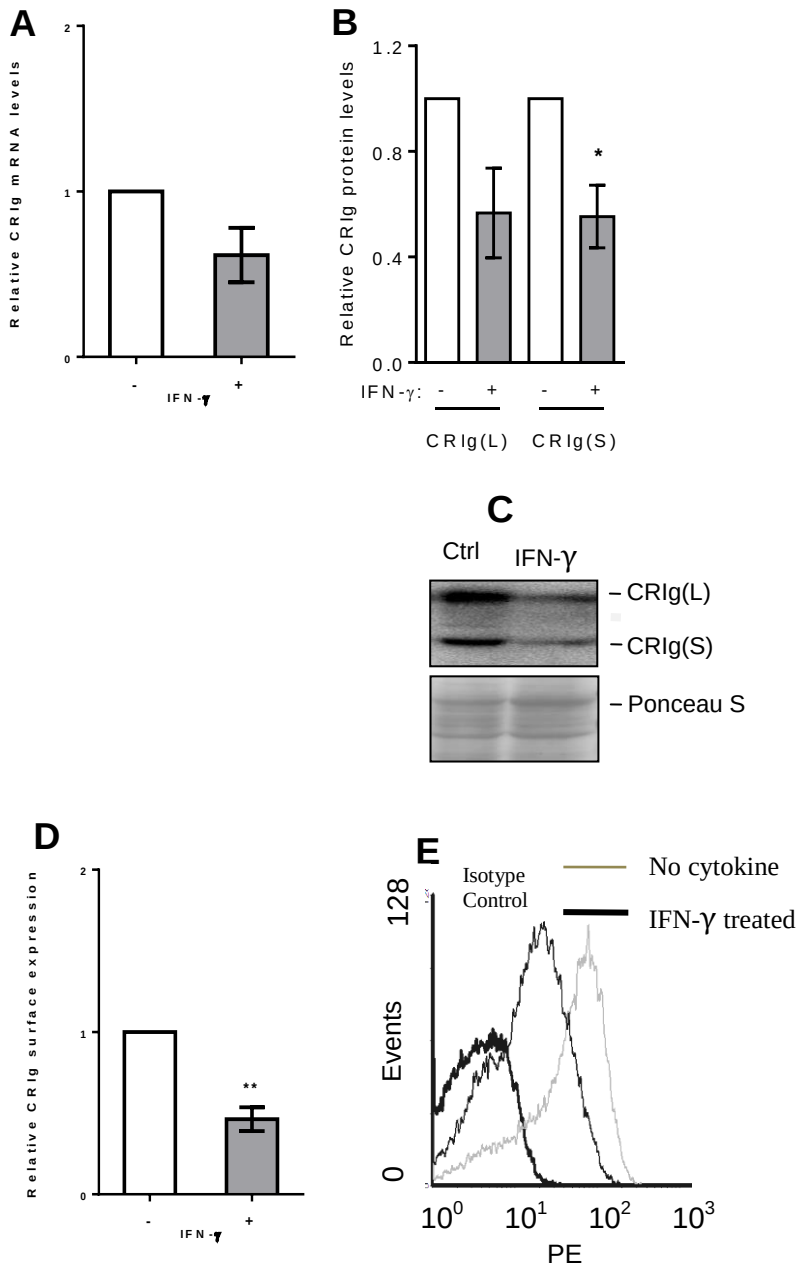

Figure 4.4: IFN-γ decreases CRIg expression in DC (see Figure 2.4 for protocol). DC were treated with 40 ng/ml of IFN-γ and after 24 h cells were examined for CRIg mRNA expression by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Western blot analysis for CRIg protein expression using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** CRIg expression on the surface of DC as determined by flow cytometry (Z39Ig, 6H8-PE). Cells were stained for CRIg or an isotype control. Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$, **$p<0.01$.

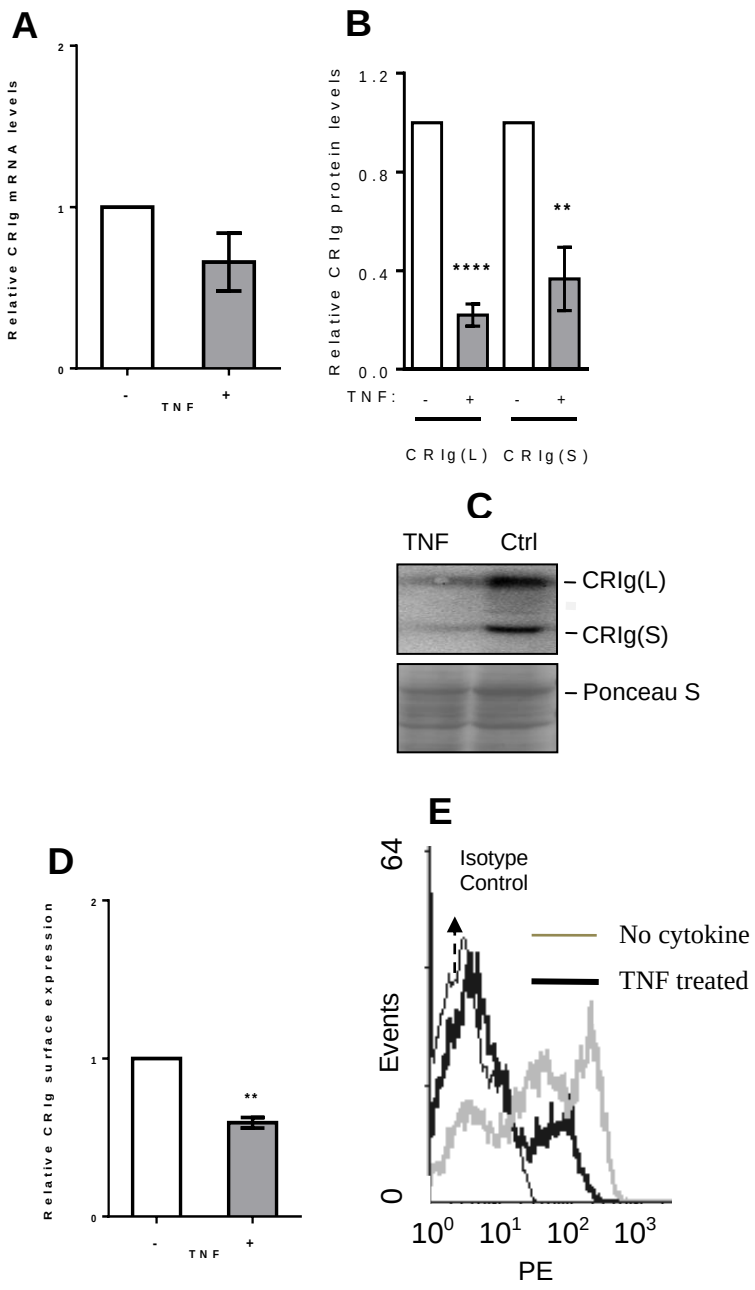

Figure 4.5: TNF down-regulated CRIg expression in DC (see Figure 2.4 for protocol). DC were incubated with 20 ng/ml of TNF for 24 h and then CRIg mRNA and protein measured. **(A)** CRIg mRNA expression was examined by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Western blot analysis for CRIg protein expression using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** Cell-surface CRIg expression measured by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, **p<0.01, ****p<0.0001.

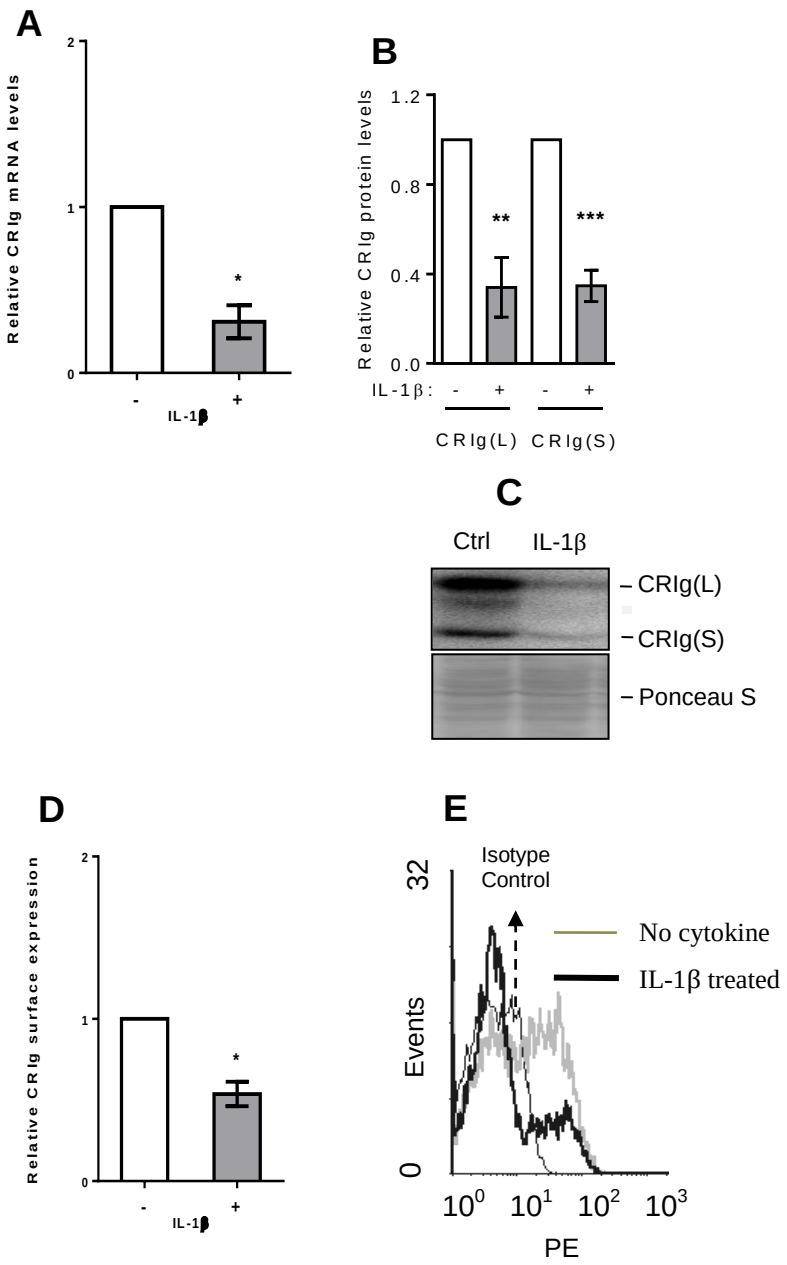

Figure 4.6: IL-1β down-regulated CRIg expression in DC (see Figure 2.4 for protocol). DC were incubated with 40 ng/ml of IL-1β for 24 h and then CRIg mRNA and protein measured. **(A)** CRIg mRNA expression was examined by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein expression by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** Cell-surface CRIg expression measured by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

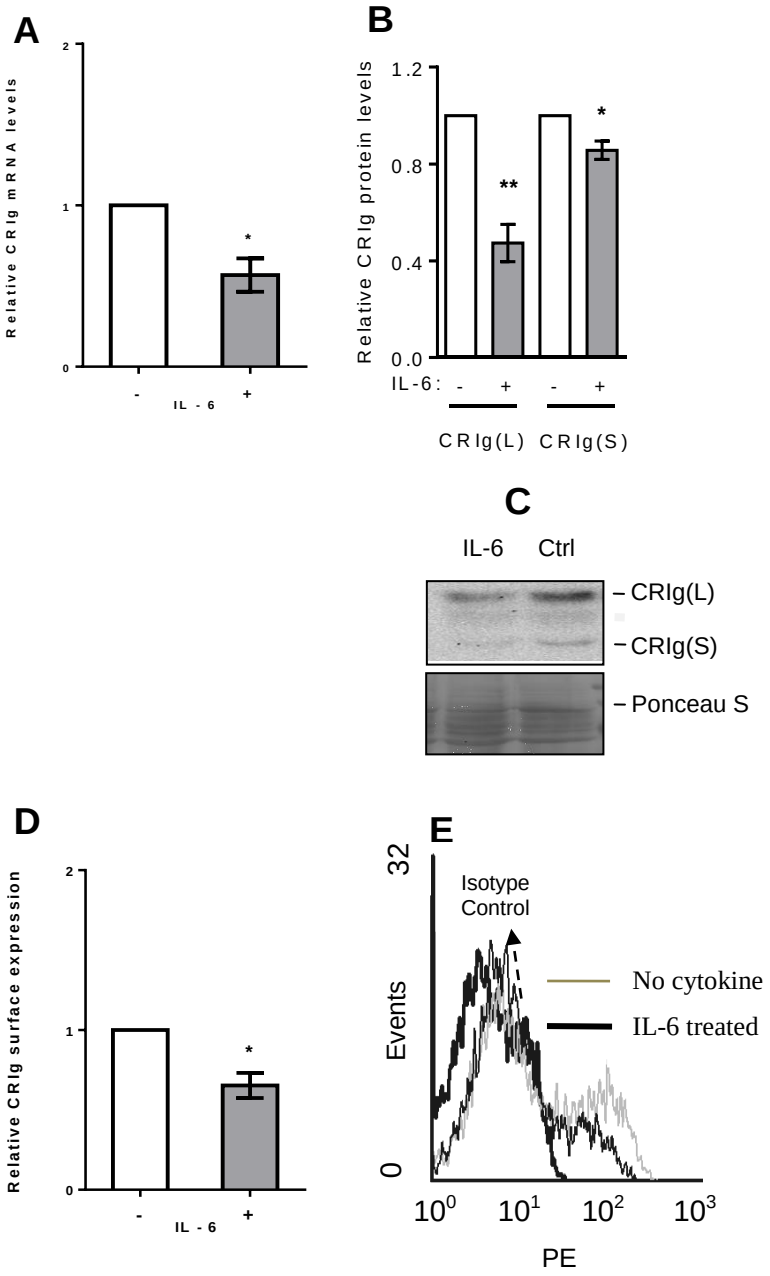

Figure 4.7: IL-6 down-regulated CRIg expression in DC (see Figure 2.4 for protocol). DC were incubated with 40 ng/ml of IL-6 for 24 h and then CRIg mRNA and protein measured. **(A)** CRIg mRNA expression was examined by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein expression by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** Cell-surface CRIg expression measured by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$, **$p<0.01$.

