Investigation of the expression and biological response of the IL-1Rrp2 receptor in human cells

By

Shilla Mutamba, MSc, FIBMS

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ABSTRACT

Dysregulated IL-1 activity has been implicated in the pathogenesis of several acute and chronic inflammatory as well as autoimmune diseases. Genomic analysis has identified additional IL-1 family members and also expanded the IL-1 receptor family. To date, there are eleven IL-1 family members and nine IL-1 receptor family members. Information regarding the biological activity and immunological role of the newer IL-1 family members is still very limited. The novel IL-1 cytokines, IL-1F6, IL-1F8 and IL-1F9 (recently renamed IL-36α, IL-36β and IL-36γ respectively) have been shown to signal via IL-1 receptor related protein 2 (IL-1Rrp2, recently renamed IL-36R). The main aims of this study were: (i) to investigate the expression of IL-1Rrp2 by human myelomonocytic and non-myelomonocytic immune cells as well as other human cells, (ii) to determine the possible function of IL-1Rrp2 and (iii) to determine the effect of IL-1Rrp2 expression on T lymphocytes. Results reported in this thesis indicate that among human myelomonocytic cells, constitutive IL-1Rrp2 expression is unique to dendritic cells (DCs). IL-1Rrp2 was expressed by monocyte- derived DCs (MDDCs) and plasmacytoid DCs (pDCs) but not by peripheral blood type 1 or type 2 myeloid DCs (mDC1 or mDC2). IL-1Rrp2 expression was regulated in response to both classical (IL-1β) and novel IL-1 cytokines (IL-1F8 and IL-1F9). Similarly to IL-1β and bacterial LPS, novel IL-1 cytokines mediated DC maturation as shown by DC phenotypic changes (e.g. upregulation of HLA-DR

expression and decreased CD1a expression following culture of DCs with IL-1F8) and cytokine production. Among non-myelomonocytic cells, constitutive IL-1Rrp2 expression was observed in lamina propria tissue, T lymphocytes, NCI/ADR-RES cells and HT 29 cells. IL-1F8-matured human DCs were fully functional as they induced proliferation of IFN-γ-producing T_H1 subsets. Results suggest that novel IL-1 cytokines play a role in inflammatory responses involving human DCs, with possible implications for inflammatory disease.

PUBLICATIONS

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Human monocytoid CD209/CD14 positive blood cells are functionally distinct from MDDCs which express IL-1Rrp2 and are phenotypically matured by IL-1F6.

Shilla Mutamba[*], Asher Allison[*], Yashwant Mahida**, Paul Barrow[*] and Neil Foster*

School of Veterinary Medicine and Science*[1] and the Institute of infection, immunity and inflammation,[**] University of Nottingham, UK

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DECLARATION

The research presented in this thesis was carried out by the author at the University of Nottingham between January 2008 and March 2011.

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CHAPTER 1: GENERAL INTRODUCTION AND BACKGROUND

1.1.0 The Interleukin-1 (IL-1) Cytokine Family: General Overview

The IL-1 cytokine family is a multifunctional cytokine family with multiple and varied effects on biological processes. IL-1 cytokines influence a wide array of physiological activities including the immune response, haematopoiesis, appetite, sleep and bone metabolism (Dinarello, 1996; Bagby, 1989; Smith *et al*., 1992; Tatakis, 1993; Fischer *et al*., 1991; Okusawa *et al*., 1988). IL-1 cytokines interact with nearly every cell type and play an essential role in host immune defence through their effect on T and B lymphocytes.

The classical IL-1 family members; IL-1α, IL-1β, IL-1Receptor antagonist (IL-1Ra) and IL-18 have been studied for a number of years. Their importance in health as well as in chronic disease has been highlighted through a number of studies. In experimental animals, for example, pre-treatment with IL-1β has been shown to reduce mortality from bacterial and fungal infections (Van der Meer *et al*., 1988). IL-1α administration has also been found to accelerate platelet recovery following chemotherapy in humans (Smith *et al*., 1993). On the other hand, studies in human patients with advanced solid malignancies have shown that dose-dependent side effects such as fever, gastrointestinal disturbances, myalgia (muscle pain), arthralgia (joint pain) and hypotension may limit the therapeutic utility of IL-1α in patients (Smith *et al*., 1992). Administration of IL-1α

to healthy animals induces physiological effects mimicking metabolic abnormalities present in human patients with catabolic diseases such as sepsis (Dinarello, 1996; Cooney *et al*., 1999a; Mitch, 2000).

It is now generally accepted that IL-1α, IL-1β and IL-18 are highly inflammatory cytokines and that their excessive production results in local tissue damage (reviewed in Dinarello, 2009). Under normal circumstances, the production and activity of IL-1 cytokines is tightly regulated and contributes to host defence mechanisms. On the other hand, dysregulated IL-1 activity has been implicated in the pathogenesis of diseases such as the septic syndrome, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), insulin-dependent diabetes mellitus (IDDM), atherosclerosis, psoriasis, asthma, cancer and many other autoimmune and/or chronic inflammatory conditions (Bistrian *et al*., 1992; Okusawa *et al*., 1988; Fischer *et al*., 1991; Fisher, 1991; Dinarello, 1996; Dinarello, 2002; Dinarello, 2003a; Dinarello 2003b; Arend, 2008).

Diseases associated with excessive IL-1 production and/activity have a huge impact on the quality of life of the sufferers. They also incur a huge social and economic burden with regards to mortality, morbidity and long-term disability. In the United States of America, sepsis has a mortality rate of 20-50% and is the second leading cause of death in non-coronary intensive care unit (ICU) patients. It is also the tenth most common cause of death overall. Care of

patients with sepsis costs $17 billion annually (Wheeler, Bernard, 1999; Angus *et al*., 2001; Martin *et al*., 2003). In the United Kingdom (UK), sepsis poses a medical challenge. The proportion of critical care admissions with severe sepsis in the first 24 hours following admission rose from 23.5% in 1996 to 28.7% in 2004 (Harrison *et al*., 2006).

The role of IL-1 in the pathogenesis of arthritis has been confirmed by *in vivo* studies (Gracie *et al*., 1999). Arthritis, in its various forms, is thought to affect 1 in 5 adults in the UK (McCormick *et al*., 1995). The total cost of arthritis to the National Health Service (NHS) and social services in the UK is believed to be in the region of £5.5 billion (Arthritis Care, 2002). Osteoarthritis affects over eight million people and is the single biggest cause of locomotor problems and the most common joint disease in the UK (de Verteuil *et al*., 2008).

About 387 000 people in the UK have RA, and 12000 new cases are diagnosed each year (Symmons *et al*., 2002). Prevalence of RA in many populations is believed to be 0.5-1%. Higher frequencies of 5.3% and 6.8% have been observed in the Pima Indians and Chippewa Indians respectively and also in a few other populations (Silman, Pearson 2002; Del Puente *et al*., 1989; Harvey *et al*., 1981). RA patients have a significantly increased risk of mortality compared with age- and sex-matched controls without RA from the same community (Gabriel, 2001). RA imposes a substantial economic burden for individuals and

health services. Direct and indirect costs per person with RA are thought to total approximately US$11 542 per year on average (Cooper, 2000).

IL-1 is now known to be a key mediator of inflammation and tissue destruction in RA (Gracie *et al*., 1999). Anti-cytokine therapeutic strategies are being employed for reducing disease severity in conditions such as RA, IBD and psoriasis. More than 300 000 people in the world are currently receiving medication which specifically blocks the biological activities of tumour necrosis factor (TNF) or IL-1. Reducing the biological activities of IL-1 and TNF is accomplished by several different, but highly specific, strategies, which involve neutralizing antibodies, soluble receptors, receptor antagonist, and inhibitors of proteases that convert inactive precursors to active, mature molecules (Dinarello, 2000; Dinarello, 2003a; Dinarello 2003b). Blocking IL-1 action through the use of Anakinra (a recombinant IL-1Receptor antagonist) has been shown to be beneficial in patients with active RA. Anakinra is now approved for the treatment of RA and is commercially supplied by Amgen as Kineret (reviewed in Fleischmann, 2002, reviewed in Furst, 2004; reviewed in Fleischmann *et al.,* 2004). It has also been found to be effective in a proportion of patients with systemic onset juvenile idiopathic arthritis and adult-onset Still's disease (Chevalier *et al.,* 2005; Church *et al.,* 2008).

Evidence of anakinra's efficacy in diseases other than RA has not been consistent. Initial studies involving psoriatic arthritis models failed to demonstrate the efficacy of anakinra, however, recent findings suggest that it may be a therapeutic option in patients with active psoriatic arthritis (Jung *et al.,* 2010). Use of recombinant IL-1Ra (rIL-1Ra) for treating sepsis has been extensively studied. Although, studies in mice, rats, rabbits and baboons with septic shock generally confirmed that administration of rIL-1Ra reduced mortality dramatically, the effects of rIL-1Ra were not uniform in all cases. Furthermore, clinical trials have not yet validated results obtained from animal models of sepsis or septic shock. Although invaluable information has been generated from studies involving animals, it is impossible for animal models to completely mimic the local and systemic picture of human sepsis (reviewed in Dinarello, 1996). Furthermore, the failure of rIL-1Ra and other anti-cytokine strategies to convincingly improve clinical outcomes in human sepsis patients suggests that there may be other cytokines or factors involved in the pathogenesis of sepsis.

In recent years, DNA database searches for IL-1 homologues have revealed that IL-1 is actually a superfamily of cytokines which are related in terms of origin, receptor structure and signal transduction pathways employed (Arend *et al*., 2008). Genomic analysis has not only revealed the existence of seven additional IL-1 family members but it has also identified an IL-1 receptor superfamily (Taylor *et al*., 2002, Barksby *et al*., 2007; Magne *et al*., 2006; Dunn *et al*., 2001; Smith *et al*., 2000; Kumar *et al*., 2000; Barton *et al*., 2000; Busfield

et al., 2000; Pan *et al*., 2001; Bazan *et al*., 1996; Lin *et al*., 2001; Debets *et al*., 2001; Blumberg *et al*., 2007; Nicklin *et al*., 2002; Sims *et al*., 2001; Schmitz *et al*., 2005; Sims, 2002; Subramaniam, Stansberg, Cunningham, 2004; Dumont, 2006).

Due to the impact of classical members of the IL-1 family on inflammatory and autoimmune diseases, the potential role of these novel IL-1 family members is now being studied and may present future opportunities for the development of novel therapeutics. As previously mentioned, anakinra (rIL-1Ra) is currently used in the treatment of RA (Furst, 2004; Cohen and Rubbert, 2003). It is, therefore, conceivable that scientific study of novel members of the IL-1 family may lead to the discovery of novel therapeutics in future.

1.1.1 IL-1 cytokine family: Introduction

Prior to its molecular identification, IL-1 had been studied for many years under various names including leukocyte endogenous mediator, haematopoietin 1, endogenous pyrogen, catabolin and osteoclast activating factor. These names give an idea of the variety of biological effects exerted by this interleukin (Sims and Smith, 2010). As already mentioned in the general background section (Section 1.1), research spanning the last three decades has revealed that IL-1 is actually a cytokine family consisting of structurally related, multifunctional

proteins which influence various cell types and play an important role as mediators of inflammatory and immune responses. Generally, IL-1 family members possess a highly conserved gene structure, including identical positioning of certain introns and a more modest preservation of key amino acid sequences that allow the folding of each protein into a twelve-stranded β-barrel (Taylor *et al*., 2002; Sims, 2002). To date, the IL-1 cytokine family comprises eleven members. As previously mentioned, four of these are commonly referred to as the classical IL-1 members while the other seven are still considered novel.

The genes for IL-1α, IL-1β and IL-1Ra are located in the same region on the long arm of human chromosome 2 (Smith *et al*., 2000) while the gene for human IL-18 is located on chromosome 11q22.2-22.3 (Nolan, Greaves and Waldmann, 1998). Despite low sequence homology, IL-18 is now widely accepted as the fourth member of the IL-1 family, not only because of the high structural similarity but also because of similarities in receptor utilisation and cytokine processing. IL-1α, IL-1β and IL-18 also share parts of a common signal transducing system which involves IL-1 receptor-associated kinases (IRAKs), MyD88, TNF receptor-associated factor (TRAF-6) and activation of the transcription factor NF-kB (Nuclear Factor-KappaB).

Genes encoding the newer members of the IL-1 cytokine family were identified by different research groups who inevitably assigned different names to the

same cytokines (Dunn *et al*., 2001; Smith *et al*., 2000; Kumar *et al*., 2000; Busfield *et al*., 2000; Barton *et al*., 2000; Pan *et al*., 2001; Lin *et al*., 2001; Debets *et al*., 2001). The genes for six of these novel IL-1 cytokines are situated in the same cluster as those for IL-1α, IL-1β and IL-1Ra on human chromosome 2 (Lander *et al*., 2001). A revised, standardised nomenclature was proposed by Sims and co-workers (2001). Under this new nomenclature, the six novel IL-1 family members whose genes reside in the same cluster as the IL-1α, IL-1β and IL-1Ra genes were named IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9 and IL-1F10. It was also suggested that the classical IL-1 members, IL-1α, IL-1β, IL-1Ra and IL-18 be renamed IL-1F1, IL-1F2, IL-1F3 and IL-1F4 respectively (IL-1F stands for IL-1family member) (Sims *et al*., 2001), however, the old naming system for the classical IL-1 family members is still widely used and accepted.

Schmitz *et al.* (2005) demonstrated that IL-33 (located on the short arm of chromosome 9 at position 24.1) has structural and functional similarities to the other IL-1 family members and is, therefore, an IL-1 cytokine. It has since been renamed IL-1F11 (Schmitz *et al*., 2005). Not much is known about the expression, biological function or regulation of expression of the novel IL-1 family members. In the following sections, current understanding regarding IL-1 family members will be discussed. The classical IL-1 family members will be discussed first, followed by emerging data regarding the newer IL-1 family members. The revised nomenclature proposed by Sims *et al.* (2001) for IL-1 family members will be used throughout this thesis when referring to the newer IL-1 family

members. For the classical members, IL-1α, IL-1β, IL-1Ra and IL-18, the original names will be adhered to as these are well established and are still commonly used in the literature.

1.1.2 Classical IL-1 cytokines

The synthesis of classical IL-1 cytokines is stimulated by a number of pro-inflammatory mediators including exposure to microbial products such as bacterial lipopolysaccharides (LPS) and exposure to proinflammatory cytokines such as Tumour Necrosis Factor alpha (TNF-α), Interferon alpha (IFN-α) and Interferon beta (IFN-β) (reviewed in Barksby *et al*., 2007) Neuroactive and inflammatory substances, cell matrix proteins, clotting factors, lipids and other biological materials also induce IL-1 synthesis (Dinarello, 1991). It has also been observed that environmental stressors such as hypoxia, ischaemia, gamma radiation and thermal injury may also stimulate IL-1 synthesis (Dinarello, 1996). In patients with sepsis, trauma and burn injury, plasma IL-1β levels correlate with both the severity of inflammation and mortality (Cannon *et al*., 1990; Casey *et al*., 1993; Eastgate *et al*., 1988; Roubenoff *et al*., 1994), thus indicating an important role for this cytokine during these pathologies.

IL-1α, IL-1β and IL-18 are secreted by various cell types including monocytes, macrophages, dendritic cells (DCs), epithelial cells, keratinocytes and synovial fibroblasts (reviewed in Dinarello, 1998). IL-1α is synthesised as a 33kDa protein

precursor (proIL-1α) which lacks a leader sequence. Proteolytic removal of the precursor domains by calcium-dependant calpain proteases results in the generation of the mature 17kDa IL-α peptide. Both proIL-1α and mature IL-1α are biologically active (reviewed in Dinarello, 1998).

IL-1β is also translated as an inactive, 33kDa, leaderless procytokine (pro IL-1β). It remains in the cytosol prior to being cleaved intracellularly by the cysteine-protease, IL-1β converting enzyme (ICE or caspase-1) (reviewed in Burns, Martinon and Tschopp J, 2003; reviewed in Dinarello, 1998). The active, mature 17kDa IL-1β is subsequently released extracellularly. It has been reported that two separate stimuli are required for efficient generation of active IL-1β. There is an initial priming stimulus (e.g. LPS) which promotes synthesis of proIL-1β and its accumulation in the cytosol. Post-translational processing of pro-IL-1β (by caspase-1) and release of mature IL-1β is initiated by a second stimulus, the absence of which leads to insufficient maturation of IL-1β (reviewed in Burns, Martinon and Tschopp J, 2003; reviewed in Dinarello, 1998). IL-18 shares primary amino acid sequences and structural features with IL-1β. It is also synthesised as a biologically inactive, 24 kDa precursor (pro-IL-18) which lacks a signal peptide (Dinarello, 1996; Dinarello, 1997). Caspase-1 cleaves pro-IL-18 to its mature, 18kDa bioactive form (Dinarello, 1996; Dinarello, 1997). There is evidence to suggest that IL-18 secretion occurs by a mechanism similar to the one observed for IL-1β (Dinarello, 2003).

1.1.2.1 The inflammasomes

It is now appreciated that caspase-1 itself is present in the cytosol as an inactive protein, pro-caspase-1 which in turn requires activation via autoproteolytic cleavage. The “secondary stimulus” that induces the production of IL-1β promotes the formation of a multi-protein molecular platform known as the inflammasome, which induces the oligomerization and activation of procaspase-1 (reviewed in Burns, Martinon and Tschopp J, 2003). The inflammasome mediates the cleavage of inactive pro-IL-1β and IL-18, as well as other proteins, into their active forms (reviewed in Schroder and Tschopp, 2010; reviewed in Barker *et al.,* 2011). Once activated, the inflammasome in turn induces activation of caspase-1 which then mediates the maturation of IL-1β. Ultimately, IL-1β is excreted into the extracellular environment (Martinon *et al.,* 2002). Extracellular adenosine triphosphate (ATP) has been found to be a strong inducer of mature IL-1β (Burns K, Martinon F, Tschopp J, 2003; Dinarello, 1998). A rapid lowering of cytoplasmic potassium ions (K^+) by stimuli other than ATP (e.g. nigericin) can also stimulate caspase-1 activation following LPS stimulation (Kahlenberg *et al.,* 2005).

Initially, the inflammasome complex was thought to affect infection and inflammation, however, recent evidence suggests that inflammasome activation influences many metabolic disorders, including atherosclerosis, type 2 diabetes, gout and obesity (reviewed in Wen *et al.,* 2012). The inflammasome consists of

an intracellular pathogen recognition receptor (PRR), the precursor procaspase-1 and the adaptor ASC (apoptosis-associated speck-like protein containing a C-terminal caspase-recruitment domain) (reviewed in Schroder and Tschopp, 2010; reviewed in Barker *et al.,* 2011). To date, four inflammasomes have been identified; NLRP1 (Nucleotide-binding, leucine-rich repeat pyrin domain containing protein 1), NLRP3 (Nucleotide-binding, leucine-rich repeat pyrin domain containing protein 3), NLRC4 (nucleotide-binding oligomerization domain, leucine rich repeat and CARD domain containing protein 4) and AIM2 (absent in melanoma 2) (reviewed in Schroder and Tschopp, 2010; reviewed in Barker *et al.,* 2011; reviewed in Wen *et al.,* 2012).

NLRP1, NLRP3 and NLRC4 contain a PRR that belongs to the Nod-like receptor (NLR) family; a family of intracellular PRRs defined by a tripartite structure (reviewed in Franchi *et al.,* 2012). NLRP3, also called NALP3 or Cryopyrin, is one of the best characterized NLR family members (reviewed in Chen *et al.,* 2011). It is encoded by the *NLRP3* (NOD-like receptor family, pyrin domain containing 3) gene, also called cold induced autoinflammatory syndrome 1 (*CIAS1*) gene (Hoffman *et al.,* 2001; reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011). Various stimuli (including muramyl dipeptide, adenosine triphosphate, toxins, *Staphylococcus aureus* or *Listeria monocytogenes*, bacterial RNA, viral infection/viral RNA) activate the NLRP3 inflammasome, triggering caspase-1 to process proIL-1β/pro-IL-18 into mature IL-1β/IL-18. In addition, metabolic substrates which accumulate in target tissues

(such as monosodium urate in gout, oxidized low-density lipoprotein in diabetes, ceramide in obesity, and cholesterol crystals in atherosclerosis) can also stimulate the NLRP3 inflammasome to release IL-1β (reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011).

1.1.2.2 IL-1 Receptor Antagonist (IL-1Ra)

The third classical IL-1 family member, IL-1Ra, functions as a specific receptor antagonist of IL-1. It was first discovered as a 22- to 25-kDa IL-1inhibitory bioactivity in the supernatants of human monocytes cultured on adherent immunoglobulin G (IgG) and in the urine of patients with fever or myelomonocytic leukaemia (reviewed in Arend *et al.,* 1998). In humans, it has been shown that the synthesis of IL-1Ra is inducible by IL-1β administration (Bargetzi *et al.*, 1993).

IL-1Ra is now understood to be a family of isoforms. The originally described isoform is now termed secreted IL-1Ra (sIL-1Ra) and is secreted as a variably glycosylated, 22-25 kDa protein. It exhibits approximately 26% amino acid sequence homology to IL-1β and 19% homology to IL-1α. It is synthesized as a precursor protein containing a classical secretory signal sequence of 25 amino acids which facilitates secretion of IL-Ra via the endoplasmic reticulum/Golgi pathway (Eisenberg *et al*., 1990). The human IL-1Ra gene contains four exons and maps to chromosome 2q13-14.1 in the vicinity of the IL-1α and IL-1β genes

(Lennard *et al.,* 1992). An 18 kDa intracellular form of IL-1Ra known as intracellular IL-1Ra (icIL-1Ra) has also been described. It is believed that there may be at least three forms of icIL-1Ra. These have been termed icIL-1Ra1, 2 and 3. They all arise from the same extended IL-1Ra gene (IL1RN) and are created by the use of alternative first exons, mRNA splicing and alternative translation initiation (reviewed in Arend *et al*., 1998; Arend, 2002).

Secreted IL-1Ra (sIL-1Ra) is primarily produced by monocytes and macrophages (reviewed in Arend *et al*., 1998). However, it can also be synthesised by any cell which produces IL-1, for example neutrophils, hepatocytes, microglial cells and other cells (reviewed in Arend *et al*., 1998). IcIL-1Ra1 is highly expressed in keratinocytes and other epithelial cells. It is also produced by stimulated monocytes and macrophages. IcIL-1Ra3 is highly expressed in hepatocytes and in the cytoplasm of bacterial lipopolysaccharide (LPS)-stimulated human neutrophils (reviewed in Arend, Palmer and Gabay, 2008). It is also present in monocytes, macrophages and keratinocytes. Although cDNA for icIL-1Ra2 has been cloned from fibroblasts, keratinocytes and human leucocytes, the predicted 25kDa icIL-1Ra2 protein has not yet been found in human cells. The *in vivo* presence of icIL-1Ra2 remains to be established (Eisenberg *et al*., 1990; reviewed in Arend *et al*., 1998; Arend, 2002). There is speculation that it may not be translated (Banda *et al*., 2005). A novel IL-1Ra variant (CGEN-R1) with a C-terminal portion that has a unique amino acid sequence has also been recently reported. CGEN-R1 was detected primarily in

the liver but was also found to be present in several human tissues. There have been speculations that it might function as an IL-1 Ra since it appears to inhibit IL-1-stimulated IL-8 (reviewed in Dumont, 2006).

As previously mentioned, IL-1 has potent, wide-ranging and potentially harmful effects on host physiology. To prevent host injury, the actions of IL-1 are normally tightly regulated, not just at the level of synthesis but also post synthesis. It is now known that, physiologically, IL-1Ra is an important regulator of IL-1 expression and of IL-1-induced responses. IL-1Ra production occurs immediately following IL-1 production. *In vitro and in vivo,* IL-1Ra is a competitive inhibitor of IL-1 receptor binding. It binds to the IL-1 receptor but because it cannot recruit the IL-1R Accessory protein (IL-1RAcP) which is crucial for signal transduction, IL-1Ra is unable to initiate intracellular signal transduction processes which normally occur after engagement of the IL-1 receptor with IL-1. Not only is IL-1Ra unable to induce intracellular signalling, but also, it blocks IL-1R1 and prevents it from interacting with IL-1α/β. IL-1Ra thus functions as a competitive inhibitor of IL-1 and is important in the regulation of the inflammatory effects of IL-1 (Smith *et al*., 2000; Sims, 2002; Dumont, 2006). It antagonizes the activities of both IL-1α and IL-1β without exerting agonist effects itself (Hannum *et al.,* 1990; Dipps *et al.,* 1991; reviewed in Arend, 2002).

Generally, the balance between IL-1 and IL-1Ra is thought to play an important

role in the normal physiology of various organs and tissues. It is even thought to regulate some aspects of the reproductive cycle (reviewed in Arend, 2002). The effect of IL-1 and IL-1Ra levels has been extensively studied in a variety of experimental animal models of disease including arthritis, infectious diseases, inflammatory bowel disease (IBD), granulomatous and fibrotic lung disorders, kidney diseases, diseases of the liver and pancreas, graft-versus-host disease (GVHD), leukaemia and different types of cancer, osteoporosis and diabetes (reviewed in Arend, 2002). Similar studies have also investigated central nervous system diseases, infectious diseases, and arterial diseases (Koch *et al*., 1992; Simón et *al*., 1998; Bendele *et al*., 1999). A consistent finding from all these studies was that either local overproduction of IL-1 and/or underproduction of IL-1Ra are a prerequisite for disease development. In these studies, therapeutic administration of IL-1Ra was found to be efficacious in preventing tissue damage (Koch *et al*., 1992; Simón et *al*., 1998; Bendele *et al*., 1999; reviewed in Arend, 2002). A summary of animal models of disease treated with recombinant IL-1Ra is given in Table 1.1.

Both the secreted and intracellular isoforms of IL-1Ra are thought to contribute to maintenance of the balance between IL-1 and IL-1Ra. There is ample evidence indicating that the single biological function of sIL1Ra is to competitively inhibit IL-1 binding to cell-surface receptors. On the other hand, the intracellular isoforms of IL-1Ra are thought to carry out additional roles inside cells (reviewed in Arend and Guthridge, 2000). It was previously suggested that

these intracellular isoforms may be storage forms that are released upon cell death to limit inflammation caused by cell debris (Muzio *et al.,* 1999). Others have suggested that the intracellular forms of IL-1Ra function as unique intracellular inhibitors which alter IL-1-inducible gene expression and abate IL-1 responses at a point downstream of the initial IL-1/IL-1 receptor interaction (Watson *et al.,* 1995).

Table 1.1 Summary of animal models of disease treated with recombinant IL-1Ra (adapted from Arend *et al*., 1998)

Collagen induced arthritis in mice
Streptococcal cell wall induced arthritis in rats
Immune complex induced arthritis in mice
Antigen induced arthritis in rabbits
Osteoarthritis in dogs
Septic shock in rabbits, baboons, rats and mice
Bacterial meningitis in rabbits
Ischaemic brain injury in rats
Experimental allergic encephalomyelitis in rats
Streptococcal cell wall induced colitis in rats
Experimental shigellosis in rabbits
Immune complex induced colitis in rabbits
Acetic acid induced colitis in rats
Lipopolysaccharide induced pleurisy in rabbits
Monocrotaline induced pulmonary hypertension in rats
Allergen induced late asthmatic reaction in guinea pigs
Bleomycin or silica induced pulmonary fibrosis in mice
Immune complex induced lung injury in rats
Ischaemia/reperfusion lung injury in rats
Crescentic glomerulonephritis in rats
Anti-glomerular basement membrane antibody induced glomerulonephritis in rats
Pre-term delivery in mice induced by IL1
Osteoclast formation and bone resorption in ovariectomised mice and rats
Hepatic fibrosis induced by dimethylnitrosamine in rats
Post-cardiac transplant coronary arteriopathy in piglets
Graft versus host disease in mice
Streptozotocin induced diabetes in mice

Although the biological roles of the intracellular IL-1Ra isoforms are still not very clear, some of the functions are starting to be elucidated. A study which sought to determine the role of icIL-1Ra1 in regulation of cytokine-induced IL-6 and IL-8 production in Caco-2 intestinal epithelial cells showed that icIL-1Ra1 inhibited IL-1-induced IL-6 and IL-8 production by inhibiting p38 MAP kinase phosphorylation and Nuclear Factor –kappaB (NF-κB) translocation (Garat and Arend, 2003). Another investigation to determine whether icIL-1Ra1mediated unique functions in keratinocyte cell lines (KB and A431) showed that icIL-1Ra1 performed an anti-inflammatory role inside cells through binding to CSN3 (the third component of the COP9 signalosome complex) with subsequent inhibition of the p38 MAPK signal transduction pathway resulting in inhibition of IL-1α-induced IL-6 and IL-8 production (Banda *et al.,* 2005).

1.1.2.3 Deficiency of IL-1Receptor Antagonist (DIRA)

Mutations in *IL1RN,* the gene encoding IL-1Ra, result in a rare autosomal recessive autoinflammatory disease of skin and bone known as Deficiency of IL-1Receptor Antagonist (DIRA) (reviewed in Ferguson and El-Shanti, 2007; Aksentijevich *et al.,* 2009). The main symptoms of the disease are skin inflammation and bone inflammation present from birth. In affected human subjects, the skin inflammation, which comes on spontaneously and can affect every part of the body, is characterized by redness, pustules and scaling. The bone inflammation typically involves the periosteum (the membrane covering the bone) and is characterized by painful bony swellings, often with the overlying

skin being reddened and warm. Many bones can be affected, including the limbs and the ribs. The nails of DIRA-patients can also become deformed (Aksentijevich *et al.,* 2009). Other organs, such as the lungs may also be involved. There is no evidence of infection, high-titre autoantibodies, or autoreactive T cells (Aksentijevich *et al.,* 2009; reviewed in Goldbach-Mansky, 2012). Untreated, DIRA may lead to severe disability and even death (Aksentijevich *et al.,* 2009).

DIRA is caused by missense and non-sense mutations in the *IL1RN* gene leading to either absence of protein expression or expression of a non-functional IL-1Ra protein (Aksentijevich *et al.,* 2009; reviewed in Goldbach-Mansky, 2012). This is in turn causes hyper-responsiveness to IL-1β stimulation and uncontrolled IL-1α/IL-1β signalling which drives overproduction of proinflammatory cytokines and chemokines ((Aksentijevich *et al.,* 2009; reviewed in Dinarello *et al.,* 2012; reviewed in Goldbach-Mansky, 2012). Unopposed IL-1 signalling due to IL-1Ra deficiency has been shown to induce both $T_H1$and IL-17-producing T_H17 cells in murine models (Koenders *et al.,* 2008). Daily injection with anakinra is the only therapy that has been effective in the treatment of DIRA. It results in rapid clearance of skin lesions and resolution of systemic inflammation (Aksentijevich *et al.,* 2009; reviewed in Dinarello *et al.,* 2012).

1.1.2.4 *IL1RN* gene polymorphism

The second intron of the *IL-1RA* gene contains an 86 base pair (86bp) tandem repeat sequence which is repeated two to six times in different people. Allele 1 (IL1RN*1) contains four copies, allele 2 (IL1RN*2) has two, allele 3 has three, allele 4 has five and allele 5 contains six copies of the 86bp tandem repeat sequence in intron 2 (reviewed in Witkin *et al.,* 2002). There are differences in the frequency of the individual alleles based on ethnic or geographical differences. Generally, allele 1 (IL1RN*1) is the most common followed by allele 2. Alleles 3, 4 and 5 occur in less than 1% of most populations. In the populations studied to date, the majority of people have been found to be either IL1RN*1 homozygotes or IL1RN*1/IL1RN*2 heterozygotes. The prevalence of IL1RN*2 homozygotes is typically less than 10% (reviewed in Witkin *et al.,* 2002; reviewed in Arend, 2002; reviewed in Dinarello, 2011).

IL-1RA genotype appears to influence the concentrations of IL-1α/IL-1β and IL-1RA proteins produced (reviewed in Witkin *et al.,* 2002; Langdahl *et al.,* 2000; El-Omar *et al.,* 2000). *IL1RN* gene polymorphisms are associated with several markers of systemic inflammation (Reiner *et al.,* 2008). IL1RN*2 homozygotes have a more prolonged and more severe proinflammatory immune response than people with other IL-1RA genotypes (reviewed in Witkin *et al.,* 2002). In addition, IL1RN*2 homozygosity has been associated with inflammatory bowel diseases, alopecia areata, psoriasis, lichen sclerosus, lupus erythematosus, vulvar vestibulitis, multiple sclerosis and coronary artery disease (reviewed in

Witkin *et al.,* 2002; reviewed in Arend, 2002; reviewed in Dinarello, 2011). *IL1RN* gene polymorphism is also associated with increased risk of osteoporotic fractures (Langdahl *et al.,* 2000), gastric cancer (El-Omar *et al.,* 2000; Zhang *et al.,* 2012), schizophrenia and other human diseases which are largely of epithelial or endothelial cell origin (reviewed in Arend, 2002; Reiner *et al.,* 2008; reviewed in Dinarello, 2011).

1.1.3 Classical IL-1 cytokines: Receptors and Cell Signalling

IL-1 cytokines exert their effects through binding specific members of the IL-1 receptor (IL-1R) family. Members of the IL-1R family have related structural features and are part of a superfamily characterised by the presence of a Toll/IL-1 receptor (TIR) module in their intracellular region. They also have one to three copies of immunoglobulin (Ig)-like domains in their extracellular region (Sims, 2002; Subramaniam, Stansberg and Cunningham, 2004; Li and Qin, 2005; Dunn and O'Neill, 2003). IL-1 family receptors which lack an intracellular TIR domain function as decoy receptors or soluble binding proteins (Dumont, 2006).

Both IL-1α and IL-1β bind to the type I IL-1R (IL-1R1) to form a complex. For signal transduction to occur, a homologue of IL-1R1, known as IL-1R accessory protein (IL-1RAcP) is recruited to this complex. Both IL-1 R1 and IL-1RAcP are expressed on numerous cell types including lymphocytes, endothelial cells, fibroblasts and other cells. Recruitment of IL-1RAcP leads to the stimulation of intracellular NF-κB and mitogen-activated protein kinase (MAPK) signalling

pathways. This results in activation of the transcription of host defence effector genes in target cells, including the production of numerous cytokines, chemokines, adhesion molecules, and enzymes such as cyclooxygenase and nitric oxide synthase (Barnes and Karin, 1997; Didierlaurent *et al.,* 2006). The transcription factors which are activated by IL-1α and IL-1β include nuclear factor-κB (NFκB), activating protein 1 (AP-1), and activating transcription factor (Barnes and Karin, 1997). NF-kB is a protein complex that controls the transcription of DNA. It is a critical transcription factor for the immune and inflammatory responses since a large number of genes are NF-kB regulated (Qian *et al.,* 2001).

Once IL-1α or IL-1β has bound to its receptor complex, the cytosolic proteins, MyD88 and Tollip, are then recruited to the receptor complex. MyD88 and Tollip function as adaptors and they in turn recruit IL-1 receptor-associated kinases (IRAKs) through a homophilic interaction of the death domains (Burns *et al.,* 2000; Didierlaurent *et al.,* 2006; Brissoni et *al.,* 2006). Following phosphorylation at the receptor complex, IRAKs then associate with tumor necrosis factor receptor-associated factor 6 (TRAF6), an ubiquitin protein ligase required for IL-1-induced JNK and NF-κB activation. Subsequently, phosphorylated IRAKs are ubiquitinated and degraded probably as a means of switching off or terminating the signal. The interaction between phosphorylated IRAKs and TRAF6 leads to activation of the downstream signalling components. Transforming growth factor-β-activated kinase 1 (TAK1), which is a member of the mitogen-activated protein

kinase kinase kinase (MAPKKK) family, and two proteins that bind to it, transforming growth factor-β-activated kinase 1 binding protein 1 (TAB1) and transforming growth factor-β-activated kinase 1 binding protein 2 (TAB2), are also thought to participate in IL-1 signalling. IRAKs are also thought to mediate the IL-1-induced translocation of TAB2 from the membrane to the cytosol (Qian *et al.,* 2001). In untreated cells, TAB2 is membrane-bound but upon stimulation with IL-1, it translocates to the cytosol and functions as an adaptor, linking TRAF6 to TAK1 and TAB1, thereby activating TAK1 (Takaesu *et al.,* 2000). It is now known that after TRAF6, has formed a complex with phosphorylated IRAKs, TRAF6 then dissociates and translocates to the cytosol to form a complex with TAK1-TAB1-TAB2. There appears to be a tight correlation between the translocation of TRAF6 and the activation of downstream signalling. It is thought that the translocation of both TAB2 and TRAF6 to the cytosol is a critical step in the formation of the TRAF6-TAK1-TAB1-TAB2 complex and in the activation of these proteins. It is still not clear how IRAKs mediate the translocation of TRAF6 and TAB2. However, formation of the TRAF6-TAK1-TAB1-TAB2 complex leads to the activation of TAK1, and the phosphorylation of TAK1, TAB1, and TAB2. Ultimately, IL-1-induced NF-κB activation occurs. IRAK phosphorylation also results in the activation of other signalling cascades such as activation of p38, JNK, and others (Qian *et al.,* 2001; Kaczorowski *et al.,* 2008).

In unstimulated cells, NF-κB is sequestered in the cytoplasm in complexes with the inhibitory IκB (Inhibitors of kB) proteins. Following stimulation with IL-1

cytokines (or other inflammatory cytokines or extracellular stimuli), the IκB proteins are phosphorylated on specific serine residues, triggering their ubiquitination and subsequent degradation through the proteasome pathway. Proteolysis of IκB releases NF-κB and allows it to translocate to the nucleus, where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase, which transcribe downstream DNA into mRNA. In turn, mRNA is translated into protein (reviewed in Perkins, 2007). Ultimately, there is a change of cell function resulting from the stimulation of transcription of genes involved in inflammatory responses as well as an increase in the surface expression of costimulatory molecules on innate immune cells.