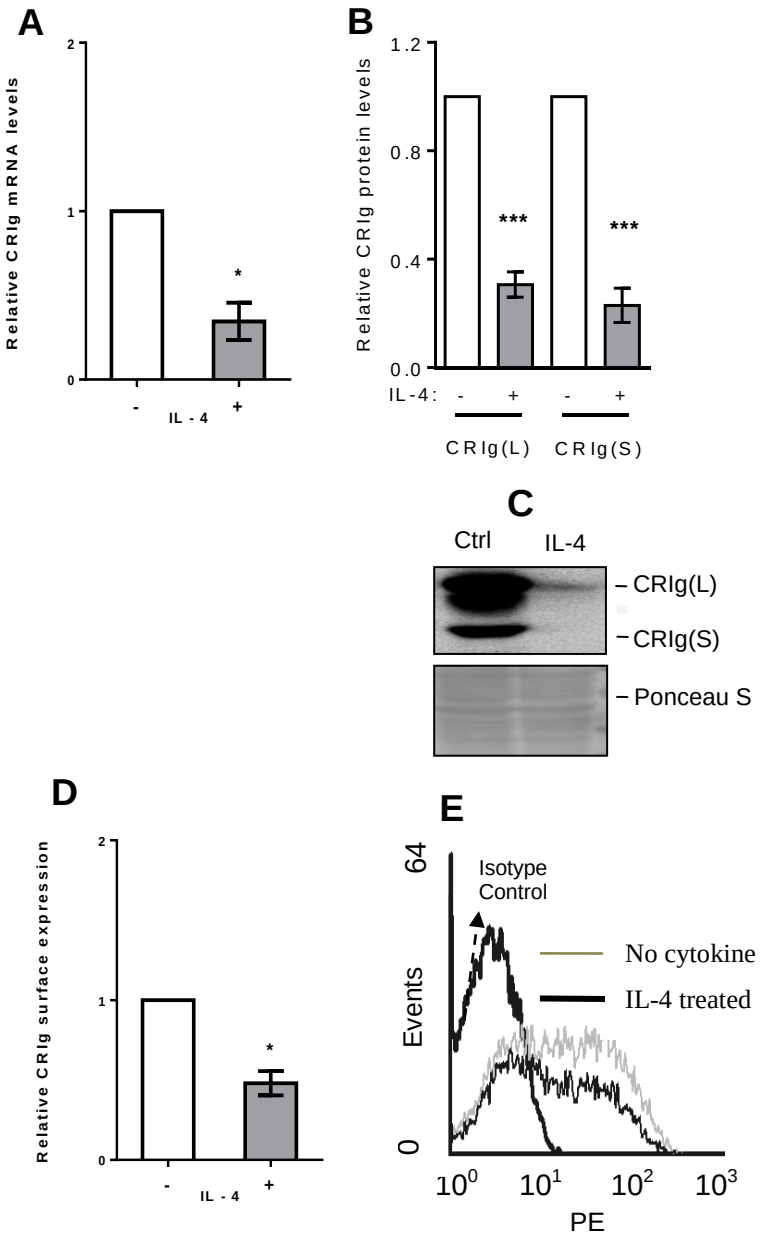

Figure 4.8: IL-4 down regulates CRIg expression in DC (see Figure 2.4 for protocol). (A) DC were treated with 40 ng/ml IL-4 for 24 h and then examined for CRIg mRNA expression by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** The levels of total CRIg protein were measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** DC were treated with IL-4 and analysed by flow cytometry for cell surface CRIg expression (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$, ***$p<0.001$.

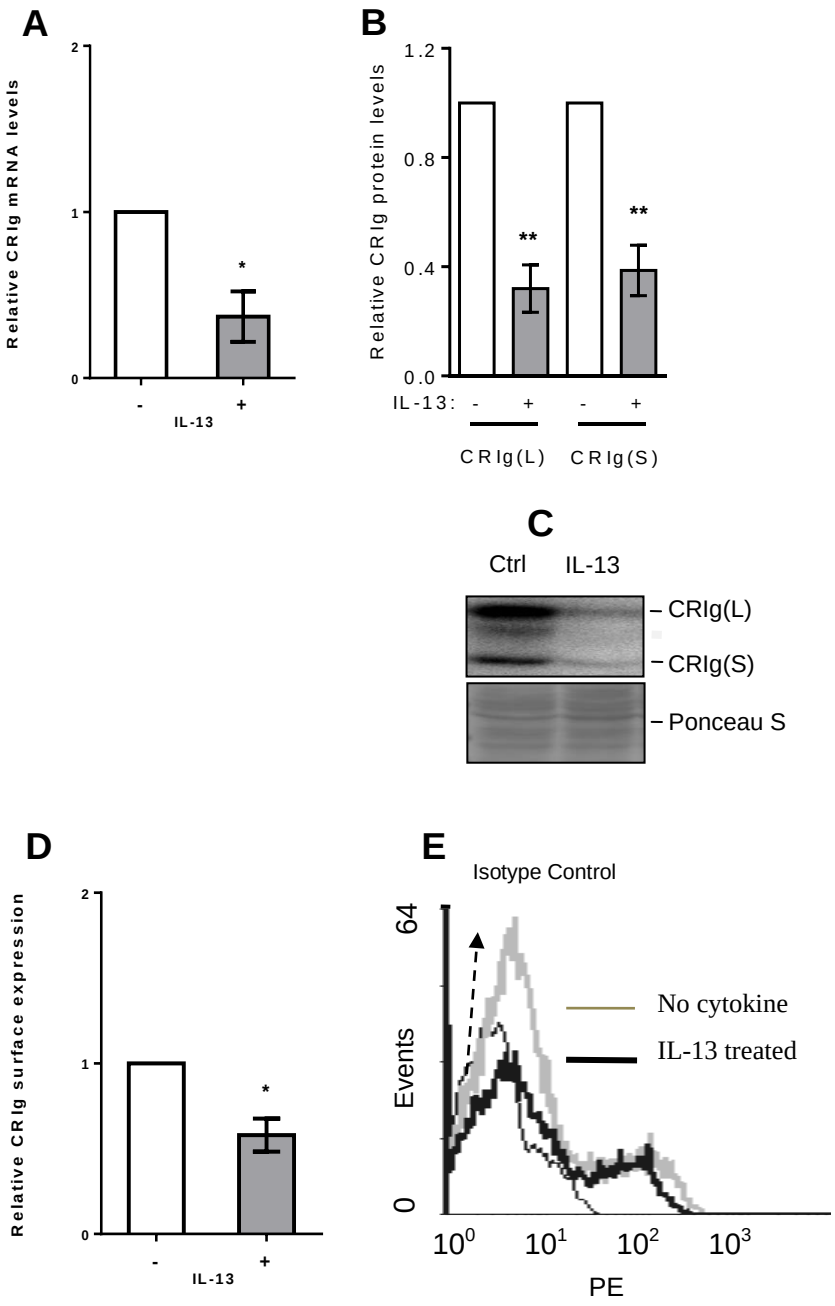

Figure 4.9: IL-13 down regulates CRIg expression in DC (see Figure 2.4 for protocol). (A) DC were treated with 40 ng/ml IL-13 for 24 h and then examined for CRIg mRNA expression by qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein levels were measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** DC were treated with IL-13 and surface CRIg expression was analysed by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$, **$p<0.01$.

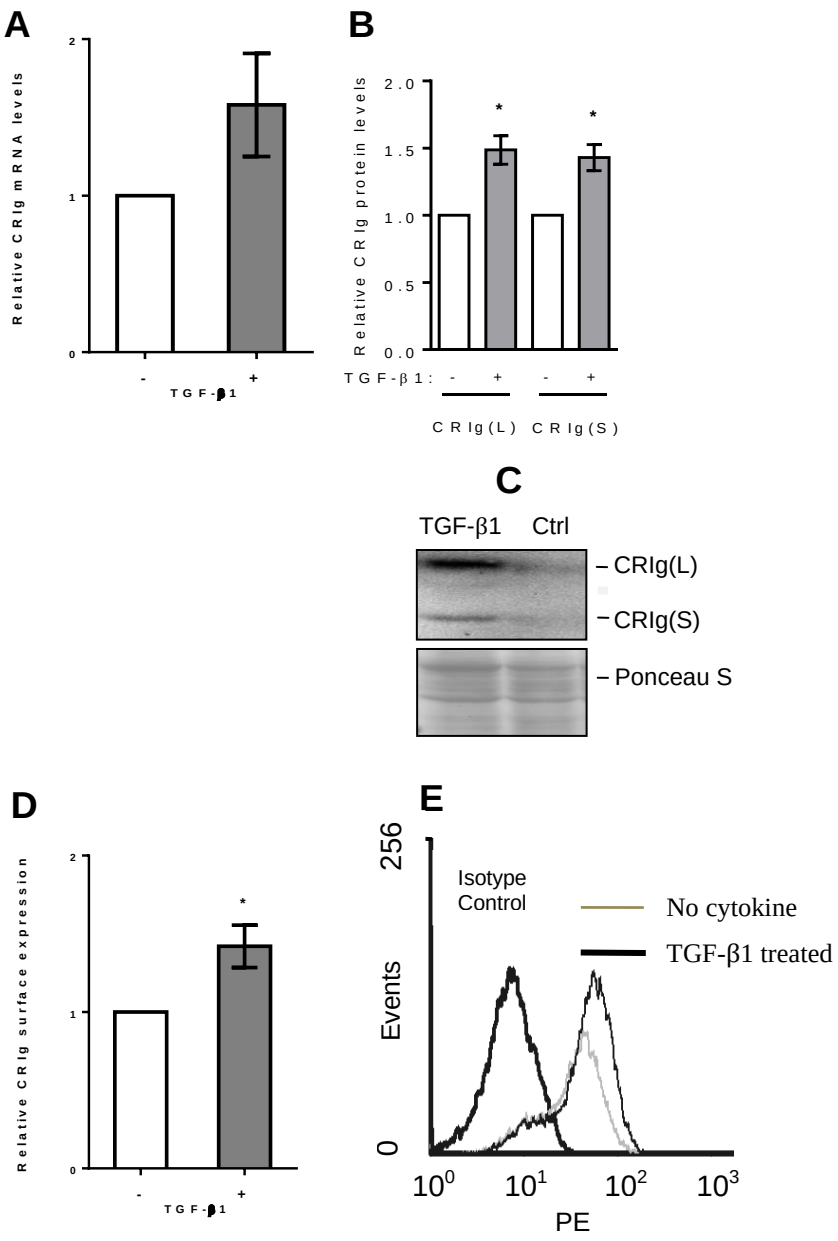

Figure 4.10: TGF-β1 up regulates CRIg expression in DC (see Figure 2.4 for protocol). (A) DC were treated with 15 ng/ml TGF-β1 for 24 h and then examined for CRIg mRNA expression using qRT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein levels were measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** DC were treated with TGF-β1 and analysed by flow cytometry for cell-surface CRIg staining (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$.

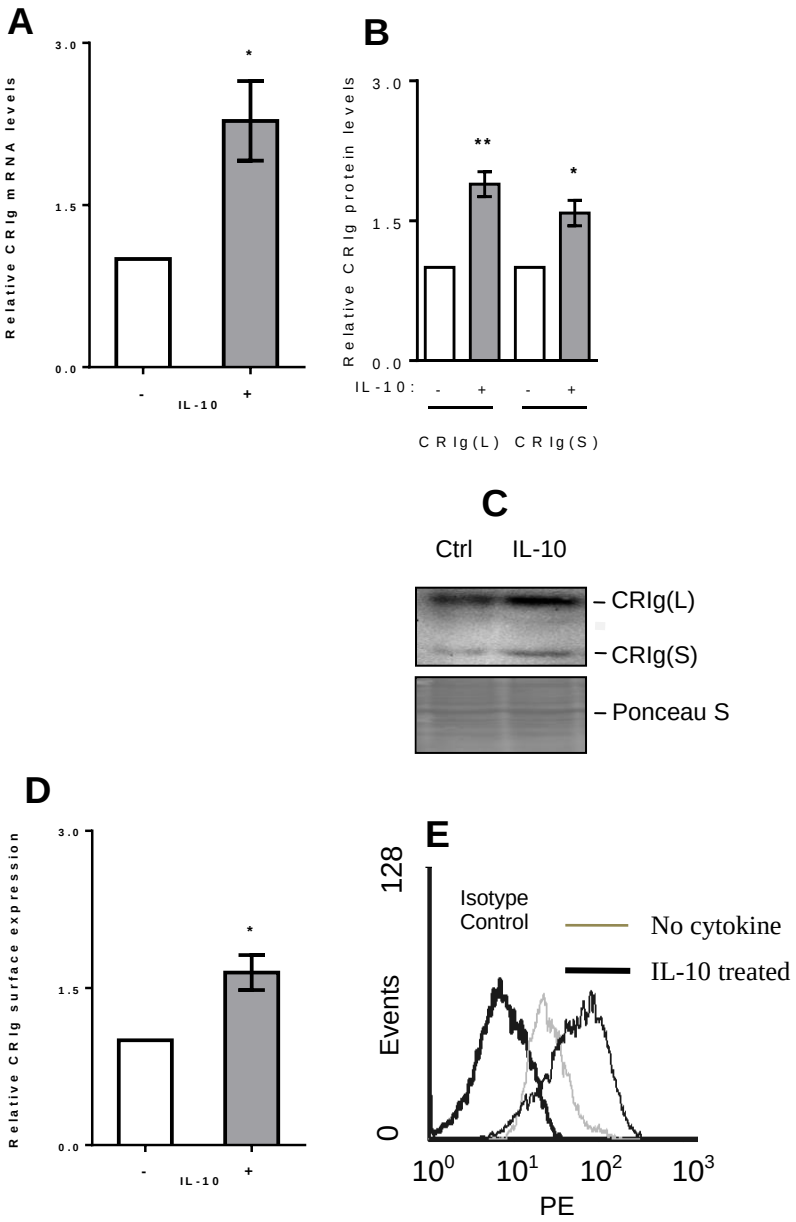

Figure 4.11: IL-10 increases CRIg expression in DC (see Figure 2.4 for protocol). DC were treated with 40 ng/ml of IL-10 for 24 h. **(A)** CRIg mRNA expression relative to housekeeping gene GAPDH was examined using quantitative RT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein was measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** Cell-surface CRIg expression were analysed by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *$p<0.05$, **$p<0.01$.