In general, stimulation of transcription of specific genes following NF-kB activation results in specific physiological responses. These could be inflammatory or immune responses, cell survival responses, or cellular proliferation (Sims, 2002; Dumont, 2006; reviewed in Perkins, 2007). A number of molecules regulate IL-1 signalling pathways. As an example, MyD88 signalling can be inhibited by MyD88s (a shorter form of the MyD88 molecule), as MyD88s expression does not induce IRAK-1 phosphorylation and it hinders IRAK4 recruitment (Burns *et al.,* 2003a). In a similar manner, IRAK-M plays a negative regulatory role by preventing release of IRAK1 and IRAK4 (Kobayashi *et al.,* 2002).

IL-1β can also bind (with a greater affinity than does IL-1α or IL-1Ra) to another IL-1receptor family member known as the type II IL-1 receptor (IL-1R2) (McMahan *et al.,* 1991; Novick *et al*., 1999). IL-1R2 is a 60-kDa protein and is found predominantly on lymphoid and myeloid cells such as monocytes, neutrophils, bone marrow cells, macrophages and B lymphocytes, myelomonocytic leukemia cells, and hairy cell leukemic cells (McMahan *et al.,* 1991; Novick *et al*., 1999; reviewed in Barksby et al., 2007). *In vitro* studies have shown that stimuli that induce IL-1 gene expression in keratinocytes also strongly induce IL-1R2 expression (Groves *et al.,* 1995). It has also been reported that IL-1-mediated inflammatory skin diseases such as psoriasis are characterized by strong epidermal expression of IL-1R2 (Groves *et al.,* 1994).

The binding of IL-1α or IL-1β to IL-1R2 does not result in signal transduction. The extracellular segment of IL-1R2 is structurally related to that of IL-1R1 in that both have three, characteristic immunoglobulin (Ig)-like domains McMahan *et al.,* 1991). Just like IL-1R1, IL-1R2 also has a transmembrane segment and a short cytoplasmic domain. However, while IL-1R1's cytoplasmic domain contains approximately 215 amino acids, IL-1R2's short cytoplasmic domain has only 29 amino acids and lacks a Toll-homology domain and, as such, is unable to initiate signal transduction (McMahan *et al.,* 1991). IL-1β binds more avidly to the non-signal-transducing IL-1R2 than it does to IL-1R1, thus, IL-1R2 is a decoy receptor for IL-1α and IL-1β. When IL-1α or IL-1β binds to the cell membrane via IL-1R2, no signal transduction occurs. Increased surface expression of IL-1R2

results in less IL-1 signalling and consequently reduces the biological response to IL-1 (Colotta *et al.,* 1993; reviewed in Colotta *et al.,* 1994).

It is thought that IL-1R2 expression is induced following IL-1-mediated inflammation as a compensatory response to down-regulate the inflammatory process (Rauschmayr *et al.,* 1997). It has been observed that cells which express high levels of IL-1R2 become unresponsive to IL-1β (Sims, 2002; Re *et al*., 1996). IL-1R2 is thought to function as a decoy receptor at two levels; the first level is the already described binding of IL-1 to cell surface-bound IL-1R2 thereby hindering the ability of IL-1 to form a signal-transducing complex with the type 1 receptor (IL-1R1) and IL-1RAcP (Colotta *et al.,* 1993; reviewed in Colotta *et al.,* 1994). The second way in which IL-1R2 is thought to function as a decoy receptor is by it being part of a trimeric complex consisting of the IL-1 ligand (IL-1α or lL-1β), IL-1R2 and IL-1RAcP. The effect of this complex is to prevent the functional IL-1 type 1 receptor from interacting with the signal-transducing IL-1RAcP chain (Lang *et al.,* 1998; Neumann *et al.,* 2000).

An extracellular, 47-kDa, soluble form of IL-1R2 also exists. It is released from the cell surface following proteolytic processing by a protease (Dower *et al.,* 1994). Soluble IL-1R2 binds to IL-1β with a higher affinity than it does to IL-1α or IL-1Ra. The binding of IL-1β to soluble IL-1R2 is almost irreversible because of a high dissociation rate and it neutralizes the biological effects of IL-1β (Dower *et*

al., 1994). While the cell surface form of IL-1R2 can only bind mature IL-1β, soluble IL-1R2 also binds the precursor form of IL-1β (pro- IL-1β) (Symons *et al.,* 1995). The distinct roles of membrane-bound IL-1R2 and soluble IL-1R2 in the control of cell activation by IL-1β are not fully understood. It is thought that all levels of regulation may occur in parallel (Neumann *et al.,* 2000). Figure 1.1 is a schematic representation of ligands and receptors of the classical IL-1 family members.

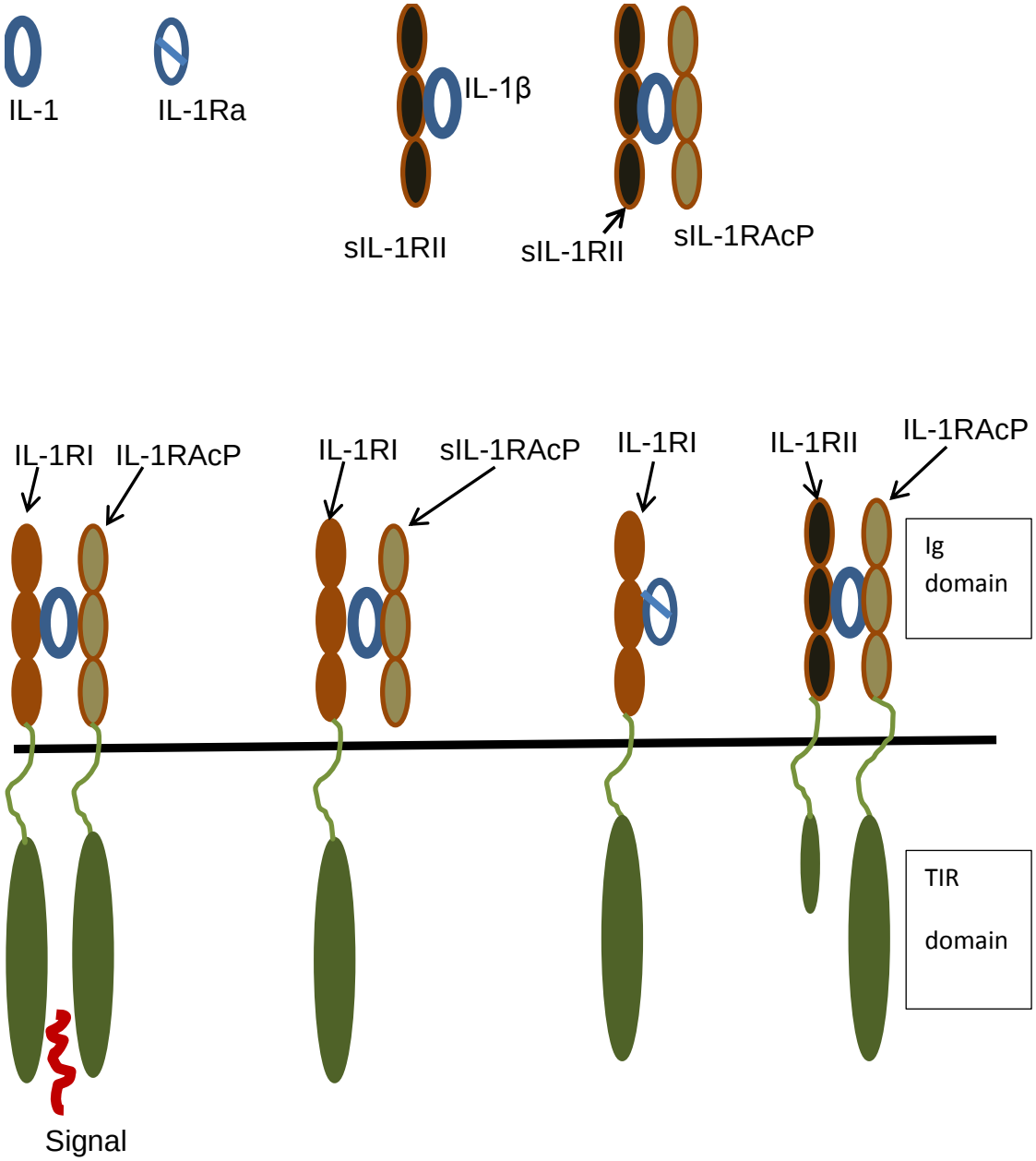


Figure 1.1 Ligands and receptors of the classical IL-1 family members. The two agonist ligands, IL-1α and IL-1β, are represented by IL-1 and the antagonist ligand by IL-1Ra. IL-1RI has a long cytoplasmic domain and, along with IL-1RAcP, activates signal transduction pathways. IL-1Ra functions as an IL-1 inhibitor by binding to IL-1R1 but not allowing interaction with IL-1RAcP. IL-1RII does not activate cells but functions as an IL-1 inhibitor both on the plasma membrane and in the cell microenvironment as a soluble receptor. IL-1RAcP can also inhibit IL-1 signals by cooperating with IL-IRII in binding IL-1 either on the plasma membrane or as a soluble molecule (adapted from Arend *et al*., 2008).

Similar to the IL-1α and IL-1β receptor complex, the IL-18 complex is a heterodimer containing an alpha (α) chain and a beta (β) chain. The IL-18Rα (also known as IL-1Rrp1) chain is responsible for extracellular binding of IL-18 while the IL-18Rβ (originally called AcPL) chain is required for signal transduction (Gracie, Robertson and McInnes, 2003, Sims, 2002). The IL-18R complex is also expressed on a variety of cells such as macrophages, neutrophils, natural killer (NK) cells, endothelial and smooth muscle cells (Gracie, Robertson and McInnes, 2003).

IL-18 also interacts with another IL-18 receptor known as IL-18 binding protein (IL-18BP). IL-18BP is a secreted protein which is distantly related to IL-1R2 and belongs to the immunoglobulin super family (Dinarello, 1996; Aizawa *et al.,* 1999; Novick *et al*., 1999). It is now known that IL-18 is down-regulated through its interaction with IL-18BP (reviewed in Barksby *et al.,* 2007; Novick *et al*., 1999). IL-18BP is constitutively expressed in many cells. Its gene expression is enhanced by IFN-γ (Kim *et al.,* 2000). Unlike the soluble IL-1α/IL-1β decoy receptor, IL-18BP has little sequence similarity with IL-18 or IL-18R (Dinarello, 1996; Aizawa *et al.,* 1999; Novick *et al*., 1999). Four human (and two murine) isoforms of IL-18BP deriving from differential splicing of the same mRNA have been identified (Kim *et al.,* 2000). IL-18BPa is the most abundant human isoform and has the greatest affinity for IL-18 (Kim *et al.,* 2000; Paulukat *et al*., 2001; Moller *et al*., 2003). IL-18BPa binds and sequesters IL-18, thereby preventing it from interacting with IL-18R. Through this competitive binding, IL-18BP acts as a

natural IL-18 inhibitor (Novick *et al*., 1999). Foster et al., (2007) reported that infection of monocytes with LPS from *Porphyromonas gingivalis* induced IL-18 and IL-18BPa secretion and that IL-18 and IL-18BPa interacted in a specific manner since addition of antibodies specific for IL-18BPa to the stimulated monocytic cell line cultures resulted in increased levels of free IL-18 (Foster *et al*., 2007). Other researchers have found that IL-18BP inhibits IL-18-induced IFN-γ and lL-8 production and NF-kB activation *in vitro* (Novick *et al*., 1999). Others have observed that LPS-induced IFN-γ production is inhibited by IL-18BP in vivo (Aizawa *et al*., 1999). Recently, another study showed that the inhibitory effect of IL-18BP on IFN-γ production can be enhanced by lL-1F7b (Bufler *et al*., 2004).

IL-18 signalling was previously thought to be similar to that for IL-1α and lL-1β and was thought to result in similar responses (reviewed in Sims, 2002). Although MyD88-IRAK-TRAF6-NF-kB signalling system is the major signalling system for IL-18, IL-1α and lL-1β, it has since been reported that lL-18 primarily signals via the MAPKp38 pathway rather than NF-kB in human epithelial cells (Lee *et al*., 2004). Since MAPK pathways are involved in cell growth, MAPK signalling is important as IL-18 is thought to exert proliferative actions on T and Natural Killer (NK) cells (Tomura *et al.,* 1998). It is now clear that different mechanisms of IL-18 gene regulation are employed, depending on the cell type. It has been suggested that the differences in the biological activities of IL-1α/β

and IL-18 can be accounted for by differences in signalling mechanisms (Lee *et al*., 2004). Figure 1.2 below is a schematic diagram showing ligands and receptors for the IL-18 family. It also shows the postulated interaction between IL-18BP and one of the newly discovered IL-1 homologues, IL-1F7. IL-1F7 will be discussed in more detail in later sections.

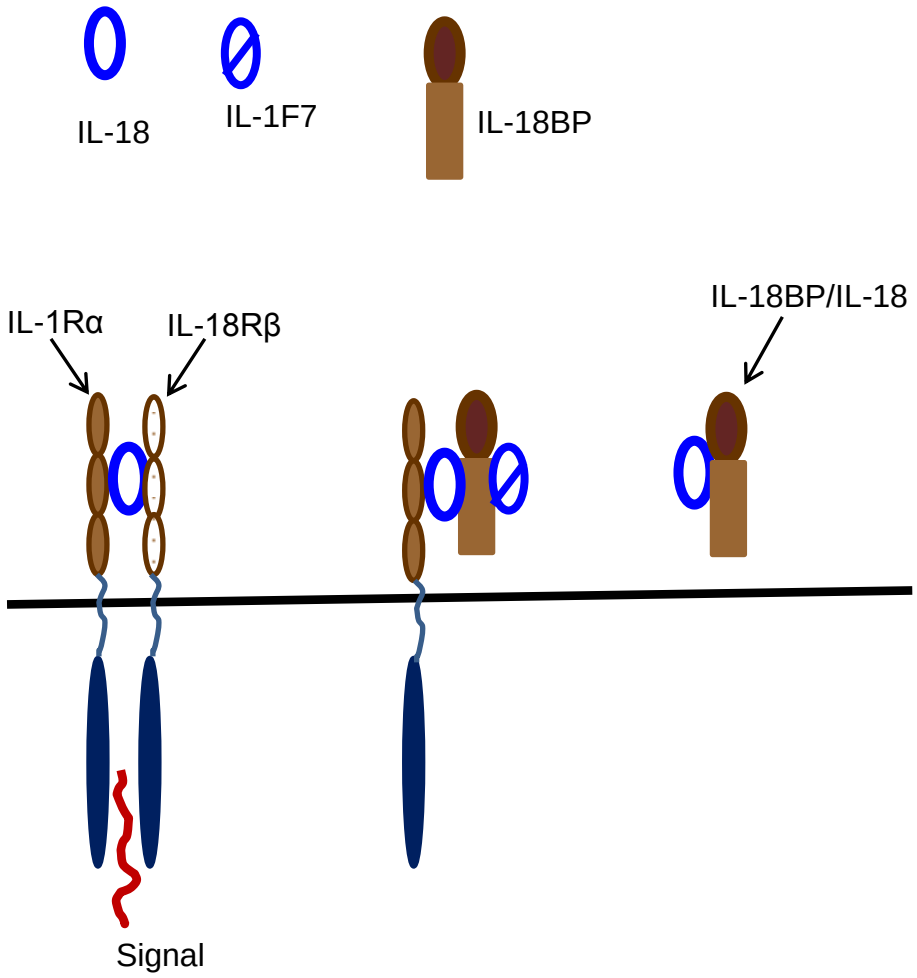


Figure 1.2 Ligands and receptors for the IL-18 family. IL-18 binds to the IL-18Rα chain, and this complex then engages the IL-18Rβ chain to initiate intracellular signals. The soluble protein IL-18BP functions as an inhibitor of IL-18 by binding this ligand in the fluid phase, preventing interaction with the IL-18Rα chain. IL-1F7 appears to enhance the inhibitory effect of IL-18BP (adapted from Arend *et al*, 2008).

1.1.4 Biological activity of classical IL-1 family cytokines

IL-1 cytokines have been shown to play an important role with regards to how a host animal responds to inflammatory, infectious or other immunological challenges (Dumont, 2006). IL-1α, IL-1β and IL-18 are primarily proinflammatory cytokines with an ability to induce expression of genes associated with inflammation and autoimmune diseases. They have pleiotropic regulatory effects on T and B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, natural killer cells, osteoblasts, fibroblasts, muscle cells, endothelial cells and epithelial cells. They therefore play an important role in both innate and adaptive immunity (Dinarello, 1984; Dinarello, 1998, Dinarello 2003c).

The most prominent role of IL-1 cytokines in inflammation is the initiation of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). Consequently, cells exposed to IL-1 (also, animals or humans injected with IL-1) produce large amounts of prostaglandin-E2 (PGE2), platelet activating factor and nitric oxide (NO) (reviewed in Dinarello 2003c). IL-1 cytokines can also increase expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM), on mesenchymal cells and vascular-cell adhesion molecule-1 (VCAM-1) on endothelial cells. Expression of VCAM-1 on endothelial cells causes inflammatory and immunocompetent cells to infiltrate the extravascular space. IL-1 cytokines also increase expression of vascular

endothelial growth factor (VEGF). VEGF is a signal protein which stimulates vasculogenesis and angiogenesis. IL-1 cytokines, therefore, play a role in tumour metastasis and blood vessel supply (reviewed in Dinarello 2003c).

It is now widely accepted that IL-1 cytokines mediate acute phase protein (APP) synthesis, adhesion molecule up-regulation, vasodilation, coagulation, production and release of matrix metalloproteinases and production of growth factors (Bagby, 1989, Smith et al., 1992; reviewed in Dinarello, 1991, 1996). IL-1 cytokines also influence other biological responses including haematopoiesis, fever, appetite control, bone metabolism and sleep (reviewed in Dinarello, 1991, 1996; Dinarello and Wolff, 1993; Bagby, 1989, Smith et al., 1992).

A number of studies have shown that administration of IL-1α to healthy animals or humans causes fever, sleep and hypotension. Fischer *et al.* (1991) evaluated how responses to sublethal endotoxaemia compared with IL-1α administration. In their study, baboons were intravenously injected with either LPS or varying amounts of IL-1α. They observed that IL-1α administration was able to reproduce most of the effects of sublethal endotoxaemia. They also reported that the effect of IL-1α depended on the dose. Generally, the most significant responses to IL-1 administration were observed at the highest dose (Fischer *et al*., 1991). In an earlier study, Okusawa *et al*. (1988) found that treating rabbits with IL-1β produced a shock-like state (Okusawa *et al*., 1988).

In addition to exerting proinflammatory effects, IL-1 cytokines are also adjuvants during antibody production. They act on bone marrow stem cells and stimulate their differentiation. In one study, pre-treatment with a single low dose of recombinant human IL-1β twenty-four hours prior to administering a lethal bacterial challenge to granulocytopenic and normal mice was found to enhance nonspecific resistance and to reduce mortality (Van der Meer *et al*., 1988).

IL-1α is known to possess antiproliferative, immunostimulatory, anti-infection, myeloprotective, and myelorestorative properties that could be beneficial in cancer treatment (Smith *et al.,* 1992). A study which sought to determine whether IL-1α could ameliorate drug-induced thrombocytopenia found that IL-1α administration post-chemotherapy can accelerate platelet recovery in human patients with advanced neoplasms. It was concluded that administration of IL-1α may be clinically useful in preventing or treating thrombocytopenia induced by chemotherapy (Smith *et al*., 1993). On the other hand, other studies in human patients with advanced solid malignancies have shown that dose-dependent side effects such as fever, gastrointestinal disturbances, myalgia (muscle pain), arthralgia (joint pain) and hypotension may limit the therapeutic utility of IL-1α in patients (Smith *et al*., 1992). It has been argued that although side effects such as chills, fever, and hypotension have been observed following administration of higher doses of IL-1α, the fact that drug tolerance develops rapidly suggests that it may be possible to administer IL-1α for more prolonged periods (for example, a few days before or after chemotherapy) (Smith *et al.,* 1993). If doses of IL-1α

that can be given safely to cancer patients are adhered to, significant, potentially beneficial haematopoietic effects occur (Smith *et al.,* 1992).

Unlike IL-1α/β, IL-18 can stimulate both pro-inflammatory (T_H1) as well as anti-inflammatory (T_H2) responses, depending on the immunological context. For example, IL-18 influences T_H1 responses by up-regulating Fas ligand expression on natural killer (NK) cells thereby enhancing their cytotoxicity and by interacting synergistically with IL-12 to stimulate the production of IFN-γ by T cells and NK cells (reviewed in Nakanishi et al., 2001). IL-18 can also act in synergy with other T_H2-stimulating factors (such as IL-2 or IL-4) to influence T_H2 responses by enhancing IL-4, IL-13 and IgE production (reviewed in Dumont, 2006; Muhl and Pfeilschifter, 2004).

Although IL-1 could potentially function as an immunoadjuvant due to its ability to up-regulate host defences, its inflammatory side effects can limit its beneficial effects (Smith *et al*., 1992). A study by Niki *et al*. (2001) showed that spontaneous arthritis developed in IL-1α transgenic mice. Since then, IL-1 has been implicated in other autoimmune-related inflammatory conditions such as colitis (Dinarello, 2002; Barksby *et al*., 2007). IL-1β has also been contraindicated in pathologies such as the septic syndrome, trauma, insulin-dependent diabetes mellitus, atherosclerosis and other diseases (Bistrian *et al*., 1992).

Studies examining the biological effects of IL-1 blockade have facilitated the understanding of the pathophysiological role that IL-1 plays in inflammatory and autoimmune conditions. In one such study, Palmer *et al.,* (2003) observed that overexpression of IL-1Ra conferred protection against collagen induced arthritis (CIA) in mice, while other studies have shown that genetic deficiency in IL-1α, IL-1β or IL-18 also offers significant protection against CIA (Saijo *et al*., 2002; Wei *et al*., 2001). The clinical benefits of IL-1 blockade through the use of Anakinra have already been highlighted.

Although IL-1α and IL-1β signal through the same receptor complex and have identical biological activities in solution, they differ in a number of ways. Table 1.2 summarises some of these differences.

Table 1.2 Summary of differences between IL-1α and IL-1β (summarised from Kurt‑Jones *et al.,* 1985; Maier *et al.,* 1994; Hazuda *et al,* 1998; Dinarello, 1996; Sims, Smith, 2010)

IL-1α	**IL-1β**
Synthesized as a 33-34 kDa, 271 amino acid pro-cytokine which is enzymatically cleaved (by calpain) into a bio-active 17 kDa, 159 amino acid mature segment and a 112 amino acid pro-sequence	Synthesized as a 31-34 kDa, 269 amino acid long precursor which is cleaved into a 116 amino acid pro-segment and a 153 amino acid, 17 kDa mature segment by IL-1 beta-converting enzyme (ICE)
Both precursor and mature forms show bioactivity. Intracellularly, pro- IL-1α appears to act through its nuclear localization signal that is found between residues 79-85 of its pro-region.	Shows bioactivity only when it is in its mature form. IL-1β precursor forms have no known biological activity.
Generally associated with the plasma membrane of the producing cell and so acts locally	Circulates systemically following secretion
Highly expressed by several cells including keratinocytes and endothelial cells, generally more widespread expression than IL-1β	Mainly produced by monocytes and macrophages
Important for priming T cells during contact hypersensitivity and for the induction of high levels of serum IgE following immunization with ovalbumin	Can circulate to the brain and is important for the induction of fever

1.1.4.1 Cryopyrin-associated Periodic Syndromes (CAPS)

Mutations in *NLRP3 (or CIAS1),* the gene which encodes NLRP3 (NALP3 or cryopyrin) (Section 1.1.2.1) causes a group of rare, autoinflammatory diseases collectively known as Cryopyrin-associated periodic syndromes (CAPS) (Hoffman *et al.,* 2001; reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011). Unlike autoimmune diseases, auto-inflammatory syndromes are characterised by a pathogenesis that does not require autoreactive T-lymphocytes or immunoglobulins to self-antigens. Instead, many auto-inflammatory syndromes are caused by abnormal regulation of cytokine signalling pathways, leading to persistent inflammation (reviewed in Hoffman, 2009). Diseases included in the CAPS category are: Familial cold autoinflammatory syndrome (FCAS; also known as familial cold urticaria), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disorder (NOMID; also known as chronic infantile neurological cutaneous and articular syndrome or CINCA) (Hoffman *et al.,* 2001; reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011).

As previously discussed (Section 1.1.2.1), NLRP3 (cryopyrin) is an intracellular sensor of pathogens or danger signals and is part of the multi-protein complex called the inflammasome which plays a key role in the generation of biologically active IL-1β and IL-18 through caspase-1 activation (Hoffman *et al.,* 2001; reviewed in Hoffman, 2009; reviewed in Wen *et al.,* 2012). CAPS have been associated with heterozygous gain of function mutations, either inherited in an

autosomal-dominant manner or developed spontaneously in the NLRP3 gene; however, mutations have not been detected in all patients (Hoffman, 2001). *NLRP3* gene mutations are believed to result in a constitutively active or hyperactive form of the NLRP3/NALP3 inflammasome complex which, in turn, causes activation of caspase 1 and hypersecretion of mature IL-1β (Hoffman *et al.,* 2001; reviewed in Hoffman, 2009). Various *NLRP3* missense mutations have been reported in CAPS, with each mutation causing a different degree of aberrant NLRP3 activation (reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011).

CAPS patients usually present with several shared clinical features, including an urticaria-like rash that appears soon after birth. All patients possess some degree of systemic inflammation in the form of fever and/or chills accompanied by elevations in acute phase reactants which are triggered by exposure to cold in patients with FCAS (reviewed in Goldbach-Mansky, 2011). In most cases, there is some involvement of the joints and eyes (Hoffman *et al.,* 2001; reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011). Although overlapping cases between FCAS and MWS or MWS and CINCA exist, there are critical features distinguishing FCAS, MWS and NOMID. FCAS, which is the mildest form of CAPS, presents with cold-induced urticarial rash, fever, and arthralgia. The symptoms of NOMID (or CINCA), the most severe form of CAPS, include neonatal-onset high fever, aseptic meningitis, mental retardation, sensory deafness, papilledema, arthritis with bone overgrowth, and secondary

amyloidosis that contributes to mortality. MWS is the intermediate phenotype (Hoffman *et al.,* 2001; reviewed in Hoffman, 2009; reviewed in Kubota and Koike, 2010; reviewed in Goldbach-Mansky, 2011).

Inhibiting IL-1β signalling (hence countering the effects of an overactive inflammasome) has proved to be highly efficacious in treating CAPS conditions, supporting findings which indicate the central role of IL-1 in the pathogenesis. The three IL-1 blocking agents currently approved for treating CAPS conditions are anakinra, rilonacept and canakinumab (Hoffman *et al.,* 2008; Alten *et al.,* 2008; reviewed in Hoffman, 2009; reviewed in Goldbach-Mansky, 2011). Anakinra is a recombinant IL-1Ra that functions as a competitive inhibitor of IL-1α and IL-1β (sections 1.1.0 and 1.1.2.2) (reviewed in Fleischmann, 2002; reviewed in Furst, 2004; reviewed in Fleischmann *et al.,* 2004), whereas rilonacept is a fusion protein that is made up of the receptor components required for IL-1 signalling (IL-1 type-1 receptor and the IL-1 receptor accessory protein) and the Fc portion of immunoglobulin G1 (IgG1). Rilonacept can bind to IL-1β with high affinity, thereby preventing its interaction with cell surface receptors (Hoffman *et al.,* 2008). Canakinumab is a fully human anti-IL-1β monoclonal antibody which selectively binds to IL-1β with no cross-reactivity with other characterised IL-1 family members. By binding to IL-1β, canakinumab prevents IL-1β from binding to the IL-1 receptor (Alten *et al.,* 2008; reviewed in Hoffman, 2009). Anakinra, rilonacept and canakinumab have proved to not only be effective at countering the inflammatory symptoms of CAPS but they have

also shown acceptable safety profiles (reviewed in Goldbach-Mansky, 2011). Understanding the role of the inflammasome and cryopyrin in other inflammatory diseases may lead to the development of more effective medication.

1.1.5 Regulation of activity: Classical IL-1 Family members

As has already been noted, depending on tissue or plasma levels, IL-1 action can either be beneficial or detrimental to the host. Although the innate response is necessary for host survival, in the presence of an excess amount of IL-1, inflammatory and autoimmune diseases may develop (reviewed in Arend, 2002). As a result, IL-1 production is a tightly controlled event. Studies suggest that under normal conditions, plasma IL-1 concentrations are below the detection range. As already highlighted in preceding sections, in addition to regulation of synthesis and secretion, regulation of IL-1 bioactivity also involves the production of IL-1 antagonists (such as IL-1Ra), the presence of decoy IL-1 receptors (for example IL-1R2 and IL-18BP), regulation of IL-1 signalling pathways and IL-1 mediated gene expression (Cooney and Shumate, 2006). The presence of such numerous pathways of IL-1 regulation underscores the need for tight control of IL-1 action. It remains to be seen whether similar regulatory mechanisms exist among the newer members of the IL-1 cytokine family. The following section will look at current understanding regarding these novel IL-1 cytokine family members.

1.2.0 Novel IL-1 family members

1.2.1 Novel IL-1 cytokines: General overview

As mentioned in previous sections, seven additional members have now been added to the IL-1 family on the basis of conservation of amino acid (aa) sequence, identity of gene structure, and predicted three-dimensional structure. It has already been noted that Sims *et al.* (2001) proposed a revised nomenclature in an effort to standardise the naming of the IL-1 family members. Under this revised nomenclature, it was suggested that the classical IL-1 family members (IL-1α, lL-1β, lL-1Ra and IL-18) be renamed IL-1F1, IL-1F2, IL-1F3 and IL-1F4 respectively (where IL-1F stands for IL-1 Family member) while the novel IL-1 family members were to be named IL-1F5 to IL-1F11 (Sims *et al*., 2001).

The intron-exon organisation of the IL-1 genes suggests that each IL-1 family member arose from a common ancestral gene that later became duplicated. Each gene codes for a protein that contains a single structural domain formed from 12 beta strands connected by loop regions arranged in a beta-trefoil structure (Nicklin *et al.,* 1994). Similar to IL-1β and IL-1Ra, the new IL-1 family members differ most from each other within these loop regions (Nicklin *et al.,* 1994). The genes for IL-1F5-F10 map to the region of chromosome 2 between the IL-1β and IL-1Ra loci (Dunn *et al*., 2001, Taylor *et al*., 2002, Barksby *et al*., 2007; Magne *et al*., 2006; Smith *et al*., 2000; Kumar *et al*., 2000; Barton *et al*.,

2000; Busfield *et al*., 2000; Pan *et al*., 2001; Bazan *et al*., 1996; Lin *et al*., 2001; Debets *et al*., 2001; Blumberg *et al*., 2007; Nicklin *et al*., 2002; Lander *et al*., 2001). The 11th member of the IL-1 cytokine family is located on the short arm of chromosome 9 at position 24.1 and was initially named IL-33. Its structural and functional properties have been observed to be similar to the other IL-1 family members. It has since been renamed IL-1F11 (Schmitz *et al*., 2005).

More recently, Dinarello *et al.* (2010) proposed that as functions have now been elucidated for several of the novel IL-1s, an individual interleukin designation should now be assigned to each of these cytokines. They argued that even though IL-1F6, IL-1F8 and IL-1F9 are encoded by distinct genes, they use the same receptor complex (IL-1Rrp2 and IL-1RAcP), they are proinflammatory and they also deliver almost identical signals. On this basis, the names IL-36α, IL-36β and IL-36γ, were proposed for IL-1F6, IL-1F8 and IL-1F9 respectively (Dinarello *et al*., 2010).

Using a similar argument, it was also proposed that natural IL-1F5 be renamed IL-36Ra (IL-36 receptor antagonist). This proposal was based on previous findings by Debets *et al*., (2001), Towne *et al*. (2004) and Blumberg *et al* (2007) which suggested that IL-1F5 binds to IL-1Rrp2 but antagonizes IL-1F6, IL-1F8 and IL-1F9 in a manner comparable to that used by IL-1Ra to antagonize IL-1α and IL-1β (Dinarello *et al.,* 2010).

IL-37 has been proposed as the new name for IL-1F7. Under this proposal, the names of the IL-1F7 splice forms would be changed from IL-1F7a, IL-1F7b and so on to IL-37a, IL-37b and so on. It was also suggested that IL-1F10 retain its IL-1F designation until its properties and biological function have been clearly elucidated, however, IL-38 was proposed as a potential new name for it (Dinarello *et al*., 2010) and has since been adopted (van de Veerdonk *et al.,* 2012). Although data regarding the newer members of the IL-1 cytokine family is starting to emerge, their biology is still less well characterised than that of the classical IL-1 family members.

Phylogenetic analysis suggests that IL-1F6, IL-1F8 and IL-1F9 (IL-36α, IL-36β and IL-36γ) (Dinarello *et al*., 2010) belong to a triplet branch of the phylogenetic tree (Blumberg *et al.,* 2007). All three cytokines lack a pro-domain (Smith *et al*., 2000) and were shown to trigger NF-kB and MAPK signalling via IL-1Rrp2 and IL-1RAcP and to induce IL-6 and IL-8 production in mammary epithelial cells (Towne *et al.,* 2004). It is now known that IL-1F6, IL-1F8 and IL-1F9 (IL-36α, IL-36β and IL-36γ) bind to IL-1Rrp2 (IL-36R) and IL-1RAcP, activating similar intracellular signals to IL-1β, while IL-1F5 (IL-36Ra) acts as an IL-1Rrp2 antagonist (IL-36Ra) (Towne *et al.,* 2004; Vigne *et al.,* 2011). In separate studies, however, IL-1F8 and IL-1F9 failed to induce typical IL-1β responses in mouse glial cells (Wang *et al.,* 2005; Berglof *et al.,* 2003). Whether or not IL-1F8 and IL-1F9 have distinct activities or indeed whether they trigger alternative pathways to MAPK and NF-kB in the brain remains to be proven. Current

understanding regarding each of the newer IL-1 family members will now be discussed in turn. For the purposes of this thesis, the classical IL-1 family members will continue to be referred to by their traditional names while the nomenclature proposed by Sims *et al*. (2001) will generally be adopted for the novel IL-1 family members, however, where necessary, the alternative names will also be quoted in brackets.

1.2.1.1 IL-1F5

In humans, IL-1F5 (recently renamed IL-36 receptor antagonist or IL-36Ra) (Dinarello *et al*., 2010) is a protein encoded by the *IL1F5* (also known as *IL-36RN*) gene (Mulero *et al.,* 1999; Smith *et al.,* 2000; Sims *et al.,* 2001). Using high throughput cDNA clone sequencing technology, Mulero *et al*. (1999) identified IL-1F5 for the first time and named it IL-1HY1. It was later identified as FIL-1δ by Smith *et al.* (2000) and IL-1L1 (Barton *et al.,* 2000). Other researchers named it IL-1RP3 (Busfield *et al.,* 2000) and IL-1δ (Debets *et al.,* 2001). Kumar *et al.,* (2000) identified a murine ortholog of this gene and named it IL-1H3. The predicted protein does not appear to contain a signal peptide or a propeptide domain at the N-terminus. Two alternatively spliced transcript variants encoding the same protein have been reported (Smith *et al.,* 2000). Besides IL‑1β, IL‑1Ra and IL‑18, IL‑1F5 is the only other IL-1 family member that has a published, experimentally determined three‑dimensional structure (Dunn *et al.,* 2003). It is widely expressed in keratinocytes, psoriatic skin, placenta, uterus, brain, thymus,

heart, kidney, monocytes, macrophages, B cells and dendritic cells (DCs) but not in fibroblasts, endothelial cells or melanocytes (Smith *et al*., 2000; Debets *et al*., 2001; Nicklin *et al*., 2002; Barton *et al*., 2000). Although IL-1F5 does not appear to show antagonistic activities towards the classical IL-1s (Barton *et al*., 2000), it has been found to show high (44%) sequence homology to IL-1Ra (Smith *et al*., 2000; Debets *et al*., 2001; Nicklin *et al*., 2002). This fuelled speculation that IL-1F5 may be a novel IL-1Ra. Indeed, Debets *et al*. (2001) found that IL-1F5 blocked the activation of NF-kB by IL-1F9 in Jurkat cells which over-expressed IL-1Receptor-related Protein 2 (IL-1Rrp2). IL-1F5 was found to be a highly specific antagonist of the IL-1 Rrp2-mediated response to IL-1F9 (Debets *et al.,* 2001). As is the case with IL-1Ra, IL-1F5 mRNA was also found to be up-regulated by treatment of human monocytic cells with phorbol esters and bacterial lipopolysaccharides (LPS) (Debets *et al.,* 2001). It was postulated that IL-1F5 could constitute part of an independent signalling system analogous to the IL-1α, IL-1β, interleukin-1 receptor type I (IL-1R1) and IL-1Ra system which is present in epithelial barriers and takes part in local inflammatory response (Debets *et al.,* 2001).

In another study, Towne and co-workers used an epithelial cell line which expressed both IL-1Rrp2 and IL-1RAcP to demonstrate that IL-1F6, IL-1F8 and IL–1F9 mediated NF-kB activation via IL-1Rrp2. They were also able to show that IL-1RAcP was necessary for signal transduction since its absence resulted in no NF-kB activation even though IL-1Rrp2 and its ligands were present,

however, investigations to establish whether IL-1F5 was an antagonist for IL-1F6, IL-1F8 or IL-1F9 (as suggested by Debets *et al.,* 2001) produced equivocal results (Towne *et al*., 2004). When Magne *et al*. (2006) tested the ability of different concentrations of recombinant human IL-1F5 to inhibit the effects of IL-1F8 on IL-6 production in human articular chondrocytes they also observed that antagonism of the effects of IL-1F8 by IL-1F5 was incomplete and not reproducible (Magne *et al.,* 2006).

Nevertheless, in a later study, Blumberg *et al*. (2007) demonstrated that IL-1F5 has antagonistic activity *in vivo*. In their study, they generated transgenic mice over-expressing IL-1F6 in their basal keratinocytes. They observed that the skin of IL-1F6 transgenic mice over-expressing IL-1F6 in basal keratinocytes is characterized by acanthosis, hyperkeratosis, the presence of a mixed inflammatory cell infiltrate, and increased cytokine and chemokine expression. They also generated mice deficient in IL-1F5 and later performed a genetic cross between the two groups of mice. They observed that expression of the IL-1F6 transgene in basal keratinocytes resulted in cutaneous lesions whose severity was worsened by co-existing IL-1F5 deficiency, suggesting that IL-1F5 plays an antagonistic role *in vivo* and that the balance between IL-1F6 agonist activity and IL-1F5 antagonist activity is critical in skin inflammation (Blumberg *et al*., 2007).

In a study involving rat glial cells and whole mouse brain, Costelloe *et al.,* (2008)

reported that IL-1F5, through its interaction with the novel receptor single immunoglobulin IL-1receptor-related (SIGIRR), antagonizes the inflammatory effects of IL-1β and LPS both *in vivo* and *in vitro* through induction of IL-4. Their study demonstrated that IL-1F5 abrogated lipopolysaccharide (LPS)-induced inhibition of long-term potentiation (LTP) in the hippocampus of IL-4 gene knock-out mice. They also found that IL-1F5 up-regulates IL-4 expression in the hippocampus, confirming earlier speculations that IL-1F5 may possess anti-inflammatory effects due to its high amino acid sequence similarity to IL-1Ra, and also suggesting, for the first time, that IL-1F5 interacts with the SIGIRR receptor in the brain (Costelloe *et al*., 2008). Another interesting observation made by this study was that in contrast to its effects in brain tissue, IL-1F5 failed to abrogate LPS-induced changes in macrophages or dendritic cells and failed to up-regulate IL-4 expression in these cells (Costelloe *et al*., 2008), suggesting that IL-1F5 may have different effects on different tissues/cells.

In a study which assessed expression of IL-1F5, IL-1F6, IL-1F8 and IL-1F9 in healthy controls, subjects with uninvolved psoriasis, and subjects with psoriasis plaque skin, increased expression of IL-1F5, IL-1F6, IL-1F8 and IL-1F9 was observed in lesional psoriatic skin compared to healthy controls (Johnston *et al.,* 2011). Other researchers have studied human keratinocytes derived from epidermal stem cells of hair follicles plucked from patients with psoriasis and also those from healthy individuals and observed that in the presence of IL-17, psoriasis-derived keratinocytes showed a significantly higher induction of the

proinflammatory IL-1 family members IL-1F6 and IL-1F9, but not of anti-inflammatory members IL-1F5, IL-1F7 or IL-1Ra (IL-1F3) compared with keratinocytes from healthy individuals (Muhr *et al.,* 2011). Both basal and IL-17-induced levels of IL-1α (IL-1F1) and IL-1β (IL-1F2) were also found to be significantly lower in psoriasis keratinocytes (Muhr *et al.,* 2011). These findings further support the potential of novel IL-1 family members as therapeutic targets in psoriatic conditions.

To address the discrepancy between their previous IL-1F5 (IL-36Ra) *in vitro* results (Towne *et al.,* 2004) and those of Debets *et al*. (2001), Towne *et al.* (2011) recently characterized several different recombinant IL-1F5 (IL-36Ra) preparations. They showed that IL-1F5 (IL-36Ra) binds to the IL-1Rrp2 receptor and is indeed an antagonist not only of IL-1F9 (IL-36γ) but also of IL-1F6 (IL-36α) and IL-1F8 (IL-36β); however, IL-1F5 activity requires removal of the amino-terminal methionine which is present in the primary translation product. Furthermore, the mechanism of antagonism by IL-1F5 (IL-36Ra) is comparable to that used by IL-1Ra to inhibit IL-1 in that a substantial molar excess is required for inhibition. In addition, it was also observed that as was the case with IL-1F5 (IL-36Ra), a particular N-terminal truncation of IL-1F6 (IL-36α), IL-1F8 (IL-36β) or IL-1F9 (IL-36γ) resulted in a 1000-10,000-fold increase in biological activity, suggesting that post-translational processing is required for full activity (Towne *et al.,* 2011). A later study showed that truncated IL-1F6, IL-1F8 and IL-1F9 were more potent than IL-1β at inducing maturation of murine bone marrow derived

DCs and subsequent inflammatory cytokine production (Vigne *et al.,* 2011). It is now appreciated that IL-1F5 inhibits the multiple proinflammatory effects induced by the agonists IL-1F6 (IL-36α), lL-1F8 (IL-36β) and IL-1F9 (IL-36γ) by preventing them from binding to the IL-1Rrp2 receptor (IL-36 receptor or IL-36R), however, the mechanism of secretion of these ligands is yet to be elucidated (Towne *et al.,* 2011).

1.2.1.1.1 *IL-1F5 (IL-36RN)* gene mutation

A recent study which sought to identify genetic abnormalities responsible for familial generalized pustular psoriasis (GPP) provided robust genetic evidence for the vital role of IL-1F5 in immune regulation in the skin (Marrakchi *et al.,* 2011). GPP is a life-threatening, multisystemic inflammatory disease comprising repeated flare-ups of sudden onset, characterized by a diffuse, erythematous, pustular rash accompanied by a high-grade fever, general malaise, and extracutaneous organ involvement (Marrakchi *et al.,* 2011). Marrakchi *et al.* (2011) performed homozygosity mapping and direct sequencing in nine Tunisian multiplex families with autosomal recessive GPP. The effect of mutations on protein expression and conformation, stability, and function was then performed. The study demonstrated that loss-of-function mutations in *IL-1F5 (IL-36RN),* the gene which encodes IL-1F5 (IL-36Ra), results in absence of functional IL-1F5 (IL-36Ra) leading to unregulated secretion of inflammatory cytokines and hence

GPP (Marrakchi *et al.,* 2011). The findings are consistent with previously mentioned mouse studies which showed that IL-1F6 (IL-36α) overexpression results in transient skin inflammation characterized by acanthosis, hyperkeratosis, and a mixed-cell infiltrate rich in neutrophils, features observed in both psoriasis vulgaris and GPP and that when crossed with IL-1F5 (IL-36Ra)-deficient mice, the skin phenotype is strikingly enhanced and persistent with extensive pustule formation (Blumberg *et al*., 2007). These data highlight IL-1/IL-36 signalling as a potential target for therapeutic intervention in GPP.

1.2.1.2 IL-1F6

The human *IL-1F6* gene was cloned from a human genomic sequence. The predicted IL-1F6 (recently renamed IL-36α) polypeptide is approximately 26% identical to IL-1Ra and its open reading frame contains neither a signal peptide nor a prodomain (Smith *et al.,* 2000). One study explored the mechanism by which murine IL-1F6 is released from bone marrow-derived macrophages (BMDMs) and compared this mechanism to that used by IL-1β. It was observed that transduced IL-1F6 is released in parallel with endogenous mature IL-1beta from LPS/ATP-treated BMDMs, but that this externalization process is not selective for cytokines since a non-cytokine, green fluorescent protein (GFP), shows similar behaviour. It was concluded that IL-1F6 is probably externalized via a stimulus-coupled mechanism comparable to that used by IL-1β (Martin *et al.,* 2009).

IL-1F6 mRNA has been detected in foetal brain, tonsils, LPS-activated monocytes, B cells, T cells and human skin (Smith *et al*., 2000; Towne *et al*., 2004). Lung cancer tissue and TNF-α treated small airway epithelium also express IL-1F6 (Dumont, 2006). Along with IL-1F8 and IL-1F9, IL-1F6 has been shown to act as an agonist by activating the pathway involving NFκB and MAPK in an IL-1Rrp2 dependent manner, suggesting that IL-1F6 signals in a similar fashion to IL-1β and IL-18 in having a binding receptor which upon ligation recruits a second receptor as a signalling component, forming an active heterodimeric receptor complex (Towne *et al.,* 2004; Towne *et al.,* 2011). An increased expression of IL-1F6, IL-1F8, IL-1F5 and IL-1F9 was observed in lesional psoriatic skin compared to healthy controls (Johnston *et al.,* 2011) and over-expression of the IL-1F6 transgene in basal keratinocytes has been shown to result in cutaneous lesions which were exacerbated by co-existing IL-1F5 deficiency (Blumberg *et al.,* 2007). It is becoming clear that IL-1F6 may play a role in the pathogenesis of psoriasis and other skin lesions.

A role for IL-1F6 in obesity was suggested by a study conducted in 2010 to establish the effect of the IL-1 cytokine family members IL-1F6 and IL-1F8 on adipocyte differentiation. Adipogenic expression levels of IL-1F6 and IL-1F8 and their effects on adipose tissue gene expression were examined. While IL-1F6 was found to be primarily present in adipose tissue resident macrophages and was also induced by inflammation, IL-1F8 was absent in both situations (van Asseldonk *et al.,* 2010). Both IL-1F6 and IL-1F8 were able to induce

inflammatory gene expression in mature adipocytes, however, only IL-1F6 inhibited PPARγ (Peroxisome proliferator-activated receptor gamma) expression (van Asseldonk *et al.,* 2010). PPARγ is a nuclear transcription factor which controls lipid metabolism in adipocytes and sensitizes these cells to insulin (reviewed in Knouff and Auwerx, 2004). Since inhibition of PPARγ expression may lead to reduced adipocyte differentiation, this suggests that IL-1F6 inhibits adipocyte differentiation and may be involved in the inflammatory link between obesity and insulin resistance. Blockade of IL-1F6 and IL-1F8 may have therapeutic potential in obesity or insulin resistance (van Asseldonk *et al.,* 2010).

1.2.1.3 IL-1F7

Until recently, very little was known about IL-1F7 (IL-37) (reviewed in Sims and Smith, 2010; Dinarello *et al*., 2010; reviewed in Tete *et al.,* 2012). It was initially identified as FIL1-zeta (FIL-1ζ) (Smith *et al.,* 2000) and was subsequently identified as IL-1H4 (Kumar *et al.,* 2000), IL-1RP1 (Busfield *et al.,* 2000) and IL-1H (Pan *et al.,* 2001). It is the only IL‑1 family member that lacks a mouse orthologue (reviewed in Sims and Smith, 2010). Among the newly-discovered IL-1 homologues, it is the only cytokine that possesses a pro-domain (pro-IL-1F7) (Kumar *et al.,* 2000). IL-1F7 is highly expressed in the testis, thymus and uterus but is also present in most other tissues (Smith *et al.,* 2000; Kumar *et al.,* 2000; Busfield *et al.,* 2000; Pan *et al.*, 2001. Mature IL-1F7 is generated following cleavage of pro-IL-1F7 by caspase-1. Caspase-4 is also able to cleave pro-IL-1F7 (Kumar *et al*., 2002).

Five isoforms of IL-1F7 were identified and named IL-1F7a-7e (Smith *et al*., 2000; Busfield *et al*., 2000; Kumar *et al*., 2002; Pan *et al*., 2001; Taylor *et al*., 2002). They were recently renamed IL-37a-IL-37e (Dinarello *et al.,* 2010). Published data indicates that the various isoforms are differentially expressed (Pan *et al*., 2001; Kumar *et al*., 2002). The best characterised of these isoforms is IL-1F7b. While this isoform shares sequence homology with IL-18, it also has a protein sequence which shows approximately 20% similarity to IL-1Ra (Kumar *et al.,* 2000). Some studies have shown that IL-1F7b is unable to bind IL-1RI but can bind IL-18Rα without inducing typical IL-18 agonist responses such as IFN-γ production (Kumar *et al*., 2002; Pan *et al*., 2001; Bufler *et al*., 2002). Kumar *et al.,* (2002) reported that neither pro-IL-1F7 nor mature IL-1F7 can bind IL-18BP; however, it has since been reported that IL-1F7b binds to IL-18BP and that this binding reduces IL-18 activity (Bufler *et al*., 2002). Figure 1.2 in section 1.1.4 shows a schematic diagram illustrating the proposed interaction between IL-1F7b and IL-18BP.

In another study, cells transfected with IL‑1F7b showed decreased pro‑inflammatory cytokine production following LPS stimulation (Sharma *et al.,* 2008). The study also found that full‑length IL‑1F7b localizes to the nucleus, suggesting that IL‑1F7 primarily acts as a negative regulator inside the cell (Sharma *et al.,* 2008). IL‑1F7 also interacts with the transforming growth factor‑β (TGFβ) signalling protein SmAD3 (Grimsby *et al.,* 2004) and it has been

suggested that this mediates the inhibitory effect of IL‑1F7 on cytokine secretion (Sims and Smith, 2010).

In one study, the anti-tumour activity of human IL-1F7 was investigated by using adenovirus-mediated transfer of the IL-1F7 gene into murine tumours (Gao *et al*., 2003). The same study reported that treatment of an established MCA205 mouse fibrosarcoma by single injection of the IL-1F7 gene resulted in significant growth suppression (Gao *et al*., 2003). Further to that, complete inhibition of tumour growth was observed following multiple injections of the IL-1F7 gene. In addition, IL-1F7b was able to confer substantial anti-tumour effects in Natural Killer T cell (NKT)-deficient mice (Gao *et al*., 2003). These recent results suggest that IL-1F7 could prove useful in tumour immunotherapy; however, the exact molecular mechanism of the anti-tumour effects of IL-1F7b is still unclear. Previously, there were speculations that functional T and B cells may be required in the process as well as IL-12-dependent adaptive immunity (Gao *et al*., 2003). IL-1F7 (IL-37) is now recognised as a natural suppressor of innate inflammatory and immune responses. It regulates inflammatory responses, mainly by inhibiting the expression, production and function of proinflammatory IL-1 family cytokines (Tete *et al.,* 2012).

1.2.1.4 IL-1F8

The sequencing of an osteoclastoma cDNA library led to the identification of IL-1F8 (IL-36β) (Kumar *et al.,* 2000; Smith *et al.,* 2000; Dinarello *et al.,* 2010). Similar to other novel IL-1homologues, IL-1F8 shows about 25% identity with IL-1Ra, however, it contains neither a signal peptide nor a prodomain (Smith *et al.,* 2000). To date, IL-1F8 has been isolated from human tonsils, bone marrow, heart, placenta, lung, testis, monocytes and B cells (Smith *et al*., 2000; Dunn *et al.,* 2001; Towne *et al*., 2004).

Similarly to IL‑1F6 and IL‑1F9, IL-1F8 signals via both IL-1RAcP and IL‑1Rrp2 (Towne *et al.,* 2004) and, as is the case with IL-1β and IL‑18, these three newer IL-1 cytokines also activate NF‑κB and MAPKs (Towne *et al.,* 2004; Towne *et al.,* 2011; Vigne *et al.,* 2011). Magne *et al*. (2006) observed that human synovial fibroblasts (hSFs) and human articular chondrocytes (hACs) expressed the IL-1Rrp2 receptor and that they also produced proinflammatory mediators in response to recombinant IL-1F8. They reported that human joint cells (hSFs and hACs) were not a major source of IL-1F8 and that joint and serum IL-1F8 levels did not correlate with inflammation. They were not able to explain the elevated IL-1F8 serum levels which they found in human samples from some healthy as well as rheumatoid arthritis (RA) donors (Magne *et al.,* 2006). It is not yet clear whether IL-1F8 plays a part in joint inflammation associated with RA. A recent study observed an increased expression of IL-1F8, IL-1F5, IL-1F6, and IL-1F9 in

lesional psoriatic skin compared to healthy controls (Johnston *et al.,* 2011), suggesting that, together with IL-1F5, IL-1F6, and IL-1F9, IL-1F8 may play a role in psoriatic conditions.

1.2.1.5 IL-1F9

IL-1F9 was initially identified from an epithelial cell cDNA library and named IL-H1 (Kumar *et al.,* 2000). It was subsequently identified as IL-1RP2 (Busfield *et al.,* 2000) and IL-1ε (Debets *et al.,* 2001) and was recently renamed IL-36γ (Dinarello *et al.,* 2010). Its predicted polypeptide sequence has neither a signal peptide nor a prodomain. It shows about 25% similarity to IL-1Ra (Kumar *et al.,* 2000). IL-1F9 mRNA and IL-1F9 protein are expressed in embryonic and epithelial tissues such as skin, lung and stomach. IL-1F9 is constitutively expressed in the placenta and in the squamous epithelium of the oesophagus (Kumar *et al.,* 2000; Debets *et al.,* 2001). Similarly to the other IL‑1 family members, IL‑1F6, and IL‑1F8, IL-1F9 signals via both IL-1RAcP and the IL‑1Rrp2 receptor and activates NF‑κB and MAPKs. As is the case with IL‑1F6, and IL‑1F8, the activity of IL-1F9 is specifically inhibited by IL-1F5 (Towne *et al.,* 2004; Towne *et al.,* 2011; Vigne *et al.,* 2011).

Inflammatory cytokines such as IFN-γ and TNF-α up-regulate IL-1F9 expression in human keratinocytes (Kumar *et al*., 2000) and murine IL-1F9 has been shown to be up-regulated *in vivo* in response to chronic contact hypersensitivity or viral

infection (Kumar *et al.,* 2000). In other studies IL-1F9, together with IL-1F5 and IL-1Rrp2, was up-regulated in skin from lesional psoriatic patients compared with skin from healthy subjects (Debets *et al*., 2001). Therefore, IL-1F9 may promote responses to injury or infection and may also play a role in the pathophysiology of skin conditions.

1.2.1.6 IL-1F10

IL-1F10 was initially named IL-1HY2. Its predicted polypeptide sequence shows approximately 32% similarity to IL-1Ra (Lin *et al.,* 2001). As is the case with most novel IL-1 homologues, IL-1F10 does not contain a signal peptide or a prodomain. IL-1F10 is widely expressed in the basal epithelia of human skin and is also expressed in the spleen and also by proliferating B cells of the tonsils (Lin *et al.,* 2001). It shares 43% amino acid sequence homology with IL-1F5 (Bensen *et al.,* 2001). Like IL-1F5, IL-F10 seems to also share some amino acid sequence homology (41%) with IL-1Ra (Taylor *et al.,* 2002; Nicklin et al., 2002; Bensen *et al.,* 2001). The genomic structure and amino acid sequence of IL‑1F10 point to a closer relationship to IL‑1Ra and IL‑1F5 than to the rest of the family. This suggests that it may have an antagonistic role. Consistent with this finding, it has been observed that recombinant IL-1F10 can bind to soluble IL-1RI with lower affinity than IL-1Ra and IL-1β (Lin *et al.,* 2001). A recent study showed that IL-10 (IL-38) binds to the IL-1Rrp2 receptor (IL-36R) and that its biological effects on immune cells are similar to the effects of IL-1F5 (IL-36 Ra) on these cells (van de Veerdonk *et al.,* 2012).

1.2.1.7 IL-1F11

IL-1F11 was originally named IL-33. It assumed the name IL-1F11 following the revised IL-1 family nomenclature proposed by Sims *et al*. (2001). IL-1F11 was discovered by Schmitz and co-workers through sequence database searches for IL-1 cytokine family members (Schmitz *et al*., 2005). Previous researchers had described it as DVS27 (a gene up-regulated in canine cerebral vasospasm) (Onda *et al.,* 1999), while others had called it “nuclear factor from high endothelial venules” (NF-HEV) (Baekkevold *et al.,* 2003). The human IL-1F11/IL-33 gene is found on chromosome 9p24.1 while the mouse gene is located in the chromosome 19qC1 region. The IL-1 family member most closely related to IL-1F11 is IL-18 (Schmitz *et al*., 2005).

IL-1F11 is widely expressed in many tissues, but its expression appears to be restricted by cell type. High levels of mouse IL-1F11 can be found in the stomach, lung, spinal cord, brain and skin. Mouse IL-1F11 was also found in lymph tissue, spleen, pancreas, kidney and heart. In human tissue, IL-1F11 was found to be highly expressed in smooth muscle cells and in bronchial epithelial cells and was also present in activated DCs and macrophages. IL-1F11 gene expression has also been shown to be induced in primary lung or dermal fibroblasts and keratinocytes activated by TNF-α or IL-1β. Stimulating T_H2-polarized naive T cell cultures with purified IL-1F11 *in vitro* induced the production of T_H2-associated cytokines such as IL-5 and IL-13 and decreased production of IFN-γ from T_H1 cells. Intraperitoneal (i.p.) administration of IL-1F11

to wild type mice caused histological signs of inflammation in the lungs and gastrointestinal tract accompanied by an increase in the number of splenic eosinophils, mononuclear cells and plasma cells and an increase in serum levels of IL-5, IgE and IgA, suggesting that IL-1F11 induces T_H2 type responses *in vivo (Schmitz et al., 2005).* There is speculation that IL-1F11 may be involved in T_H2-mediated immune responses, including asthma (and other inflammatory airway diseases) allergy, parasitic helminth infections and possibly inflammatory bowel disease (IBD) (reviewed in Dinarello, 2005c; reviewed in Dinarello, 2009).

It is now known that IL-1F11 plays a key role in the biology of allergy-associated innate immune cells and that it also augments T_H2 cell responses. In addition to its activity on human mast cells, IL-1F11 is capable of activating human basophils, polarized T cells, invariant Natural killer T cells (iNKT cells) and Natural Killer (NK) cells (Smithgall *et al.,* 2008). Although IL-1F11 induces predominantly T_H2-skewed immune responses, it can promote both T_H1 and T_H2 immune reactions depending on the actual cytokine and cellular environment (Smithgall *et al.,* 2008). By amplifying both T_H1- and T_H2-type responses through its activity on human basophils, allergen-reactive T_H2 cells, iNKT and NK cells, IL-1F11 emulates the biology of the IL-1 family member, IL-18 (reviewed in Smith, 2011; Sims and Smith, 2010).

There are speculations that as is the case with IL-1α and high-mobility group

protein B1 (HMGB-1), IL-1F11 may be a cytokine with dual function since studies have suggested that IL-1F11 may function as both a traditional cytokine and also as an intracellular nuclear factor with transcriptional regulatory properties (Carriere *et al.,* 2007). Localization of IL-1F11 to the nucleus of endothelial cells is mediated by its amino terminus, which contains a nuclear localization signal and a homeodomain (helix-turn-helix-like motif) which can bind to heterochromatin (Carriere *et al.,* 2007). The physiological role of IL-1F11 as a nuclear factor is not fully understood; however, it is thought to be involved in transcriptional repression by binding to the H2A-H2B acidic pocket of nucleosomes and regulating chromatin compaction by promoting nucleosome–nucleosome interactions (Roussel *et al.,* 2008).

Initially, it was assumed that IL-1F11 was synthesized as a biologically inactive precursor which was subsequently activated by caspase-1 cleavage at amino acid 112 under pro-inflammatory conditions (Schmitz *et al.,* 2005). More recent data suggests that IL-1F11 is cleaved by caspases-3 and -7 at amino acids 175 and 178 respectively, and is not a physiological caspase-1 substrate as originally thought (Ali *et al,* 2010). It has also been shown that full length IL-1F11 is biologically active at the ST2 receptor indicating that proteolytic processing is not required to induce its cytokine activity. Available data suggest that full length biologically active IL-1F11 may be released during necrosis, but during apoptosis IL-1F11 is cleaved by caspases-3/7 leading to inactivation of its pro-inflammatory properties (Ali *et al,* 2010; reviewed in Palmer and Gabay, 2011). This has led to

the idea that full length IL-1F11 may function as an endogenous danger signal. On the other hand, inactivation of IL-1F11 during apoptosis may serve as a control mechanism to avoid simultaneous damage to host tissues by the pro-inflammatory action of IL-1F11 (reviewed in Miller and Liew, 2011).

IL-F11 exerts its biological effects by interacting with the receptors ST2 (also known as interleukin 1 receptor-like 1 (IL-1RL1)) and IL-1RAcP. This interaction leads to activation of intracellular molecules in the NF-κB and MAPK signalling pathways which drive production of T_H2 cytokines (e.g. IL-5 and IL-13) from polarized T_H2 cells (Smithgall *et al.,* 2008). The severe pathological changes observed in mucosal organs following administration of IL-1F11 are thought to occur following the induction of type 2 cytokines by IL-1F11 *in vivo* (Schmitz *et al.,* 2005; Chackerian *et al.,* 2007).

Following on from the finding that cardiac fibroblasts synthesise IL-1F11 and that IL-1F11 markedly antagonises angiotensin II– and phenylephrine-induced cardiomyocyte hypertrophy (Sanada *et al.,* 2007), data is also starting to emerge about the role of the IL-1F11/ST2 pathway in the cardiovascular system (Seki *et al.,* 2009). The soluble isoform of the IL-1F11 receptor, soluble ST2 (sST2), is a mechanically induced cardiomyocyte protein and is considered to be an unfavourable prognostic marker in myocardial infarction, congestive heart failure and trauma/sepsis shock patients (Sanada *et al.,* 2007). Soluble ST2 has been

shown to block the antihypertrophic effects of IL-1F11. This suggests that sST2 functions in myocardium as a soluble decoy receptor (Sanada *et al.,* 2007; Seki *et al.,* 2009). On the other hand, IL-1F11 and the transmembrane ST2 isoform (also known as ST2L) may have a protective role in atherosclerosis, obesity and cardiac remodelling (Sanada *et al.,* 2007; Seki *et al.,* 2009; reviewed in Miller and Liew, 2011).

1.2.2 Synthesis of novel IL-1 family members

As already mentioned, very little is known about novel IL-1 synthesis, regulation of expression or biological activity. There is also very little published data regarding the signalling pathways involved. Although it has been observed that IL-1F6, IL-1F8 and IL–1F9 are all up-regulated in response to LPS in human monocytes; the exact signalling pathways employed in the regulation of these responses have not yet been elucidated (Towne *et al*., 2004). It is now known that for some of the classical IL-1 cytokines (such as IL-1β), RNA stability and translational control form part of the regulatory mechanism (Brook *et al.,* 2006). It has already been mentioned that post-translational modification of IL-1α may be important for its biological activity (Kobayashi *et al.,* 1988; Brook *et al*., 2006). It is not yet clear whether this kind of regulation occurs with novel IL-1 cytokines.

It has already been noted that IL-1α, IL-1β and IL-18 are synthesised as pro-cytokines from which mature forms are generated following proteolytic cleavage.

For IL-1β and IL-18, this intracellular processing by caspase-1 is crucial because these cytokines are only biologically active in their mature form (Dinarello, 1998). Recent work by Towne *et al.* (2011) shows that truncation of IL-1F6, IL-1F8 and IL-1F9 (IL-36α, IL-36β, and IL-36γ) enhances their activity, suggesting that post-translational processing is required for full activity, however, the proteases responsible for cleavage of these ligands *in vivo* have not yet been identified. It is also unclear whether a single protease is responsible for cleavage of IL-1F6, IL-1F8 and IL-1F9 (Towne *et al.,* 2011). Although Kumar *et al*., (2002) observed that IL-1F7b is processed by caspase-1, no caspase-1 cleavage sites have been identified for IL-1F5, IL-1F6, IL-1F8, IL-1F9 or IL–1F10 (Kumar *et al*., 2000; Smith *et al*., 2000; Lin *et al*., 2001; reviewed in Miller and Liew, 2011). Further work is needed to clarify the mechanisms involved in the synthesis and secretion of these novel IL-1 cytokines.

1.2.3 Receptors for the novel IL-1 family members

IL-1 family members exert their function through binding to cell surface receptors on target cells (reviewed in Sims, 2002; reviewed in Subramaniam, Stansberg and Cunningham, 2004). IL-1 receptors are part of a structurally-related receptor superfamily characterised by the presence of a Toll/IL-1 receptor (TIR) module in the intracellular region and by the presence of one to three copies of immunoglobulin (Ig)-like domains in their extracellular region. To date, 11 members of this receptor family have been identified (reviewed in Sims, 2002; reviewed in Subramaniam, Stansberg, Cunningham, 2004; reviewed in Dumont,

2006). These include T1/ST2 (Klemenz *et al*., 1989; Tominaga, 1989), IL-1 receptor-related protein (IL-1Rrp)1 (Parnet *et al*., 1996), three immunoglobulin domain-containing IL1 receptor-related-1 (TIGIRR-1) (Sana *et al*., 2000; Born *et al*., 2000), the IL-1 receptor accessory protein-like (IL1RAPL) molecule (Born *et al*., 2000 and Carrie *et al*., 1999) and IL-1 receptor-related protein 2 (IL-1Rrp2) (Lovenberg *et al*., 1996).

In accordance with conventional IL-1 receptors, all of these newly discovered receptors possess three extracellular immunoglobulin (Ig)-like domains and an intracellular Toll/IL-1 receptor-related (TIR) domain (reviewed in Subramaniam, Stansberg, Cunningham, 2004). To date, none of these novel receptors has been shown to bind IL-1β, IL-1α or IL-1ra (Lovenberg *et al*., 1996; Berglof *et al*., 2003).

1.2.3.1 IL-1Rrp2

IL-1 Receptor-related protein-2 (IL-1Rrp2) is also known as IL-1 receptor-like 2 (IL-1RL2) (Lovenberg *et al.,* 1996) and was recently renamed IL-36 receptor (IL-36R). Rat and human cDNAs for IL-1Rrp2 were first identified and isolated in 1996 (Lovenberg *et al.,* 1996). The protein encoded by both of these cDNAs was found to be 561 amino acids long and to exhibit 42% and 26% overall identity with the IL-1 type 1 and type 2 receptors, respectively (Lovenberg *et al.,* 1996). A predominant expression of IL-1Rrp2 was observed in the lung and epididymis

and lower levels were detected in the testis and cerebral cortex. Expression of IL-1Rrp2 in rat brain appeared to be non-neuronal and associated with the cerebral vasculature and the receptor was found to be incapable of high affinity binding to either recombinant human IL-1α or recombinant human IL-1β (Lovenberg *et al.,* 1996).

Debets *et al* (2001) observed that IL-1Rrp2 binds IL-1F9 as a ligand and that this binding leads to the activation of transcription factor NF-kB. They also reported that IL-1F5 inhibited the IL-1Rrp2-mediated NF-kB response induced by IL-F9 and proposed that IL-1F5 could be a receptor antagonist. In a later study, Towne *et al.* (2004) reported that in addition to IL-1F9, the receptor also binds IL-1F6 and IL-1F8 and that this binding stimulates NF-kB and MAPK activation and induces expression of IL-6 in human cell lines. They also observed that, as is the case with IL-1α and IL-1β signalling, IL-1F6, IL-1F8 and IL-1F9, in addition to IL-1Rrp2, also require IL-1RAcP as an accessory subunit. The same study also investigated the hypothesis that IL-1F5 could be a receptor antagonist as proposed by Debets *et al*. (2001). Contrary to the findings of the earlier study, this later study found that inhibition of IL-1F6, IL-1F8 or IL–1F9 by IL-1F5 was incomplete and equivocal (Towne *et al*., 2004). In a separate study, Berglof *et al*. (2003) reported that mouse astrocytes and microglial cells expressed IL-1Rrp2 and that exposure to bacterial lipopolysaccharides (LPS) strongly reduced this expression.

Magne *et al.* (2006) tested the ability of different concentrations of recombinant human IL-1F5 to inhibit the effects of IL-1F8 on IL-6 production in human articular chondrocytes (hACs). They observed that antagonism by IL-1F5 was incomplete and not reproducible. This finding confirmed observations that had been made by Towne *et al.* (2004). It has since been shown that IL-1F5 (IL-36Ra) is indeed an antagonist of IL-1F6, IL-1F8 and IL-1F9 (IL-36α, IL-36β and IL-36γ) but that full antagonist activity requires removal of the N-terminal methionine present in the primary translation product. Furthermore, a substantial molar excess is required for inhibition (Towne *et al.,* 2011). Previous studies had failed to show IL-1F5 antagonism (Towne *et al.,* 2004; Magne *et al.,* 2006) probably because an N-terminal tag had been used to facilitate purification of IL-1F5 (Towne *et al.,* 2011).

Blumberg *et al*. (2007) reported that expression of IL-1Rrp2, IL-1F5 and IL-1F6 is increased in human psoriatic skin. They also showed that IL-1F5 has antagonistic activity *in vivo* since co-existing IL-1F5 deficiency exacerbated the skin inflammation exhibited by IL-1F6 transgenic mice (Blumberg *et al*., 2007). In a more recent study, it was proposed that IL-1Rrp2 and its ligands may contribute to the cytokine network in psoriasis. This study demonstrated that application of an irritant can rapidly induce psoriatic-like skin inflammation in phenotypically normal skin from 2–3-month-old mice transgenic for IL-1F6 (Blumberg *et al.,* 2010). Furthermore, it was shown that cytokines known to be involved in human psoriasis (such as IL-17, IL-22, and IL-23) are overexpressed

in this psoriasis model and that they can induce IL-1F6, which, in turn, can induce IL-17, IL-22, and IL-23, thereby establishing a self-amplifying gene-expression loop. When psoriatic lesional human skin was transplanted onto mice with Severe Combined Immunodeficiency (SCID) prior to treatment with an antibody to IL-1Rrp2, anti-IL-1Rrp2 antibody reduced the epidermal hyperplasia and other skin changes associated with psoriasis (Blumberg *et al.,* 2010). Observations made in this study suggest that agents which block signalling through IL-1Rrp2 could be useful therapeutic tools for psoriatic conditions.

1.2.3.2 ST2

The ST2 gene is found on the long arm of Chromosome 2 (2q12). It encodes two glycoproteins of the interleukin 1 receptor (IL-1R) family. The larger protein is a membrane-anchored (transmembrane) receptor (ST2 or ST2L), which is very similar to the type I IL-1R, and whose predominant sites of expression are mast cells and T_H2 cells (Iwahana *et al.,* 1999). It has 3 extracellular IgG domains, a single transmembrane domain, and an intracellular SIR domain homologous to (Toll like Receptors) TLRs and other IL-1Rs. The smaller, soluble, secreted isoform (sST2) corresponds to the extracellular domain of the ST2 receptor and is produced by fibroblasts and osteoblasts in low quantities and in much higher amounts after stimulation with growth factors, proinflammatory cytokines, and in response to oncogene expression (Iwahana *et al.,* 1999; Weinberg *et al.,* 2002).

It lacks the transmembrane and intracellular domains. Both ST2L and sST2 are biomechanically induced in cardiomyocytes (Iwahana *et al.,* 1999; Weinberg *et al.,* 2002; Sanada *et al.,* 2007).

For many years, ST2 (also known as Interleukin 1 receptor-like 1 or IL-1RL1, as previously mentioned) was an orphan receptor belonging to the interleukin-1 receptor family (Klemenz *et al.,* 1989; Tominaga, 1989). It was later identified by Schmitz *et al*. (2005) as being part of the receptor complex through which IL-1F11 mediates its T_H2-like biological effects. Schmitz *et al*. (2005) observed that IL-1F11 administration led to NF-kB and MAPK activation in cells which expressed ST2, such as mast cells and T_H2 cells. They also observed that IL-1F11-initiated- NF-kB activation resulted in the production of IL-4, IL-5 and IL-13 (Schmitz *et al*., 2005). Although ST2 was shown to be part of the IL-1F11 receptor complex, for a while, the second (signal-transducing) chain of this receptor complex was not identified. However, there were suggestions that the single immunoglobulin IL-1R-related (SIGIRR) protein or IL-1RAcP could be possible candidates (Schmitz *et al*., 2005).

It has since been shown that IL-F11 exerts its biological effects by interacting with the receptors ST2L and IL-1 Receptor Accessory Protein (IL-1RAcP). This interaction leads to activation of intracellular molecules in the NF-κB and MAPK signalling pathways which drive production of type 2 cytokines (e.g. IL-5 and IL-

13) from polarized T_H2 cells (Chackerian *et al.,* 2007). The soluble ST2 isoform, sST2, acts as a decoy receptor and association of sST2 with IL-1F11 blocks ST2L-dependent signalling and the immunological and cardiac effects of IL-1F11 (Sanada *et al.,* 2007). The cardioprotective role played by the IL-1F11/ST2L system is beginning to be elucidated. Through ST2L-mediated signalling, IL-1F11 appears to counteract cardiac myocyte hypertrophy which is induced by angiotensin II or phenylephrine (Sanada *et al.,* 2007).

The finding that IL-1F11 signals via ST2 and induces Th2-associated cytokines is consistent with the role that ST2 plays in Th2 development, as previously suggested by Townsend *et al*. (2000). In that study, mice with a deficiency in T1/ST2 expression were generated in an effort to clarify the roles of T1/ST2 in T helper cell type 2 (Th2) responses. The responses of T1/ST2-deficient mice to immunological challenges normally characterized by a Th2-like response were then compared with those generated by wild-type mice. It was found that in the absence of T1/ST2 expression, the levels of Th2 cytokine production were severely impaired after immunization. This study showed that ST2 plays a crucial role in the development of Th2-like cytokine responses (Townsend *et al*. (2000).

1.2.4 Biological Functions of novel IL-1 family members

Although novel IL-1 cytokines have been found to be structurally related to the classical IL-1 cytokines, significant research is underway to determine whether

novel IL-1 cytokines influence immune responses in the same way as the classical IL-1 family members. The severe pathophysiological effects of dysregulated IL-1 expression have already been highlighted. Studies suggest that with the classical IL-1 cytokines, very tiny amounts of IL-1 are needed to activate the relevant IL-1 receptor complex and to initiate IL-1-mediated effects (Dinarello, 1991; 1996; 2005; Smith *et al*., 1992). On the other hand, research involving novel IL-1 cytokines seems to suggest that they are only biologically active at much higher concentrations than those observed for classical IL-1 family members such as IL-1β (Debets *et al*., 2001; Towne *et al*., 2004). The significance of this finding is not yet clear.