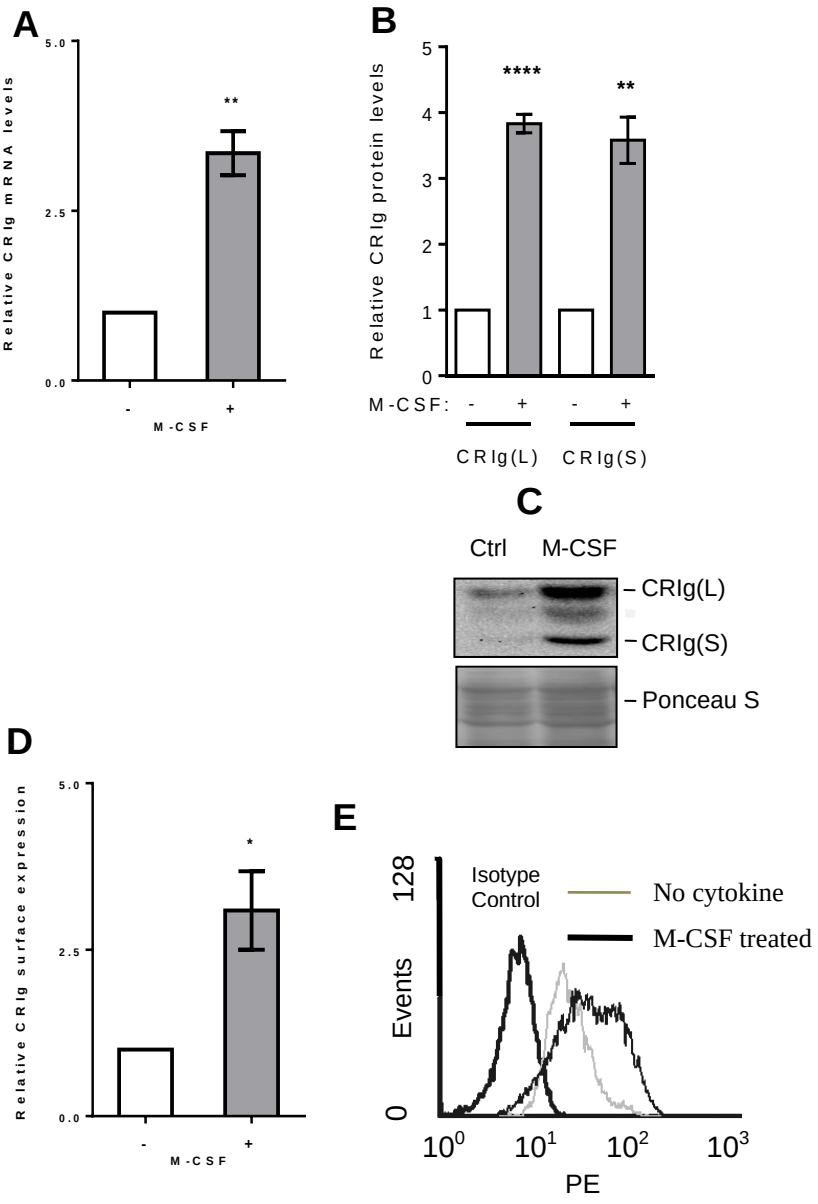

Figure 4.12: M-CSF increases expression of CRIg in DC (see Figure 2.4 for protocol). DC were treated with 40 ng/ml of M-CSF for 24 h. **(A)** CRIg mRNA expression relative to housekeeping gene GAPDH was examined using quantitative RT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein was measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** Cell-surface CRIg expression were analysed by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ****p<0.0001.

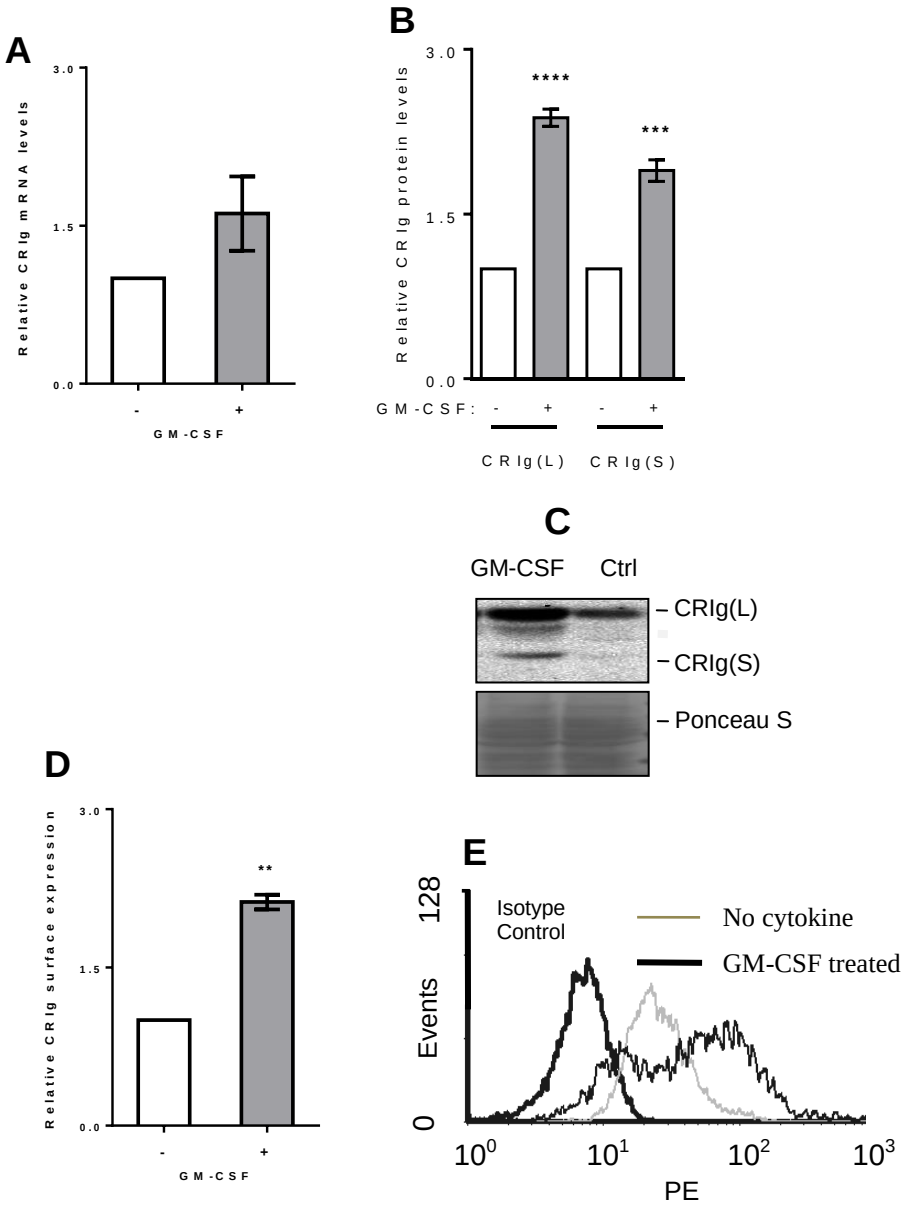

Figure 4.13: G**M-CSF up regulates expression of CRIg in DC (see Figure 2.4 for protocol).** DC were treated with 40 ng/ml of GM-CSF for 24 h. **(A)** CRIg mRNA expression relative to housekeeping gene GAPDH was examined using quantitative RT-PCR. Data are expressed as fold-change over GAPDH-normalised CRIg mRNA. **(B, C)** Total CRIg protein was measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. **(D, E)** Cell-surface CRIg expression were analysed by flow cytometry (Z39Ig, 6H8-PE). Data are expressed as fold-change in mean fluorescence intensity over isotype control (IgG1). E shows a representative experiment as a histogram. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, **p<0.01, ***p<0.001, ****p<0.0001.

4.3.4 Effect of cytokines on CR3/Cd11b and CR4/Cd11c expression in DC

To gain a better understanding of the consequences of the modulation of CRIg by cytokines it was important to examine whether these cytokines also caused changes to the classical complement receptors CR3, CR4 thus CD11b and CD11c mRNA levels were also measured in DC. The DC were treated for 24h with the cytokines and then CD11b and Cd11c mRNA measured by qRT-PCR.

The cytokines differentially regulated the expression of CR3 and CR4 complement receptors in DC (Figure 4.14). While LT-α increased the expression of CR3 and decreased CR4 (Figure 4.14 A), IFN-γ up regulated expression of CR4 (Figure 4.14 B). IL-4 induced a decrease in CR3 and CR4 expression but IL-13 caused a significant increase in both of these receptors (Figure 4.14 C, D).

The pro-inflammatory cytokines, in particular TNF and IL-1β caused a decrease in CD11b and CD11c mRNA expression in DC (Figure 4.14 E, F, G). TGF-β1 induced a decrease and IL-10 caused a marked decrease in CD11b and CD11c mRNA expression in DC (Figure 4.14 H, I). Since dexamethasone was found to be a strong regulator of CRIg expression, we examined its effects on CD11b and CD11c mRNA expression in DC. Treatment of DC with dexamethasone results in a significant decrease in CD11b and CD11c mRNA expression in DC (Figure 4.14 J). DC incubated with M-CSF showed decreased CD11b and CD11c mRNA expression whereas GM-CSF caused a dramatic increase in these receptors, in particular CD11c expression (Figure 4.14 K, L).

The finding that IL-10, TGF-β1 and dexamethasone increase DC CRIg expression but decrease CR3 and CR4 is of interest as these agents are all known to promote the development of tolerogenic DC. This contrasts with TNF and IFN-γ.

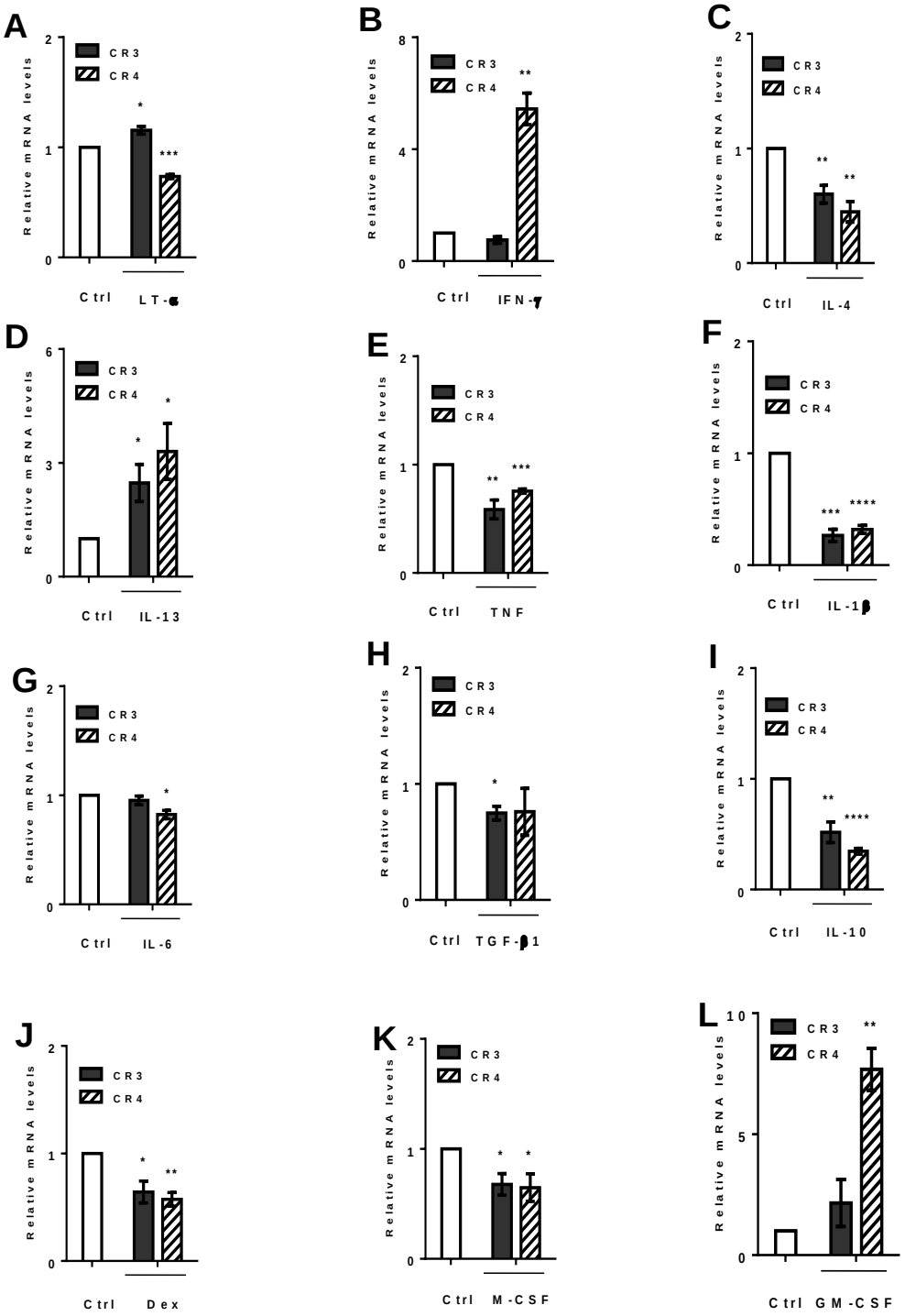

Figure 4.14: The effect of cytokines on CR3/CD11b and CR4/CD11c expression in DC (see Figure 2.5 for protocol). Monocytes were differentiated to DC by culturing in the presence of GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). After 5 days the cells were treated with LT-α **(A)** (40 ng/ml) or IFN-γ **(B)** (40 ng/ml) or IL-4 **(C)** (40 ng/ml) or IL-13 **(D)** (40 ng/ml) or TNF **(E)** (20 ng/ml) or IL-1β **(F)** (40 ng/ml) or IL-6 **(G)** (40 ng/ml) or TGF-β1 **(H)** (15 ng/ml) or **(I)** IL-10 (40 ng/ml) or dexamethasone **(J)** (50 ng/ml) or M-CSF **(K)** (40 ng/ml) or GM-CSF **(L)** (40 ng/ml) for 24 h and then the level of CD11b and CD11c mRNA were measured using qRT-PCR. Data are expressed as fold-change over GAPDH-normalized CD11b and CD11c mRNA. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

4.3.5 Expression of an additional form of CRIg in DC

Analysis of lysates on Western blot showed that MDM expressed the L and S forms. However in DC the same anti-CRIg monoclonal antibody revealed another protein which migrated between these two forms, tentatively referred to as intermediatory or I. The results presented in Figure 4.15 demonstrate that this form was similarly regulated by cytokines. LT-α, IFN-γ, IL-4, IL-13, TNF, IL-1β, IL-6 decreased expression and IL-10, TGF-β1, M-CSF, GM-CSF and dexamethasone increased expression of all three forms.

The finding of an additional form is not surprising since there have been 5 different spliced forms of CRIg reported (Table 4.1) (Langnaese et al., 2000, Helmy et al., 2006, Vogt et al., 2006, Guo et al., 2010, Tanaka et al., 2012).

Table 4.1: Different spliced forms of CRIg.

	CRIg variants
1	VSIG4 (uc031tjt.1) at chrX:65241580-65259967 – Homo sapiens V-set and immunoglobulin domain containing 4 (VSIG4), transcript variant 5, mRNA. This variant has 347 amino acids. (NM_001257403.1)
2	VSIG4 (uc011moy.2) at chrX:65241580-65259967 – Homo sapiens V-set and immunoglobulin domain containing 4 (VSIG4), transcript variant 3, mRNA. This variant has 227 amino acids. (NM_001184831.1)
3	VSIG4 (uc004dwj.3) at chrX:65241580-65259967 – Homo sapiens V-set and immunoglobulin domain containing 4 (VSIG4), transcript variant 4, mRNA. This variant has 321 amino acids. (NM_001184830.1)
4	VSIG4 (uc004dwi.2) at chrX:65241580-65259967 – Homo sapiens V-set and immunoglobulin domain containing 4 (VSIG4), transcript variant 2, mRNA. This variant has 305 amino acids. (NM_001100431.1)
5	VSIG4 (uc004dwh.2) at chrX:65241580-65259967 – Homo sapiens V-set and immunoglobulin domain containing 4 (VSIG4), transcript variant 1, mRNA. This variant has 399 amino acids. (NM_007268.2)

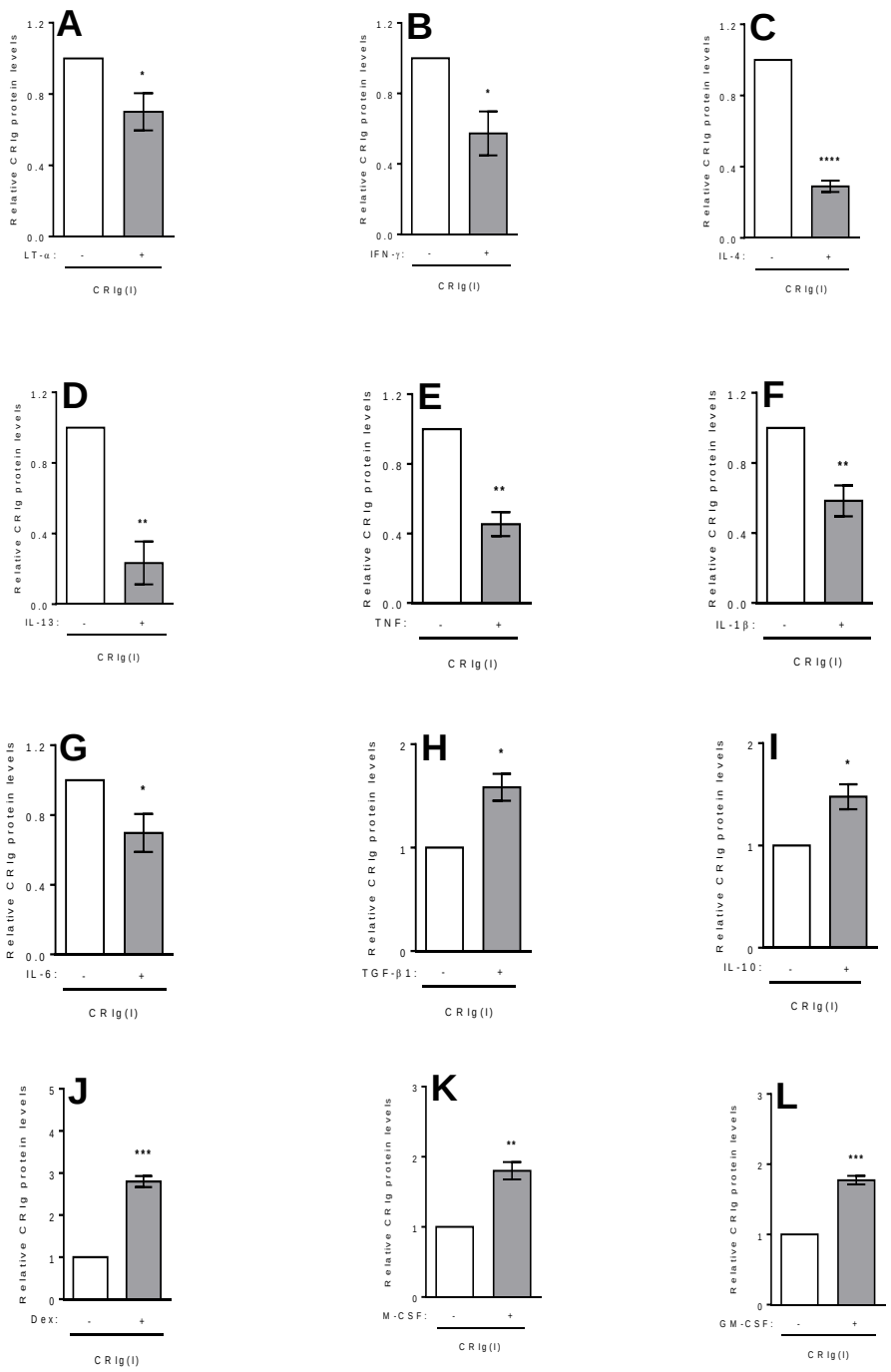

Figure 4.15: The effect of cytokines on CRIg expression (intermediate band I) in DC (see Figure 2.4 for protocol). Monocytes were differentiated to DC by culturing in the presence of GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). After 5 days the cells were treated with LT-α **(A)** (40 ng/ml) or IFN-γ **(B)** (40 ng/ml) or IL-4 **(C)** (40 ng/ml) or IL-13 **(D)** (40 ng/ml) or TNF **(E)** (20 ng/ml) or IL-1β **(F)** (40 ng/ml) or IL-6 **(G)** (40 ng/ml) or TGF-β1 **(H)** (15 ng/ml) or **(I)** IL-10 (40 ng/ml) or dexamethasone **(J)** (50 ng/ml) or M-CSF **(K)** (40 ng/ml) or GM-CSF **(L)** (40 ng/ml) for 24 h and then total CRIg protein was measured by Western blot using anti-CRIg monoclonal antibody (clone 3C9). A representative Western blot of total protein lysates is shown in figures (4.4 – 4.15) with Ponceau staining showing consistency of protein load. Data are expressed as fold-difference in CRIg band intensity as determined by densitometry. Data are presented as means ± SEM of three experiments, each conducted with cells from different individuals, *p<0.05, **p<0.01, ***p<0.001.

4.4 Summary

This work demonstrates that DC generated from monocytes treated using IL-4 and GM-CSF express CRIg mRNA and protein. Western blot analysis revealed that the L form of CRIg was most prominent in DC. Interestingly an additional band migrating with lower molecular weight to the L form was also observed. This is not surprising as other spliced variants of CRIg have been described (Guo et al., 2010, Tanaka et al., 2012).

CRIg expression in DC was found to be regulated by cytokines (Table 4.2). TGF-β1, IL-10, M-CSF and GM-CSF caused an up regulation of CRIg expression and LT-α, IFN-γ, IL-4, IL-13, TNF, IL-1β and IL-6 depressed expression (Table 4.2). The effects of cytokines were seen at CRIg mRNA and protein level as well changes to cell surface expression. The data indicates firstly that regulation by cytokines occurs at a pre-transcriptional level and secondly that these cytokines may regulate DC function via CRIg modulation.

The results also show that cytokines also regulated the expression of CR3 and CR4 on DC. As CR3 and possibly CR4 influence the adaptive immune response (Ben Nasr et al., 2006, Haniffa et al., 2015, Eberl et al., 2015), cytokines may influence the immune responsiveness of the DC in this manner. LT-α, IL-13 and GM-CSF up regulated the expression of CD11b mRNA and IFN-γ, IL-13 and GM-CSF increased expression of CD11c mRNA in DC. However the remaining cytokines down regulated this expression (Table 4.2). Thus the role of these cytokines in adaptive immunity needs to take into consideration their ability to also modulate CR3 and CR4 expression in DC, in the context of changes to CRIg expression.

The finding that IL-10 and TGF-β1 caused an increase in CRIg expression on DC is of interest and importance in adaptive immunity and immune responsiveness. Tolerogenic dendritic cells (tDC) can be generated by immunosuppressive cytokines

including IL-10, TGF-β (Geissmann et al., 1999, Steinbrink et al., 2002, Torres-Aguilar et al., 2010, Tai et al., 2011), and immunomodulatory drugs such as dexamethasone (Unger et al., 2009). Since tDC are being considered as a therapeutic strategy in autoimmune inflammatory diseases (Morelli and Thomson, 2007, Thomas, 2013), these findings might be helpful in developing tDC for this purpose. The regulatory and immuno-suppressive cytokine IL-10 caused a substantial increase in CRIg mRNA and corresponding CRIg protein in human DCs.

The results reveal for the first time the expression of the L and S forms of CRIg in human DC. In addition this work demonstrates that the anti-CRIg antibody detects an additionally sized protein, most likely relating to another spliced form of CRIg. At this stage the significance of the expression of these different forms is not known. Cytokines regulated all of these forms in a similar manner. Since CRIg expression relates to changes in immune responses these cytokines may at least in part be acting by altering CRIg expression on DC. It is interesting that these agents, cytokines which induce tDC, IL-10, TGF-β1 and dexamethasone not only increased CRIg expression on DC but depressed CR3 and CR4 expression (Table 4.2).

Table 4.2: Effect of cytokines on CRIg, CR3 and CR4 expression in DC.

Cytokine	CR expression		
	CRIg	**CR3**	**CR4**
LT-α	↓	↑	↓
IFN-γ	↓	↓	↑
IL-4	↓	↓	↓
IL-13	↓	↑	↑**i**
IL-10	↑	↓	↓**t**
TGF-β1	↑	↓	↓**t**
TNF	↓	↓	↓
IL-1β	↓	↓	↓
IL-6	↓	-	↓
M-CSF	↑	↓	↓**t**
GM-CSF	↑	↑	↑
Dexamethasone	↑	↓	↓**t**

t = tolerogenic DC (tDC); i = immunogenic DC (iDC)

The ↑ and ↓ arrows represent an increase and a decrease in receptor expression respectively. The – represent no change in mRNA levels.

CHAPTER FIVE

Protein Kinase Cα Regulates the Expression of Complement Receptor Immunoglobulin in Human Monocyte-Derived Macrophages

Chapter 5 Authorship

This work was accepted for publication in Journal of Immunology on 23rd of January 2015. The final version is therefore presented in the thesis in JI format.

Title: Protein kinase Cα regulates the expression of complement receptor immunoglobulin in human monocyte-derived macrophages

Yeufang Ma: Co-ran the experiments, developed the knock down method, collated data and checked statistics

Kanchana Usuwanthim: Assisted with experimental design and experimental runs and writing of the manuscript

Usma Munawara: Co-ran the experiments, collated data, drew graphs and assisted with writing of the manuscript

Alex Quach: Supervised aspects of the experiments and collation of data, drew graphs and ran statistics on data, and assisted with the writing of the manuscript

Nick N Gorgani: Advised on the measuring of CRIg and contributed to the interpretation of the data and writing of the manuscript

Catherine A. Abbott: Co-supervised the project and contributed to data interpretation and writing of the manuscript

Charles S Hii: Contributed to experimental design, data interpretation and writing of the manuscript

Antonio Ferrante: Initiated and co-supervised the project and contributed to experimental design, data interpretation and led the preparation of the manuscript

Protein Kinase Cα Regulates the Expression of Complement Receptor Ig in Human Monocyte–Derived Macrophages

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The complement receptor Ig (CRIg) is selectively expressed by macrophages. This receptor not only promotes the rapid phagocytosis of bacteria by macrophages but also has anti-inflammatory and immunosuppressive functions. Previous findings have suggested that protein kinase C (PKC) may be involved in the regulation of CRIg expression in human macrophages. We have now examined the role of PKCα in CRIg expression in human monocyte-derived macrophages (MDM). Macrophages nucleofected with plasmid containing short hairpin RNA against PKCα showed markedly reduced expression of PKCα, but normal PKCζ expression, by Western blotting analysis, and vice versa. PKCα-deficient MDM showed increased expression of CRIg mRNA and protein (both the long and short form), an increase in phagocytosis of complement-opsonized *Candida albicans*, and decreased production of TNF-α and IL-6. TNF-α caused a marked decrease in CRIg expression, and addition of anti-TNF mAb to the TNF-α–producing MDMs increased CRIg expression. PKCα-deficient macrophages also showed significantly less bacterial LPS-induced downregulation of CRIg. In contrast, cells deficient in PKCα showed decreased expression of CR type 3 (CR3) and decreased production of TNF-α and IL-6 in response to LPS. MDM developed under conditions that increased expression of CRIg over CR3 showed significantly reduced production of TNF-α in response to opsonized *C. albicans*. The findings indicate that PKCα promotes the downregulation of CRIg and upregulation of CR3 expression and TNF-α and IL-6 production, a mechanism that may promote inflammation. ***The Journal of Immunology*****, 2015, 194: 2855–2861.**

Members of complement receptor (CR), TLR, and scavenger receptors, as well as C-type lectins, are among the groups of cell surface proteins that initially recognize opsonized-pathogen or pathogen-associated molecular patterns. Recently, a new protein, CR Ig (CRIg) coded by V-set and Ig domain–containing protein 4 (*VSIG4*), has been added to this list (1–4). CRIg represents a singular CR with structure and properties distinct from those of classically known CR, such as CR type 3 (CR3) (4). The expression of CRIg on subpopulations of macrophages has been widely reported (2, 4).

CRIg, a high-affinity CR, is readily available to phagocytose complement-opsonized bacteria as well as soluble complement breakdown products (5). In contrast, CR3-mediated uptake of complement-opsonized bacteria requires divalent cations and 37˚C temperature, preactivation of CR3, and multivalent interactions with the opsonized particles. This requirement is supported by the findings that CRIg promotes a rapid mechanical clearance of blood-borne bacteria engulfed by Kupffer cells via the bile duct and gut (4). Furthermore, CRIg expression has been associated with protection against inflammation in several chronic inflammatory diseases. Helmy et al. (4) reported that mice deficient in CRIg not only were incapable of effectively clearing *Listeria monocytogenes* and *Staphylococcus aureus* but also experienced a cytokine storm and died earlier than wild-type litermates. They described the complement-binding properties of CRIg and developed a CRIg–Fc fusion protein capable of inhibiting alternative complement pathway activation (6–8) and providing protection against experimental arthritis (9). The immunosuppressive action of CRIg on T cells has also been highlighted (10, 11). Others have reported its anti-inflammatory properties in experimental autoimmune uveoretinitis (12), intestinal ischemia/reperfusion-induced injury (9), and immune-mediated liver injury (13). Thus identification of mediators that regulate CRIg expression should provide better insights into understanding the inflammatory reaction.

Previous studies using activators and a pharmacological inhibitor of protein kinase C (PKC) have shown that PKC activation and inhibition depresses and increases, respectively, CRIg expression in human macrophages (14). One of these PKC activators was arachidonate, a product as well as activator of cytosolic phospholipase A_2 ($cPLA_2$) (15). Arachidonate, when applied exogenously to cells, causes the activation of PKC isozymes such as PKCα and stimulates the activity of $cPLA_2$ (16). This activity contrasts with that of dexamethasone, which increases CRIg expression (14) but inhibits $cPLA_2$ activity/expression (17). Furthermore, PKCα not only is a major PKC isoform in macrophages (18) but also phosphorylates/activates $cPLA_2$ (19). These findings

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Abbreviations used in this article: $cPLA_2$, cytosolic phospholipase A_2; CR, complement receptor; CR3, CR type 3; CRIg, CR Ig; MDM, monocyte-derived macrophage; PKC, protein kinase C; RT-PCR, real-time PCR; shRNA, short hairpin RNA; *VSIG4*, V-set and Ig domain–containing protein 4.

led us to hypothesize that PKCα may be involved in regulating CRIg expression in human macrophages. We have now used PKCα-specific short hairpin RNA (shRNA) to examine the role of this PKC isozyme in CRIg expression in human monocyte-derived macrophages (MDM) and delineated this relative to CR3 expression, phagocytosis, and cytokine production in MDM. Evidence is presented that PKCα plays a role in downregulating CRIg expression but upregulating the expression of CR3 and TNF and IL-6 production.

Materials and Methods

Reagents

Mouse mAbs against PKCα, PKCζ, and CRIg (detects both the long and short forms) were purchased from Santa Cruz Biotechnology (Dallas, TX). The ECL kit was from PerkinElmer (Waltham, MA). Recombinant human TNF-α was from Prospec (Rehovot, Israel), and the anti–TNF-α mAb (2TNF-H34A) was purchased from Thermo Fisher Scientific (Rockford, IL). The anti-CD11b mAb was from Abcam (Cambridge, U.K.). Dexamethasone, mouse mAbs against GAPDH, predesigned shRNA specific for PKCα (GenBank accession no. NM_002744) (http://www.ncbi.nlm.nih.gov/gene/?term=NM_002744), and nontargeting control shRNA and RPMI 1640 tissue culture medium were purchased from Sigma-Aldrich (St. Louis, MO). The Human Macrophage Nucleofection Kit was from Amaxa (Lonza, Walkersville, MD). Tissue culture petri dishes were obtained from Sarstedt (Postfach, Nümbrecht, Germany). Cytometric Beads Array Flex Sets were obtained from BD Biosciences (San Jose, CA).