It has also been observed that while haematopoietic cells are the main source of IL-1β and IL-18, they do not appear to be the main source of the newer IL-1 family members (Debets *et al.,* 2001; Towne *et al.,* 2004). IL-1F6, IL-1F8 and IL–1F9 and their receptor, IL-1Rrp2 are most highly expressed in the skin and in gastric mucosa, suggesting a role in immune/inflammatory responses involving the skin and gastrointestinal tract (Debets *et al*., 2001; Towne *et al*., 2004; Barksby *et al*., 2007).Further studies are also needed to establish whether IL-1F8 plays an inflammatory role in RA as suggested by some findings (Magne *et al.,* 2006).The precise biological role of IL-1F11 and the exact mechanism by which it interacts with its receptor (ST2) also needs clarification.

In view of the already-mentioned role of IL-1 cytokines in health and pathological processes, there is clearly a case for further research in order to elucidate whether or not novel IL-1s and their receptors behave like typical IL-1 cytokines. The need to fully understand their synthesis, regulation of expression and bioactivity cannot be overemphasised.

1.3.0 Dendritic cells (DCs)

1.3.1 DCs: Background

DCs are professional antigen presenting cells with a unique ability to activate naive T cells thereby providing a link between innate and adaptive immunity (reviewed in Banchereau and Steinman, 1998). They can also capture and retain unprocessed antigen and can transfer this antigen to naive B cells to initiate a specific antibody response (Wykes *et al.,* 1998). Maturation of dendritic cells (DC) is a prerequisite for induction of adaptive immunity (Fig. 1). Mature DCs have the capacity to both induce clonal expansion of T cells and also to direct differentiation of T helper (T_H) effectors through secretion of cytokines which favour the development of different effector Th cells (Cella *et al*., 1996; reviewed in Cella *et al*., 1997; reviewed in Banchereau and Steinman, 1998; Reise e Sousa *et al*., 1999, reviewed in Banchereau *et al*., 2000). Cytokines such as IL-12 and IL-4 are believed to be critical for differentiation of activated T_H cells into interferon IFN-γ-producing T_H1 effector cells or IL-4/IL-5/IL-10-producing T_H2 effector cells (Macatonia *et al*., 1993; Abbas *et al*., 1996; Reis *et al*., 1999). CD40 ligand (CD40L) and microbial products are known to rapidly

induce DCs to secrete large amounts of IL-12 which in turn leads to induction of T_H1 responses (Cella *et al.,* 1996; reviewed in Cella *et al.,* 1997; Wesa and Galy, 2002).

Classical IL-1 cytokines such as IL-1β (IL-1F2) are also known to mediate DC maturation in an autocrine/paracrine fashion during bacterial infection (Macatonia *et al*., 1995; Cella *et al*., 1996; Koch et al., 1996; Cella *et al*., 1997; Banchereau and Steinman, 1998; Reise e Sousa *et al*., 1999). In addition to their effect on T cells, mature DCs also trigger a natural response to invading pathogens by activating macrophages, natural killer (NK) cells, NK-T cells and eosinophils (Lipscomb and Masten, 2002; Reis e Sousa, 2004).

1.3.2 DC types and DC precursors

Several different types of DCs and DC precursors have so far been described. These include Langerhans cells (LCs), dermal/interstitial DCs, interdigitating reticulum cells in lymphoid organs as well as blood DCs (reviewed in Banchereau *et al.,* 2000; reviewed in Lipscomb, and Masten, 2002. It is believed that the different DCs differ in terms of origin, maturation state, morphology, localization, function and phenotype (Galy *et al.,* 1995; reviewed in Banchereau *et al*., 2000; Lipscomb and Masten, 2002; Shortman and Liu, 2002). Human DCs are found as precursor populations in bone marrow and blood and as more mature forms in lymphoid and non-lymphoid tissues (Romani *et al.,* 1994;

O'Doherty *et al.,* 1994; Romani *et al.,* 1996). Various combinations of growth factors (such as granulocyte macrophage-colony stimulating factor (GM-CSF), tumour necrosis factor (TNF), IL-4, transforming growth factor (TGF) and IL-3) favour the development of myeloid and lymphoid lineage DCs from bone marrow progenitors and blood precursors (reviewed in Lipscomb, and Masten, 2002) as Figure 1.3 shows. Identification of subset-specific blood dendritic cell antigens (BDCA) in recent years has greatly facilitated DC research (Dzionek *et al*., 2000).

Myeloid $CD34^+$ progenitors are thought to differentiate into monocytes ($CD14^+$, $CD11c^+$ DC precursors) which give rise to immature DCs in response to GM-CSF and IL-4, and to macrophages in response to macrophage colony stimulating factor (M-CSF) (the interstitial pathway) (Caux *et al.,* 1992; Sallusto, and Lanzavecchia, 1994; reviewed in Banchereau *et al*., 2000). Myeloid progenitors differentiate into $CD11c^+$ $CD14^-$ precursors, which yield Langerhans cells in response to GM-CSF, IL-4 and transforming growth factor (TGF) β, and macrophages in response to M-CSF. These later precursors are able to spontaneously differentiate into DCs in cultures (reviewed in Banchereau *et al*., 2000). The $CD14^-$ $CD11c^-$ IL-$3R\alpha^+$ DC precursor (also called pDC2, IFNα-producing cell, or plasmacytoid T cell; a possible equivalent to the murine lymphoid DCs) probably originates from the lymphoid $CD34^+$ progenitor (reviewed in Banchereau *et al*., 2000). A blood cell population with a comparable phenotype has been shown to yield T cells in foetal thymic organ cultures.

$CD11c^-$ $IL3R\alpha^+$ DC precursors differentiate into immature DCs in response to IL-3. The immature cells differentiate to mature cells in response to cytokines (MCM, monocyte-conditioned medium) or pathogen products (LPS or DNA) (reviewed in Banchereau *et al*., 2000). Table 1.3 shows the main phenotypic features of peripheral blood dendritic cells (PBDCs) and monocytes.

There is speculation that distinct types of DCs induce different types of immune responses (T_H1 or T_H2) (Pulendran *et al.,* 1999). It is starting to become clear that myeloid DCs (mDCs) and pDCs show interesting differences not only in terms of phenotypic markers but also with regards to cytokine secretion pattern, chemokine receptor expression and pathogen recognition receptor (PRR) expression. This suggests differences in functional properties among the DC subsets (Sallusto and Lanzavecchia, 2002; reviewed in Banchereau *et al*., 2003). The issue of which DCs regulate or influence which T cell response still needs clarification. Some researchers think that DC maturation stage has a big influence on the resulting T cell response (Lutz and Schuler, 2002) but others think that DC lineage is a critical parameter (Palucka and Banchereau, 2002; Grabbe *et al*., 2000). It is highly likely that a combination of factors is at play. There is also a possibility that DC lineages possess a unique flexibility which allows for lineage adaptability. Clearly, the intricacies of DC biology still need to be unravelled.

Table 1.3 Immunophenotype of PBDC subsets and monocytes (adapted from Robinson *et al.,* 1999)

Surface antigen	CD11c– DC (Lymphoid)	CD11c+ DC (Myeloid)	CD 14++ Monocytes
Progenitor			
CD34	±	±	–
DC-associated			
CD1a	–	–	–
CD83	–	–	–
Myeloid			
CD13	–	+	++
CD14	–	±	++
CD33	±	+	++
Lymphoid			
CD2	–	+	±
CD4	++	+	±
CD5	±	±	±
CD8	–	–	–
CD10	±	–	–
FcR			
CD16	–	–	+
CD32	–	++	++
CD64	±	+	++

Table 1.3 (continued) Immunophenotype of PBDC subsets and monocytes

(adapted from Robinson *et al.,* 1999)

Surface antigen	CD11c– DC (Lymphoid)	CD11c+ DC (Myeloid)	CD 14++ Monocytes
Adhesion			
CD11a	+	+	++
CD11b	–	–	++
CD11c	–	+	++
CD44	+	+	+
CD54	+	+	+
CD62L	++	+	±
E-cadherin	+	+	+
CLA	+	++	+
MHC			
Class I	+	+	+
HLA-DR	++	+++	+
HLA-DQ	++	+++	+
Co-stimulation			
CD40	–	±	+
CD80	–	–	+
CD86	–	±	+
Activation			
CD45RA	++	±	+
CD45RO	–	+	+
Cytokine receptors			
CD25	–	–	±
CD115	–	±	++
CD123	++	+	±

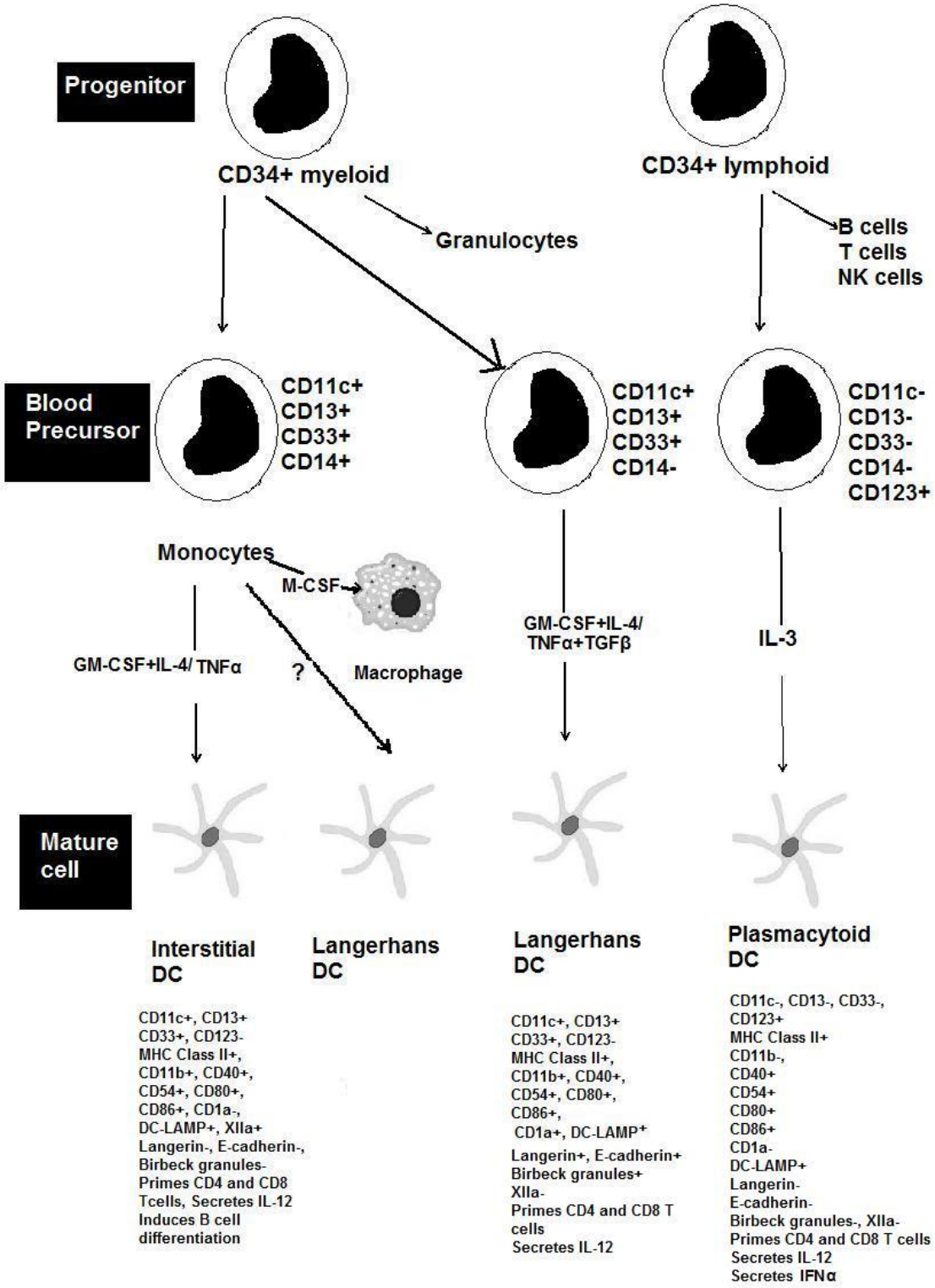


Figure 1.3 Schematic diagram showing derivation of human dendritic cell (DC) subsets from CD34+ myeloid and lymphoid progenitors. Phenotypic and functional characteristics defining each DC subset are shown below each DC subset. MHC= major histocompatibility complex; IFN= interferon (adapted from Lipscomb and Masten, 2002).

1.3.3 Human Peripheral Blood Dendritic Cells (PBDCs)

Human peripheral blood (PB) has been shown to contain a heterogeneous mixture of at least two main distinct types/subsets of DC precursors (O'Doherty *et al*., 1994; Strobl *et al*., 1998; Robinson *et al*., 1999). The two main DC subsets have been named myeloid (m) and plasmacytoid (p)/lymphoid. Myeloid and lymphoid (plasmacytoid) DCs evolve from myeloid or lymphoid precursors respectively. Both subsets lack expression of several lineage markers for monocytes, lymphocytes and natural killer cells. Myeloid DCs are $CD123^{dim}$ and $CD11c^{bright}$ (O'Doherty *et al*., 1994; Strobl *et al*., 1998; Robinson *et al*., 1999). They are further subdivided into type 1 mDCs (mDC1) and type 2 mDCs (mDC2) (Dzionek *et al.,* 2000; Grabbe *et al.,* 2000).

1.3.3.1 Type 1 myeloid dendritic cells (mDC1)

Type 1 mDCs make up about 0.6 % of all peripheral blood mononuclear cells (PBMCs) and are the major subset of mDCs in human blood (Dzionek *et al.,* 2000; Grabbe *et al.,* 2000). They are CD1c /BDCA-1^{+} and show a monocytoid morphology. They also express myeloid markers such as CD13 and CD33 as well as Fc receptors such as CD32, CD64 and FceRI. Furthermore, they were determined to be $CD4^{+}$, Lin (CD3, CD16, CD19, CD20, $CD56)^{-}$, $CD2^{+}$, $CD45RO^{+}$, CD141 $(BDCA\text{-}3)^{low}$, CD303 $(BDCA\text{-}2)^{-}$, and CD304 (BDCA-4/Neuropilin-$1)^{-}$.[1] (Dzionek *et al.,* 2000). A minor proportion of mDC1 expresses CD14 and CD11b. The CD1c (BDCA-1) marker is also found on $CD1a^{+}$ dendritic

cells generated *ex vivo* from monocytes or haematopoietic precursor cells (Dzionek *et al.,* 2000). In blood, apart from mDC1, a subset of B cells also expresses CD1c (BDCA-1) (Dzionek *et al.,* 2000; Grabbe *et al.,* 2000; Brigl and Brenner, 2004).

1.3.3.2 Type 2 myeloid dendritic cells (mDC2)

Type 2 mDCs are a minor subpopulation of mDCs constituting about 0.04 % of all PBMCs in human blood. They express high levels of CD141 (BDCA-3) (Dzionek *et al.,* 2000). They are CD141/BDCA-3^{++}, CD11c^{dim}, CD123^{-}, CD1c (BDCA-1)$^{-}$and CD4^{+}. They lack lineage markers such as CD3, CD14, CD16, CD19, CD20 and CD56 but express myeloid markers such as CD13 and CD33. They are also monocytoid in appearance. The CD141/BDCA-3 antigen is also expressed at a much lower level on mDC1 cells, plasmacytoid dendritic cells, monocytes, and granulocytes in blood (Dzionek *et al.,* 2000). In contrast to mDC1, mDC2 cells do not express CD2 and Fc receptors such as CD32, CD64, and FceRI. Furthermore, mDC2 cells differ from mDC1 cells in terms of Toll-like receptor (TLR) expression, cytokine production, and T helper cell polarization (Dzionek *et al*., 2000; Grabbe *et al.,* 2000; Liu, 2001a; Liu 2001b).

1.3.3.3 Plasmacytoid dendritic cells (pDCs)

Plasmacytoid dendritic cells (pDCs**)** possess a plasmacytoid morphology, express CD45RA, are $CD123^{bright}$, $CD11c^{-}$ and they depend on IL-3 for their survival and differentiation into mature DC (Dzionek *et al*., 2000; Penna *et al.,* 2002). They can produce high amounts of Type 1 interferon (IFN) (IFN-α, and IFN-β) on stimulation by several viruses (Bruno *et al*., 1997; Strobl *et al*., 1998; Rissoan *et al*., 1999; Liu, 2005; Penna *et al.,* 2002). This feature earned them the name “interferon-producing cells” (IPC) before their dendritic cell nature was demonstrated (Bruno *et al*., 1997; Strobl *et al*., 1998; Rissoan *et al*., 1999; Liu, 2005). Expression of the pre-T Cell Receptor (TCR) α -chain by pDCs is considered evidence that they arise from lymphoid precursors (Rissoan *et al*., 1999; Dzionek *et al*., 2000). However, recent data from mouse studies suggests that pDCs can arise from both lymphoid and myeloid precursors both *in vivo* and *in vitro* and that the myeloid pathway is a major pathway for pDC development *in vivo* (Harman, 2006; Karsunky *et al.,* 2005). There is clearly a need for further investigation into the mechanisms behind DC lineage specification and/or DC plasticity.

1.4 Justification of current study

There is growing evidence showing that the classical IL-1 cytokines play a pathogenic role in diseases such as RA, periodontitis, sepsis, insulin-dependent diabetes mellitus, IBD, atherosclerosis and others (Bistrian *et al*., 1992; Graves and Cochran, 2003; Gracie, 1999). Based on the structural similarities between the novel IL-1 cytokines and the classical IL-1 cytokines, it is possible that some of these newly identified IL-1 cytokines may also play a role in the pathophysiology of conditions such as the septic syndrome. It has been observed that for some inflammatory disorders such as RA, inhibiting IL-1 production, signalling and bioactivity can have beneficial effects (Braddock and Quinn, 2004; Dinarello, 2005; Moller and Villiger, 2006). Clinical trials have shown that Anakinra (a recombinant form of sIL-1Ra) has an acceptable safety profile and that it reduces the signs and symptoms of active disease and joint destruction in patients with RA (Furst, 2004; Cohen and Rubbert, 2003) and it is now commercially available as Kineret (Cohen and Rubbert, 2003).

At the moment, there is very limited information regarding the novel IL-1 cytokines. It is not yet clear whether or not they behave like typical IL-1 cytokines. There is a need to fully understand their synthesis, regulation of expression and bioactivity. Although new members of the 11-member IL-1R family have been identified, their expression patterns, regulation and biological functions have not been well characterized (Sims, 2002; Subramaniam, Stansberg, Cunningham, 2004; Sims, Smith, 2010). It is not clear how some of

these orphan receptors interact with the novel IL-1 cytokines. A better understanding of the immunobiology of the novel IL-1 cytokines and their receptors will help shed more light into the pathogenesis of several immune and inflammatory diseases. Such an understanding may in turn lead to the development of novel therapeutic agents.

1.5 Aims of current study

- To investigate the expression of IL-1Rrp2 in human dendritic cell subsets, other human immune and non-immune cells
- To establish if human dendritic cells (DCs) respond to the novel IL-1 cytokines IL-1F8 and IL-1F9
- To assess the effect of established mediators of DC maturation (bacterial LPS, recombinant IL-1β and imiquimod) on IL-1Rrp2 expression in human DCs. (Imiquimod is a toll like receptor-7 agonist known to induce type I interferon in plasmacytoid DCs) (Gibson *et al.,* 2002)

The aims of this study were achieved by:

- measuring the expression of IL-1Rrp2 mRNA in human DCs and other myelomonocytic cells (Chapters 3)
- measuring IL-1Rrp2 expression in human T cells, lamina propria tissue and other human cells and cell lines (Chapter 4)
- stimulating human DCs with different concentrations of IL-1F8 and IL-1F9 and assessing effect on phenotypic profile and cytokine production (Chapters 3)
- stimulating human DCs with bacterial LPS, recombinant IL-1β and imiquimod and assessing expression of IL-1Rrp2 mRNA (Chapter 3)
- assessing the effect of IL-1F8-matured DCs on T cells in terms of T cell proliferation and cytokine profile (Chapter 3)

1.6 Challenges encountered

The recombinant IL-1F8 and IL-1F9 reagents used in this study were kindly donated by Amgen Corporation, Seattle, WA, USA; however they were only supplied in small quantities. In addition, the ligands showed very weak biological activity (as they were non-truncated) and had to be used at very high concentrations. As a result, the range of investigations that could be pursued was restricted. Amgen researchers (Towne *et al.,* 2011) and others (Vigne *et al.,* 2011) have since demonstrated that truncation of IL-1F6, IL-1F8 and IL-1F9 (IL-36α, IL-36β, and IL-36γ) enhances their activity (Towne *et al.,* 2011). Unfortunately, the truncated ligands were not yet available when the work outlined in this thesis was carried out.

CHAPTER 2: MATERIALS AND METHODS

2.1.0 Routine Cell culture experiments

Cell culture experiments were performed using aseptic techniques. Appropriate personal protective equipment was used throughout. Work was carried out in a Class II Biological Safety Cabinet according to University Health and Safety regulations. Equipment, gloved hands, work area inside the Safety Cabinet and reagent bottles were decontaminated using 70% (v/v) ethanol in sterile water prior to cell manipulation. As much as possible, sterile, single-use consumables were used for cell culture experiments. Where it was necessary to use non-disposable glassware, this was sterilised by autoclaving at 121°C for 20 minutes prior to use. To prevent mycoplasma contamination and to inactivate complement, serum used for cell culture was heat-inactivated (heated to 56°C in a water bath for 30 minutes with swirling every 5 to 10 minutes) prior to use. All solutions and cell culture media were also sterilised by passing them through a Thermo Scientific Nalgene 0.2µm pore size Disposable Sterile Filter Unit (Fisher Scientific, Loughborough, UK) prior to use. Furthermore, to minimise bacterial contamination, culture media was supplemented with Penicillin-Streptomycin solution (working concentration of 100 IU/ml penicillin, 100 µg/ml streptomycin). Cultures and media were examined daily for evidence of gross bacterial or fungal contamination. Cells were grown at 37°C in a humid 5% CO_2 incubator. Cells that were not in use were stored at -80°C. For long term storage, cells were

stored in liquid nitrogen at -196°C. All equipment used was routinely cleaned, sanitised, maintained and serviced according to manufacturer and University Health and Safety specifications. Each cell culture experiment was performed in duplicate on at least three independent occasions.

2.1.1 Routine cell counting and assessment of viability

An Improved Neubauer Haemocytometer was used to determine cell numbers. This type of haemocytometer has two counting chambers, each 0.1 mm deep. Each chamber is engraved with a laser-etched grid containing nine large squares demarcated by triple white lines. The rulings of the haemocytometer grid cover 9 mm^2 in a 3x3 square. Typically, 10µl of cell suspension were mixed with 10µl of 0.4% Trypan Blue to make a 1:2 dilution. However, if cell concentrations were too high, suitable dilution factors were used to ensure that cells were evenly distributed (and not overlapping) on the counting grid. 10µl of the cell suspension/Trypan Blue mix was then gently pipetted onto the edge of the cover slip and allowed to run onto the chamber under the cover slip by capillary action. Using a fresh pipette tip, another 10µl of the cell suspension/Trypan Blue mix was loaded onto the second counting chamber. The haemocytometer was then placed on the microscope stage of a Leica DM IL inverted microscope (Leica Microsystems, Milton Keynes, UK) and the counting grid was brought into focus using the x10 objective lens. Once the grid and cells were in focus, the magnification needed to recognize the desired cell type was determined. Using a hand tally counter, cells in the large centre square of each chamber (see Figure

2.1**)** were then counted. The central square of the haemocytometer grid is ruled into 25 squares. Each of the 25 squares contains 16 smaller squares. The area of each smaller square is 0.0025 mm^2 and each group is separated by triple lines, the middle one of which is the boundary. The area of the central square is: 25 x 16 x 0.0025 = 1 mm^2 and the volume is: 1 mm^2 x 0.1 mm = 0.1mm^3 or 0.1µl (since 1 ml = 1000 µl= 1000 mm^3).

To ensure accuracy of counts, the following counting pattern was adhered to: Cells falling on the bordering triple lines were only counted if they were on either the top or on the left lines of each square. Cells touching the bottom and the right hand side lines were excluded (see Figure 2.2). To work out the number of cells/ ml, the following formula was used:

Number of cells counted per ml = number of cells counted per square mm x dilution factor x 10^4 (where 10^4 is the conversion factor needed to convert 10^{-4}ml to 1ml). Cells in both sides (chambers) of the haemocytometer were counted and the average of the two counts was taken. The total number of cells was calculated using the following formula:

Total cells = cells/ml x volume of original suspension

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It does not interact with the cell unless the cell membrane is damaged (Mishell and

Shiigi, 1980). To assess cell viability, cells looking faint or dark blue within the grid being counted were counted as dead (non-viable) while unstained cells were counted as viable. Cell dilutions and Trypan Blue dye addition were performed immediately prior to counting since viable cells may absorb Trypan blue over time. To calculate percentage cell viability, the following formula was used:

$$\% \text{ cell viability} = \frac{\text{Live cell count (unstained cells)}}{\text{Total cell count (Trypanblue - stained and unstained cells)}} \text{ X } 100$$

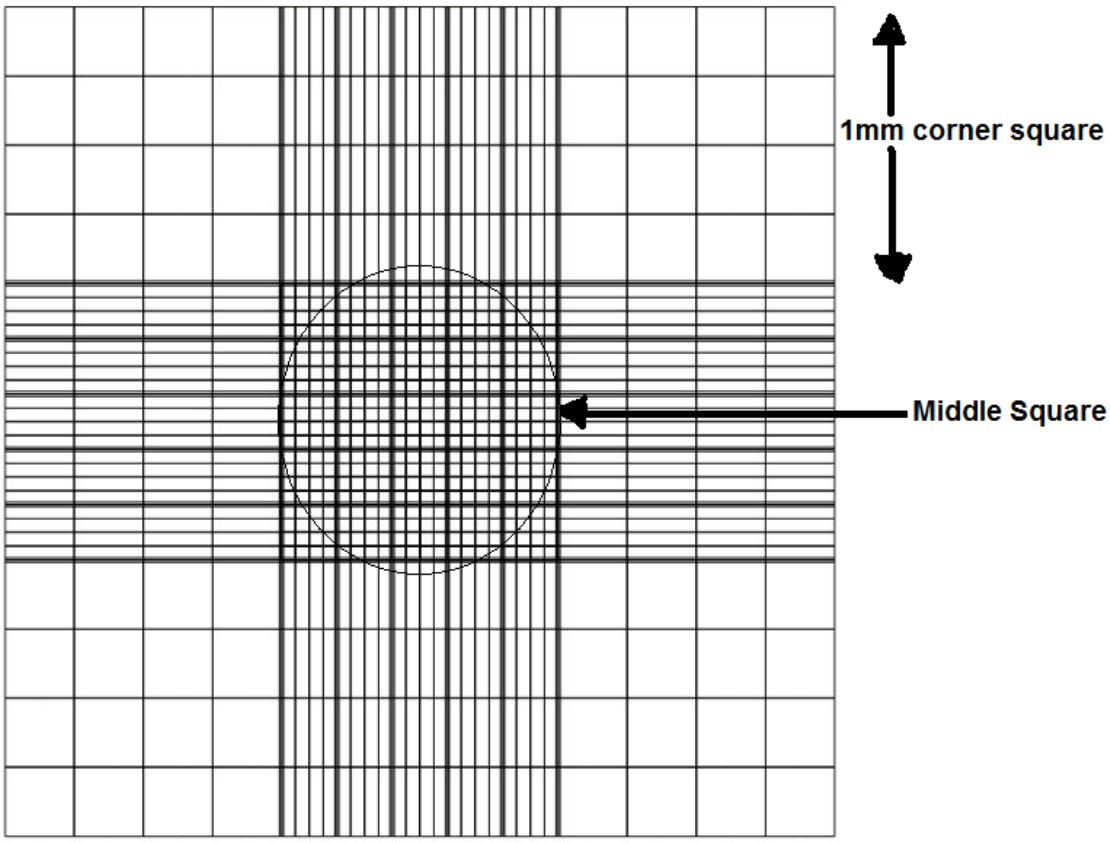


Figure 2.1 Diagram showing the appearance of a haemocytometer grid when viewed under the microscope

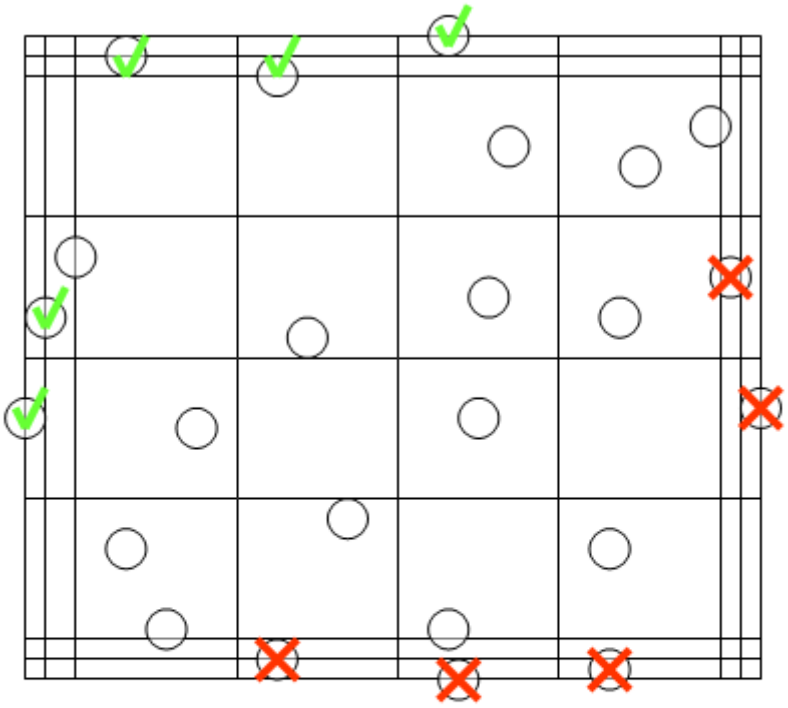


Figure 2.2 Diagram showing one of the 25 squares making up the central square of a haemocytometer. Each square contains 16 smaller squares as shown in the diagram. To ensure consistency in counting, cells that were on a boundary line between two squares were only counted if they were on the upper boundary line or the left-hand side boundary line (indicated by green tick) but not if they were on the lower boundary line or the right-hand side boundary line (indicated by a cross).

2.1.2 Endotoxin assays

A chromogenic Limulus Amebocyte Lysate (LAL) assay (Kinetic-QCL, ™Lonza, USA) was performed on IL-1 reagents at Amgen and at the University of Nottingham. Assay sensitivity was between 0.005 and 50.0 EU/ml. The test is done on a sterile, disposable 96 well plate and is based on the principle that gram negative bacterial endotoxin (LPS) in a sample catalyses the activation of a proenzyme in the Limulus Amebocyte Lysate (LAL). The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme then catalyses the cleavage of paranitroaniline (pNA) from the colourless substrate Ac-Ile-Glu-Ala-Arg-pNA. The yellow pNA released is measured photometrically (at 405 nm) continuously throughout the incubation period by the assay operating software (WinKQCL^{TM}) on the microplate reader. Using the initial absorbance reading of each well as its own blank, the reader determines the time required for the absorbance to increase 0.200 absorbance units. This time is termed Reaction Time. The WinKQCL^{TM} software automatically performs a log/log linear correlation of the Reaction Time of each standard with its corresponding endotoxin concentration. The concentration of endotoxin in a sample is calculated from its Reaction Time relative to the Reaction Time of solutions containing known amounts of endotoxin standard. The following equations summarise the reaction:

Proenzyme $\xrightarrow{\text{Endotoxin}}$ Enzyme

Ac-Ile-Glu-Ala-Arg-pNA substrate + water $\xrightarrow{\text{Enzyme}}$ Peptide + pNA

Results showed LPS concentrations of 0.216 for human recombinant IL-1F8 and 0.130 for human recombinant IL-1F9, which were equivalent to <<0.01 Pg/ml in 100 ng IL-1 protein used in cell culture. Similar results were obtained on these reagents by the manufacturers (Amgen Corporation, Seattle, WA, USA) prior to shipment. Other reagents used in the study also showed negligible endotoxin levels (see Table 2.1). To cause DC maturation, endotoxin (LPS) concentrations of 100ng/ml are required (Zhou *et al.,* 2006). Since the concentrations of endotoxin in the reagents used is <<0.05 Pg/ml even at the highest concentration used in this study (500ng/ml), it is highly unlikely that such low levels would have interfered with the results obtained. Any stimulatory or other effect seen is very likely to have been caused by the reagents themselves. As an additional test for LPS contamination, a THP-1 functional status assay was performed, which showed that THP-1 cells did not produce TNF-α in response to IL-1 reagents but that they did produce TNF-α when stimulated with bacterial lipopolysaccharide (see Appendix 1).

Table 2.1 Endotoxin assay results. Concentration of endotoxin, independently determined by Amgen Corporation (Seattle, WA, USA) and University of Nottingham, in IL-1F8 and IL-1F9 protein aliquots and other reagents used in all experiments.

Reagent	EU/mg protein	
Amgen	Endotoxin-free water	0.002
	IL-1F8	0.209
	IL-1F9	0.092
Nottingham University	Endotoxin-free water	<0.01
	IL-1F8	0.216
	IL-1F9	0.13
	IL-1β	<0.005
	IFN-γ	0.005
	IL-4	<0.005
	GM-CSF	0.010

2.1.3 Human IL-1F8 and IL-1F9 reagents

Human non-truncated IL-1F8 and IL-1F9 reagents were supplied by Amgen Corporation, Seattle, USA. They were produced using the following procedure: Human IL-1F8 and IL-1F9 were cloned into pGEX4T-1 (Amersham Biosciences) as N-terminal glutathione *S*-transferase fusions. A Factor Xa recognition sequence was placed immediately upstream of the first methionine of each IL-1F gene. The resulting constructs were expressed in *Escherichia coli* DH10B by induction with 100 mM isopropyl-1-thio-β-D-galactopyranoside. Lysates from cultures were run over glutathione-Sepharose (Novagen, Madison, WI) columns to capture the glutathione *S*-transferase/IL-1F fusion proteins and then subjected to on-column cleavage with Factor Xa (Novagen). The cleaved IL-1F proteins were eluted with PBS, and the Factor Xa was removed using a specific affinity agarose (Novagen). The IL-1F proteins were further purified by size exclusion chromatography using a Superdex 75HR column. Purified proteins were then quantitated by amino acid analysis (Towne *et al.,* 2004).

2.2.0 Cells

2.2.1 THP-1 cells: Cell culture

Promonocytic THP-1 cells were purchased from the European Collection of Cell Cultures, Salisbury, UK). THP-1 cells were cultured in RPMI 1640 medium

supplemented with 10% v/v heat-inactivated Fetal Calf Serum (FCS), L-glutamine (2mM), penicillin (100 U/ml), streptomycin (100µg/ml) and maintained in a humidified incubator at 37^{o}C and 5% CO_2. During cell culture, viability was assessed by the Trypan Blue Exclusion method (Mishell and Shiigi, 1980). Viability was >95% prior to use. Cell numbers were monitored using an improved Neubauer haemocytometer. Cells were fed with fresh medium twice a week. They were kept at a density of 400 000 cells/ml in each 75cm^2 flask and medium was changed every 2 to 3 days. Cells were sub-cultured when numbers exceeded 800 000 cells/ml but were not allowed to exceed 1x10^6 cells/ml. Spare cells were suspended in freezing mixture (5% DMSO, Sigma-Aldrich, Poole, UK) and stored at -80 ^{o}C. For stimulation assays, THP-1 cells were used at passages between 5 and 10.

2.2.1.1 THP-1 Functional Status test

To confirm that THP-1 cells which were used as negative controls for IL-1Rrp2 mRNA expression in Quantitative Real Time PCR experiments were fully functional, their response to LPS stimulation was investigated (Essner *et al*, 1990; Agarwal *et al*., 1995; Asakura *et al*., 1996; Dedrick and Conlon, 1995; Jones *et al*., 2003; Foster *et al*., 2005; Lackman and Cresswell, 2006) as described in Appendix 1.

2.2.2 NCI/ADR-RES cells (OVCAR-8 ovarian adenocarcinoma cell line)

NCI/ADR-RES cells were a kind gift from Professor Susan Watson, University of Nottingham. Cells were maintained at 37^{o}C in a humidified atmosphere of 95%

air and 5% CO2 in a culture medium containing RPMI 1640 (Fisher Scientific, Loughborough, UK) supplemented with heat-inactivated FCS (10%v/v), Penicillin-Streptomycin (1%), HEPES sodium salt (15 mM) (Fisher Scientific, Loughborough, UK) and L-glutamine (2mM) (Sigma-Aldrich, Poole, UK).
For experiments, cells were harvested using trypsin (0.2%) and EDTA (0.02%) in PBS prior to rinsing with the appropriate medium (low-speed centrifugation). Cell viability was determined by haemacytometer counting after staining with Trypan blue as previously described (Chapter 2, Section 2.1.1). Cell viability was >95% prior to use.

2.2.3 HT 29 cells

The human colonic epithelial carcinoma cell line, HT-29, was a kind gift from Professor Susan Watson, University of Nottingham. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with glucose (25mM), Sodium Bicarbonate (44mM), Transferrin (10mg/l), sodium pyruvate (1mM), L-glutamine (2mM), 0.1 mM non-essential amino acids (NEAA) and 10% FCS. Cell cultures were maintained in humidified incubators at 37°C, 5% CO_2. The medium was changed every day to prevent glucose exhaustion.