Preparation of human MDM

MDM were prepared as described previously (20). Monocytes were isolated from blood buffy coats of healthy donors (Australian Red Cross Blood Service, Adelaide, South Australia) by centrifugation on Hypaque-Ficoll medium and then adherence was carried out according to Human Macrophage Nucleofection instructions (Amaxa), except that culture dishes (150 mm in diameter) were coated with plasma in lieu of poly-D-lysine. After plating for 1 h, the adherent cells were cultured for 5 d to allow them to differentiate into MDM in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in an incubator at 37˚C, 5% CO_2 air mixture. Medium was replaced on two occasions during this period. To generate MDM with increased expression of CRIg, the monocytes were cultured in the presence of 50 ng/ml dexamethasone for 5–6 d (14). The macrophages were carefully dislodged by a 15-min incubation in detachment buffer (lidocaine/EDTA/PBS) (20) at 37˚C, followed by pipetting over the monolayer. The preparation consisted of ~99% MDM.

Knockdown of PKCα

Knockdown of PKCα was achieved using an Amaxa nucleofector and a Human Macrophage Nucleofection Kit. For ~10^6 macrophages, 4 μg nontargeting control shRNA or PKCα-specific shRNA was added to each cuvette, and the cells were transfected using program Y-010 according to the manufacturer's instructions. After transfection, MDMs were cultured for 24 h before harvesting to assay functional responses. An aliquot of the cultures was used to confirm the knockdown of PKCα by Western blot analysis. Cell viability was also monitored by the trypan blue dye exclusion. Cell viability was retained at ~90%, which is consistent with the statement made by the Nucleofection Kit information document (Amaxa).

Macrophage stimulation with LPS

Transfected MDM were harvested using detachment buffer and plated in a 96-well plate at 1×10^5 cells per well. LPS was added to cells at 10 ng/ml final concentration (21). The plate was incubated in 37˚C for 24 h, and the supernatants were harvested for cytokine measurements. To examine the effects of LPS on CRIg expression in PKCα-deficient MDM, cells were plated in 40- × 10-mm dishes with 2 ml medium. After incubation with 10 ng/ml LPS at 37˚C for 24 h, cells were harvested, and CRIg protein levels were determined by Western blot analysis.

Cytokine assays

IL-6, TNF-α, and IL-1β levels were determined using cytometric bead array according to the manufacturer's instructions and as described previously (22). In brief, 50 μl capture bead suspension and 50 μl PE detection reagent were added to an equal amount of sample or standard dilution and incubated for 2 h at room temperature in the dark. Subsequently, samples were washed and centrifuged at 200 × *g* at room temperature for 5 min. The supernatant was discarded, and 150 μl wash buffer was added. Samples were analyzed on a BD FACSCanto System (BD Biosciences).

Phagocytosis assay

The phagocytosis assay was performed essentially as described previously (14). At 24 h post transfection with shRNA, MDM were washed and detached with detachment buffer. Then 1×10^5 heat-killed *Candida albicans* were added to 5×10^4 MDM in a final volume of 0.5 ml HBSS. Complement-containing human blood group AB serum was added to a final concentration of 10%. In addition, serum that had been depleted of complement by treatment with the fungi was used as control serum. The standard routine sheep RBC–hemolysin hemolytic assay (total complement hemolytic activity) was used to gauge the presence and absence of complement activity in the treated and nontreated serum. The macrophages were incubated for 45 min at 37˚C on a rocking platform. The unphagocytosed fungi were removed by differential centrifugation at 175 × *g* for 5 min, and then the MDM in the pellet were resuspended and cytocentrifuged on a microscope slide and stained with Giemsa. The number of particles in phagocytic vacuoles was then determined (14).

TNF-α–induced cell adhesion assay

To examine the ability of the anti–TNF-α mAb to block TNF activity, we examined its effects in a standard TNF-α–induced neutrophil adhesion model, as described previously (23). Neutrophils were prepared from whole blood from normal volunteers, using the single-step centrifugation density gradient system (23). Cell adherence was assessed using a flat-bottom well of microtiter plates coated with autologous human plasma. Neutrophils were added to wells (5×10^5) and then 25 IU TNF-α that had been treated with the isotype control or the anti-TNF mAb. After 30 min of incubation at 37˚C, cell adhesion was quantitated by the rose bengal staining method.

Measurement of CRIg and CD11b mRNA levels

Total RNA was isolated with an RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instruction and used for the synthesis of cDNA with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction. Real-time (RT)–PCR was performed in 96-well plates in triplicate reactions on an iQ5 RT-PCR detection system, using iQ SYBR Green Supermix (Bio-Rad Laboratories) and 500 nmol/l of each primer pair for CRIg, CD11b, and housekeeping gene GAPDH. Results were analyzed using iQ5 optical system software version 2.1. (Bio-Rad Laboratories). Forward and reverse primers used for amplification of human CRIg (5′-TCCTGGAAGTGCCAGAGAGT-3′ and 5′-TGTACCAGCCACTTCACCAA-3′) and CD 11b (5′-CCTGGTGTTCTTGGTGCCC-3′ and 5′-TCCTTGGTGTGGCACGTACTC-3′) were designed using Oligo Perfect Designer (Invitrogen, Carlsbad, CA) (14).

Western blot

Western blots were conducted essentially as previously described (16). Cells were lysed, and equal amounts of total cell lysates (20 μg for GAPDH, 30 μg for PKCα, and 50 μg for PKCζ and CRIg) were resolved by SDS-PAGE and transferred to 0.45-μm nitrocellular membranes (Bio-Rad Laboratories). After blocking, the membranes were incubated overnight at 4˚C with Abs (1:1000) with gentle shaking. After three washes, the membrane was incubated with anti-mouse mAbs conjugated to HRP (1:2000) for 1 h at room temperature. After three washes with 5% milk buffer, the blots were developed using ECL reagents and were analyzed on Chemi-Doc XRS system (Bio-Rad Laboratories) using Quantity One program.

CRIg expression on the macrophage surface

CRIg expression on the MDM surface was performed as previously described (14). Briefly, MDM were harvested and 2×10^5 cells were used for examination of CRIg expression. Cells were blocked with 10 μg/ml Intragam human Ig and 5% human AB serum and incubated with PE-labeled anti-human CRIg Ab (Santa Cruz Biotechnology) or IgG1 isotype (BD Biosciences) for 30 min at 4˚C. The cells were analyzed by flow cytometry using a BD FACSCanto system (BD Biosciences).

Statistical analysis

Data were analyzed by two-tailed or one-tailed Student test or one-sample *t* test (Graphpad Prism).

Results

Generation of PKCα-deficient MDM

Because knockdown of PKCα has not been reported previously in MDM, initial studies were undertaken to standardize the technology. After culturing monocytes for 5 d, the generated macrophages were subjected to knockdown of PKCα. The cells were nucleofected with plasmid containing shRNA against PKCα. Controls in which cells were either not nucleofected or nucleofected with nontargeting shRNA were run in parallel. The results showed that the amount of PKCα in the cells was reduced by ~85%, determined by Western blot, in cells nucleofected with PKCα-targeting shRNA (Fig. 1A). The specificity of the knockdown was demonstrated by a normal expression of PKCζ in these cells (Fig. 1B) and vice versa (Fig. 1C). This finding was also supported by normal expression of GAPDH (Fig. 1B, 1C).

CRIg expression is increased in PKCα-deficient MDM

The effect of reducing PKCα levels in MDM on CRIg expression was then examined. The results showed that PKCα-deficient MDM expressed significantly increased amounts of CRIg mRNA (Fig. 2A). This increase in CRIg expression was also evident at the protein level, as determined by Western blot analysis (Fig. 2B), and in cell surface expression of CRIg, determined by flow cytometry (Fig. 2C). The data show that human MDM express both spliced forms of CRIg but that the long form CRIg predominates. The MDM deficient in PKCα showed an increase in phagocytosis of complement-opsonized *C. albicans* particles (Fig. 2D). Control incubations using serum depleted of complement activity showed that in the absence of complement the serum failed to promote significant phagocytosis in both normal and PKCα-depleted MDMs (Fig. 2D).

It is unlikely that the effects of the shRNA on PKCα were caused by an IFN response. When we analyzed the IFN response by the expression of 2′,5′-oligoadenylate synthetase, a well-known indicator of IFN response (24), there was no significant difference between cells subjected to mock-nucleofection (no vector), those nucleofected with control shRNA, and those with PKCα shRNA (data not shown).

Regulation of CR3 expression by PKCα

To further elaborate on the relationship between PKCα, CRIg, and CR expression, we sought to assess the effects of PKCα depletion on the expression of CR3 (CD11b/CD18), the classical CR. The data demonstrated that PKCα-deficient MDM had decreased expression of CD11b mRNA (Fig. 3A) and protein (Fig. 3B), compared with cells that received nontargeting shRNA or were not nucleofected (Fig. 3).

Regulation of CRIg expression by TNF-α

Examination of CRIg expression in macrophages treated with TNF-α showed that the cytokine caused a marked decrease in CRIg expression over a concentration range of 1–20 ng/ml (Fig. 4A). In comparison, IL-1β and IL-6 produced less of an effect (Fig. 4B, 4C). In contrast, TNF-α caused a significant increase in CR3 expression in MDMs (Fig. 4D). Both IL-1β and IL-6 had no effect on CR3 expression (Fig. 4D).

Of interest, we found that PKCα-deficient MDMs, which showed increased CRIg expression, produced reduced amounts of TNF-α and IL-6, but not IL-1β (Fig. 4E), which, taken together

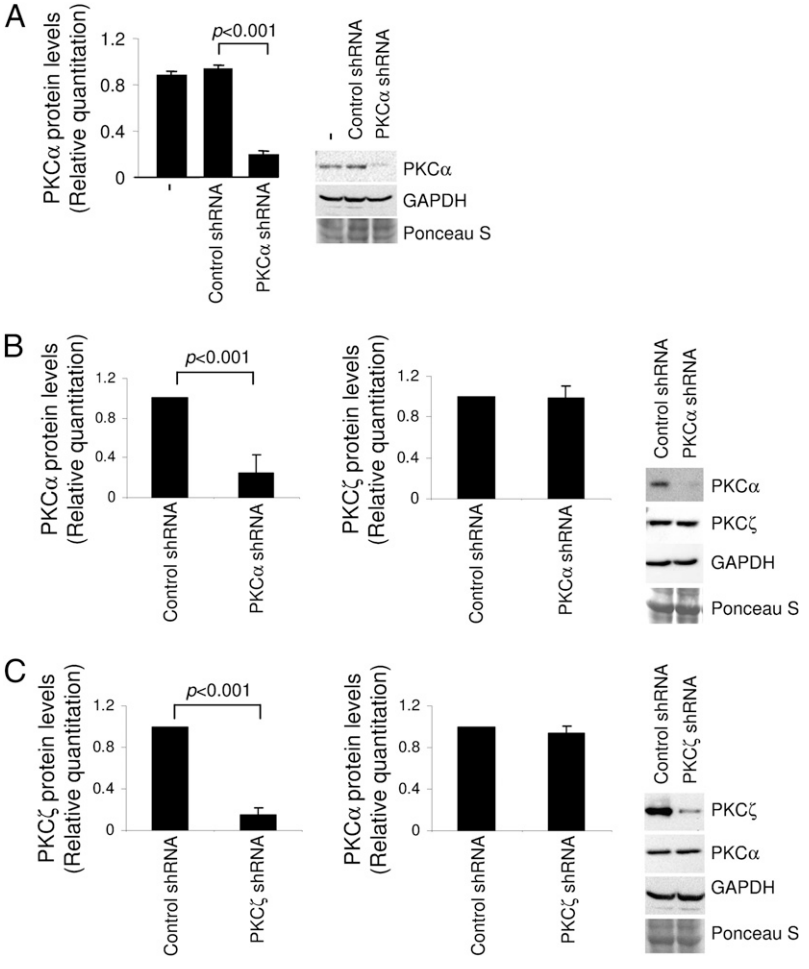

FIGURE 1. Generation of PKCα-deficient human MDM. Cells were nucleofected with shRNA targeting PKCα or nontargeting shRNA (control shRNA) and without shRNA (as mock-nucleofection control). After nucleofection, the MDM were cultured for 24 h and the expression of PKCα was determined by Western blotting. (**A**) Reduced expression of PKCα, by Western blots, in MDM nucleofected with shRNA targeting this isozyme of PKC. (**B**) MDM nucleofected with shRNA to PKCα, showing normal expression of PKCζ. (**C**) MDM nucleofected with shRNA to PKCζ, showing normal expression of PKCα, but reduced PKCζ. The results are expressed as mean ± SEM of three to four experiments, each conducted with cells from a different individual, and as a representative Western blot of data.

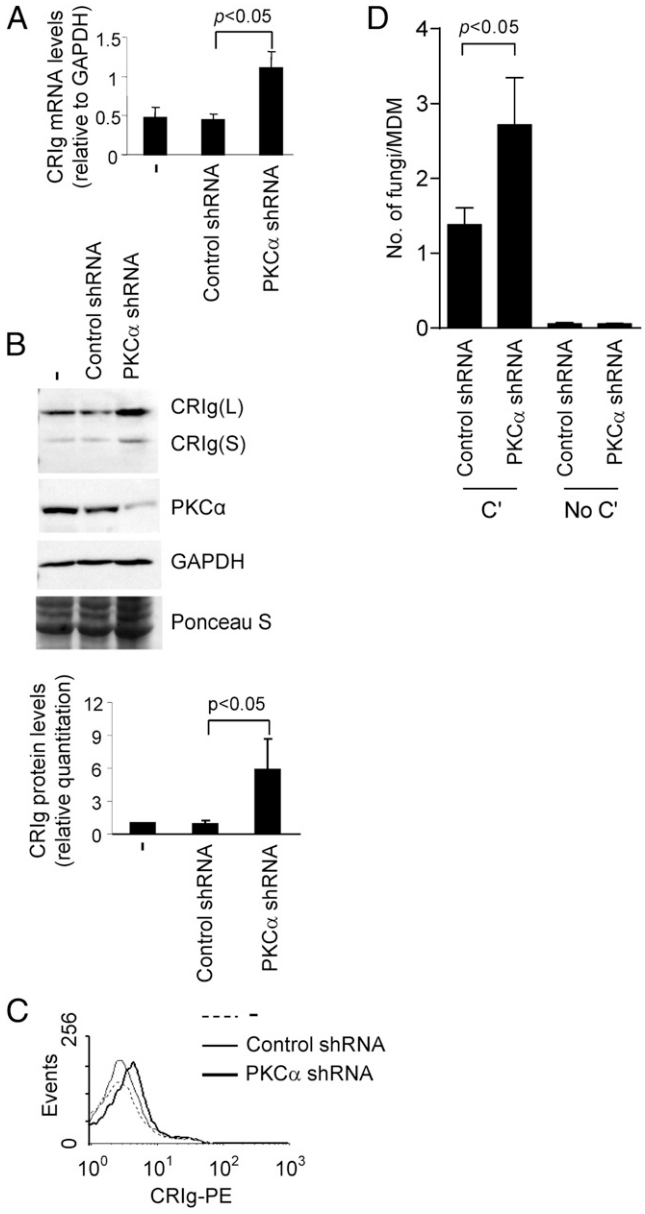

FIGURE 2. CRIg expression in PKCα-deficient MDM. Cells were nucleofected with shRNA to PKCα and then, after 24-h culture, examined for levels of CRIg expression. (**A**) CRIg mRNA levels relative to GAPDH measured by real-time PCR. (**B**) CRIg protein levels (L, long; S, short) measured by Western blot and (**C**) by flow cytometry. (**D**) Phagocytosis of opsonized yeast particles by PKCα-deficient MDM. The data are expressed as the stimulated minus the nonstimulated base lines. Data are presented as mean ± SEM of three separate experiments, each conducted with cells from a different individual. C′, complement.

with results in Fig. 4A–C, suggest that TNF-α may be central to the regulation of CRIg expression in a paracrine or autocrine manner. To gain evidence for this idea, we examined whether the addition of anti–TNF-α mAb could increase the expression of CRIg in MDM. The Ab was examined for the ability to inhibit TNF action in TNF-induced cell adhesion assay. The anti-TNF mAb added to cells, together with TNF-α, inhibited the TNF-α–induced cell adherence function (adherence arbitrary units, mean ± SEM: isotype control/untreated = 0.033 ± 0.001; TNF-α–treated = 0.260 ± 0.027; TNF-α and anti–TNF-α mAb–treated = 0.034 ± 0.004). The addition of anti–TNF-α mAb to the MDMs caused a marked increase in CRIg expression (Fig. 4F) in a similar manner to that seen by knocking down PKCα (Fig. 2B).

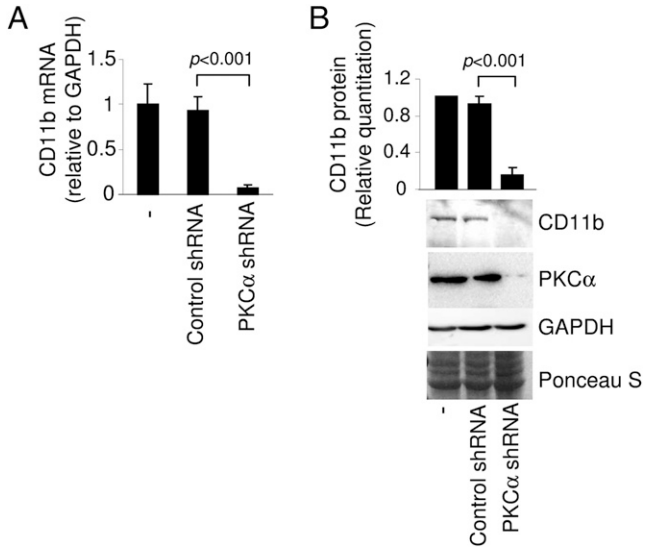

FIGURE 3. Decreased expression of CD11b in PKCα-deficient MDM. Cells were nucleofected with shRNA to PKCα and then, after 24 of culture, examined for levels of CD11b by (**A**) mRNA and (**B**) protein by Western blot. A representative blot is shown also. Results are presented as mean ± SEM of three experiments, each conducted with cells from a different individual.

Role of PKCα in LPS-induced TNF-α, IL-1β, and IL-6 production and CRIg modulation

To better understand the role of PKCα and CRIg in the inflammatory reaction, we examined cytokine production by PKCα-deficient MDM. The MDM were nucleofected with PKCα-targeted shRNA, nontargeted shRNA, or not nucleofected. These MDM were then stimulated with LPS and examined for cytokine production. The data presented in Fig. 5 demonstrate that cells deficient in this PKC isozyme produced markedly less TNF and IL-6 in response to LPS (Fig. 5). Although MDM produce substantially less IL-1β than do monocytes, we found that the production of this cytokine was not significantly affected by PKCα knockdown in the MDM (Fig. 5C).

Furthermore, the data showed that PKCα regulates the LPS-induced changes to CRIg expression in macrophages (Fig. 5D). Control, nonnucleofected, and control shRNA nucleofected cells displayed similar levels of CRIg expression in MDM, which, when treated with LPS, showed almost complete absence of CRIg (Fig. 5D). The PKCα-deficient MDM showed increased CRIg expression, and this was not decreased when the cells were treated with LPS (Fig. 5D).

Phagocytosis via CRIg tames TNF-α production by MDM

To attempt to determine the relationship between the relative levels of CRIg to CR3 expression and TNF-α responses in macrophages, we used a previously published model that involves the differentiation of monocytes in the presence of dexamethasone (14). When monocytes were cultured under these conditions, the MDM showed a marked increase in CRIg expression (14) and no change in CR3 expression (Fig. 6A). When these MDM were challenged with opsonized heat-killed *C. albicans*, the production of TNF-α was markedly decreased compared with that in control MDM, which expressed less CRIg (Fig. 6B). The macrophages expressing CRIg showed an increase in phagocytosis of complement-opsonized *C. albicans* (Fig. 6B).

Discussion

Our data demonstrate that human PKCα-deficient MDM can be successfully generated by nucleofection with PKCα-targeted

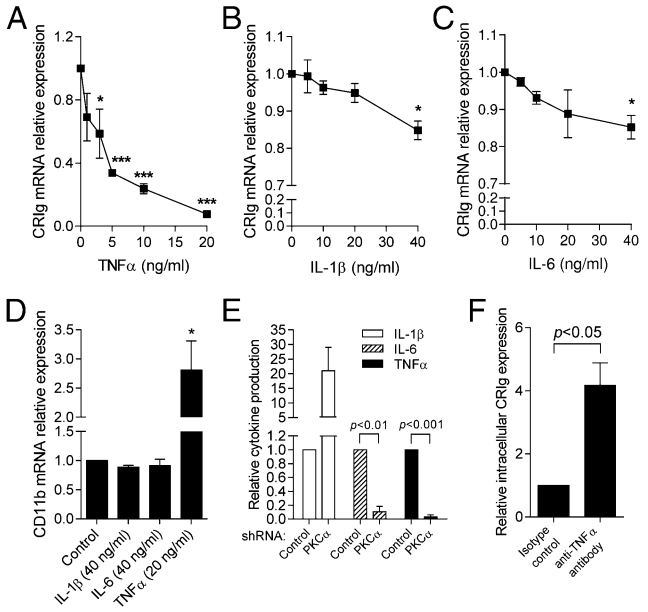

FIGURE 4. The effects of TNF-α, IL-1β, and IL-6 on CRIg (**A**–**C**) and CD11b (**D**) expression. MDM were treated with the cytokines for 24 h, and then the CRIg and CD11b mRNA was measured. (**E**) Cytokine production by PKCα-deficient MDM. Nucleofected MDM were cultured for 24 h before examining for cytokine protein expression (**F**) Effects of anti-TNF mAb on CRIg expression. The nucleofected MDM were cultured for 24 h and then treated with anti-TNF Ab or isotype control for a further 24 h before examining for CRIg expression. Results are expressed as changes relative to control values and presented as mean ± SEM of three (A–C, E, and F) or four (D) experiments, each conducted with cells from a different individual. Statistical analyses: $*p < 0.05$, $***p < 0.001$ compared with control.

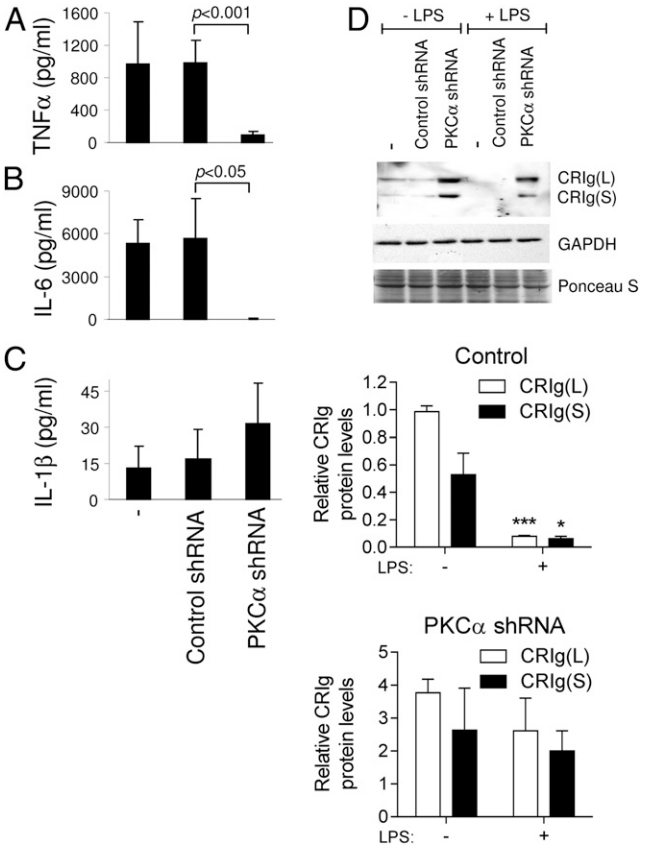

FIGURE 5. Cytokine production by PKCα-deficient MDM. Cells were nucleofected with shRNA to PKCα and cultured for 24 h. LPS-induced TNF-α (**A**), IL-6 (**B**), and IL-1β (**C**) expression was then examined at 24 h. Note that all MDM were checked and shown to display PKCα knockdown. (**D**) shows that the ability of LPS to downregulate CRIg expression is, for the most part, prevented in PKCα-deficient MDM. *Bottom panel* shows pooled densitometric data obtained from PKCα-deficient MDM that had been incubated in the absence or presence of LPS. The results are expressed as mean ± SEM of three experiments, each conducted with cells from a different individual. $*p < 0.05$, $***p < 0.001$.

shRNA, retaining appropriate levels of cell viability. The evidence strongly indicated that PKCα was specifically depleted in these cells, as not only were the GAPDH levels in these cells not affected but also the levels of another PKC family member, PKCζ, were not affected (Fig. 1).

Because the PKCα-deficient MDM showed increased expression of CRIg mRNA and protein (Fig. 1), the results suggest that this isozyme of PKC downregulates CRIg expression, possibly through a transcriptional regulation of the complement protein. This idea would be supported by our finding that both spliced variants, the long form CRIg and short form CRIg, were controlled by PKCα (Fig. 1). The predominance of the longer form was evident. Both forms bind C3b and iC3b as soluble proteins on the surface of bacteria/particles, despite the fact that although both possess identical IgV domains, the shorter form lacks an IgC domain (4, 6).

These findings are in agreement with previous reports showing that the PKC activator PMA caused a decrease in CRIg expression (14). Other agonists that induced PKC activation, arachidonate (14) and LPS (4), also caused a decrease in CRIg expression. The PKC pharmacological inhibitor GF109203X was also found to prevent arachidonate, an activator of PKCα (18), from inducing a decrease in CRIg expression (14). Our studies have now revealed that the downregulation of CRIg expression in MDM by LPS could be reduced by making the cells PKCα deficient (Fig. 5D).

Our results significantly increase our understanding of the pathways involved in the regulation of CR in macrophages. Examination of CR3 expression in these macrophages demonstrated that expression of CR3 was differentially regulated by PKCα compared with CRIg (Fig. 3). PKCα, although decreasing the expression of CRIg, promoted the expression of CD11b/CR3. Because we found that increasing CRIg by targeting PKCα increased phagocytosis of complement-opsonized fungi, the results suggest that this increased phagocytosis by the PKCα-deficient macrophages is most likely due to CRIg and not CR3. This idea indicates that PKCα may promote its proinflammatory effects through the reciprocal control of these CR. Indeed, the data demonstrated that when MDM were generated to show increased expression of CRIg over CR3, phagocytosis was increased but TNF-α production in response to the opsonized fungi was significantly reduced (Fig. 6). This finding suggests that there may be interplay between these CR. The idea is consistent with the view that engagement of CRIg leads to phagocytosis and physical clearance of bacteria, avoiding a cytokine storm associated with particles engulfed via CR3 (4).

A proinflammatory role of PKCα is further supported by our findings that PKCα promotes the production of the proinflammatory cytokines TNFα and IL-6 in response to LPS (Fig. 5). This result is consistent with data from a previous study in murine RAW264.7 macrophages that inhibition of PKCα function with a dominant negative PKCα mutant caused a significant reduction in TNF-α production in response to LPS (25). In comparison, we found that LPS-induced IL-1β production is independent of PKCα because production of this cytokine was not significantly changed in PKCα-deficient cells. In human monocytes, it has previously been reported that PKCδ and perhaps PKCα (at very

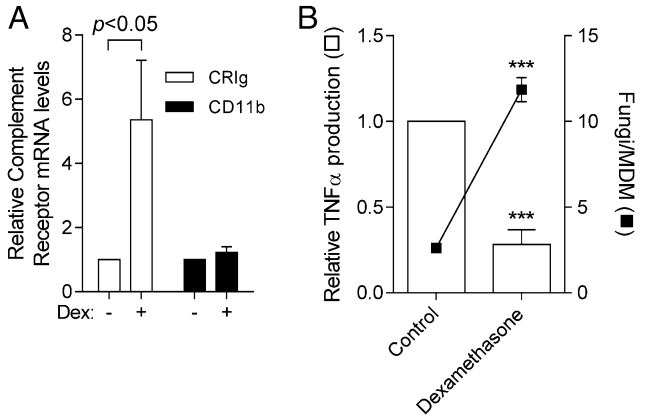

FIGURE 6. TNF-α production in MDM expressing increased CRIg over CR3. (**A**) Expression of CRIg and CR3 in MDM derived from monocytes cultured for 5 d in the presence or absence of 20 nM of dexamethasone. (**B**) Production of TNF-α (□) by the MDM after challenge with complement-opsonized killed *C. albicans*. Cytokine production was measured in the culture fluids 24 h after stimulation. The level of phagocytosis of opsonized *C. albicans* by MDM is expressed as the number of engulfed fungi per macrophage (■). Results are expressed as changes relative to control values and presented as mean ± SEM of three experiments. $*p < 0.001$.