2.2.4 Human ileal lamina propria cells: Analysis of IL-1Rrp2 mRNA expression by qRT-PCR

After obtaining informed consent from three different patients, fresh, histologically normal mucosal samples surplus to clinical requirements were

obtained by Professor Yash Mahida (Institute of Infection, Immunity and Inflammation, Queen's Medical Centre, Nottingham) from human terminal ileum resected for tumour. Ethical committee approval was provided by the Nottingham Research Ethics Committee. Mucosal samples were obtained at least 5 cm from the tumour. The tissue was transported to the laboratory in Hanks medium, gently wiped with sterile gauze to remove most of the adherent mucus and debris and immediately processed. Lamina propria cells were isolated from mucosal samples using a modification of the ethylenediamine tetra-acetic acid (EDTA) collagenase technique of Bull and Bookman, as previously described (Bull and Bookman, 1977; Gibson *et al.,* 1985). Briefly, strips of mucosa were washed, dissected and incubated in 1 mmol/l dithiothreitol solution (DTT; Sigma, Poole, Dorset, UK) for 20 minutes at room temperature. To remove the epithelial cells, mucosal strips were treated three times with 5 mmol/l ethylenediamine tetra-acetic acid (BDH, Poole, Dorset, UK) at 37°C for half an hour. After the final wash the mucosa was minced into 1 mm pieces and digested for three hours at 37°C in RPMI 1640 (Fisher Scientific (Loughborough, UK) supplemented with 1mg/ml collagenase (from Clostridium histolyticum, Sigma, Poole, UK) and 10% v/v foetal calf serum (Fisher Scientific (Loughborough, UK). The digested tissue was passed through a sterile nylon mesh (Sigma-Aldrich, Dorset, UK) to obtain the lamina propria cells and exclude undigested tissue. Depending on down-stream applications, the isolated lamina propria cells were suspended in appropriate medium at a density of $1x10^6$ cells/ml. Cell viability, assessed using the Trypan Blue Dye Exclusion method, varied between 90 and 100%.

To stabilize RNA prior to extraction, samples for qRT-PCR analysis were immediately pelleted and suspended in RNA*later* RNA Stabilization Reagent according to the Manufacturer's recommendations (QIAGEN, Crawley UK). Total RNA was extracted from the cells, reverse-transcribed into cDNA and amplified in a typical real time PCR reaction. A relative quantification analysis using GAPDH as the reference gene and IL-1Rrp2 as the target gene was performed on a LightCycler® 480 analyser. HT 29 cells and THP-1 cells were used as positive and negative controls respectively. Each analysis was performed in triplicate on samples from three different patients.

2.3.0 Isolation of cells from human peripheral blood

2.3.1 Human peripheral blood buffy coat preparations

Human peripheral blood buffy coat preparations were used for the isolation of peripheral blood mononuclear cells (PBMCs). Preparations were purchased from The National Blood Service, Sheffield, and were received within 24 hours of blood donation. They were kept at room temperature during storage and transport. Peripheral blood monocytes, lymphocytes, granulocytes and dendritic cells were isolated from buffy coat preparations using standard techniques as described in the relevant sections below. Monocyte-derived dendritic cells were generated from isolated peripheral blood monocytes as described in the relevant section below.

2.3.2 Isolation of human peripheral blood mononuclear cells (PBMC)

Peripheral blood buffy coat preparations obtained from the National Blood Service (Sheffield) were used as starting material for the isolation of peripheral blood mononuclear cells. Buffy coat blood is a concentrated leukocyte fraction derived from venipuncture donations obtained from healthy blood donors. It is generally enriched for white blood cells (leukocytes) but contains trace amounts of red cells and other blood components. If desired, it may be further purified. Purchased buffy coat preparations used in this study had an average volume of 50ml.

A standard Histopaque®-1077 (Sigma, UK) density gradient centrifugation technique was used for PBMC isolation (Boyum, 1968). Histopaque®-1077 is a sterile, endotoxin tested solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/ml. It facilitates separation of blood cells according to their densities and enables rapid recovery of mononuclear cells from small volumes of whole blood. To improve isolation of pure PBMC, the purchased buffy coat samples were treated as whole blood. Briefly, the following method was used: 25ml of Histopaque®-1077 was pipetted into each of 4 sterile 50 ml conical centrifuge tubes and allowed to reach room temperature. On a separate rack, a buffy coat unit was split into 2x 25ml aliquots in sterile 50 ml conical centrifuge tubes. 25ml of buffy coat was then diluted 1:1 with phosphate buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) in sterile 50 ml conical centrifuge tubes. 25 ml of diluted buffy coat was then slowly layered on

top of each Histopaque®-1077 layer. The centrifuge tube was kept at a 45 degree angle and the sample was allowed to run down the side of the tube until it was filled to the 50 ml mark. Care was taken to keep a clear separation between the Histopaque®-1077 medium and the blood layer prior to centrifugation. The sample tubes were centrifuged at 400 x g for exactly 30 minutes at room temperature (25^{o}C) in a swinging bucket rotor with the brake off. After 30 minutes, tubes were carefully removed from the centrifuge while ensuring that the layering was not disturbed. Figure 2.3 is a schematic diagram showing isolation of peripheral blood mononuclear cells (PBMCs) from human peripheral blood by density gradient centrifugation.

As the diagram (Figure 2.3) shows, centrifugation of the blood/Histopaque®-1077 mixture for 30 minutes at 400g resulted in rapid sedimentation of red blood cells to the bottom as they were aggregated by the polysucrose in Histopaque®-1077. Granulocytes became slightly hypertonic, which increased their sedimentation rate, resulting in them pelleting at the bottom of the centrifuge tube. PBMCs formed a band at the interface between the Histopaque®-1077 and the plasma. The upper platelet-rich plasma layer was aspirated off to within 0.5-1 cm from the opaque interface containing the PBMCs. Using a sterile plastic pipette, the opaque PBMC or buffy coat interface was carefully transferred into a new 50 ml centrifuge tube, avoiding carryover of the lower clear Histopaque®-1077 layer. PBMCs from all sample tubes were pooled into one 50 ml centrifuge tube. The tube was filled to 50 ml with 1X PBS (at room temperature), and gently

mixed by inversion prior to centrifugation at 300g for 10 minutes at room temperature to remove platelets and excess Histopaque®-1077. Using a 50 ml pipet, the supernatant was carefully removed and discarded. To remove any contaminating red cells, the PBMC pellet was gently loosened using a sterile plastic pipette and resuspended in 2ml of 1X Red Blood Cell (RBC) Lysis buffer (Biolegend, Cambridge, UK). Immediately after adding the lysing solution, the tube was gently vortexed prior to incubating at room temperature, protected from light, for 10 minutes. The RBC Lysis buffer was quenched by filling up the tube with PBS and centrifuging at 300 x g for 10 minutes. The supernatant was aspirated off and discarded without disturbing the PBMC pellet. The pellet was resuspended in the appropriate buffer (1X PBS, supplemented RPMI 1640 medium or staining buffer, depending on further applications). Sample preparation procedures were evaluated by assessing cell viability and by performing a cell count as previously described. PBMC yields ranged between 3 x 10^8 and 8x10^8 per buffy coat. Average cell viability was found to be >90%.

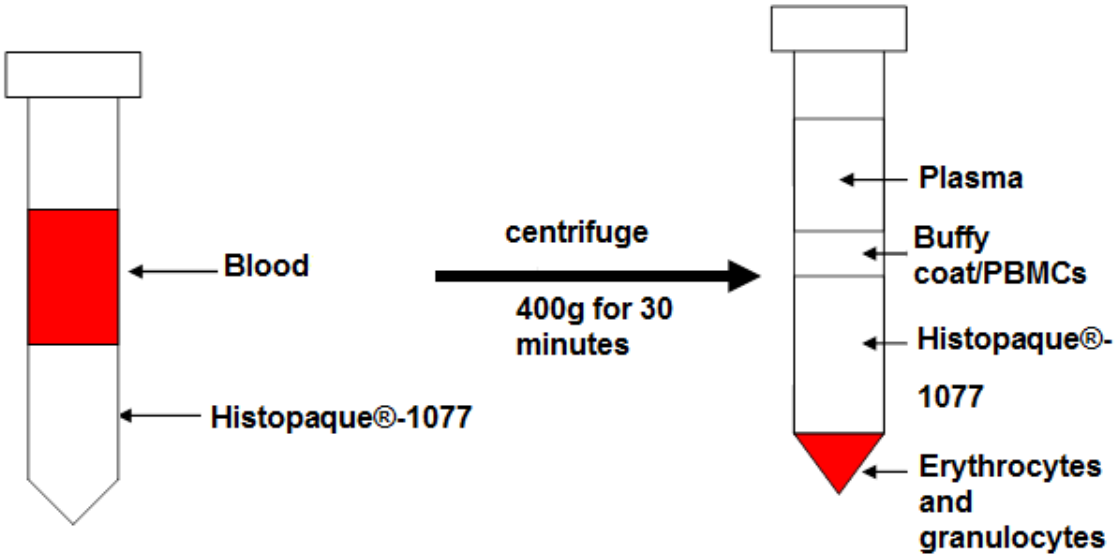


Figure 2.3 Schematic diagram showing isolation of PBMCs from human peripheral blood by density gradient centrifugation using Histopaque®-1077. The diagram shows layers observed in a representative test tube containing peripheral blood (purchased buffy coat blood) and Histopaque®-1077 before and after centrifugation at 400g for 30 minutes.

2.3.3 Isolation of Monocytes from human PBMCs by adherence

PBMCs obtained from human peripheral blood buffy coats as described in previous sections were the starting material for monocyte isolation. Following removal of any remaining platelets by washing with PBS, PBMCs were resuspended at a density of 1 x 10^6 cells/ ml in RPMI 1640 medium supplemented with 2mM L-Glutamine, 10% (v/v) heat-inactivated Foetal Calf Serum (FCS) and 100 U/ml Penicillin-Streptomycin (made up of 100 IU/ml penicillin and 100 µg/ml streptomycin) (Sigma, Poole, UK). Cells were plated on 6-well plastic culture plates in a volume of 3ml per well. Plates were incubated at 37°C in a humidified, 5% CO_2 incubator for 2 hours. After 2 hours, plates were visually inspected under an inverted microscope to ensure that monocytes had adhered to the bottom of the plastic wells. When a distinct carpet of cells was observed at the bottom of the wells, the supernatant, containing non-adherent lymphocytes was gently pipetted off and discarded. To aid removal of any residual non-adherent cells, the adherent monocytes in each well were gently washed three times with 3ml of fresh supplemented RPMI 1640 medium. Adherent monocytes were then removed by gently scraping with a sterile plastic cell scraper before being counted and resuspended in appropriate media at an appropriate density for each experiment. Cell morphology was confirmed by microscopy and purity of monocyte preparations was judged by flow cytometry.

2.3.4 Isolation of lymphocytes

2.3.4.1 Isolation of lymphocytes from human PBMCs using Histopaque®-1077 density gradient centrifugation

PBMCs isolated from purchased human buffy coat samples as previously described were resuspended at a density of 1 x 10^6 cells/ ml in RPMI 1640 medium supplemented with 2mM L-Glutamine, 10% (v/v) heat-inactivated Foetal Calf Serum (FCS) and 100 U/ml Penicillin-Streptomycin (made up of 100 IU/ml penicillin and 100 µg/ml streptomycin) (Sigma, Poole, UK). Cells were plated on 6-well plastic culture plates in a volume of 3ml per well. Plates were incubated at 37°C in a humidified, 5% CO_2 incubator for 2 hours. After 2 hours, plates were visually inspected under an inverted microscope to ensure that monocytes had adhered to the bottom of the plastic wells. When a distinct carpet of cells was observed at the bottom of the wells, the supernatant, containing non-adherent lymphocytes was gently pipetted off, washed in fresh RPMI 1640 (at 300g for 10 minutes), counted, assessed for viability and resuspended in appropriate medium at the right concentration for each experiment. Cell morphology was confirmed by microscopy.

2.3.4.2 Isolation of Lymphocytes using ACCUSPIN™ Histopaque®-1077 centrifuge tubes

Total lymphocytes were isolated from PBMCs by density gradient centrifugation using sterile- filtered ACCUSPIN™ Histopaque®-1077 centrifuge tubes as per

manufacturer's instructions (Sigma-Aldrich, Poole, UK). ACCUSPIN System-Histopaque®-1077 centrifuge tubes have two chambers which are separated by a porous high-density polyethylene barrier ("frit"). Anticoagulated whole blood (purchased buffy coats) was added to the upper chamber without mixing with the Histopaque®-1077 separation medium in the lower chamber prior to centrifugation. On centrifugation, the blood descended through the frit and made contact with the Histopaque®-1077 below the frit, giving a clear separation of the blood components. Lymphocytes and other PBMCs remained at the plasma-Histopaque®-1077 interface while erythrocytes and granulocytes pelleted at the bottom of the ACCUSPIN tube. The isolated PBMCs were resuspended at a density of 1 x 10^6 cells/ ml in RPMI 1640 medium supplemented with 2mM L-Glutamine, 10% (v/v) heat-inactivated Foetal Calf Serum (FCS) and 100 U/ml Penicillin-Streptomycin (made up of 100 IU/ml penicillin and 100 µg/ml streptomycin) (Sigma, Poole, UK). Cells were plated on 6-well plastic culture plates in a volume of 3ml per well. Plates were incubated at 37°C in a humidified, 5% CO_2 incubator for 2 hours. After 2 hours, plates were visually inspected under an inverted microscope to ensure that monocytes had adhered to the bottom of the plastic wells. When a distinct carpet of cells was observed at the bottom of the wells, the supernatant, containing non-adherent lymphocytes was gently pipetted off, washed three times (at 300g for 10 minutes) in fresh RPMI 1640 prior to cell counting and assessment of viability using previously stated techniques. Cell morphology was confirmed by microscopy (Figures 2.6 and 2.7).

2.3.5 Isolation of granulocytes from human PBMCs by density gradient centrifugation

Density gradient centrifugation (using Histopaque®-1077) was carried out on purchased buffy coat samples as already described. The polysucrose in Histopaque®-1077 aggregated red blood cells thereby increasing their density. Centrifugation of the blood/Histopaque®-1077 mixture for 30 minutes at 400g resulted in red blood cells sedimenting rapidly to the bottom of the centrifuge tube. Granulocytes became slightly hypertonic, which increased their sedimentation rate, resulting in them pelleting at the bottom of the centrifuge tube. PBMCs formed a band at the interface between the Histopaque®-1077 and the plasma. The plasma, PBMC and Histopaque®-1077 layers were carefully removed by aspiration. The remaining mixture at the bottom (containing red blood cells and granulocytes) was washed by topping it up to 50 ml with 1X PBS (at room temperature) and centrifuging at 300g for 10 minutes at room temperature. Using a 50 ml pipet, the supernatant was carefully removed and discarded.

Red blood cells were removed by lysis using the RBC Lysis buffer (Biolegend, Cambridge, UK) as previously described. In brief the red blood cell/granulocyte pellet was gently loosened using a sterile plastic pipette and resuspended in 2ml of 1X RBC Lysis buffer. Immediately after adding the lysing solution, the tube was gently vortexed and then incubated at room temperature, protected from light, for 10 minutes. The RBC Lysis buffer was then quenched by filling up the

tube with PBS and centrifuging at 300 x g for 10 minutes. The supernatant was aspirated off and discarded without disturbing the granulocyte pellet. A cell count and cell viability assessment was carried out as previously described. The pellet was then resuspended in the appropriate buffer depending on further applications. Cell morphology was confirmed by phase contrast microscopy and purity was confirmed by flow cytometry. Neutrophils had granules in their cytoplasm and a conspicuous nucleus consisting of three to five lobules.

2.3.6 Isolation of cells from PBMC using the AutoMacs™ Separator

2.3.6.1 Isolation of $CD14^+$ monocytes from PBMCs using the AutoMacs™ Separator

In addition to isolating monocytes from purchased peripheral blood/buffy coat samples using density gradient centrifugation followed by adherence as already described, CD14+ monocytes were also isolated from buffy coat samples using the AutoMacs™ Separator (Miltenyi Biotec Ltd., Surrey, UK). The AutoMacs ™ Separator is a bench top automated magnetic cell sorter for the isolation of virtually any cell type from any species based on the MACS® Technology. Up to 4×10^9 pure target cells per sample can be isolated within minutes. MACS® Technology is based on MACS MicroBeads (50-nm super paramagnetic particles that are conjugated to highly specific antibodies against a particular antigen on the cell surface, MACS Separators, and MACS Columns. MACS MicroBeads are super-paramagnetic particles of approximately 50 nanometers in

diameter which are coupled to highly specific antibodies or proteins. Since they are composed of a biodegradable matrix, they do not need to be removed from cells after the separation process. MACS MicroBeads are not known to interfere with subsequent experiments or to alter structure, function, or activity status of labelled cells (Miltenyi Biotec 1999; 2006b).

MACS Technology takes place within MACS Columns. When a MACS Column is placed in a MACS Separator, a strong permanent magnet, a high-gradient magnetic field is induced on the column matrix. The induced magnetic field is strong enough to retain cells labelled with minimal amounts of MACS MicroBeads. Unlabelled cells pass through and can be collected. Labelled cells are released after removal of the column from the magnet. MACS technology facilitates the isolation of highly pure labelled and unlabelled cell fractions by positive selection or depletion (negative selection) respectively. The isolated cells can immediately be used for further experiments (Miltenyi Biotec 1999; 2006b).

Anti-CD14 microbeads (Miltenyi Biotec, Surrey, UK) were used for the positive selection of human monocytes from PBMCs according to the manufacturer's instructions. The principle of the isolation is that $CD14^+$ cells are first magnetically labelled with anti-CD14 microbeads. The cell suspension is then loaded onto a MACS® Column which is placed in the magnetic field of an

AutoMacs™ Separator. The magnetically labelled cells are retained within the column while the unlabelled cell fraction (depleted of $CD14^+$ cells) runs through. Following removal of the column from the magnetic field, the magnetically labelled $CD14^+$ cells are eluted as the positively selected cell fraction (Dzionek, 2000; Miltenyi Biotec, 2007b).

Briefly, the following protocol was used: 10^7 PBMCs were resuspended in 80µl of MACS buffer (0.5 % BSA, 2 mM EDTA in PBS). 20µl CD14 microbeads were then added to the suspension. The sample was mixed and incubated on a rotator for 15 minutes at 4°C. Following incubation, cells were washed by adding 2ml of buffer and centrifuging at 300g for 10 minutes prior to being resuspended in 500 µl MACS buffer. The suspension was then loaded onto a MACS® Column on an AutoMacs™ Separator. The positive selection program was selected and the positive fraction (CD14+ cells) was collected, washed by adding 2ml of buffer and centrifuging at 300g for 10 minutes. It was then resuspended in appropriate buffer for downstream investigations. An aliquot was also taken for FACS analysis to confirm purity of isolation. Cytospin preparations of monocytes isolated using CD14 microbeads were also made and stained with DAPI nuclear stain. The monocyte nucleus (Figure 2.4) was identified as being mostly kidney-shaped, bean-shaped or horseshoe-shaped but occasionally amoeboid-shaped or almost segmented (William, 2005).

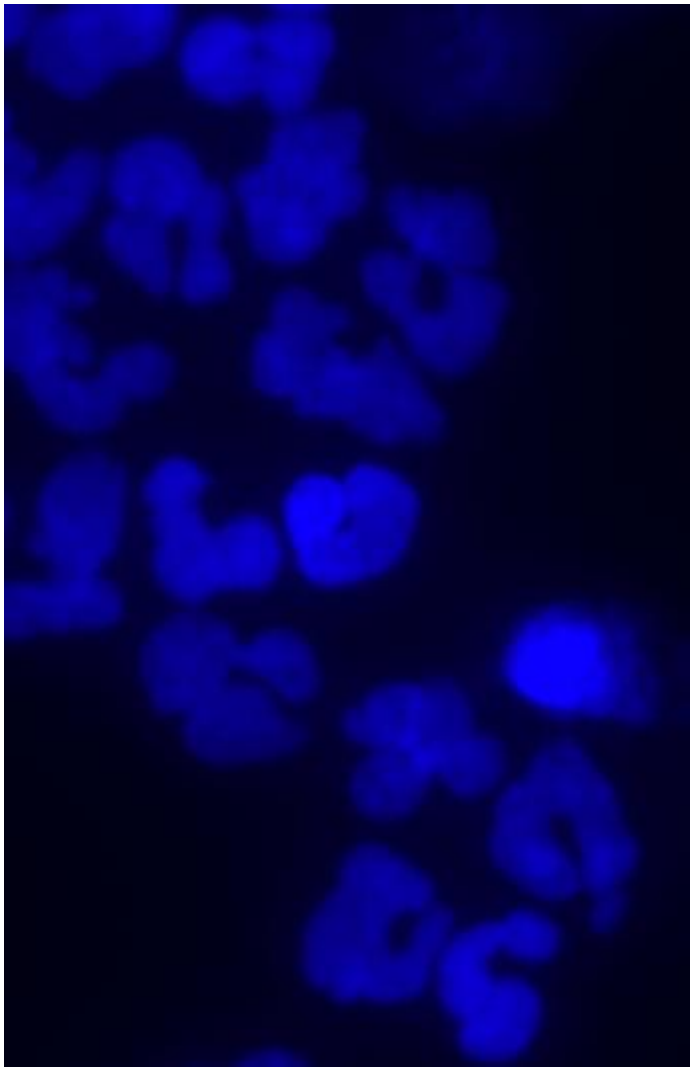


Figure 2.4 CD14+ cells (fluorescence microscopy). Fluorescence microscopy image (1000x magnification) of a cytospin preparation of monocytes isolated using CD14 microbeads and stained with DAPI nuclear stain. The photographs show that the monocyte nucleus is highly variable in shape. It is mostly kidney-shaped, bean-shaped or horseshoe-shaped but it may also be amoeboid-shaped or almost segmented (William, 2005).

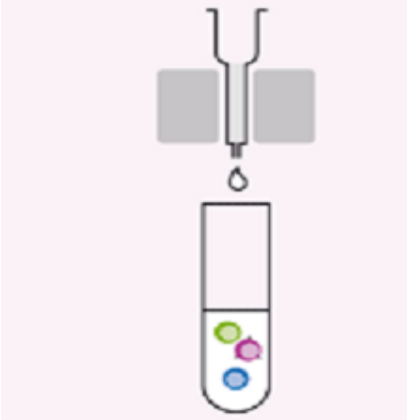
2.3.6.2 Isolation of type 1 myeloid dendritic cells (mDC1s) from PBMCs using the AutoMacs™ Separator

Human type 1 myeloid dendritic cells were isolated from PBMCs using the human CD1c/BDCA-1 magnetic cell separation kit (Miltenyi Biotec Ltd., Surrey, UK) according to the manufacturer's protocol. In brief, separation of mDC1s on the AutoMacs™ involved two magnetic separation steps. In the first step, CD1c/BDCA-1-expressing B cells were magnetically labelled with anti-CD19 microbeads and subsequently depleted by separation over a MACS® Column which is placed in the magnetic field of an AutoMacs™ Separator. In the second step, CD1c/BDCA^{+} myeloid dendritic cells in the B-cell-depleted flow-through fraction were indirectly magnetically labelled with biotin-conjugated CD1c antibodies and Anti-Biotin MicroBeads as previously described (Dzionek, 2000; Miltenyi Biotec, 2008). The purity of isolation was assessed by flow cytometry using appropriate antibodies. Cell viability was assessed using the Trypan blue dye exclusion test as previously described. Figure 2.11 is a schematic diagram showing the isolation of type 1 myeloid DCs (mDC1) from peripheral blood using the CD1c (BDCA-1)$^{+}$ Dendritic Cell Isolation Kit.

Magnetic labelling of CD19+ cells

CD19+ B cells are magnetically labelled with anti-CD19 microbeads.

CD1c/BDCA^{+} myeloid dendritic cells are labelled with biotin-conjugated CD1c antibodies.

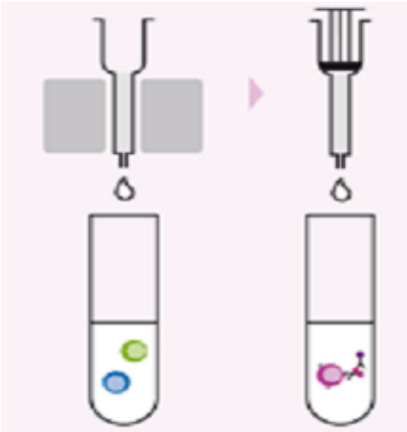


Depletion of CD19+ cells

Cells are separated over a MACS Column placed in the magnetic field of a MACS® separator. The B cell-depleted, pre-enriched CD1c/BDCA+ myeloid DC fraction is collected in the column flow-through.

Magnetic labelling of CD1c/BDCA-1+ cells

Pre-enriched CD1c/BDCA+ myeloid DCs are indirectly magnetically labelled with anti-biotin microbeads



Positive selection of CD1c/BDCA-1+ cells

Magnetically labelled CD1c/BDCA-1+ myeloid DCs are retained on the column. The column is removed from the separator and CD1c/BDCA-1+ myeloid DCs are eluted.

Figure 2.5 Isolation of mDC1 cells. Schematic diagram showing the isolation of type 1myeloid dendritic cells from peripheral blood using the CD1c (BDCA-1)$^{+}$ Dendritic Cell Isolation Kit (adapted from Miltenyi Biotec, 2008).

2.3.6.3 Isolation of type 2 myeloid dendritic cells (mDC2) from PBMCs using the AutoMacs™ Separator

Human type 2 myeloid dendritic cells were positively selected from PBMCs using the human CD141/BDCA-3$^+$ magnetic cell separation kit (Miltenyi Biotec Ltd., Surrey, UK) according to the manufacturer's protocol. In brief, CD141/BDCA-3$^+$ cells were magnetically labelled with anti-CD141/BDCA-3 microbeads. The cell suspension was loaded onto a MACS® column which was then placed in the magnetic field of an AutoMacs™ Separator. The magnetically labelled CD141/BDCA-3$^+$ cells were retained on the column while the unlabelled CD141/BDCA$^-$ cells were collected in the flow-through. The column was then removed from the magnetic field and the CD141/BDCA-3$^+$ cells were eluted and once gain separated over a new column to achieve highest purities (Dzionek, 2000; Miltenyi Biotec, 2006c). The purity of isolation was assessed by flow cytometry using appropriate antibodies. Cell viability was assessed using the Trypan blue dye exclusion test as previously described.

2.3.6.4 Isolation of Plasmacytoid Dendritic cells (pDCs) from PBMCs using the AutoMacs™ Separator

Human plasmacytoid DCs (pDCs) were isolated from PBMCs using the Diamond Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec Ltd., Surrey, UK) according to the manufacturer's protocol. Briefly, separation of pDCs on the

AutoMacs™ involved two magnetic separation steps. In the first step, all non-pDC cells were magnetically labelled with a cocktail of biotin-conjugated antibodies and anti-biotin Microbeads for depletion over a MACS Column. In the subsequent positive selection step, the enriched pDCs were directly magnetically labelled with anti-CD304 (BDCA-4/Neuropilin-1) Microbeads. The purity of isolation was assessed by flow cytometry using appropriate antibodies. Cell viability was assessed using the Trypan blue dye exclusion test as previously described.

2.3.6.5 Isolation of CD209+/CD14+ cells from PBMCs using the AutoMacs™ Separator

The isolation of human $CD209^{+}/CD14^{+}$ cells from PBMCs was done in two steps. In the first step, CD14+ monocytes were isolated by negative selection using the human Monocyte Isolation Kit II (Miltenyi Biotec Ltd., Surrey, UK) according to the manufacturer's instructions. Briefly, non-monocytes were indirectly magnetically labelled using a cocktail of biotin-conjugated antibodies and anti-biotin Microbeads. Highly enriched unlabelled monocytes were obtained by depletion of the magnetically labelled cells. In the second step, CD209+ cells were positively isolated from the CD14+ cell population using the CD209 (DC-SIGN) MicroBead Kit (Miltenyi Biotec Ltd., Surrey, UK) as per manufacturer's instructions. Briefly, CD209/DC-SIGN+ cells were first magnetically labelled with

anti-CD209/DC-SIGN microbeads. The cell suspension was loaded onto a MACS® column which was then placed in the magnetic field of an AutoMacs™ Separator. The magnetically labelled CD14+ CD209/DC-SIGN+ cells were retained within the column while the unlabelled CD14+ CD209/DC-SIGN$^-$ cells were collected in the flow-through. After removing the column from the magnetic field, the CD14+ CD209+ cells were eluted as the positively selected cell fraction. Cell purity was confirmed by staining a fraction of the isolated CD209+CD14+ cells with appropriate fluorescent antibodies. Cell viability was assessed using the Trypan blue dye exclusion test as previously described.

2.3.6.6 Isolation of CD3+ T cells

Human CD3+ T cells were positively selected from PBMCs using anti-CD3 paramagnetic beads (CD3 MicroBeads kit, Miltenyi Biotec Ltd., Surrey, UK) according to the manufacturer's instructions. Briefly, CD3+ cells were first magnetically labelled with anti-CD3 microbeads. The cell suspension was loaded onto a MACS® column which was then placed in the magnetic field of an AutoMacs™ Separator. The magnetically labelled CD3+ T cells were retained on the column while the cell fraction depleted of CD3+ cells was collected in the flow-through. Cell purity was confirmed by staining a fraction of the isolated T cells with a fluorescent anti-CD3 antibody (CD3: FITC). Cell viability was assessed using the Trypan blue dye exclusion test as previously described.

2.4.0 Differentiation and culture of monocyte-derived dendritic cells (MDDCs)

Blood products from healthy donors were obtained from the National blood transfusion service (Sheffield, UK) as already described. Isolation of peripheral blood monocytes (PBMs) was performed using differential centrifugation on Histopaque®-1077 as described in previous sections. PBMs were differentiated into MDDCs using previously reported methods (Sallusto and Lanzavecchia, 1994). Briefly, monocytes were cultured in six well plates at a concentration of $1x10^6$ cells/ml in RPMI 1640 media supplemented with heat-inactivated FCS (10%v/v), Penicillin-Streptomycin (1%), L-glutamine (2mM) (Sigma-Aldrich, Poole, UK), GM-CSF (50 ng/mL) (Sigma-Aldrich, Poole, UK) and IL-4 (10 ng/mL) (Sigma-Aldrich, Poole, UK). To investigate the effect of IL-4 on IL-1Rp2 expression, GM CSF (50 ng/mL) and IL-4 concentration ranges from 1, 10 and 50 ng/mL were used. All cells were maintained at 37◦C in 5% CO2 for 5 days, with media and cytokine change every 2 days. After 5 days the cells became non-adherent and were analysed for MDDC phenotype and morphology.

2.5.0 Differentiation and culture of monocyte-derived macrophages (MDMs)

PBMs were cultured for 4 days with GM-CSF (50 ng/mL) without IL-4, under the same experimental conditions used for MDDCs (stated above). After 5 days the morphological appearance of adherent macrophages were observed by phase-contrast microscopy using a TCS SP2 UV confocal laser-scanning microscope

(CLSM) (Leica Microsystems, UK). IL-1Rrp2 expression was subsequently measured in MDMs by Fluorescent Activated cell Sorting Analysis (FACS) and quantitative real time PCR (qRT-PCR). In other experiments, MDMs were washed and stimulated for 2 days with IL-4 (10 ng/mL) prior to microscopic and qRT-PCR analyses.

2.6.0 Immunofluorescence Assays

2.6.1 Fluorescence Activated Cell Sorting (FACS) analyses

FACS analyses were performed by standard methods. Briefly, cells were harvested and the total cell number was determined. Cells were pelleted by centrifuging at 300g for 10 minutes and the culture medium (supernatant) was discarded. 10ml FACS buffer (Phosphate buffered saline (PBS) pH 7.4; BSA (1% w/v); EDTA (2 mM)) was added to the cell pellet followed by centrifugation at 300g for 10 minutes. After discarding the supernatant, the cell suspension was adjusted to a concentration of 1×10^6 cells/ml with FACS buffer. 100 µl of cell suspension was aliquoted into the required number of test tubes. To block Fc receptors on the cell surface thereby reducing non-specific antibody binding, cells were incubated in FACS buffer containing human serum (10% v/v) for 15 minutes at room temperature on an orbital shaker prior to incubation with relevant antibodies (see Table 2.2). To detect intracellular antigens, a cell permeabilisation step (incubation with 0.5% Triton X for 10 minutes at room temperature) was done prior to staining. This step was omitted in FACS

analyses for the detection of cell surface antigens.

For direct immunofluorescence staining (where the fluorochrome was directly linked to the primary antibody), blocking of Fc receptors was followed by incubation, in the dark, for 45 minutes with the relevant antibody at the appropriate concentration (determined by preliminary optimisation studies) on ice, on a shaker. Cells were then washed three times by adding 2ml of FACS buffer and centrifuging at 300g for 10 minutes each time. This was done to remove unbound antibody. As a negative control, isotype matched control samples were also included and these were incubated for 45 minutes with an appropriate isotype matched control antibody only but not with the primary antibody (Table 2.2). Isotype control antibodies have no relevant specificity and help to distinguish non-specific "background" staining from specific antibody staining.

After washing cells and discarding the supernatant, cells were resuspended in 0.2 ml of FACS buffer prior to analysis on a FACSCanto II analyser (Becton Dickinson, USA). Samples were acquired using the BD FACSDiva™ (BD Biosciences, USA) and analysed using the WinMDI 2.8 software. Cell viability was assessed by propidium iodide uptake (20 μg/mL for 10 min) via FACS analysis.

For indirect immunofluorescence staining (where a fluorochrome on a secondary antibody was used to visualize the primary antibody), sample preparation, including Fc receptor blocking, was done as already stated above. The primary antibody was then added at the appropriate concentration and the sample was incubated for 45 minutes in the dark under the conditions that have already been mentioned above. Cells were then washed with 2ml of FACS buffer and centrifuged at 300g for 10 minutes. After discarding the supernatant, cells were incubated for 45 minutes on ice, in the dark, with an appropriate secondary antibody (Table 2.2). Cells were washed, resuspended in 0.2 ml of FACS buffer and analysed as described above. To test for non-specific binding of the secondary antibody, secondary antibody control cells were stained as above with the secondary antibody only but not with the primary antibody.

2.6.1.1 FACS analyses for IL-1Rrp2 expression

Cells were resuspended in FACS buffer at a concentration of $1x10^6$ cells/ml as described above (Section 2.6.1). Test cells were blocked in FACS buffer containing human serum (10% v/v) for 15 min prior to incubation for 45 minutes with 10µg/ml mouse anti-human IL-1Rrp2 antibody (M145, Amgen Corporation, Seattle, WA) followed by incubation for 45 minutes with the detection/secondary antibody (10µg/ml allophycocyanin (APC)-conjugated goat anti-mouse IgG (Biolegend, UK). Secondary antibody control cells were incubated for 45 minutes with 10µg/ml APC-conjugated goat anti-mouse IgG only and not with the primary antibody. Cells were then analysed using a FACSCanto II analyser (Becton

Dickinson, USA). Samples were acquired using the BD FACSDiva™ (BD Biosciences, USA) and analysed using the WinMDI 2.8 software. Cell viability was assessed as already stated and was found to be >90% in all cases.

2.6.1.2 Analysis of MDDC phenotype and maturation status

Following co-culture of PBMs with GM CSF and IL-4 for 5 days, changes in expression of CD1a, CD11C, CD14, HLA-DR and CD83 were measured by FACS analyses (as previously stated) using antibodies listed in Table 2.2. The phenotype of these cultured cells was $CD11C^{high}$, $CD1a^{high}$, $HLA\text{-}DR^{high}$ and $CD14^{low}$, consistent with their differentiation to immature MDDCs (see Figure 2.12).

Prior to assessing the effect of mature MDDCs on T cell populations (section 2.8.0), the surface expression of CD40 and CD80 on IL-1F8-matured MDDCs was measured by FACS analyses (as previously stated) to further confirm the ability of IL-1F8 to induce MDDC maturity. The antibodies used are listed in Table 2.2.

Table 2.2 Concentration of antibodies used in FACS analyses. Unless otherwise stated, antibodies were ordered from Abd Serotec (Oxford, UK) or BD Biosciences (Oxford, UK).