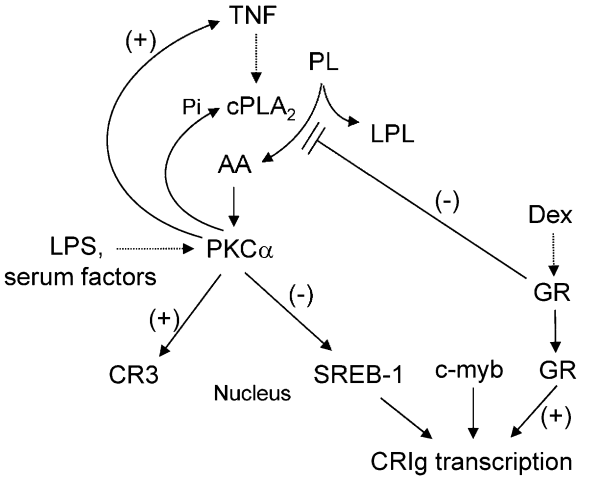

FIGURE 7. Diagrammatic representation of the proposed pathways of regulating CRIg and CR3 expression in macrophages. PKC-activating agents (AA, arachidonate; LPS) and dexamethasone exert their antithetical effects on CRIg expression via their opposing actions on a common target, $cPLA_2$. The PLA_2 is phosphorylated and activated by PKCα, releasing arachidonate to suppress CRIg expression. Arachidonate can also stimulate $cPLA_2$ activity through its ability to stimulate PKCα activation, creating a signaling loop, which also regulates CR3 expression. TNF-α, produced in response to LPS, activates $cPLA_2$. Dexamethasone downregulates $cPLA_2$ expression, resulting in reduced production of arachidonate. PKC could target downstream transcription factors such as SREBP-1, whereas dexamethasone can directly affect *VSIG4* transcription as well as downregulating $cPLA_2$ expression. (+), stimulatory; (−), inhibitory; GR, glucocorticoid receptor; LPL, lysophospholipid; Pi, phosphorylation; PL, phospholipid; SREBP-1, sterol regulatory element-binding protein-1.

high inhibitor concentrations) may regulate IL-1β production in response to LPS (26) or to PMA (27). However, these studies were conducted using rottlerin, a weak PKCδ inhibitor with poor specificity (28). Our data argue against PKCα as a regulator of IL-1β production in human macrophages.

The proposed pathways of regulating CRIg and CR3 expression are illustrated in Fig. 7. We speculate that the antithetical effects of PKC-activating agents and dexamethasone on CRIg expression are mediated via their opposing actions on a common target, $cPLA_2$. Activated PKCα phosphorylates and activates $cPLA_2$ (19), resulting in the release of arachidonate, which suppresses CRIg expression (14). Our studies have demonstrated that arachidonate can also stimulate $cPLA_2$ activity (29), most likely owing to its ability to stimulate PKCα activation (16, 18), giving rise to a regulatory signaling loop (30). TNF-α, produced in response to LPS, activates $cPLA_2$ (31), explaining the cytokine's ability to inhibit CRIg expression. Consistent with this concept, LPS and TNF-α, which depress CRIg expression but upregulate CR3 expression in macrophages, promote the expression and activation of $cPLA_2$ (31–33). The signaling loop involving $cPLA_2$, arachidonate, and PKCα also promotes CR3 expression. In contrast, glucocorticoids, including dexamethasone, downregulate $cPLA_2$ expression and inhibit the enzyme in monocytes and macrophages (17, 34). The concept that $cPLA_2$ acts as a point of convergence for the positive (up-) and negative (down-) regulators of CRIg expression has enabled us to identify PKCα as a negative regulator of CRIg expression. Possible downstream targets of PKCα that regulate CRIg expression are the transcription factors that modulate the transcription of *VSIG4* that codes for CRIg. Although there have been no reports on the promoter regions of *VSIG4*, in silico data and nucleotide sequence analysis of the upstream elements of the transcription start site of *VSIG4* have identified regulatory *trans*-acting factor binding sites for factors such as glucocorticoid receptor, c-myb, and SREBP-1 (Genecard database). The expression of SREBP-1 has recently been reported to be negatively regulated by classical PKC isoforms such as PKCα in Hep G2 cells in response to 3,5,3′-triiodothyronine (35). It would be of interest to determine, in future studies, whether PKCα negatively regulates CRIg expression by downregulating SREBP-1 expression, resulting in reduced CRIg transcription. Dexamethasone could also exert a more direct action via the glucocorticoid receptor.

The relative differences in potency of the ability to decrease MDM CRIg expression by TNF-α compared with IL-1β/IL-6 suggest that the PKCα effects are likely to be operating via the generation of TNF-α. In addition, in these, MDM TNF-α production was regulated by PKCα. Thus the addition of anti-TNF Ab to the MDM resulted in a similar upregulation of CRIg expression (Fig. 4F), as was seen in macrophages in which PKCα had been knocked down (Fig. 2). It is tempting to speculate that a paracrine/autocrine action of TNF-α is operating to regulate CRIg expression. This idea may have relevance to anti-TNF therapy in rheumatoid arthritis, as CRIg-expressing macrophages have been identified in synovial tissue from rheumatoid arthritis patients (36).

The data have revealed for the first time, to our knowledge, that PKCα plays a key role in the regulation of expression of CRIg in human macrophages. Because PKCα depletion upregulates CRIg expression but downregulates CR3 expression and IL-6/TNF production, our findings imply that activation of PKCα downregulates CRIg expression but upregulates CR3 expression and IL-6/TNF production. This idea suggests that PKCα is a proinflammatory intracellular signaling molecule. It is therefore tempting to speculate that the molecule could be a target for developing anti-inflammatory therapies.

Disclosures

The authors have no financial conflicts of interest.

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CHAPTER SIX

Discussion

6.1 Introduction

The major aim of this thesis was to examine the effect of cytokines on CRIg expression in human monocytes differentiating into macrophages, human MDM and in human DC utilising an *in vitro* human monocyte culture system. These effects of cytokines on CRIg were compared to those on classical complement receptors CR3, CR4. This work demonstrated that cytokines can significantly alter the expression of CRIg, CR3 and CR4. Cytokines can increase or decrease the expression of CRIg on macrophages and this is likely to have important implications in the understanding of the role of cytokines in innate and adaptive immunity. Thus CRIg expression becomes a control point in the inflammatory reaction. Cytokine-induced increase or decrease in CRIg expression correlated with increase or decrease in phagocytosis, respectively. Because of the critical functions played by CRIg in infection, immunity and inflammation, these results suggest that cytokines have the potential to modify inflammation and resistance to microibial pathogens by modulating the CRIg receptor expression, hence identifying a mechanism by which cytokines regulate immune responses and inflammation. CRIg expression by cytokines appears to be modulated at a pre-transcriptional level as the changes were evident at the mRNA level.

Cytokines not only influenced the level of expression of CRIg following their differentiation from monocytes but also regulated the expression of CRIg on mature macrophages and DC to provide a second control point by which cytokines could modify macrophage microbial killing, inflammation and immune responsiveness. This provides a significant development in our understanding of the mechanisms of the inflammatory reaction and immune responsiveness. The ability of cytokines to alter expression of CRIg on DC identifies a significant development in the understanding of

the regulation of adaptive immunity, as a consequence of generating tolerogenic versus immunogenic DC (Morelli and Thomson, 2007, Thomas, 2013).

Besides the already well established fact that CRIg has has distinct functions to CR3/CR4 this thesis demonstrates that these two types of receptors are differentially regulated by cytokines. By differentially expressing these receptors macrophages may display quite different characteristics. Not surprisingly there has been an interest in examining the levels of $CRIg^+$ versus $CR3^+$ macrophages in inflammatory sites in organs and tissues such as synovial tissue, intestine and liver (Tanaka et al., 2008, Tanaka et al., 2012). The relative expression of CRIg versus CR3/CR4 is likely to contribute to the role macrophages play in inflammation, during infection and in also autoimmunity.

One of the significant findings of this thesis was the identification of a key signalling molecule, PKCα in the regulation of CRIg expression. PKCα regulates CRIg mRNA expression and macrophages made deficient in this PKC isozyme were not amenable to down regulation of CRIg expression by agents such as lipopolysaccharide (LPS). There were two interesting outcomes from a more in depth study on properties of $CRIg^+$ macrophages. Firstly our results demonstrate that by directing phagocytosis via CRIg versus CR3 resulted in significantly reduced TNF production. This provides support for the view that when bacteria uptake occurs via CRIg it avoids the generation of a "cytokine storm". Secondly, TNF appears to be central to regulation of CRIg expression in a 'pool' of cytokines and mediators. By using anti-TNF neutralising antibody, CRIg expression by macrophages was substantially increased. It is tempting to speculate from the above results that other cytokines and mediators cause changes in CRIg expression by promoting or inhibiting TNF production. This may be the reason why anti-TNF therapy is successful in rheumatoid arthritis (RA).

6.2 Cytokines regulate the expression of CRIg in macrophages

The work described in this thesis demonstrates that cytokines regulate the expression of CRIg during the development of monocytes to macrophages, supporting and extending previous observations in our laboratory (Gorgani et al., 2011). This work provides further support for the hypothesis that CRIg expression may be a control point in infection and immunity, through which cytokines control macrophage function. The cytokines tested could be divided into a group which caused an increase in CRIg expression, LT-α, IL-1β, IL-6, IL-10, GM-CSF, M-CSF and those which caused a decrease, IFN-γ, TNF, TGF-β1, IL-4 and IL-13 (Table 3.1). Thus identifying for the first time the cytokine patterns which regulate CRIg expression in macrophages and also revealing new and unexpected properties for some of these cytokines, relevant to their role in resistance against infection. Previously our laboratory reported that dexamethasone increased CRIg expression and enhanced macrophage-mediated phagocytosis of complement opsonised *C. albicans* (Gorgani et al., 2011) but IFN-γ decreases CRIg expression and caused reduced phagocytosis of fungi (Gorgani et al., 2011). The present study demonstrated that IL-4 caused a decrease in the expression of CRIg protein and reduced in the phagocytosis of *C. albicans*, which matches previous work which reported that IL-4 caused a decrease in the phagocytosis and killing of *Plasmodium falciparum* infected red blood cells by macrophages (Kumaratilake and Ferrante, 1992).

The immuno-suppressive cytokine IL-10 caused a substantial increase in CRIg mRNA (Gorgani et al., 2011) in human monocytes developing into macrophages and the work in this thesis confirmed that it also increased CRIg protein levels (Figure 3.4). Interestingly, this cytokine was as effective as the anti-inflammatory agent, dexamethasone in increasing the levels of CRIg in human macrophages (Gorgani et al.,

2011) (Figure 3.4). In contrast another regulatory cytokine, TGF-β1, which shares properties with IL-10, profoundly decreases CRIg mRNA (Gorgani et al., 2011) and protein expression in macrophages (Figure 3.4).

While TNF, IL-1β and IL-6 share many biological activities and are often termed as pyrogenic cytokines, their effects on macrophage CRIg expression differed. TNF caused a decrease and IL-1β and IL-6 increased CRIg expression. Since TNF has been shown to be a therapeutic target for RA (Feldmann et al., 1994, Redlich et al., 2002, Ritchlin et al., 2003, Scardapane et al., 2012) and CRIg is protective in this disease (Katschke et al., 2007, Tanaka et al., 2008), the finding that the cytokine inhibits the expression of CRIg in macrophages (Figure 3.6), suggests that the cytokine may be causing its effects, in part, through the modulation of CRIg expression. In Chapter 5 it was found that TNF caused these effects via activation of PKCα and those macrophages treated with anti-TNF antibody showed increased expression of CRIg (Figure 4, Chapter 5). It is therefore tempting to speculate that one important action of anti-TNF therapy is to prevent the loss of CRIg expression induced by TNF in RA and thereby improve phagocytic uptake of microbial pathogens, a possible reason as to why patients on anti-TNF therapy do not experience the expected wider increase in susceptibility to infection.

GM-CSF and M-CSF regulate monocytes-macrophage differentiation and function (Hamilton, 2008). Chapter 3 demonstrated that macrophages developing under the influence of these growth factors show increased expression of CRIg. These changes were found both at mRNA and protein level. This is conducive with reports that GM-CSF and M-CSF alter macrophage functional responses (Hamilton, 2008, Naito, 2008). The findings suggest an important role for CSF in defence against infection that may be due to an increase in the phagocytosis of bacteria as a consequence of increases in CRIg expression. This finding is worth further exploration.

Although, the laboratory have previously reported that IFN-γ, IL-4, IL-10 and TGF-β1 altered CRIg expression, this was assessed only at the mRNA level (Gorgani et al., 2011). Thus there was essentially no information as to the effects of cytokines on CRIg protein expression let alone on the two different spliced forms of CRIg. In this thesis by measuring CRIg protein by Western blotting, the fate of both spliced forms of the receptor could be followed. The present studies revealed that the CRIg(L) and CRIg(S) were similarly regulated by these cytokines. While both forms are found in human macrophages, murine macrophages possess only the latter form (Helmy et al., 2006). Thus the finding that cytokines regulate the CRIg(S) form is also relevant to the murine models of infection and immunity and inflammation.

Cytokine networks play an important role in regulating inflammation and those tested in our present study act on the macrophage, a cell which is central to the pathogenesis and possibly resolution of chronic inflammatory diseases (Feldmann et al., 1996). Cytokines are known for their differences in either promoting or protecting against these diseases. In this thesis only the effects of individual cytokines on CRIg, CR3 and CR4 expression were examined *in vitro*. Therefore it is important to acknowledge that during inflammatory diseases multiple cytokines will be at play which limits the extent to which conclusive statements can be made from the present research results. Nevertheless, it is tempting to speculate that CRIg may be one of the control points in these inflammatory diseases through which cytokines and other intercellular acting inflammatory mediators act. Indirect support for this view can be derived from the findings that $CRIg^{+}$ macrophages disappear from inflammatory sites and with the intensity of inflammation e.g. in experimental and clinical arthritis, IBD and Type 1 diabetes (Vogt et al., 2006, Katschke et al., 2007, Tanaka et al., 2008, Tanaka et al., 2012, Jung et al., 2012, Fu et al., 2012). IFN-γ causes a marked decrease in CRIg expression, in line with its reported effects in the pathogenesis of RA (Baccala et al.,

2005) and atherosclerosis (Frostegard et al., 1999, Gupta et al., 1997, Whitman et al., 2000, Buono et al., 2003, Pamir et al., 2012). IFN-γ is present in RA patient's synovium (Baccala et al., 2005). CD4 T cells in RA patients contribute to the pathogenesis by producing IFN-γ (Rodeghero et al., 2013, Park et al., 2014). Several other studies reported the atherogenic effects of IFN-γ (Frostegard et al., 1999, Gupta et al., 1997, Whitman et al., 2000, Buono et al., 2003, Pamir et al., 2012).

It was interesting to find that the Th2 cytokines, IL-4 and IL-13 both caused a decrease in expression of CRIg at the mRNA and protein level. IL-4 and IL-13 exhibit similarities in structural and functional properties (Zurawski and de Vries, 1994). In human monocytes the activity of IL-13 is very similar to that of IL-4 possibly because of the IL-4Rα, a predominant signalling chain common to both receptors (Hart et al., 1999), however other reports suggested that with monocyte differentiation, the configuration of IL-4 and IL-13 cell surface receptors altered (Hart et al., 1993, Hart et al., 1995a, Hart et al., 1995b, Bonder et al., 1999). Cytokines levels have been reported to play a role in the pathogenesis of depressive disorder. Depressed patients had higher serum levels of IL-13 (Hernandez et al., 2008), however few studies showed that IL-13 levels were unaltered in depressed individuals (Simon et al., 2008, Hallberg et al., 2010). Furthermore, non-obese depressed individuals had higher serum levels of IFN-γ and TNF and IL-13 compared to non-obese non-depressed individuals, while IFN-γ was significantly elevated in obese depressed individuals (Schmidt et al., 2014). In previous studies IFN-γ was also associated with the pathogenesis of Type 1 diabetes (Campbell et al., 1991, Pradhan et al., 2001). Another report showed that IL-13 and TGF-β1 were also detected in the immune islet infiltrates in animal models and human pancreatic samples (Jorns et al., 2013).