Antibody	**Concentration (µg/ml)**
Mouse anti-human CD14:PE	5
Mouse anti-human HLA-DR:PE	5
Mouse anti-human CD1a:FITC	1
Mouse anti-human CD11c:FITC	1
Mouse anti-human CD83:FITC	5
Mouse anti-CD80: FITC	5
Mouse anti-human CD40: PE	5
Mouse anti human CD40:Alexa Fluor® 488	5
Mouse IgG2a:Alexa Fluor® 488	5
Mouse IgG2a:PE (Isotype control CD14/CD40)	5
Mouse IgG3: PE (Isotype control HLA.DR)	5
Mouse IgG1:FITC (Isotype control CD1a)/CD80	1
Mouse IgG2a:FITC (Isotype control CD11c	1
Mouse IgG2a:FITC (Isotype control CD83)	5
Mouse anti-human IL-1Rrp2 (M145) (Amgen Corporation, Seattle, WA, USA)	10
Goat anti-mouse IgG:APC (Biolegend, Cambridge UK)	10

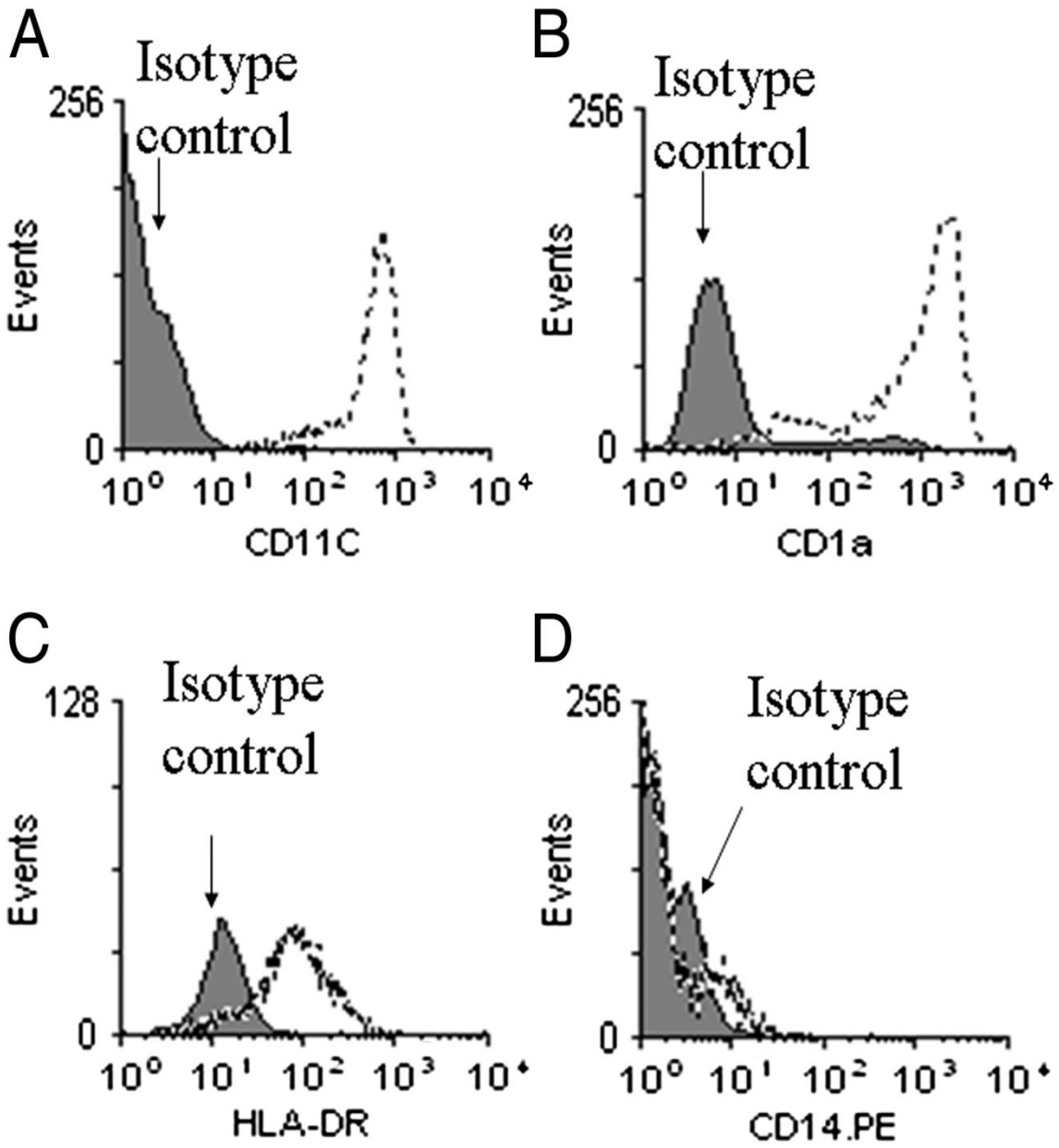


Figure 2.6 MDDC phenotypic characteristics. Histograms show phenotypic characteristics following conversion of human PBMs to immature monocyte-derived dendritic cells (MDDCs). PBMs were co-cultured with GM CSF and IL-4 for 5 days prior to phenotypic examination using flow cytometry. Differentiation to an MDDC phenotype is shown by measurement of the following parameters: (A) $CD11C^{high}$; (B) $CD1a^{high}$; (C) $HLA\text{-}DR^{high}$ and (D) $CD14^{low}$. Grey shaded areas correspond to the control. Data are representative of at least three independent experiments.

2.6.1.3 Analysis of IL-1Rrp2 expression and maturation status following culture with IL-1 cytokines

Initially, expression of IL-1Rrp2 on the cell membranes of promonocytes, PBMs, MDMs and MDDCs was analysed using FACS analysis as previously stated (Sections 2.6.1 and 2.6.1.1). To assess IL-1-dependent maturation of MDDCs, changes in HLA-DR and CD1a expression were assessed by FACS analysis on the surface of day 5 (Immature) MDDCs that had been washed three times in PBS prior to incubation with IL-1F8 or IL-1F9 (100 ng/ml) for 48 h. As a positive control, day 5 MDDCs were incubated under the same conditions for 48 h with IL-1β (100 ng/ml) or IFN-γ (100 U/ml). As a negative control, spontaneous maturation of MDDCs was also assessed. Parallel experiments were performed in which day 5 MDDCs were washed prior to culture in fresh RPMI 1640 media, which did not contain cytokines, for a further 48 h prior to measurement of CD1a and HLA.DR by the FACS analysis. As an additional measurement of maturation, the expression of CD83 by day 5 and day 7 MDDCs was compared with the expression of CD 83 by day 5 MDDCs which had been stimulated with IL-1F8 or IL-1F9 for 48 h under the same experimental conditions stated above.

Cells were gated as R1 (immature) and R2 (mature) populations according to parameters set for immature MDDCs, which had been washed and then cultured for a further 48 h without cytokine stimulation (unstimulated controls). These parameters were then applied to all subsequent FACS data so that direct comparisons could be made between the numbers of cells in each R1 and R2

population following different treatments. Prior to co-culture of IL-1F8 matured MDDCs with CD3+ T lymphocytes, CD40 and CD80 expression on the surface of IL-1F8-stimulated MDDCs was measured by standard FACS analysis using antibody concentrations shown in Table 2.2. Each experiment was repeated on more than three separate occasions.

2.6.2 Fluorescence microscopy for IL-1Rrp2 detection

To assess IL-1Rrp2 expression in various human cells by immunofluorescence microscopy, harvested cells were rinsed with PBS and resuspended at a concentration of 1 x 10^5 cells/ml in PBS prior to being fixed by incubation with 4% v/v paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. After washing cells with PBS, 500µl of cell suspension was used to make cytospin preparations using a Cytospin 4 centrifuge (Thermo Scientific, UK) and following standard techniques. Slides were left to air dry prior to permeabilising cells with 0.5% Triton X for 10 minutes at room temperature. After rinsing three times in PBS for 3 minutes each time, slides were incubated with 50-100ul of human AB serum for 10 minutes at room temperature to block non-specific Fc Receptor binding. This was followed by incubation with 25µg/ml mouse anti-human IL-1Rrp2 antibody (M145, Amgen Corporation, Seattle, WA) for 45 minutes. After rinsing slides as previously stated, slides were incubated with the secondary/detection antibody (10µg/ml FITC-conjugated Goat anti-mouse IgG) for 30 minutes in the dark. Isotype control cells were incubated with secondary antibody only. Unstained cells were also included as controls. After rinsing slides

as before, a drop of ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, UK) nuclear stain was placed on the slides and the slides were cover-slipped and left to dry in the dark for at least 5 minutes. Slides were then analysed under a Leica fluorescence microscope (Leica Microsystems, UK).

Immunofluorescence is a technique which allows the visualization of a specific protein or antigen in cells or tissue sections by binding a specific antibody chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC). There are two main types of immunofluorescence staining methods; direct immunofluorescence staining (where the primary antibody is labelled with fluorescence dye) and indirect immunofluorescence staining (where a secondary antibody labelled with fluorochrome is used to recognize a primary antibody). Immunofluorescence staining can be performed on cells fixed on slides and on tissue sections. Immunofluorescence stained samples are examined under a fluorescence microscope or confocal microscope.

2.7.0 Molecular techniques

2.7.1 RNA Extraction

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. The RNeasy procedure is a well-established technology for total RNA purification. It combines the selective

binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer which immediately inactivates RNases. This ensures purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30–100 µl water. The RNeasy procedure facilitates the purification of RNA molecules longer than 200 nucleotides. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded (QIAGEN, 2006).

In brief, the appropriate number of harvested cells (typically 1 x 10^6) was pelleted by centrifuging for 5 min at 300 x g and the supernatants were discarded. 350µl of RLT Buffer (containing 10 µl β-mercaptoethanol (β-ME) per ml Buffer RLT) was added to the cell pellet to lyse the cells. The lysate was then homogenized by passing it at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. This was followed by the addition of 350µl of 70% ethanol to the homogenized lysate and thorough mixing (using a sterile pipette). The sample was then transferred to an RNeasy spin column (placed in a 2 ml collection tube) and centrifuged for 15 seconds at 12000 rpm. After

discarding the flow-through, on-column DNase digestion was done to eliminate genomic DNA contamination (see below). This was followed by the addition of 700 µl Buffer RW1 to the RNeasy spin column and centrifuging for 15 seconds at 12000 rpm to wash the spin column membrane. After discarding the flow-through, 500 µl of Buffer RPE was added to the RNeasy spin column and the tube was centrifuged for 15 seconds at 12000 rpm to wash the spin column membrane. The flow through was discarded before adding another 500 µl of Buffer RPE was added to the RNeasy spin column and the tube was centrifuged for 2 minutes at 12000 rpm to wash and dry the spin column membrane, ensuring that no ethanol (which could interfere with downstream reactions) was carried over during RNA elution. The RNeasy spin column was then placed in a new 2 ml collection tube and centrifuged at 12000 rpm for 1 min to eliminate any possible carryover of Buffer RPE. This was followed by placing the RNeasy spin column in a new 1.5 ml RNAse-free Eppendorf tube, adding 30µl RNase-free water directly to the spin column membrane and centrifuging for 1 min at 12000 rpm to elute the RNA. Purity and concentration were analysed using a Nanodrop (ND1000) spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) according to manufacturer's instructions (see below). Prior to cDNA synthesis and other down-stream applications, RNA was stored at -80°C.

2.7.2 On-column DNase Digestion using RNase-free DNase set

The purpose of DNase digestion is to prevent genomic DNA from contaminating the extracted RNA. Lysis and homogenization of the sample and binding of RNA

to the RNeasy membrane are performed according to the standard protocols. After washing with a reduced volume of Buffer RW1 (which contains a small amount of guanidine thiocyanate and ethanol), the RNA is treated with DNase I while bound to the RNeasy membrane. The DNase I is removed by a second wash with Buffer RW1. Washing with Buffer RPE (which contains a non-chaotropic salt and ethanol) and elution of RNA are then performed according to the standard protocols (QIAGEN, 2006).

Briefly, cells were harvested, lysed, homogenized and loaded onto the RNeasy spin column as stated in previous sections (see Section 2.8.1 above). Prior to performing the first wash step, 350 µl of Buffer RW1 was added to the RNeasy spin column and the tube was centrifuged for 15 s at 12000 rpm to wash the spin column membrane. After discarding the flow-through, 80 µl of DNase I incubation mix (consisting of 10 µl DNase I stock solution and 70 µl Buffer RDD) was added directly to the RNeasy spin column membrane and the tube was placed on the bench top at room temperature (20–30°C) for 15 minutes. This was followed by the addition of 350 µl Buffer RW1 to the RNeasy spin column, centrifugation for 15 seconds at 12 000 rpm and discarding of the flow-through prior to continuing with the first Buffer RPE wash step in the RNA extraction protocol as already described in Section 2.8.1.

2.7.3 RNA Quantification and determination of purity

Concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in a Nanodrop (ND1000) spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) according to the manufacturer's instructions. The ratio of the readings at 260 nm and 280 nm (A260/A280) was used to provide an estimate of purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. RNA with an A260/A280 ratio greater than 1.8 was considered to be pure (QIAGEN, 2006) and was used for reverse transcription and subsequently for quantitative real time PCR (qRT-PCR).

2.7.4 Complementary DNA (cDNA) synthesis

Prior to qRT-PCR analysis, RNA was first reverse-transcribed into complimentary DNA (cDNA) in a reverse transcription (RT) reaction using the Transcriptor First Strand cDNA Synthesis Kit (Roche, West Sussex, UK) as per supplier's instructions. A random hexamer primer was used as detailed in the supplier's manual. The total reaction volume was 20 µl made up of Transcriptor Reverse Transcriptase reaction buffer (4 µl), Deoxynucleotide (dNTP) mix (2 µl), random hexamer primer (2 µl), protector RNase inhibitor (0.5 µl), Transcriptor Reverse transcriptase (0.5 µl) and a maximum of 11 µl total RNA (up to 1µg of total RNA was used). The following program was used on the thermal cycler: 10 minutes at 25^{o}C, 30 minutes at 55^{o}C, 5 minutes at 85^{o}C then hold at 4^{o}C. All samples were run in duplicate.

2.7.5 Quantitative Real Time PCR (qRT-PCR)

The LightCycler® 480 Probes Master Kit (Roche, West Sussex, UK) was used for qRT-PCR and the manufacturer's guidelines for use with LightCycler® 480 Multiwell Plate 96 were followed. Gene-specific primers were obtained from Invitrogen, (Paisley, UK) through Fisher Scientific (Loughborough, UK). 5-carboxyfluorescein (FAM)-labelled probes were obtained from Roche Diagnostics (West Sussex, UK). Gene-specific primers and probes were designed using the Roche Universal Probe Library (UPL) Assay Design Centre software (primer and probe sequences used are shown in Table 2.3). To allow differentiation of the amplified cDNA from contaminating genomic DNA, intron-spanning primers (primers that annealed to exon sequences on both sides of an intron or on exon/exon boundaries) were designed (Roche Diagnostics Corporation, 2007).

Template cDNAs (2 µl) were amplified in a PCR reaction mix (25 µl total volume) containing an appropriate 5-carboxyfluorescein (FAM)-labelled hydrolysis probe (Universal ProbeLibrary probe), FastStart Taq DNA Polymerase, reaction buffer, deoxynucleotide triphosphate (dNTP) mix, PCR-grade water, $MgCl_2$ and gene-specific primers according to the LightCycler® 480 Probes Master kit (Roche, West Sussex, UK). The following volumes were used: 12.5 µl LightCycler® 480 Probes Master (2x concentration), 2 µl cDNA, 1.5 µl forward primer, 4.5 µl reverse primer, 0.88 µl hydrolysis probe and 3.63 µl PCR-grade water. The working concentration for all primers and probes was 5 µM. The absence of DNA

contamination in RNA preparations was tested by including RNA samples that had not been reverse transcribed. A negative no template control (NTC) and a positive calibrator sample were also included with each run. For each target and control (housekeeping) gene, a standard curve was constructed from running serial dilutions of relevant positive standards covering six points in order to determine the efficiency of each gene (Figure 2.13 shows a representative standard curve). All samples were analysed in triplicate. Amplifications were performed using a LightCycler® 480 Real-Time PCR analyser (Roche, West Sussex, UK) as per manufacturer's recommendations. The PCR cycling program used for IL-1Rrp2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes was: Pre-incubation (1 cycle; 95^{o}C for 10 minutes), Amplification (45 cycles; 95^{o}C for 10 seconds, 60^{o}C for 40 seconds and 72^{o}C for 1 second) and Cooling (1 cycle; 40^{o}C for 10 seconds).

For quantification purposes, a relative quantification method based on the relative changes in mRNA expression of the target gene versus a reference (or housekeeping) gene was employed. GAPDH was used as the reference gene for this purpose. Relative expression of the target gene to the reference gene was calculated using the LC480 software program (Roche, West Sussex, UK). The Advanced Relative quantification efficiency corrected calculation model (E-method) was used according to the LC 480 user manual. (Pfaffl, 2001; 2004; Roche Applied Sciences, 2006; Roche Applied Sciences, 2008; Roche Diagnostics Ltd., 2008).

A Relative Quantification analysis compares two ratios: the ratio of a target DNA sequence to a reference DNA sequence in an unknown sample, and the ratio of the same two sequences in a standard sample called a “calibrator”. The “Target” is the nucleic acid of interest (specific RNA or DNA sequence); while the “Reference” is a nucleic acid that is found at constant copy number in all samples and serves as endogenous control which normalizes for sample-to sample differences. The “Calibrator” is typically a positive sample with a stable ratio of target to-reference and is used to normalize all samples within one run, but in addition provides a constant calibration point between several LightCycler ® 480 System runs. The result is expressed as a normalized ratio. Calibrator Normalized ratio =

$(\text{Concentration of target/concentration of Reference})_{\text{in sample}}$ **:** $(\text{Concentration of target/concentration of Reference})_{\text{in calibrator}}$

Relative Quantification is the best technique for determining gene expression and gene dosage since it allows users to easily compare the expression behaviour of a target gene under at least two conditions (e.g., disease-free / diseased or untreated / treated). By dividing the concentration of the target in each sample by the concentration of a reference in the same sample, this method corrects the sample for differences in quality and quantity caused by variations in initial sample amount, variations in nucleic acid recovery, possible RNA degradation of sample material, differences in sample and/or nucleic acid quality, variations in sample loading/pipetting errors and/or variations in cDNA

synthesis efficiency (Roche Diagnostics Ltd., 2008).

A Relative Quantification analysis can be performed on an experiment that has an amplification program and that has the appropriate sample types. It can be performed on a single-colour or on a dual-colour experiment. A Relative Quantification analysis is based on the assumption that the concentration of DNA at a sample's crossing point is the same for every sample containing the same target DNA. This is the DNA concentration necessary for the LightCycler® 480 instrument to detect a signal above background noise. Each sample may require a different number of cycles to reach the crossing point, depending on the initial concentration of DNA in the sample.

At the end of the experiment, each sample's DNA concentration may vary, depending on how many cycles were completed by that sample after the crossing point was reached. The analysis uses the sample's crossing point (expressed as a cycle number), the efficiency of the reaction, the number of cycles completed, and other values to determine how much the DNA concentration must have increased for each sample by the end of the amplification. The analysis uses these calculations to compare the samples and generate the ratios. The final ratio resulting from the calibrator normalized Relative Quantification is only a function of PCR efficiency and of the determined crossing points. It does not require the knowledge of absolute copy numbers at

the detection threshold and thus the analysis does not determine the actual concentration of DNA in the samples. The calculation of the calibrator-normalized ratio does not require a standard curve in each LightCycler® 480 run (Roche Diagnostics Ltd., 2008).

The Efficiency Method (E-method) used by Roche Applied Sciences for calculation of the normalized ratio is based on true efficiency values and can produce more accurate relative quantification data than the previously used $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001; Pfaffl, 2001; 2004) since it can compensate for efficiency differences between target and reference either within an experiment or between experiments. It uses the individual PCR efficiencies of target and reference gene for calculating the final normalized relative ratio. Efficiency is determined via linear/non-linear standard curves (Pfaffl, 2001; 2004; Roche Applied Sciences, 2006)

The LightCycler 480® Software provides two different analysis modes for Relative Quantification approaches: Basic and Advanced Analysis. The Basic Analysis mode is based on the well-accepted ΔΔCT-Method (Livak and Schmittgen, 2001) while the Advanced Analysis mode represents a manual, flexible mode providing sophisticated software algorithms and excellent tools for the most demanding research projects. The Advanced Relative Quantification method not only allows determination of the relative ratio, but it extends and

improves this concept by enabling crossing point (Cp) calling via either Fit points or Second Derivative Maximum methods, reference analysis via either In-Run references or External Reference experiments, pairing rules for multiple target/reference genes, the use of standards and the use of subsets. An Advanced Relative Quantification experiment contains target and reference unknowns, target and reference standards (optional), target and reference calibrators (optional) and target and reference negatives (optional). The references can be measured in the same or in a separate experiment (Roche Diagnostics Ltd., 2008).

Table 2.3 Primer and probe sequences used in the current study (*UPL No. = Universal Probe Library Probe Number)

GENE NAME	**Genbank Accesion Number**	**Forward primer Sequence**	**Reverse Primer Sequence**	***UPL No.**
Human TNF α	BC028148	CAGCCTCTTCT CCTTCCTGAT	GCCAGAGGGC TGATTAGAGA	29
Human GAPDH	NM_002046	CTCTGCTCCTCC TGTTCGAC	ACGACCAAATCC GTTGACTC	60
Human IL-1Rrp2	AF284434	GCTGGAGTGTCC ACAGCATA	GCGATAAGCCCT CCTATCAA	24

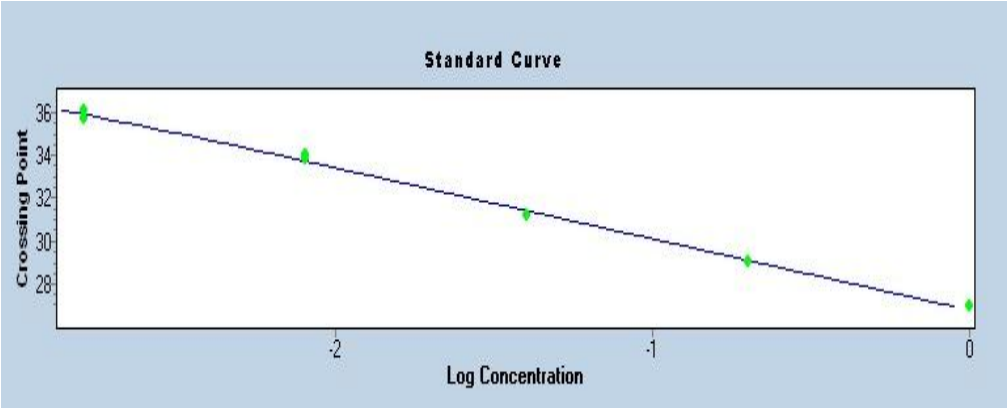


Figure 2.7 Representative GAPDH gene RT-PCR standard curve. (Efficiency: 2.000, Slope: 3.306, Error: 0.0219, Y-intercept: 26.82, Link: 0.00188). The standards used were serial five-fold dilutions of cDNA from a monocyte derived dendritic cell (MDDC). Standards were constructed to span five data points. The concentrations chosen for the standard curve reflected the expected concentration range of the target. All samples were analysed in duplicate using the LightCycler® 480 instrument. The slope of the standard curve describes the kinetics of the PCR amplification. The LightCycler® 480 software automatically calculated efficiency using the formula: $E = 10^{-1/slope}$ The Error value is the mean squared error of the single data points fit to the regression line. It is a measure of the accuracy of the quantification result based on the standard curve. An acceptable error value should be < 0.2.

2.7.6 Evaluation of PCR products by agarose gel electrophoresis

PCR products were evaluated by electrophoresis on 2% agarose gels. Briefly, 100ml agarose solution was prepared by measuring 2g agarose into a conical flask and adding 100ml of 1x TAE (Tris-acetate-EDTA) buffer. The mixture was microwaved at medium heat for 2-3 minutes until all the agarose was dissolved and the solution was clear. The solution was allowed to cool to about 55-60 ^{0}C before adding 1-2µl of Ethidium bromide (concentration 10mg/ml) (10ug/µl). A 10-well comb was placed in a gel tray about 1 inch from one end of the tray ensuring that the teeth were about 1-2mm above the surface of the tray. The gel solution was slowly poured into the gel tray to a depth of about 5mm. Any bubbles were pushed away to the side using a disposable tip. The gel was allowed to solidify for about 30 minutes at room temperature (when set, the gel changed colour from clear to opaque) before the comb was gently removed. The gel tray was covered with 1x TAE buffer (until wells were submerged). 2µl of 6x gel loading buffer was added to 10µl of DNA sample and mixed in well. 10µl of mixed DNA sample was loaded into appropriate wells. The first and last wells (first and last lanes) were loaded with 10µl of a 1kb molecular weight marker (DNA ladder). The gel tank was closed and the power source switched on. The gel was run at 100V for about 60 minutes (until bromophenol blue tracking dye had run ¾ of the length of the gel). When the run was complete, the gel-tank was switched off and unplugged and the gel was carried in a holder to the dark-room to view on a long wave UV transilluminator.

Agarose gel electrophoresis is a rapid technique used to identify, quantify and purify nucleic acids based on their differential separation according to their size and charge when an electric current is passed through them. Separation is based on the principle that DNA molecules are negatively charged in a neutral to basic solution (1X TAE buffer solution) due to dissociation of the phosphate backbone. During electrophoresis they migrate towards the positively charged electrode. The electrophoretic mobility of DNA molecules depends on the voltage and the composition of the electrophoresis buffer. Small DNA fragments migrate more rapidly in the gel matrix compared to large fragments, resulting in molecule separation based on size. After running the gel, the DNA fragments can be seen with a UV light due to the Ethidium bromide dye in the gel. Ethidium bromide dye adheres to the DNA in the gel and fluoresces when viewed under a UV light and can be photographed. Before a DNA sample is loaded onto a gel, it is mixed with an appropriate loading buffer or loading dye solution which contains Glycerol (to ensure that the sample easily sinks into the well), EDTA (which binds divalent metal ions that may interfere with electrophoresis and also stops metal-dependent enzymatic reactions such as DNA degradation by nucleases) and tracking dyes (such as bromophenol blue) to monitor the progress of electrophoresis by the migration of the dyes.

The principle of agarose gel electrophoresis is that DNA fragments are separated according to their weight by using electricity. DNA has a sugar backbone that is negatively charged in a neutral to basic solution, the

buffer solution, which is 1X TBE. After running the gel the fragments can be seen with a UV light. The reason fragments can be seen with UV light is due to the Ethidium bromide dye that has been added to the gel. This dye will adhere to the DNA in the gel and when the gel is finished it can be viewed under a UV light and can be photographed.

To help detect the presence of an expected DNA molecule/fragment and to estimate its size, a molecular weight marker (DNA ladder) is used as a reference and is added to specific wells (usually lane 1 and lane 10) in each gel electrophoresis run. A DNA ladder is a solution containing DNA molecules/fragments of different lengths. A 1kb DNA ladder was used in the present study. The 1 kb DNA Ladder contains discrete DNA fragments ranging from about 300 base pairs (bp) to 10,000 bp.

2.8.0 T cell proliferation studies

2.8.1 MDDC-induced T cell Proliferation

Stimulated MDDCs were co-cultured with allogeneic CD3+ lymphocytes and the WST-1 cell proliferation assay (Roche Diagnostics Ltd., West Sussex, UK) was used to measure T cell proliferation. The Roche WST-1 cell proliferation reagent is a colorimetric assay designed to measure the relative proliferation rates of cells in culture. The assay principle is based on the cleavage of the stable tetrazolium salt WST-1 into a coloured formazan dye by mitochondrial

dehydrogenase enzymes which are present in viable cells. The amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

Briefly, prior to co-culturing stimulated MDDCs with CD3+ T lymphocytes, $1.44x10^6$ cells/well day-5-MDDC were stimulated for 48 hours in 12- well cell culture plates with one of the following: Complete RPMI 1640 culture medium only (RPMI 1640 culture medium containing 10% heat-inactivated FCS, 2mM L-glutamine and 1% Penicillin-Streptomycin) (negative control) or complete RPMI 1640 supplemented with one of the following: IL-1F8 100ng/ml, IL-1F8 500ng/ml, IL-1β 10ng/ml, IL-1β 100ng/ml, LPS 10ng/ml or LPS 100ng/ml (Lipopolysaccharides from Escherichia coli 0127:B8, Sigma-Aldrich, Poole, Dorset, UK). Cultures were performed in duplicate. Incubation was performed at 37^oC and 5% CO_2. At the end of the culture period, cells were harvested and checked for viability and maturity prior to further culture with CD3+ T cells.

The stimulated, mature MDDCs (or unstimulated negative controls) were then plated at a concentration of $6x10^4$ cells/well stimulated and co-cultured with $6x10^5$ cells/well CD3+ T cells (isolated from a different donor buffy coat using CD3+ microbeads as already described) in 1ml of complete RPMI 1640 medium for 96 hours in 24-well culture plates at 37^oC and 5% CO_2. This gave an MDDC (stimulator cells)**:** CD3+ T cell (responder cells) ratio of 1:10 per well. As a

positive control, CD3+ T cells were also cultured with the positive control, Concanavalin A (Con A) (75 µg/ml) (Sigma-Aldrich, Poole, UK) in separate wells. Con A is a plant mitogen known for its ability to induce T cell proliferation *in vitro*. All cultures were performed in triplicate and repeated on three separate occasions. After 96 hour incubation as described above, MDDC/T cell and Con A/T cell cultures were harvested. Supernatants were collected and frozen for cytokine analysis. Remaining MDDC/T cell suspensions were then treated as follows: 100µl cell suspension was aliquoted onto appropriate well on a 96 well plate, 10 µl cell proliferation reagent WST-1 was added to each well and the mixture was incubated in a humidified chamber at 37^{o}C and 5% CO_2 for 1½ hours. After the incubation period, the formazan dye formed (following cleavage of the stable tetrazolium salt WST-1 by mitochondrial dehydrogenase enzymes present in viable cells) was quantified by measuring absorbance at 450 nm on a Labtech LT-4000 ELISA Plate Reader. The measured absorbance (against a background/blank control) directly correlated to the number of viable cells in each well. All samples were cultured in triplicate and repeated on three separate occasions. The mean absorbance of each co-culture was divided by the mean absorbance of the unstimulated negative control and expressed as a proliferation ratio.

2.8.2 pDC-induced T cell proliferation

To assess the effect of IL-1F8-matured pDCs on T cell proliferation, pDCs isolated from peripheral blood using the Diamond Plasmacytoid Dendritic Cell Isolation Kit as previously described were plated at a density of 1.16 x 105 cells/well on 6 well plates and cultured for 24 hours in complete RPMI 1640 medium supplemented with one of the following: IL-1F8 (500 ng/mL), E. coli 0127:B8 LPS (100 ng/mL) or imiquimod (50 µg/mL). After 24 hours, cells were harvested and checked for viability and maturity prior to further culture with CD3 + T cells. To measure the effect of stimulated pDCs on T cell proliferation, the WST-1 cell proliferation assay was performed according to the manufacturer's instructions. Briefly, $4x10^4$ cells per well stimulated, pDCs were cultured with $4x10^5$ cells/well CD3+ T cells (isolated from a different donor buffy coat using CD3+ microbeads as previously described) in 1ml of complete RPMI 1640 medium for 48 hours in 24-well culture plates and incubated at 37oC and 5% CO2. The ratio of stimulator cells (pDCs) to responder cells (CD3+ T cells) was 1:10 per well. As a positive control, CD3+ T cells were also cultured with Con A (75 µg/ml) in separate wells. At the end of the culture period, 100µl of each pDC/T cell suspension was aliquoted into appropriate wells on a sterile 96 well microtitre plate. 10 µl of Cell Proliferation reagent WST-1 was added to each well and the plate was incubated for 1½ hours at 37oC and 5% CO2. After gently shaking the plate, the absorbance of the samples at 450nm was measured against a blank control (containing culture medium and cell proliferation reagent only) using a Labtech LT-4000 ELISA Plate Reader. The measured absorbance

directly correlated to the number of viable cells in each well. All cultures were performed in triplicate and repeated on three separate occasions. The mean absorbance of each co-culture was divided by the mean absorbance of the unstimulated negative control and expressed as a proliferation ratio.

2.9.0 Measurement of cytokine profiles: ELISA Techniques

To assess whether stimulating MDDCs with IL-1F8 produced functionally mature DCs, immature day 5 MDDCs were plated in six well culture plates at a concentration of 4 x 10^5 cells/ml and stimulated for 48 hours with IL-1F8 (50ng/ml, 200ng/ml, 400 ng/ml, or 500ng/ml), IL-1β (10ng/ml) or *E. coli* 0127:B8 LPS (500 ng/ml). Negative control cells were cultured in cytokine-free complete RPMI 1640 medium for the same period. At the end of the culture period, supernatants were collected from the wells and IL-12 p70 and IL-18 concentrations were measured in the supernatant of stimulated or unstimulated DCs by specific enzyme-linked immunosorbent assay (ELISA) methods.

ELISA was also used to measure the production of IFN-α by IL-1F8 or IL-1β-stimulated pDCs. In this study, pDCs were stimulated with IL-1F8 or IL-1β (100 ng/mL) prior to removal of supernatants after 48 h. As a positive control, cells were cultured in media containing imiquimod (50 µg/ml) (3 M Healthcare, Loughborough, UK) and, as a negative control, immature MDDCs were washed and cultured in media only for a further 48 h. To determine T-cell phenotypes

differentiated by IL-1-matured MDDCs, analysis of IL-10 and IFN-γ concentrations were performed using standard ELISA analyses in supernatants obtained after 96 h co-culture of MDDCs with CD3+ lymphocytes.

2.9.1 IL-12p70 ELISA

IL-12p70 was measured using a solid phase sandwich ELISA (BD OptEIA™IL-12 (p70) human ELISA kit, BD Biosciences, UK) performed according to the manufacturers' instructions. The sensitivity of the IL-12 p70 ELISA was < 1 pg/ml. The BD OptEIA™IL-12 (p70) test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay) which utilizes a monoclonal antibody specific for IL-12 (p70) coated on a 96-well plate. Standards and samples are added to the wells, and any IL-12 (p70) present binds to the immobilized antibody. The wells are washed and a mixture of biotinylated anti-human IL-12 (p70) antibody and Streptavidin-horseradish peroxidase is added, producing an antibody-antigen-antibody “sandwich”. The wells are again washed and a substrate solution is added, which produces a blue colour in direct proportion to the amount of IL-12 (p70) present in the initial sample. The Stop Solution changes the colour from blue to yellow, and the wells are read at 450 nm.

Briefly, lyophilized recombinant human IL-12 (p70) was reconstituted and serially diluted to make IL-12 (p70) standard solutions with the following concentrations: 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml and 7.8

pg/ml. 100 µl standard or sample was added to an appropriate well on a 96 well plate. Each well had been pre-coated with anti-human IL-12 (p70) monoclonal antibody by the manufacturer. The plate was covered with plate sealer and incubated for 2 hours at room temperature. Contents of wells were decanted and wells washed by filling with at least 300 µl/well wash buffer and then decanting. This washing procedure was repeated 4 times to make a total of 5 washes. After the last wash, the plate was blotted on absorbent paper to remove any residual buffer before adding 100 µl Working Detector (containing biotinylated anti-human IL-12 (p70) monoclonal antibody and Streptavidin-horseradish peroxidase conjugate with Bovine Serum Albumin) to each well. Wells were covered with Plate Sealer and incubated for 1 hour at room temperature. This was followed by decanting contents of wells and washing as already described but with a total of 7 thorough washes. 100 µl of Substrate Solution (containing hydrogen peroxide in buffered solution and tetramethylbenzidine (TMB) in organic solvent) was added to each well and the plate was incubated without Plate Sealer for 30 minutes at room temperature in the dark. After adding 50 µl of Stop Solution (containing 1M phosphoric acid) to each well the absorbance was read on a Labtech LT-4000 ELISA Plate Reader at 450 nm within 30 minutes of stopping the reaction on a Labtech LT-4000 ELISA Plate Reader (with Manta Data analysis software) (Labtech International Ltd, East Sussex, UK) . To eliminate background absorbance, the absorbance of the blank control (sample containing ELISA diluent only and no target antigen) was subtracted from that of the standards and samples. A standard curve was computer generated from the absorbance values of the IL-12p70 standards. A standard curve was done for

each assay run. Using computer-aided linear regression analysis, the concentration of IL-12p70 in test samples was automatically calculated and displayed by the Labtech LT-4000 ELISA Plate Reader. Figure 2.14 shows a typical standard curve for the BD OptEIA™ Human IL-12 (p70) ELISA.

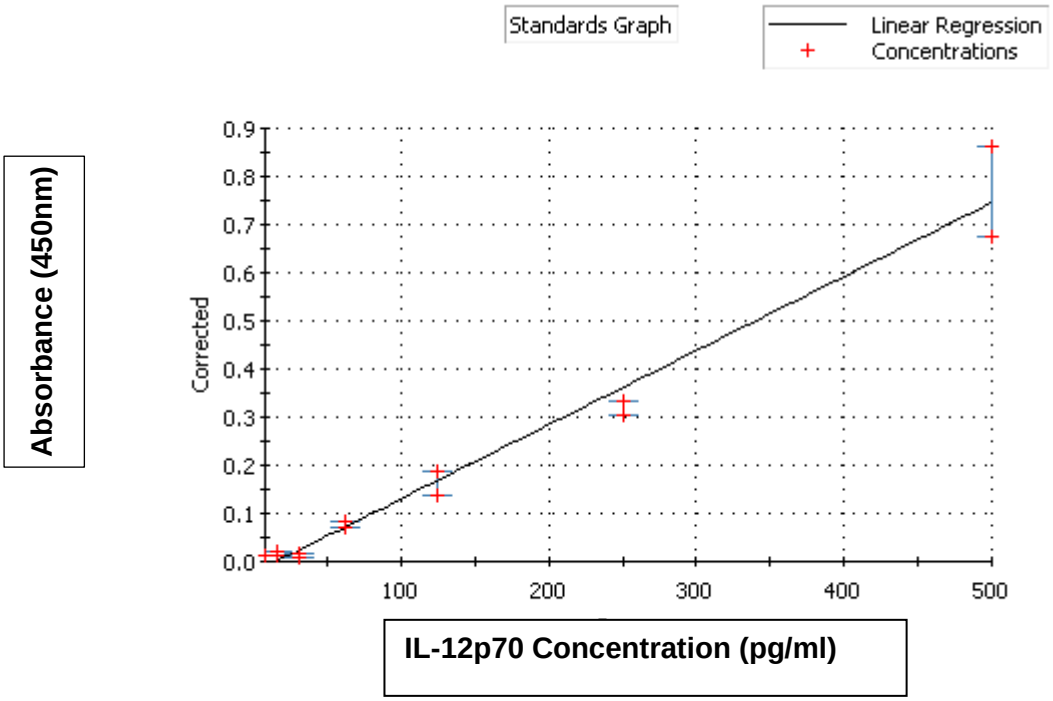


Figure 2.8 IL-12p70 Standard Curve (ELISA). A representative standard curve for the BD OptEIA™ Human IL-12 (p70) ELISA.

2.9.2 IL-18 ELISA

IL-18 was measured using the Human IL-18 sandwich ELISA kit (R & D Systems, Abington, UK) according to the manufacturers' instructions. The sensitivity of the IL-18 ELISA was < 1 pg/ml. The Human IL-18 ELISA Kit measures human IL-18 by sandwich ELISA. The assay uses two monoclonal antibodies against two different epitopes of human IL- 18. In the wells coated with anti-human IL-18 monoclonal antibody, samples to be measured or standards are incubated. After washing, a peroxidase-conjugated anti-human IL-18 monoclonal antibody is added into the microwell and incubated. After another washing, the peroxidase substrate is mixed with the chromogen and allowed to incubate for an additional period of time. An acid solution is then added to each well to terminate the enzyme reaction and to stabilize the developed colour. The optical density (O.D.) (absorbance) of each well is measured at 450 nm using a microplate reader. The concentration of human IL-18 is calibrated from a dose response curve based on reference standards. To eliminate background absorbance, the absorbance of the blank control was subtracted from that of the standards and samples. Using computer-aided linear regression analysis (Manta Data analysis software) (Labtech International Ltd, East Sussex, UK), the concentration of IL-18 in test samples was automatically calculated from the standard curve and displayed by the Labtech LT-4000 ELISA Plate Reader.

2.9.3 Interferon (IFN)-α ELISA

IFN-α concentration was measured in the supernatant of stimulated or unstimulated pDCs using a sandwich ELISA immunoassay (*VeriKine*^{TM} Human

IFN-α ELISA Kit, supplied by R & D Systems, Abington, UK) performed according to the manufacturers' instructions. The *VeriKineTM* Human IFN-α ELISA kit uses the sandwich immunoassay technique for the quantitative measurement of IFN-α in media. As before, the concentration of human IFN-α in test samples was automatically calculated from a dose response curve (absorbance versus concentration) based on reference standards. To eliminate background absorbance, the absorbance of the blank control was subtracted from that of the standards and samples. The concentration of IFN-α in test samples was automatically calculated and displayed by the Labtech LT-4000 ELISA Plate Reader software.

2.9.4 IFN-γ ELISA

IFN-γ ELISA was performed using the BD OptEIA™ Human IFN-γ ELISA Kit II (BD Biosciences, Oxford, UK) as per manufacturer's instructions. The BD OptEIA™ Human IFN-γ ELISA is a solid phase sandwich ELISA which utilizes a monoclonal antibody specific for IFN-γ coated on a 96-well plate. Standards and samples are added to the wells, and any IFN-γ present binds to the immobilized antibody. The wells are washed and Streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IFN-γ antibody is added, producing an antibody-antigen-antibody "sandwich". The wells are again washed and TMB substrate solution is added, which produces a blue colour whose intensity is directly proportional to the amount of IFN-γ present in the initial sample. The Stop Solution changes the colour from blue to yellow, and the microwell

absorbances are read at 450 nm. The concentration of human IFN-γ in test samples was automatically calculated from a dose response curve (absorbance versus concentration) based on reference standards. To eliminate background absorbance, the absorbance of the blank control was subtracted from that of the standards and samples. The concentration of IFN-γ in test samples was automatically calculated and displayed by the Labtech LT-4000 ELISA Plate Reader software.

2.9.5 IL-10 ELISA

IL-10 levels in supernatants from the MDDC/CD3+ T cell co-cultures were measured using the BD OptEIA™ Human IL-10 ELISA Kit II (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. The BD OptEIA™ Human IL-10 ELISA is a solid phase sandwich ELISA which utilizes a monoclonal antibody specific for IL-10 coated on a 96-well plate. Standards and samples are added to the wells, and any IL-10 present binds to the immobilized antibody. The wells are washed and streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IL-10 antibody is added, producing an antibody-antigen-antibody "sandwich". The wells are washed again and TMB substrate solution is added, which produces a blue colour whose intensity is directly proportional to the amount of IL-10 present in the initial sample. The Stop Solution changes the colour from blue to yellow, and the microwell absorbances are read at 450 nm. As previously explained, the concentration of human IL-10 in test samples was automatically calculated from a dose response curve

(absorbance versus concentration) based on reference standards. To eliminate background absorbance, the absorbance of the blank control was subtracted from that of the standards and samples. The concentration of IL-10 in test samples was automatically calculated and displayed by the Labtech LT-4000 ELISA Plate Reader software.

2.10 Statistical Analysis

The Roche LightCycler® 480 Relative Quantification Software was used to calculate relative and calibrator-normalized expression ratios using an automated mathematical computation devised by Pfaffl (Pfaffl, 2001; 2002; 2004) and to display these as the mean of performed replicates. The Roche LightCycler® 480 Relative Quantification Software also calculated all means, standard deviation of means, medians and standard deviations of crossing points (CPs) and target/reference ratios. Data are expressed as mean ± standard deviation. Each value is the mean of at least three independent experiments with each analysis being performed in triplicate (or in duplicate in some cases). An analysis of variance (ANOVA) test with a one-way classification was used to calculate significant differences between test and control samples following cytokine ELISA analyses and the difference between the number of cells, within total cell populations, expressing CD1a or HLA-DR following stimulation with IL-1 cytokines and IFN-γ. A p value of < 0.05 was considered statistically significant. Tukey's test was used post hoc to determine significance between individual means. Minitab software was used for all statistical analyses.

CHAPTER 3: IL-1RRP2 EXPRESSION AND BIOLOGICAL RESPONSE IN HUMAN MYELOMONOCYTIC CELLS

3.1 Introduction

Typically, IL-1β (as well as IL-1α) exerts its effects on target cells by binding to the type 1 interleukin-1 receptor (IL-1R1) to form a complex. This binding leads to recruitment of the signal-transducing IL-1R accessory protein (IL-1RAcP) to the complex which in turn results in the activation of intracellular nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways. The end result is the transcription of host defence effector genes in target cells; including the production of numerous cytokines, chemokines, adhesion molecules, and enzymes such as cyclooxygenase and nitric oxide synthase (Barnes and Karin, 1997; Didierlaurent *et al.,* 2006). The type 2 IL-1 receptor (IL-1R2) acts as a decoy receptor for IL-1α and IL-1β and thus suppresses IL-1 biological activities (Dinarello, 1991; Colotta *et al.,* 1993; reviewed in Colotta *et al.,* 1994; Lang *et al.,* 1998; Neumann *et al.,* 2000; reviewed in Dinarello, 2009). As previously mentioned, the activities of IL-1α and IL-1β are also naturally down-regulated by IL-1 receptor antagonist (IL-1Ra) (Hannum *et al.,* 1990; Dipps *et al.,* 1991; Smith *et al*., 2000; reviewed in Arend, 2002; Sims, 2002; Dumont, 2006).

It has been shown that novel IL-1 cytokines do not signal via the classic IL-1R1 receptor (Busfield *et al.,* 2000; Debets *et al.,* 2001; Lin *et al.,* 2001; Pan *et al.,* 2001; Towne *et al.,* 2004). IL-1F6, IL-1F8 and IL-1F9 have been shown to signal via a receptor complex comprising IL-1Rrp2 and IL-1RAcP (Debets *et al.,* 2001; Towne *et al.,* 2004). Thus, IL-1Rrp2 expression is central to the functioning of these novel IL-1 family members.

Prior to the current project, there was no available data showing expression of IL-1Rrp2 by human myeloid immune cells. Lovenberg *et al.* (1996) reported that IL-1Rrp2 is highly expressed in rat meninges, choroid plexus and cerebral cortex. IL-1Rrp2 expression has also been observed in several human cell lines (Towne *et al.,* 2004). The highest IL-1Rrp2 expression in humans has been observed in skin while lower levels were found in prostate, ovary, thyroid, uterus, liver, kidney, lung, and trachea. IL-1Rrp2 is either not present or is expressed at very low levels in human testis, heart, spleen, and small intestine (Towne *et al.,* 2004). Human synovial fibroblasts and human articular chondrocytes express IL-1Rrp2 and produce inflammatory mediators in response to stimulation by IL-1F8 (Magne *et al.,* 2006). This suggests that IL-1Rrp2 expression may play an important role in some human diseases.

Indeed, Blumberg *et al*. (2007) reported that expression of IL-1Rrp2, IL-1F5 and IL-1F6 is increased in human psoriatic skin. More recently, a follow-up study showed that treatment with anti-IL-1Rrp2 antibody reduced the epidermal hyperplasia and other skin changes associated with psoriasis in mice (Blumberg

et al., 2010), suggesting that agents which block signalling through IL-1Rrp2 could be useful therapeutic tools for psoriatic conditions and related skin pathologies.

Classical IL-1 family members (IL-1α, IL-1β, IL-18 and IL-1Ra) are essential for the complete functioning of all human myeloid immune cells. Not only are they mostly synthesized by myelomonocytic cells (monocytes, macrophages and DCs); they also regulate these and other immune cells (Dinarello, 1996; Dinarello, 1997). Typically, classical IL-1 cytokines (e.g. IL-1β) stimulate maturation of DCs; a critical event in the induction of adaptive immunity. Immature DCs in peripheral tissue mature as they migrate to draining lymph nodes following stimulation by pathogens, pathogen-associated molecules or cytokines (reviewed in Mellman *et al.,* 1998). Many studies have shown that IL-1β (IL-1F2) acts as a potent maturation signal for DCs (Banchereau and Steinman, 1998; Nakahara *et al., 2005;* Guo *et al.,* 2003; Wesa and Galy*,* 2002), however, there is no published data on the expression of IL-1Rrp2 by human myelomonocytic cells or the effect of novel IL-1 cytokines, which act via this receptor, on the function of these cells.

The specific aim of the work outlined in this chapter was to investigate IL-1Rrp2 expression by human myelomonocytic immune cells and to determine whether IL-1F8 and/or IL-1F9 function as maturation signals for these cells. THP-1 cells were used as a negative control in all qRT-PCR experiments

investigating IL-1Rrp2 mRNA expression in this study (Chapters 3 and 4). The decision to use THP-1 cells as a negative control for IL-1Rrp2 expression was based on published reports which stated absence of IL-1Rrp2 expression in THP-1 cells (Magne *et al.,* 2006. Our own preliminary studies also confirmed these findings. However, there was a need to confirm that THP-1 cells used in this study were fully functional. Prior to using THP-1 cells as negative controls for IL-1Rrp2 expression in qRT-PCR experiments, their functional status was tested using an established system; measuring TNF-α mRNA expression by THP-1 cells in response to bacterial (*E. coli* 0127:B8 LPS) stimulation (Essner *et al*, 1990; Agarwal *et al*., 1995; Asakura *et al*., 1996; Dedrick and Conlon, 1995; Jones *et al*., 2003; Foster *et al*., 2005; Lackman and Cresswell, 2006). Results of that preliminary test are shown in Appendix 1 of this thesis.

3.2 Results

3.2.1 Expression of IL-1Rrp2 mRNA in human myelomonocytic cells

Quantitative Real-Time PCR (qRT-PCR) analysis was performed on human promonocytes (THP-1 cells), peripheral blood monocytes (PBM), monocyte-derived macrophages (MDM), monocyte-derived dendritic cells (MDDCs) and peripheral blood dendritic cell subsets to assess expression of IL-1Rrp2 mRNA. Cells were isolated, cultured and harvested as previously stated (Chapter 2). RNA was extracted and quantified prior to cDNA synthesis as previously described. Quantitative RT-PCR for the IL-1Rrp2 gene and GAPDH gene (control) was then performed as stated in Chapter 2 (Sections 2.7.0-2.75).

Confirmation of the qRT-PCR product was made by gel electrophoresis (2% agarose) (Section 2.7.6) which showed presence of the expected IL-1Rrp2 RT-PCR product (Figure 3.1).

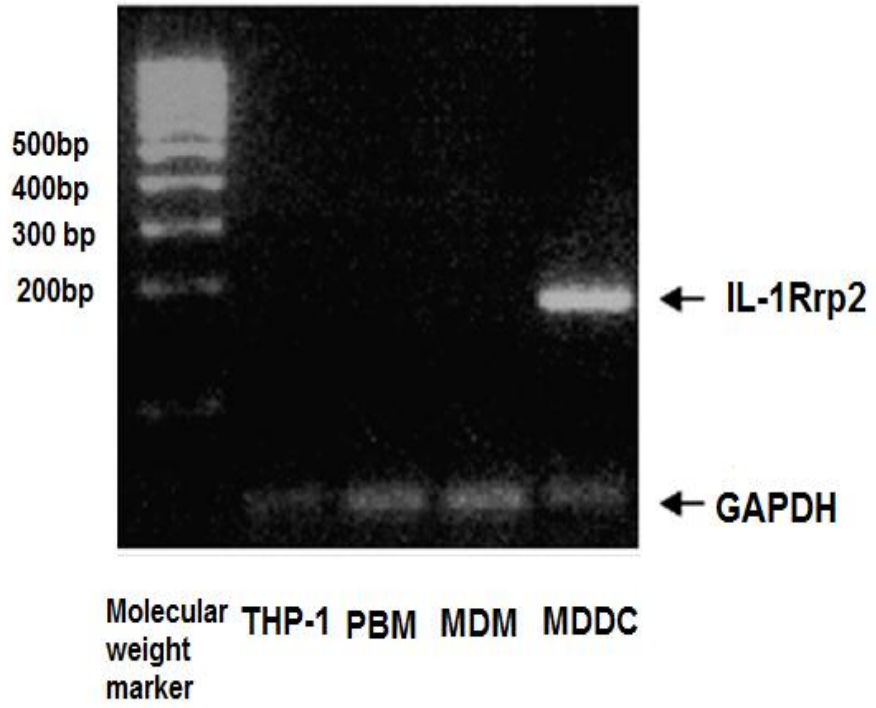


Figure 3.1 Representative agarose gel electrophoresis results confirming IL-1Rrp2 gene expression in MDDC. Results show presence of expected IL-1Rrp2 PCR product.

3.2.2 Human MDDCs express IL-1Rrp2 mRNA

qRT-PCR analysis showed that IL-1Rrp2 mRNA is expressed by human monocyte-derived dendritic cells (MDDCs) but not by promonocytes (THP-1 cells), peripheral blood monocytes or monocyte-derived macrophages (MDMs) (Figure 3.2). MDMs only express IL-1Rrp2 if IL-4 (10ng/ml) is added to the culture and incubated for 48 hours prior to analysis. An infinite increase in IL-1Rrp2 mRNA expression (relative to GAPDH) was observed in MDDCs generated by culturing peripheral blood monocytes with GM-CSF (50ng/ml) and IL-4 (10ng/ml) for 5 or 7 days. Similar results were also obtained when 5-day old monocyte derived macrophages (MDMs) were stimulated with IL-4 (10ng/ml) for 48 hours (Figure 3.2). The morphological appearance of a good number of the IL-4-stimulated MDMs resembled that of MDDCs (Figure 3.3).

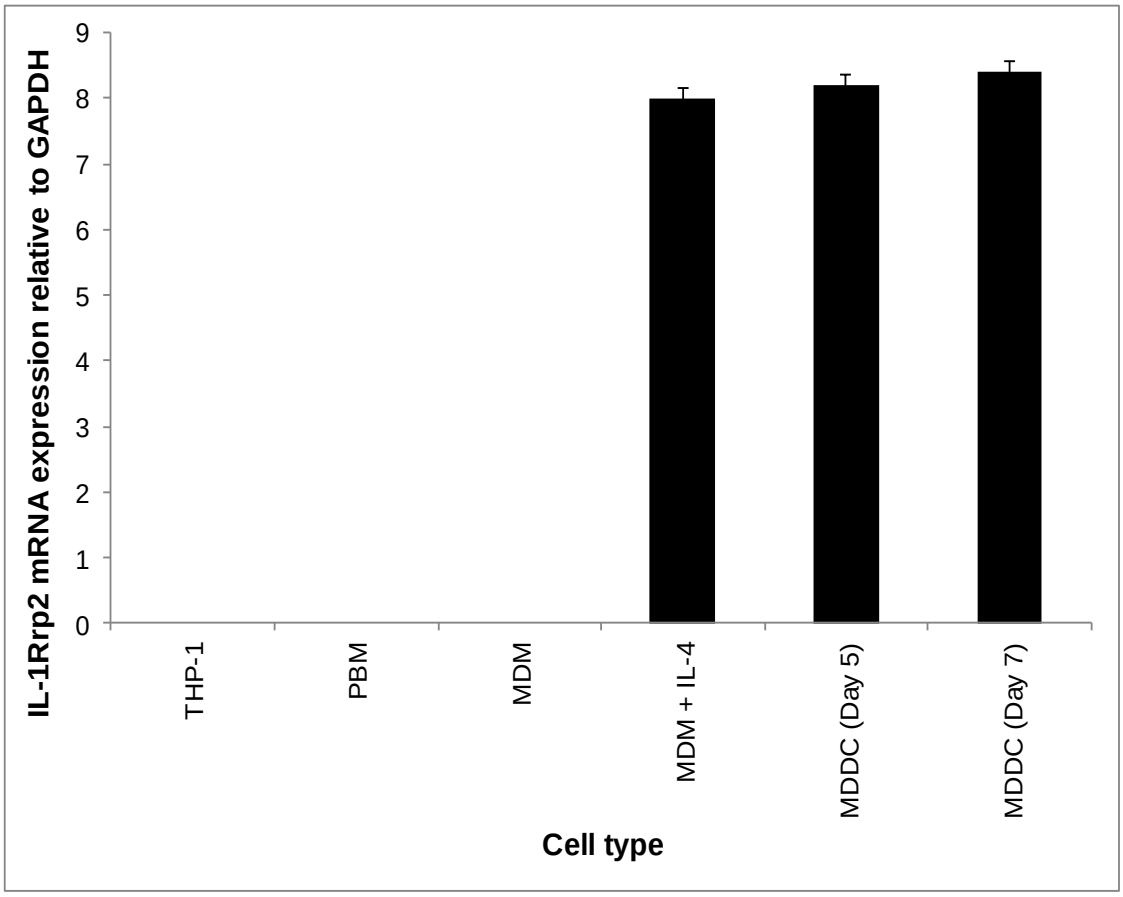


Figure 3.2 IL-1Rrp2 mRNA expression in THP-1 cells, PBM, MDM and MDDCs. Graph shows IL-1Rrp2 mRNA expression (qRT-PCR) in human promonocytic cells (THP-1), peripheral blood monocytes (PBM), monocyte derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC). IL1Rrp2 is expressed by human MDDCs but is not expressed by promonocytes, peripheral blood monocytes or monocyte-derived macrophages. MDMs only express IL-1Rrp2 following incubation with IL-4 (10ng/ml) for an extra 48 hours prior to analysis. Bar chart shows mean data from at least three independent experiments. Error bars show standard deviation (1SD) from the mean.

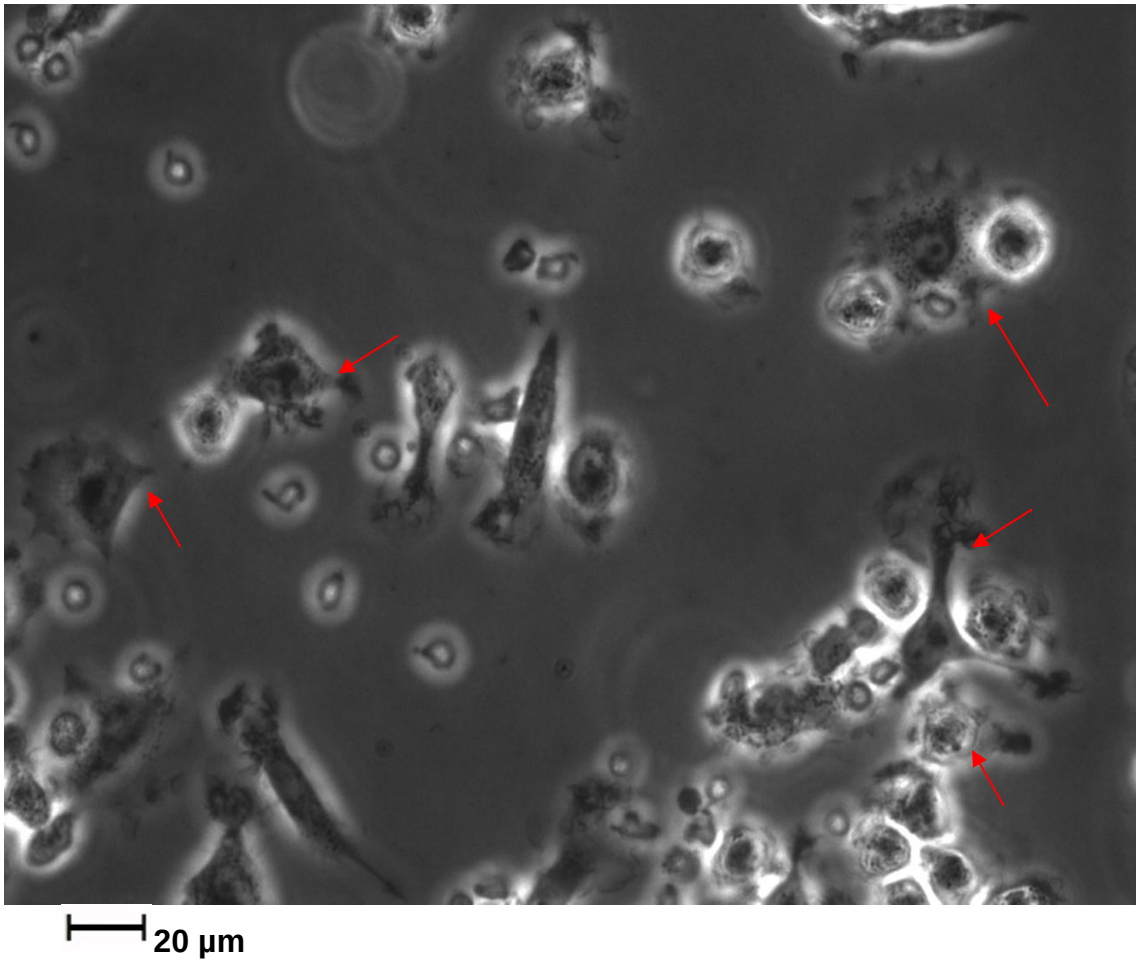


Figure 3.3 Morphology of monocyte derived macrophages following culture with IL-4 for 48 hours. Phase contrast microscopy image (original magnification x 400) showing the morphology of monocyte derived macrophages following culture with IL-4 for 48 hours. The majority of cells show typical dendritic cell morphology (presence of dendrites as shown by the red arrows). Scale bar represents 20 µm.

3.2.3 IL-4 dose-dependently increases MDDC differentiation and IL-1Rrp2 expression

When populations of peripheral blood monocytes (PBM) were cultured for 5 days with GM-CSF (50ng/ml) and variable concentrations of IL-4 (1, 10 or 50 ng/ml), lowest IL-1Rrp2 mRNA expression was measured in PBM cultures containing 1ng/ml IL-4 while highest IL-1Rrp2 mRNA expression was obtained in cultures containing 50 ng/ml (Figure 3.4). These results indicate that IL-4 increases IL-1Rrp2 expression in a dose-dependent manner. However, increase in IL-4 concentration (and concurrent increase in IL-1Rrp2 expression) was consistent with increased numbers of MDDCs in these cultures, ascertained by morphological analysis (Figures 3.5 and 3.6).

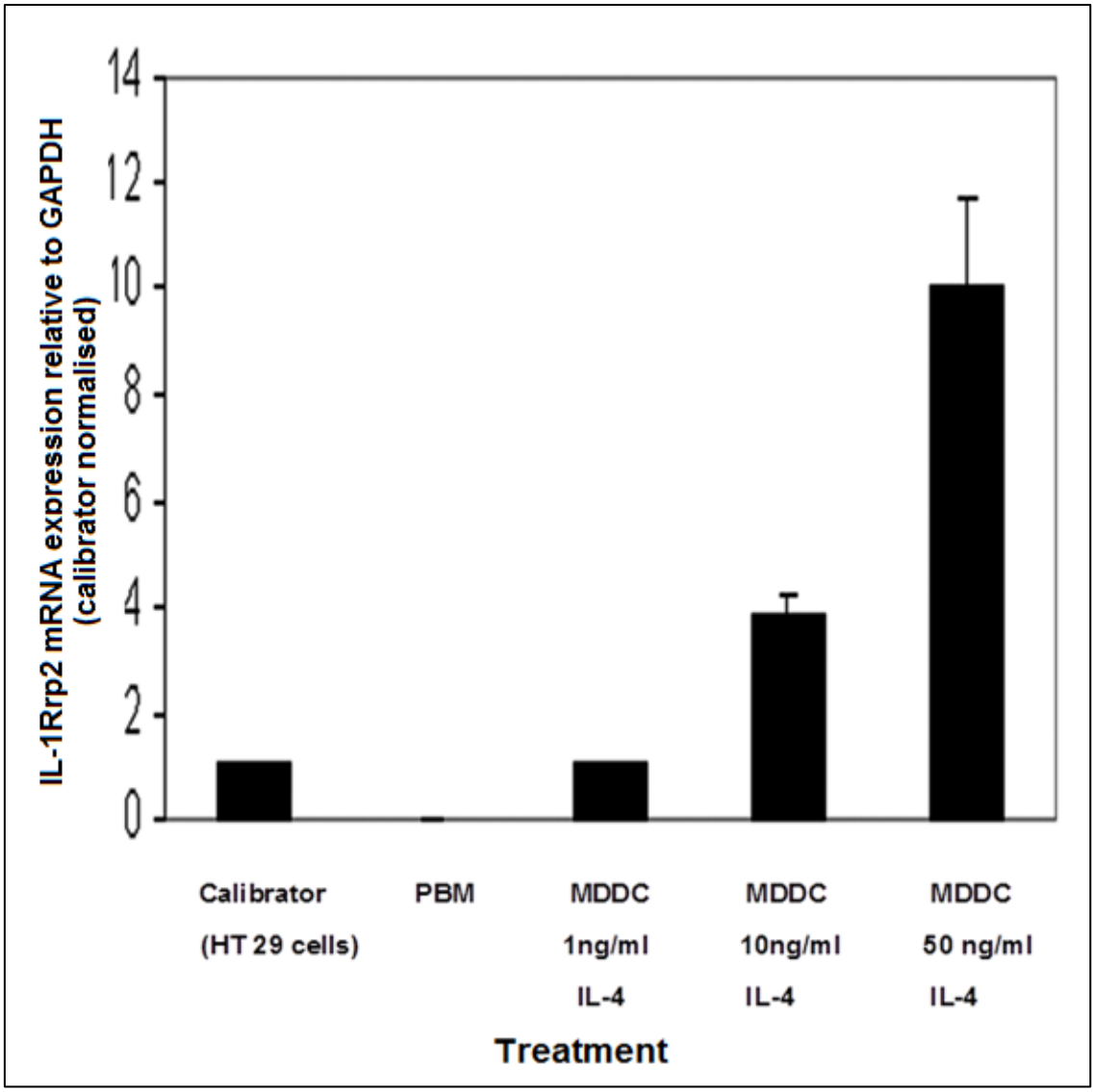


Figure 3.4 IL-4 dose-dependently increases MDDC differentiation and IL-1Rrp2 expression. qRT-PCR analysis of IL-1Rrp2 mRNA expression in HT29 cells (calibrator), peripheral blood monocytes (PBM) and MDDCs co-cultured in the absence/presence of the indicated concentrations of IL-4. Results show that IL-4 dose-dependently increases MDDC differentiation and IL-1Rrp2 expression. Data shown are means of experimental data obtained on five separate occasions. Error bars show mean ± SD of each mean.

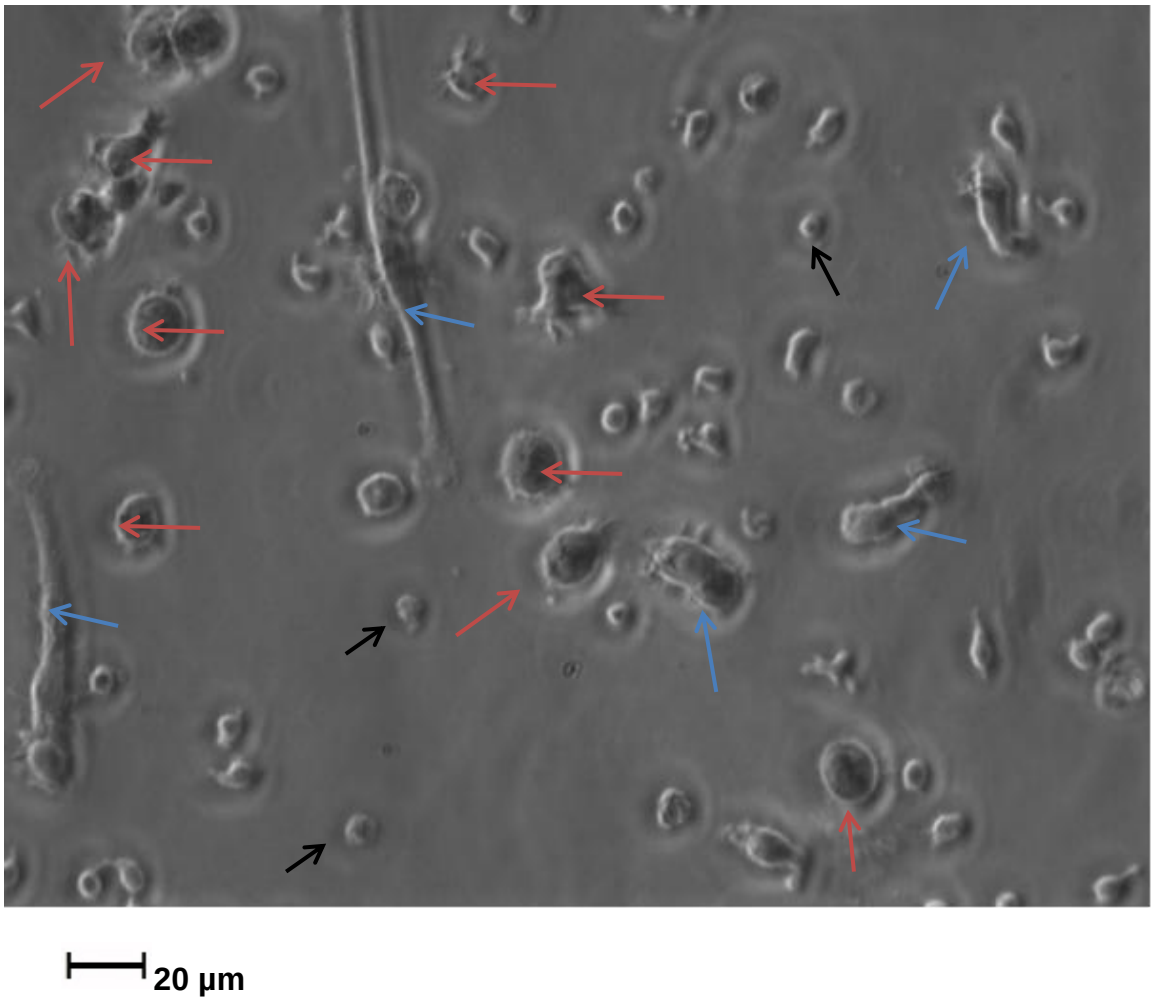


Figure 3.5 Peripheral blood monocytes following 5 day culture with GM-CSF and IL-4. Phase contrast microscopy image (original magnification x400) of peripheral blood monocytes cultured with GM-CSF (50ng/ml) and IL-4 (1 ng/ml) for 5 days. Image shows presence of monocyte derived macrophages (blue arrows), MDDCs with visible dendrites (red arrows) and undifferentiated peripheral blood monocytes (black arrows). Image is representative of at least three independent experiments with similar results. Scale bar represents 20 µm.

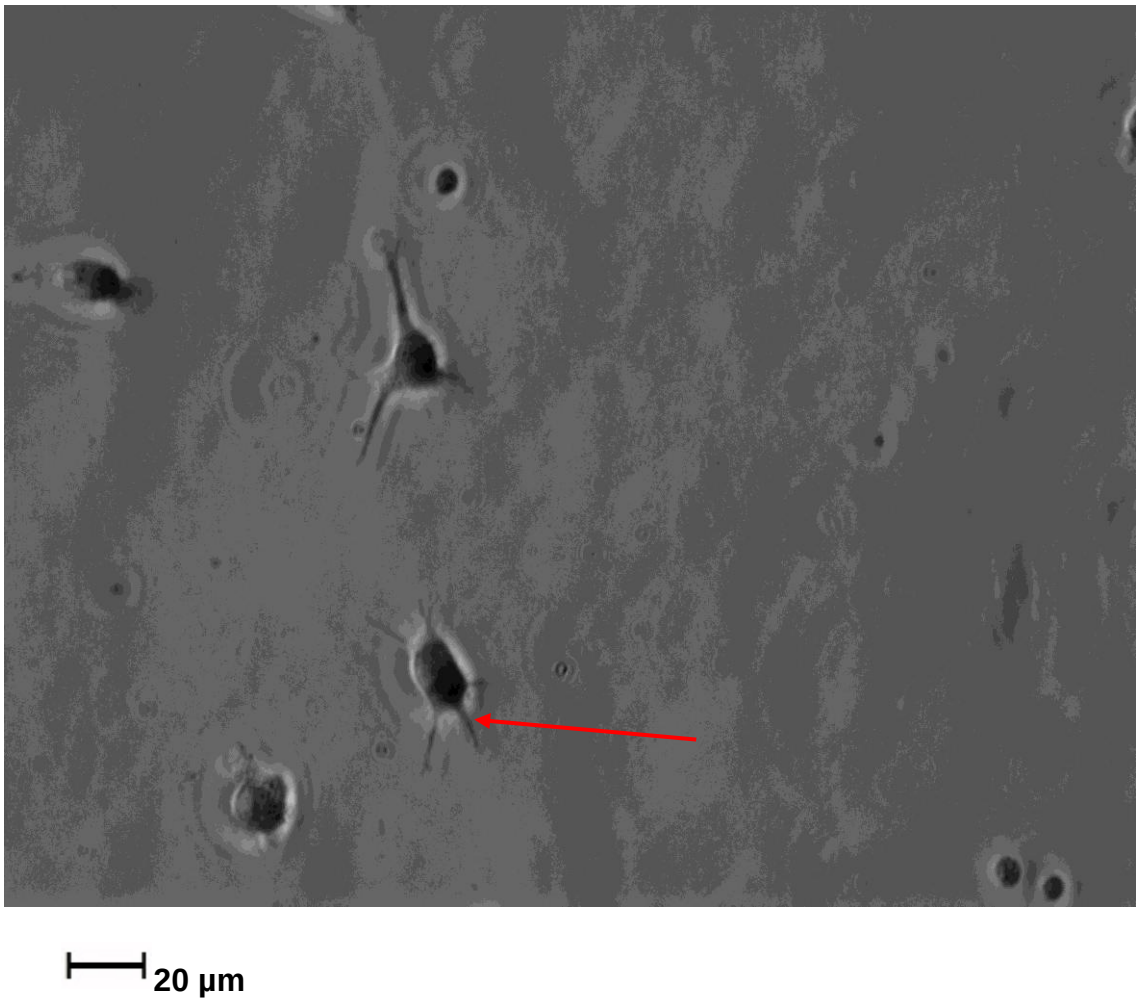


Figure 3.6 Light microscopy MDDC morphology. MDDCs with typical observable dendrites (shown by red arrow) were observed using a Leica DM IL LED (Leica Microsystems, (UK)) inverted microscope (400x magnification) when peripheral blood monocytes were cultured with GM-CSF and 10 ng/ml IL-4 for 5 days. There were more MDDCs with characteristic dendritic morphology than that observed when 1ng/ml IL-4 was used. Image is representative of at least three independent experiments with similar results. Scale bar represents 20 µm.

3.2.4 FACS analysis showing IL-1Rrp2 protein expression on the surface of MDDCs

Having shown that MDDCs express IL-1Rrp2 mRNA, the presence of the IL-1Rrp2 protein on the surface of MDDCs was confirmed by flow cytometry (FACS analysis) using standard methods as previously described in Chapter 2 (Section 2.6.1.1). Briefly, $1x10^6$ test cells per group were resuspended in FACS buffer (BSA (1% w/v); EDTA (2 mM)) and 100µl of the suspension was aliquoted into the appropriate number of tubes. Fc receptors on cells were blocked in FACS buffer containing human serum (10% v/v) for 15 min prior to incubation for 45 minutes with either 10µg/ml mouse anti-human IL-1Rrp2 antibody (M145, Amgen Corporation, Seattle, WA) followed by incubation for 45 minutes with the detection/secondary antibody (10µg/ml allophycocyanin (APC)-conjugated goat anti-mouse IgG (Biolegend, UK)) or incubation for 45 minutes with 10µg/ml APC-conjugated goat anti-mouse IgG only (isotype/secondary antibody control cells). Cells were then analysed using a FACSCanto II analyser (Becton Dickinson, USA). Samples were acquired using the BD FACSDiva™ software (BD Biosciences, USA) and analysed using the WinMDI 2.8 software. Cell viability was assessed by propidium iodide uptake (20 µg/ml in PBS for 10 minutes).

Surface expression of IL-1Rrp2 was not increased above that measured for isotype/secondary antibody control levels on the cell membranes of promonocytes, peripheral blood monocytes (PBMs) or monocyte-derived

macrophages (MDMs) (Figure 3.7). However, a progressive increase in IL-1Rrp2 signal (starting from day 2) above that measured for the isotype control was observed (Figure 3.8) when MDDCs were generated by culturing PBMs with GM-CSF (50ng/ml) and IL-4 (10ng/ml) for five days.