It has been reported that there is an association of IL-4 gene 70bp VNTR and MTHFRC677T polymorphism in the development of RA (Inanir et al., 2013).

Furthermore, it has been suggested that IL-4 and its receptor could play a role in the pathogenesis of RA (Prots et al., 2006). Similarly, IL-13 is also identified as a risk locus for psoriatic arthritis (Bowes et al., 2011). Thus the ability of IL-4 and IL-13 to down regulate CRIg in human macrophages identifies new properties which may have implication in the pathogenesis of various disorders. This may be a mechanism by which macrophages promote pathogenesis induced by helminths such as schistosomes and other Th2 mediated inflammation such as that seen in allergy and the observation could be given consideration in future research.

IL-10 caused a substantial increase in CRIg expression at both the mRNA and protein level. This is consistent with its protective and anti-inflammatory effects observed in several murine arthritis models and its praised therapeutic potential in arthritis (Fellowes et al., 2000, Finnegan et al., 2003). However there are other effects of the cytokine which could also contribute to its protective effects, namely IL-10 down regulates class II major histo-compatibility complex molecule expression, inhibits the production of pro-inflammatory cytokines (de Waal Malefyt et al., 1991) and decreases release of metalloproteases (Lacraz et al., 1995). Similarly the findings of increased CRIg expression on exposure to IL-10 are conducive with its athero-protective effects in several experimental models (Mallat et al., 1999, Pinderski Oslund et al., 1999, Pinderski et al., 2002, Von Der Thusen et al., 2001, Caligiuri et al., 2003).

Variation in $CRIg^{+}$ macrophages has been reported in several other autoimmune diseases including Type 1 diabetes (Fu et al., 2012) and IBD (Tanaka et al., 2012). These levels appeared to relate to the intensity of the inflammation observed. The role of cytokines in these diseases is partly defined as essential elements of the regulatory network. Our data illustrates that cytokines may be the mechanism that cells use to alter levels of CRIg and control inflammation. $CRIg^{+}$ macrophages were markedly reduced in the large intestine of patients with IBD and in mice with the experimental form of the

disease (Tanaka et al., 2012). Fu et al showed that the enhanced expression of CRIg on $CRIg^+$ macrophages has a protective effect on the pathogenesis of Type 1 diabetes; indicated a potential therapeutic benefit in treating this disease (Fu et al., 2012). In experimental autoimmune liver fibrosis, the level of CRIg expression was found to correlate with disease activites, in a reciprocal manner (Jung et al., 2012).

6.3 Cytokines modulate CRIg expression in mature macrophages

In Chapter 3 it was also demonstrated that cytokines not only affected expression of CRIg on developing macrophages but also regulated the expression of this receptor on mature macrophages, indicative of events in tissues. CRIg expression in MDM was regulated by cytokines; however most of the cytokines caused a down-regulation of the receptor. Only LT-α and M-CSF induced an up regulation similar to the anti-inflammatory agent dexamethasone. The other cytokines: IFN-γ, TNF, IL-1β, IL-6, IL-4, IL-13, TGF-β1, IL-10 and GM-CSF down regulated CRIg expression. This result demonstrates that mature macrophages are amenable to cytokine-induced modulation of CRIg expression but that their actions are substantially different when compared to those observed during the development of macrophages (Table 4.1). This then becomes a second control point in inflammation through which cytokines may have their influence on macrophages in tissues.

The changes in CRIg in MDM correlated with their altered rates of phagocytosis of complement opsonised *C. albicans* (Chapter 3). The laboratory has previously demonstrated that components of microbial pathogens stimulate human lymphocytes to produce LT-α but not TNF (Ferrante et al., 1990). Thus this may be a first line of defence for tissue located macrophages to increase their phagocytic ability. As previously demonstrated by Helmy et al (2006), once phagocytosis has been initiated by

liver macrophages (Kupffer cells), CRIg expression is dramatically reduced. This is most likely due to the release of cytokines, in particular TNF which decrease CRIg expression (see Chapter 5).

Because most of the cytokines examined caused a decrease in CRIg expression on mature macrophages, it is inevitable that those monocytes which respond to tissue infection and damage and mature to macrophages will be susceptible to the action of these cytokines and may be a reason why CRIg expressing macrophages are low at inflammatory sites (Vogt et al., 2006). However as the inflammation resolves macrophages expressing CRIg re-appear at these sites (Gorgani N N, personal communication).

6.4 CRIg and adaptive immunity

It has already been suggested that CRIg participates in adaptive immunity (Zang and Allison, 2006, Vogt et al., 2006, Xu et al., 2009, Fu et al., 2012). This may be caused by a direct inhibition of lymphocyte responses (Vogt et al., 2006) but others have reported that CRIg expression in DC may lead to an immunosuppressed response or tolerance (Xu et al., 2009, Fu et al., 2012). The results in Chapter 4 showing that cytokines alter the expression of CRIg on DC are of interest and potential importance. CRIg expression on DC was increased by TGF-β1, IL-10, M-CSF and GM-CSF. In comparison, LT-α, IFN-γ, IL-4, IL-13, TNF, IL-1β and IL-6 decreased expression (Table 4.1). In this manner the cytokines could participate in tolerogenic versus immunogenic responses, respectively through their ability to alter expression of CRIg on DC.

tDC can be generated by immuno-suppressive cytokines including IL-10, TGF-β (Geissmann et al., 1999, Steinbrink et al., 2002, Torres-Aguilar et al., 2010, Tai et al., 2011), and immunomodulatory drugs such as dexamethasone (Unger et al., 2009). This

has relevance to attempts to develop tDC therapy (Morelli and Thomson, 2007, Thomas, 2013). It has been reported that the induction of TGF-β and IL-10 in dendritic cells attenuated the severity of IBD, by using astilbin in dextran sodium sulphate (DSS)-induced murine colitis model (Ding et al., 2014). Astilbin, possess anti-inflammatory properties and immunosuppressive activity (Huang et al., 2011, Ding et al., 2014) and was associated with the regulation of DC function both *in vivo* and *in vitro* (Ding et al., 2014). The administration of astilbin led to increased levels of IL-10^+ DCs and TGF-β^+ DCs and decreased the number of IL-$1\beta^+$ DCs (Ding et al., 2014). Other reports also reveal the immunosuppressive effects of astilbin (Cai et al., 2003, Yi et al., 2008), showing stimulation of IL-10 and decreased production of TNF and IFN-γ in contact dermatitis (Fei et al., 2005), and reduced TNF production in Jurkat cells (Yi et al., 2008). In this thesis the regulatory and immuno-suppressive cytokine IL-10 caused a substantial increase in CRIg mRNA and corresponding CRIg protein in macrophages and DC. Dexamethasone treated DC (dxDC) generate tDC that have reduced alloantigenic capacity, higher IL-10 secretion can inhibit Th2 differentiation of naïve $CD4^+$ T cells in NRL-allergic patients (Escobar et al., 2014).

In Chapter 4 another protein band was identified in the Western blots in DC which does not correspond to the two forms of CRIg. As this protein was identified by a CRIg specific monoclonal antibody, it is likely that this represents another spliced variant of CRIg which has been called intermediatory (I) in this thesis. This protein was also regulated by cytokines.

6.5 Cytokines induce changes to CR3 and CR4 expression on macrophages

In Chapter 3 it was also demonstrated that the cytokines which altered CRIg expression in macrophages, caused changes to the expression of CR3 and CR4. It is evident from

these results that some cytokines had different effects on these three receptor types (Table 3.1). Because these receptors perform different functions their differential expression caused by cytokines will have an impact on the final response precipitated during microbial interaction as well as inflammation. While IL-4 and TGF-β1 promoted the development of CR3 expressing macrophages, the development of CR4 expressing macrophages was promoted by LT-α, IFN-γ, IL-4, IL-13, IL-1β and TGF-β1. Thus although CR3 may be decreased on macrophages subjected to LT-α, IFN-γ, IL-13 and IL-1β their phagocytic function is likely to be retained through the up regulation of CR4 by these cytokines. In comparison to this scenario, IL-4 and TGF-β1 promote the development of macrophages with increased expression of both CR3 and CR4; increasing the potential phagocytic capability of the macrophage. These *in vitro* models, mimick the monocyte invasion of tissues and their development into macrophages to interact with complement opsonised microbial pathogens or altered self-tissues. Macrophage development towards cells with lower phagocytic activity may occur when the same cytokines cause a decrease in expression of both CR3 and CR4. Cytokines which gave rise to this decrease were TNF, IL-10, GM-CSF, M-CSF and IL-6.

Examination of effects of cytokines on human monocytes developing into mature macrophages and MDM, demonstrated a different pattern of increases and decreases in CR3 and CR4. The ability of cytokines to regulate these receptors provides a second point of regulating macrophage function in inflammation, depending on the inflammation type and cytokines generated. The cytokines LT-α, IFN-γ, IL-1β, IL-6, M-CSF and GM-CSF decreased CR3 expression on macrophages. This decrease in CR3 cannot be compensated by CR4, in the case of M-CSF and GM-CSF as these inhibit both the development of $CR4^{+}$ macrophages and decreased CR4 expression on mature macrophages.

Cytokines also had effects on CR3 and CR4 expression on DC. However in this case there was a uniform down regulation effect on the two receptors apart from IFN-γ, GM-CSF and IL-13 which caused an increase. Presumably cytokines which cause an increase in CRIg expression together with reduced CR3/CR4 expression may promote a tolerogenic functional phenotype e.g. IL-10, TGF-β1, GM-CSF and dexamethasone.

6.6 Concluding remarks

6.6.1 Highlights from the thesis

1. A range of cytokines TGF-β1, IL-10, M-CSF, GM-CSF, LT-α, IFN-γ, IL-4, IL-13, TNF, IL-1β and IL-6 which are known to be generated during infection and immunity, autoimmune inflammation and allergy and known to modulate macrophage function were found to regulate CRIg expression on macrophages, as summarised in Tables 3.1 and 4.1.
2. The cytokines examined altered the expression of CRIg in human monocytes developing into macrophages, and the expression of CRIg on MDM and on DC. This has enabled us to place perspectives on how cytokines may work via CRIg in innate and adaptive immunity. It is evident from this thesis that there exists two points of inflammation control. In the first control point cytokines promote or inhibit the infiltrating monocytes to develop into macrophages expressing different levels of CRIg. While at the second control point cytokines regulate CRIg expression levels on mature macrophages. This suggests that cytokines regulate CRIg expression in tissue resident macrophages, perhaps maintaining homeostasis to enable the first and effective encounter with bacteria and then further controlling the mature macrophages arising during inflammation.

3. Because CR3 and CR4 interact with complement components as does CRIg, their co-expression with CRIg is of functional importance since CRIg differs in function and delivers a more efficient phagocytosis system and immunosuppressive axis of adaptive immunity. This thesis revealed for the first time the modulation of the development of $CR3^+$ and $CR4^+$ macrophages from monocytes by cytokines and their modulation on mature macrophages.

    - LT-α promotes CRIg expression at both control points in association with decreased expression of CR3 and to some extent CR4. This should enable effective phagocytosis of bacteria with limited pathology. A similar effect can be deduced for M-CSF.
    - In comparison, IFN-γ depressed CRIg and CR3/CR4 expression, essentially at both control points. The consequences of this IFN-γ induced down regulation of complement receptors is not clear but would indicate that the cytokine *per se* is likely to reduce the phagocytic activity of macrophages.
    - If emphasis is placed on the second control point of inflammation then it is evident from the data presented here that the majority of the cytokines, apart from LT-α and M-CSF caused a decrease in CRIg expression in MDM. The cytokines IL-4, TGF-β1, IL-1β and IL-6 caused the down regulation of all three complement receptors, suggesting that these may compromise bacterial phagocytosis. Further analysis of these findings showed that IL-13, IL-10 and TNF while promoting phagocytosis by increasing CR3 and CR4 expression are also likely to induce a highly inflammatory response (Schif-Zuck et al., 2011), particularly as they cause a co-decrease in CRIg expression.

4. While in MDM only LT-α and M-CSF increased CRIg expression, in DC CRIg expression was increased by TGF-β1, IL-10, M-CSF, GM-CSF. Thus these cytokines may promote the development of tolerogenic DC, particularly TGF-β1, IL-10 and M-CSF which co-decreased CR3 and CR4 (see Chapter 4).

5. The relationship between CRIg expression and phagocytosis was examined for MDM. The results showed that the cytokines caused a corresponding change in phagocytosis of complement opsonised *C. albicans*, irrespective of the changes in CR3 and CR4. These findings support the view that CRIg is the most important phagocytosis promoting receptor for complement opsonise microbial pathogens (Helmy et al., 2006, Gorgani et al., 2008).

6. The effects of cytokines were evident both at the level of CRIg mRNA and protein. This suggested that the main control is at a pre-transcriptional level. Indeed in the case of TNF the down regulatory effects in MDM was at the level of PKCα (Chapter 5).

7. This thesis demonstrated the presence of both spliced forms of CRIg in human macrophages, L and S forms. In addition another spliced form of CRIg appeared to be present in DC. All of these were similarly affected by cytokines, supporting the above statement that effects were occurring at the transcriptional level.

8. The effects displayed by cytokines such as, TNF versus IL-10 on CRIg, CR3 and CR4, along with their effects on DC versus MDM would at least in part explain their pathogenesis-versus protection-inducing properties in diseases such as RA. Indeed the data presented here showed that TNF was a major autocrine controlling cytokine for down regulating CRIg expression and that this could be prevented by adding anti-TNF antibody to macrophages.

6.6.2 Limitations of the research and future directions

1. First and foremost, because of time pressures the changes in complement receptors were not fully studied in regards to the co-responding functional activities of phagocytosis, cytokine production and antigen-presenting function, although some functional aspects were presented in Chapter 3 and Chapter 5.
2. The finding of an additional protein band in Western blots following staining with anti-CRIg antibody identified potentially another spliced form of CRIg but its identity was not elucidated or any reasoning for why it is selectively expressed in DC.
3. The mechanisms by which cytokines cause opposing effects on CRIg expression versus CR3 and CR4 was not examined.
4. The effect of combined addition of cytokines (and also the combined addition of dexamethasone and cytokines) was not given consideration in the present study but is of key importance as cytokines are produced in certain pattern types.
5. These results have been generated using an *in vitro* model and care needs to be taken in trying to extrapalate these findings to an *in vivo* inflammatory reaction.
6. Finally, the monocyte/macrophage populations were not absolutely pure, raising the potential for cytokines to act via the contaminating cells such as T cells to indirectly affect the expression of CRIg on macrophages.

Acknowledging the limitations listed above the work described in this thesis not only increases our knowledge of the immune-biology of CRIg but is also likely to lead to a better interpretation of action of anti-inflammatory drugs including anti-TNF therapy in diseases such as rheumatoid arthritis (RA).

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