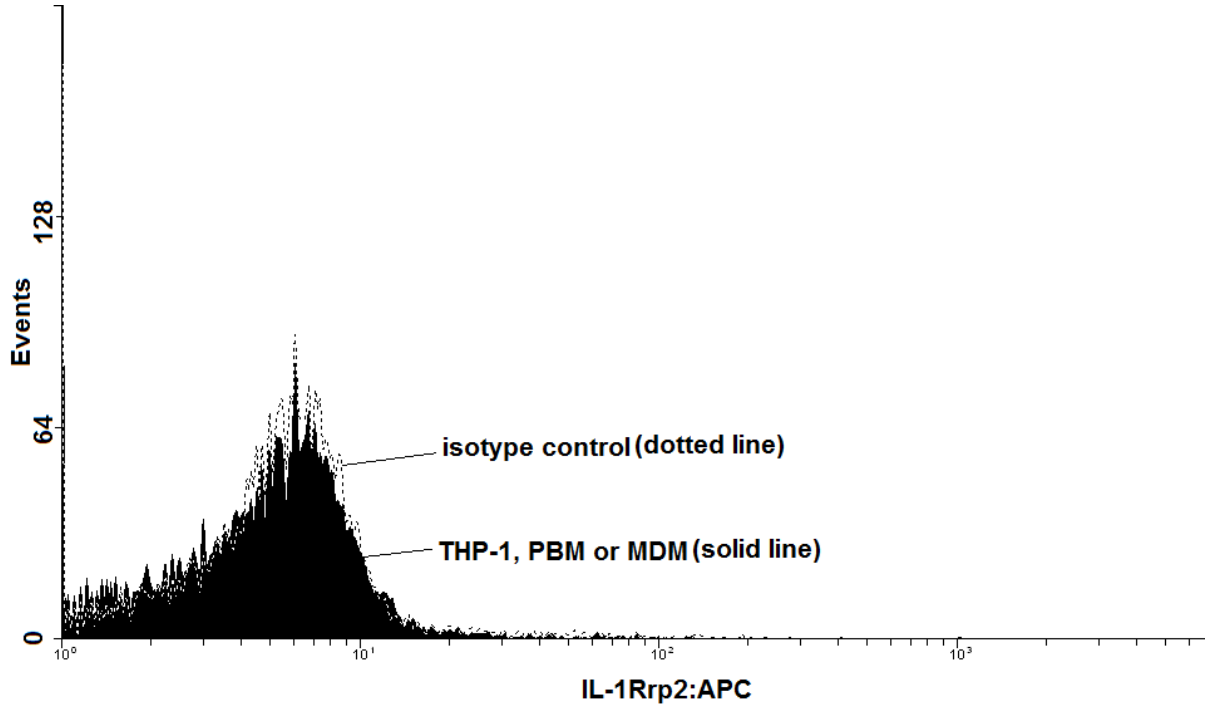


Figure 3.7 Promonocytes, PBM and MDM do not express IL-1Rrp2. Typical FACS analysis histogram obtained when promonocytic THP-1 cells, peripheral blood monocytes and monocyte-derived macrophages (represented by test sample in histogram above) were stained with either 10µg/ml mouse anti-human IL-1Rrp2 antibody (M145, Amgen Corporation, Seattle, WA) plus 10µg/ml allophycocyanin (APC)-conjugated goat anti-mouse IgG or with 10µg/ml APC-conjugated goat anti-mouse IgG only (isotype control). The histogram shows that promonocytic THP-1 cells, peripheral blood monocytes or monocyte-derived macrophages do not express IL-1Rrp2 on the cell surface. Histogram is representative of at least three independent experiments with similar results.

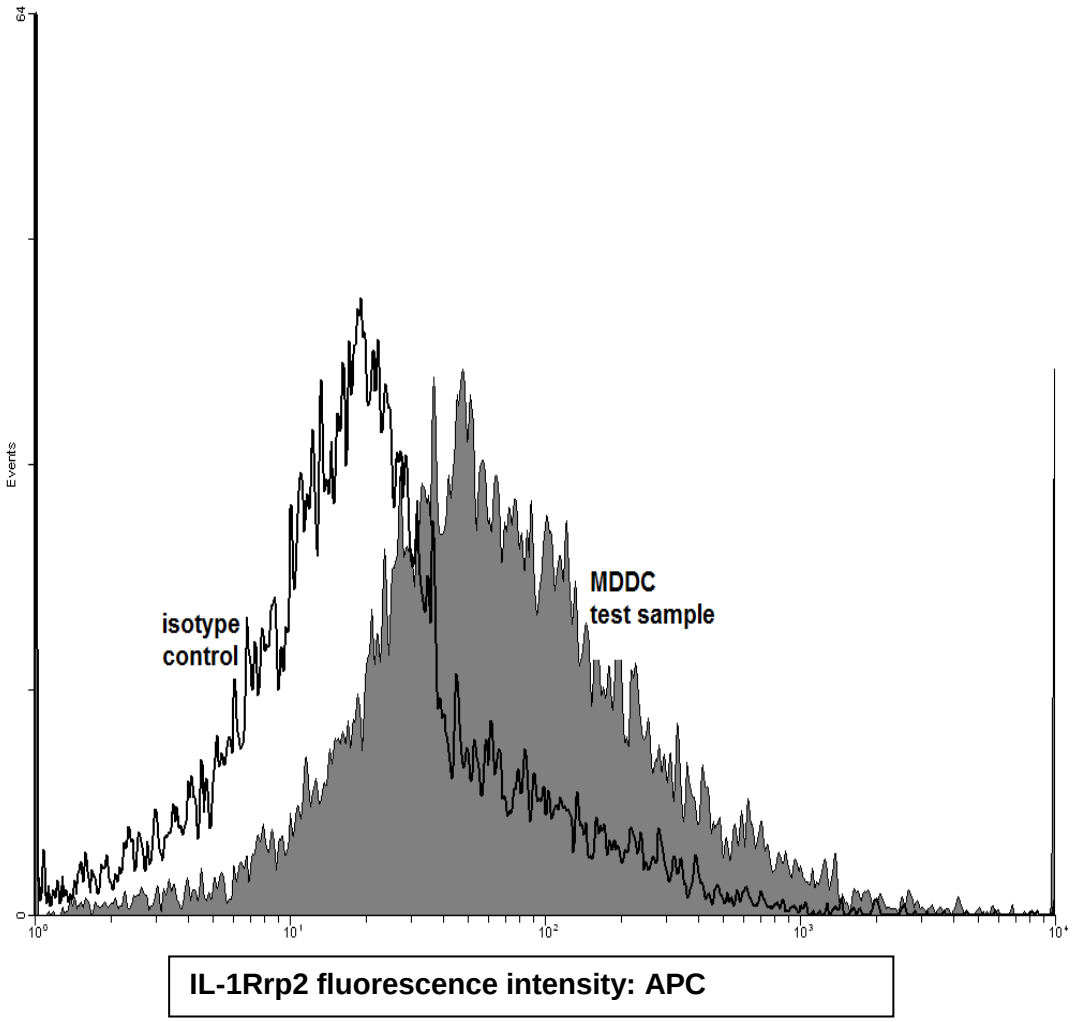
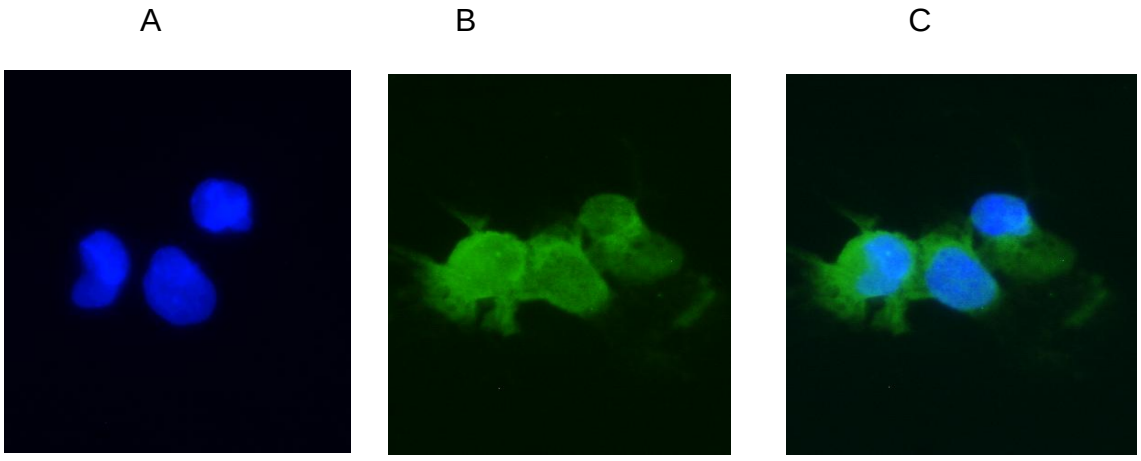


Figure 3.8 MDDCs express IL-1Rrp2 on the cell surface (FACS analysis). Typical FACS analysis histogram showing that monocyte derived dendritic cells (MDDCs) express IL-1Rrp2 on the cell surface. Cells were stained with either 10µg/ml mouse anti-human IL-1Rrp2 antibody (M145, Amgen Corporation, Seattle, WA) plus 10µg/ml allophycocyanin (APC)-conjugated goat anti-mouse IgG or with 10µg/ml APC-conjugated goat anti-mouse IgG only (isotype control). Histogram is representative of at least three independent experiments with similar results.

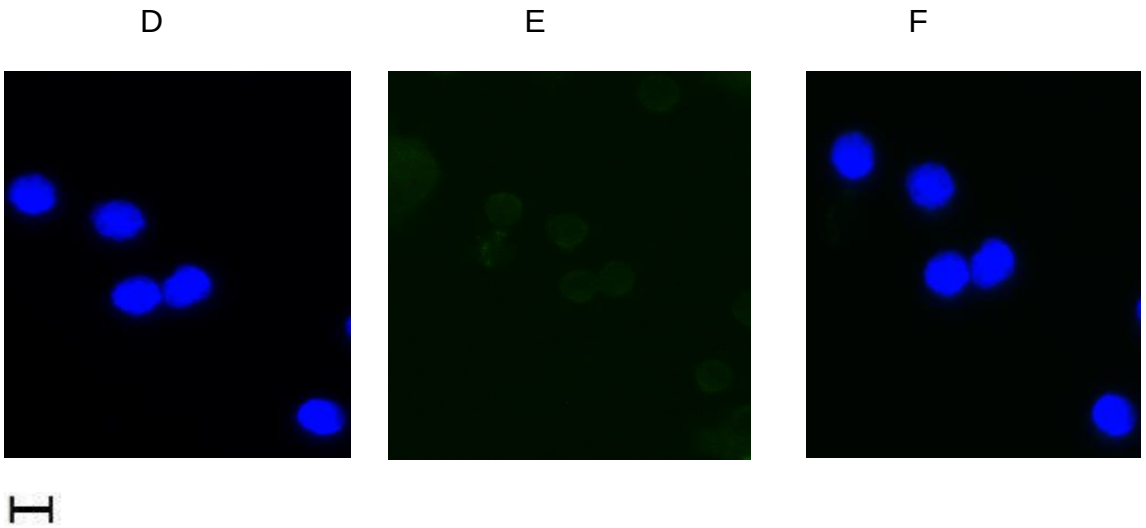
3.2.5 Expression of IL-1Rrp2 protein in MDDCs (Fluorescence microscopy)

Fluorescence microscopy was used to further ascertain expression (and location) of the IL-1Rrp2 protein in MDDCs. MDDCs were cultured as described previously (Chapter 2). Cultured cells were harvested and processed for fluorescence microscopy as previously described (Section 2.6.2). Briefly, cytospin preparations were made from MDDC cell suspensions and the cells were stained for fluorescence microscopy using a monoclonal primary antibody specific for IL-1Rrp2 (M145, Amgen Corporation, Seattle, WA) followed by staining with a secondary detection antibody (FITC-conjugated Goat anti-mouse IgG). Cells were counterstained with DAPI nuclear stain prior to being analysed using a Leica fluorescence microscope (Leica Microsystems, UK) as described in Section 2.6.2. The images are shown below. Images are representative of at least three independent experiments.

MDDC sample:



Negative (secondary antibody only) control:



DAPI Nuclear stain Mouse anti-human IL-1Rrp2 (FITC-conjugated) Overlay image

Figure 3.9 MDDCs express IL-1Rrp2 (fluorescence microscopy). Images show that IL-1Rrp2 is localised to the cell membrane of human MDDCs following incubation with an IL-1Rrp2 mAb followed by staining with a FITC-conjugated secondary antibody and DAPI nuclear stain (images A-C). The negative control sample (D-F) was incubated with secondary antibody and DAPI only but not with the primary IL-1Rrp2 mAb. The overlay images are shown by C and F. Images are representative of five independent experiments with similar results. Scale bar represents 20 µm.

Compared with the negative control (images D-F), results confirm that human MDDCs (images A-C) express IL-1Rrp2 protein on the cell surface (Figure 3.9). The blue images (A and D) show nuclear staining with DAPI while images B and E show immuno-cytochemical localisation of IL-1Rrp2 protein (green-staining due to the FITC-labelled secondary antibody binding to the IL-1Rrp2 monoclonal antibody which is in turn bound to IL-1Rrp2 in the cells). Image C is an overlay image of A and B and shows localization of IL-1Rrp2 to both the cytoplasm and cell membrane of MDDCs. In contrast, image F, which is an overlay of images D and E respectively, shows no immunoreactivity occurring when MDDCs are incubated with secondary antibody only.

3.2.6 Both IL-1F8 and IL-1F9 can induce MDDC maturation

Immature 5-day-old MDDCs were plated at a density of 4 x 10^5 cells/ml on 6 well plates and cultured for 48 hours with 100ng/ml recombinant human IL-1F8 (rhIL-1F8), 100ng/ml recombinant human IL-1F9 (rhIL-1F9) (Amgen Corporation, Seattle, WA) or with positive control (100ng/ml recombinant human IL-1β (rhIL-1F2) (R & D Systems, UK) or 100ng/ml IFN-γ)). Negative control cells were cultured without IL-1 cytokines. The effect of IL-1 cytokines on MDDC maturation was assessed by FACS analysis and compared with relative isotype controls as explained in Section 2.6.1.3 and cells were gated as R1 (immature) and R2 (mature) populations according to parameters set for immature MDDCs. The results are shown below (Figures 3.10).

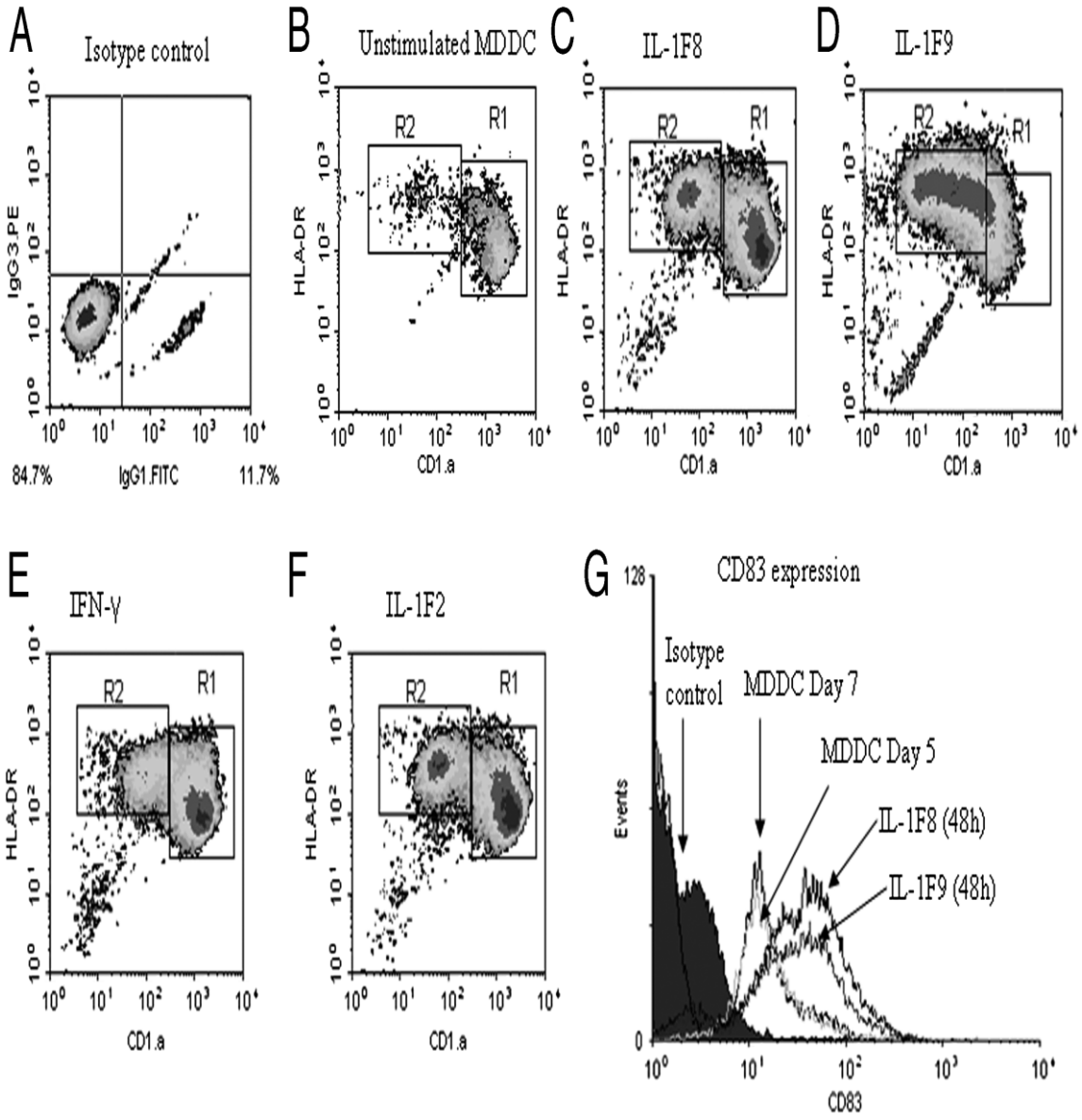


Figure 3.10 IL-1F8 or IL-1F9 induces maturation of human MDDCs. IL-1F8 or IL-1F9 induces maturation of human MDDCs. Immature MDDCs (day 5) were cultured in media alone (A and B); Media plus IL-1F8 (100 ng/mL) (C); Media plus IL-1F9 (100 ng/mL) (D); Media plus IFN-γ (100 ng/mL) (E) or Media plus IL-1F2 (100 ng/mL) (F) for 48 h and then stained with either (A) isotype control Abs or (B–F) mAbs for HLA-DR and CD1a. Boxed regions indicate the $CD1a^{high}$ $HLA\text{-}DR^{low}$ (R1) and the $CD1a^{low}$ $HLA\text{-}DR^{high}$ (R2) populations. Both R1 and R2 gates are identical in all plots and were set according to data obtained from the unstimulated MDDC population. (G) shows FACS histograms of CD83 expression by MDDCs matured by culture with IL-1F8 or IL-1F9, relative to unstimulated MDDCs and isotype controls. Data are representative of at least three independent experiments.

MDDCs that had been cultured for 7 days without IL-1 cytokines (unstimulated/negative controls) contained on average 15% of cells in the R2 population ($CD1a^{low}$/$HLA\text{-}DR^{high}$) which expressed increased HLA-DR and decreased CD1a compared with the R1 (immature) population ($CD1a^{high}$/$HLA\text{-}DR^{med}$) which on average was about 77% of the total cell population (Figure 3.10B). However, incubation with IL-1F8 clearly separated MDDCs into two distinct populations with R1 containing 61% and R2 (mature population) containing 33% of the total cell population (Figure 3.10C). When MDDCs were cultured with IL-1F9 there was a greater horizontal spread of cells between R1 and R2 and although CD1a expression was clearly reduced in a large number of these cells (~50%) there was a less clear effect on HLA-DR expression, although a clear increase in HLA-DR was measured in cells with the lowest expression of CD1a within the R2 population (Figure 3.10D). Positive controls also induced maturation with a mean 28% of cells in the R2 population following IFN-γ culture (Figure 3.10E) and a mean of 32% of cells in the R2 population following IL-1β (IL-1F2) culture (Figure 3.10F). CD83 expression was also increased following culture of immature MDDCs with IL-1F8 or IL-1F9, when compared with CD83 expression by unstimulated day 5 or day 7 MDDCs (Figure 3.10G). The numbers of cells in R2 populations were statistically analysed following incubation with IFN-γ, IL-1β or IL-F8 and compared with unstimulated controls and the results are shown in the graph below (Figure 3.11).

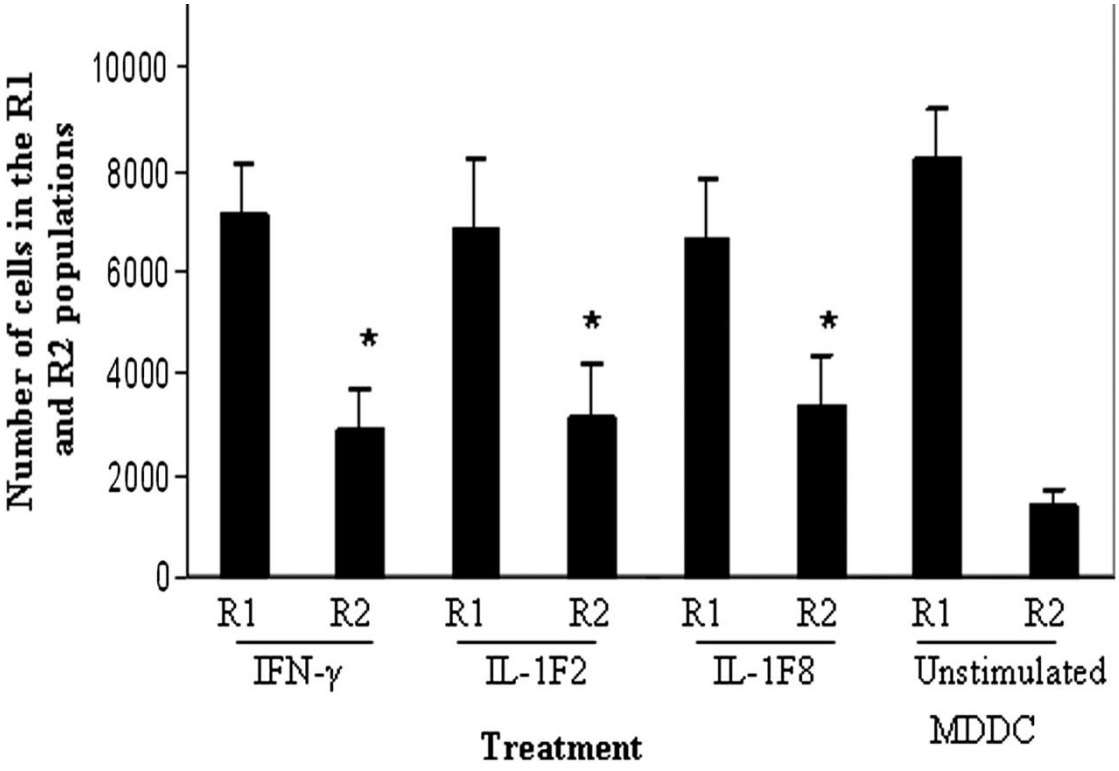


Figure 3.11 IL-1F8 stimulates a significant increase in the number of mature (CD1a^{low}HLA-DR^{high}) MDDCs. The number of cells in the R1(CD1a^{high} HLA-DR^{low} immature) and R2 (CD1a^{low}HLA-DR^{high} mature) populations generated after culture of immature MDDCs (day 5) stimulated with IFN-γ, IL-1β (IL-1F2) and IL-1F8 for 48 h as determined by flow cytometry are shown. For comparison, R1 and R2 populations are also shown for immature MDDCs (day 5) cultured for a further 48 h without cytokine (unstimulated control). *$p < 0.05$=statistical increase in the number of cells in the mature (R2) population compared with the unstimulated (control) MDDC population. Data shown are mean± SD of at least three independent experiments with similar results.

IL-1F8, IFN-γ or IL-1β significantly increased ($p < 0.05$) the size of the R2 population above that measured in unstimulated controls (Figure 3.11). However, due to the spread of cells across the R1 and R2 populations following IL-1F9 culture, we were unable to perform the same analysis for this cytokine.

3.2.7 Regulation of IL-1Rrp2 mRNA expression in MDDCs stimulated with IL-1F8 or IL-1β

In separate experiments, qRT-PCR was performed to investigate whether IL-1Rrp2 mRNA expression is upregulated in MDDCs stimulated with rhIL-1F8, rhIL-1β or *E. coli* 0127:B8 LPS. Immature day 5 MDDCs generated from peripheral blood monocytes as previously described were plated at a density of 4 x 10^5 cells/ml on 6 well plates and cultured for 48 hours with IL-1F8 (100ng/ml, 200ng/ml or 500ng/ml), IL-1β (10ng/ml or 50ng/ml), *E. coli* 0127:B8 LPS (50ng/ml)(positive control). Negative control cells were left unstimulated and were cultured for a further 48 hours in cytokine-free medium. Cells were harvested and processed for qRT-PCR to assess IL-1Rrp2 mRNA expression as previously described (Chapter 2). The qRT-PCR results obtained are shown below (Figure 3.12).

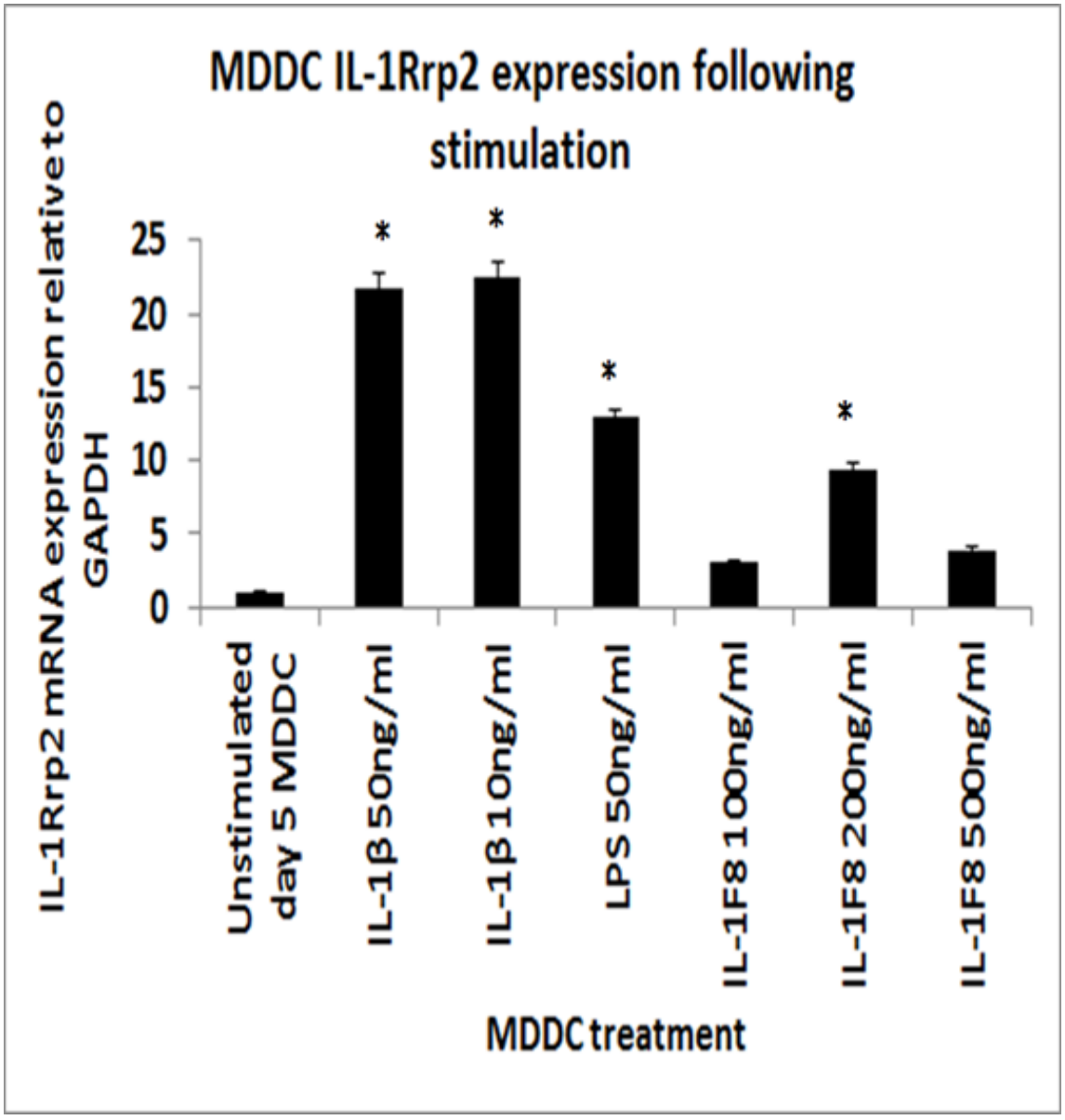


Figure 3.12 Regulation of IL-1Rrp2 mRNA expression in MDDCs stimulated with IL-1F8. Quantitative RT-PCR results showing that IL-1Rrp2 mRNA expression is significantly upregulated (*$p < 0.05$ relative to unstimulated MDDCs) in immature day 5 MDDCs stimulated with 200ng/ml IL-1F8 in a comparable manner to the up-regulation caused by *E. coli* 0127:B8 LPS or IL-1β. Lower or much higher concentrations of IL-1F8 (100ng/ml or 500ng/ml) still induce an increase in IL-1Rrp2 expression but this increase is not statistically significant. Results shown are means ± SD of three independent experiments with similar results. Each analysis was performed in triplicate.

Real time PCR results (Figure 3.12) show that there is no significant fold increase in IL-1Rrp2 mRNA expression above unstimulated day 7 MDDCs in MDDCs stimulated with 100ng/ml or 500ng/ml IL-1F8. When 200ng/ml IL-1F8 is used as a stimulant, there is a 10-fold increase in MDDCs IL-1Rrp2 expression relative to the unstimulated control and this increase is comparable to the up-regulation in IL-1Rrp2 mRNA expression caused by *E. coli* 0127:B8 LPS (about 13-fold). IL-1β (10ng/ml or 50ng/ml) induce the greatest up-regulation in IL-1Rrp2 mRNA expression (more than 20-fold) compared to unstimulated MDDCs.

3.2.8 Cytokine production by IL-1F8-stimulated MDDCs

Functionally mature DCs are known to produce IL-18 (IL-1F4) and IL-12p70 in response to relevant stimuli (reviewed in Reis e Sousa, 2006). IL-12p70 is a critical cytokine required for T_H1 polarization (reviewed in Hunter, 2005). T_H cells are essential regulators of adaptive immune responses and inflammatory diseases. They have little or no cytotoxic or phagocytic activity but are involved in activating and directing other immune cells (reviewed in Mosmann and Coffman, 1989; reviewed in Romagnani, 1994). They also play a role in the induction of autoimmune and allergic diseases (Sutton *et al.,* 2006).

Up until 2005, the production of specific cytokines by antigen-presenting cells such as mature DCs was thought to direct the differentiation of naïve T cells to specific T_H1 or T_H2 subsets and the cytokine profile within the DC-T cell

microenvironment was believed to be a critical factor in determining the phenotype of the subsequent specific response (reviewed in Mosmann and Coffman, 1989; reviewed in Romagnani, 1994). Although the cytokine environment at the time of antigen encounter is a major element influencing T_H cell differentiation, it is now apparent that T_H cell polarisation is also influenced by the antigen type, antigen affinity to the T-cell receptor (TCR), type of available costimulatory molecules and by a sophisticated network of transcription factors (reviewed in Zhu and Paul, 2010).

After activation by (TCR)-and cytokine-mediated signalling, naive $CD4^+$ T cells may differentiate into various T_H cells which influence different types of immune responses; T_H1, T_H2, T_H17, inducible T-regulatory (iTreg) cells, T_H9, T_H22 as well as other functional subsets including memory T cells (Harrington *et al.,* 2005; Sutton *et al.,* 2006; reviewed in Zhu and Paul, 2008; reviewed in Locksley, 2009; reviewed in Zhu and Paul, 2010).

Differentiated T_H cell subsets secrete different cytokines that have autocrine and paracrine functions to engage other leucocytes including macrophages, mast cells, eosinophils, neutrophils, natural killer (NK) cells, and B cells. In addition, they can express different profiles of cell-surface molecules that determine their effector cell capacity. These include adhesion molecules and chemokine receptors that determine their capacity to home to and within lymphoid or peripheral tissues where offending target antigens may reside (reviewed in Romagnani, 1994; Sutton *et al.,* 2006; reviewed in Zhu and Paul, 2008; reviewed

in Locksley, 2009; reviewed in Zhu and Paul, 2010). Not only do different human T_H cells secrete different cytokines, they also show different responsiveness to cytokines and also play different roles in host immune protection. In addition, they can also promote different immunopathological reactions (reviewed in Romagnani, 1994; Sutton *et al.,* 2006; reviewed in Zhu and Paul, 2008; reviewed in Locksley, 2009; reviewed in Zhu and Paul, 2010). The development of T_H cell subsets involves a complex cytokine network. More than one cytokine is generally required for differentiation to a particular phenotype. Furthermore, cytokines which promote differentiation to one lineage may suppress the development of other lineages (reviewed in Zhu and Paul, 2010). There is growing evidence suggesting the existence of plasticity in CD4+ T_H cell lineage differentiation (reviewed in Locksley, 2009).

T_H1 cells mediate immune responses against intracellular pathogens and are responsible for both humoral and cell-mediated immune responses. They typically secrete interferon-gamma (IFN-γ), IL-2 and tumor necrosis factor (TNF)-β, cytokines which are usually associated with inflammation. The development of T_H1 cells is favoured by the presence of IFN-γ, IFN-α, TGF-β, and IL- 12 (reviewed in Romagnani, 1994; Stobie *et al.,* 2000). IL-12 plays a crucial role in both the induction and sustenance of a primary T_H1 response *in vivo* (Stobie *et al.,* 2000), however, its absence does not automatically induce T_H2 polarisation (Jankovic *et al.,* 2002). Most DC populations are able to produce IL-12 in response to activation by various stimuli, such as infection with pathogens or exposure to their products. CD40 ligation is also considered to be a major

inducer of IL-12 production) (Cella, 1996; Stobie et al., 2000). IFN-γ and IL-4 are potent enhancers of the production of IL-12p70 (the bioactive form of IL-12) (Kaliński *et al.,* 1999; Kaliński *et al.,* 2000) while IL-10 and other factors suppress IL-12 production by DCs (Kaliński *et al.,* 1998; Kaliński *et al.,* 2000; Ebner *et al.,* 2001).

IL-12 is now understood to be a family of cytokines made up of IL-12, IL-23, IL-27 and IL-35. Each member is a heterodimeric complex consisting of two subunits whose expression is regulated independently. The biologically active form of the original IL-12 family member, IL-12 (or IL-12p70) is a covalently linked heterodimer composed of a light chain (IL-12p35) and a heavy chain (IL-12p40). On their own, the IL-12p70 subunits do not possess IL-12 bioactivity; however, studies have shown that homodimers and monomers of the p40 subunit (IL-12p40) may act as IL-12p70 antagonists. The IL-12 receptor encompasses IL-12Rβ1 and IL-12Rβ2, both of which have homology to glycoprotein (gp) 130 (Langrish *et al.,* 2005; reviewed in Hunter, 2005; Sutton *et al.,* 2006; reviewed in Gabay and McInnes, 2009).

The IL-23 heterodimer consists of the IL-12p40 component of IL-12 and the IL-23p19 component, which is homologous to IL-12p35. The receptor for IL-23 is formed by the association of IL-12Rβ1 and IL-23R. IL-27 is a heterodimeric cytokine consisting of Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28, which are related to IL-12p40 and IL-12p35, respectively. It binds a receptor

composed of gp130 and WSX1 (Langrish *et al.,* 2005; reviewed in Hunter, 2005; Sutton *et al.,* 2006). The last member of the IL-12 family, IL-35, is a heterodimer consisting of IL-12p35 and EBI3 (reviewed in Hunter, 2005; Sutton *et al.,* 2006; reviewed in Gabay and McInnes, 2009).

Through the induction of IFN gamma (IFN-γ) production, IL-12 family members play critical roles in regulating the inflammatory response. Each member plays a role in mediating T cell-dependent immunity. IL-12 and IL-27 are involved in T_H1 differentiation which is essential for host defense and tumor suppression (Langrish *et al.,* 2005). The functions of IL-35 are still to be elucidated; however, preliminary data from mouse studies suggest that it is primarily concerned with T_{reg} effector function (reviewed in Gabay and McInnes, 2009). Although IL-12 (IL-12p70) and IL-23 share a common IL-12p40 subunit, the two cytokines exert very different biological effects. While IL-12 is essential for the differentiation of naive T cells into IFN-γ–producing T_H1 cells (and hence for antimicrobial responses and tumour suppression) as already highlighted, IL-23 promotes the differentiation, survival and expansion of T_H17 CD4+ cells, a T cell population characterized by the production of IL-17, IL-17F, IL-21, IL-10, TNF, IL-6, IL-22 and other cytokines (Langrish *et al.,* 2005; Sutton *et al.,* 2006).

T_H17 cells regulate both innate and adaptive immune responses. They are also known to play an inflammatory pathological role in organ-specific autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (Langrish *et al.,* 2005). Sutton *et al.* (2006) demonstrated that IL-1α or IL-1β acts directly on

murine T cells in synergy with IL-23 to promote IL-17 secretion in the presence or absence of T cell receptor engagement. TNF-α can also synergize with IL-23 to induce IL-17, however, this is dependent on IL-1. Furthermore, in mice lacking the IL-1 type 1 receptor, IL-17 production and development of experimental autoimmune encephalomyelitis (EAE) is compromised, suggesting that IL-1 signalling is crucial for T_H17 cell differentiation (Sutton *et al.,* 2006). It has since been shown that inter-regulation between T_H17 cytokines and the novel IL-1 family members, IL-1F6, IL-1F8 and IL-1F9 (IL-36α, IL-36β and IL-36γ) is involved in driving cytokine expression in psoriatic tissues (Blumberg *et al.,* 2010; Carrier *et al.,* 2011).

IL-12 and IL-18 (also known as IL-1F4 and originally known as the interferon-inducing factor) synergistically induce interferon-γ production in T cells. IL-12 up-regulates expression of the IL-18 receptor on interferon-γ-producing T cells. Although IL-18 does not induce the development of T_H1 cells, it is vital for the effective induction and activation of T_H1 cells by IL-12 (reviewed in Okamura *et al.,* 1998; Sabatté *et al.,* 2007). Depending on the surrounding cytokines, IL-18 can induce both T_H1 and/or T_H2 responses. In addition to enhancing IL-12-driven T_H1 immune responses, IL-18 can also stimulate T_H2 immune responses in the absence of IL-12 (reviewed in Nakanishi *et al.,* 2001).

In humans, T_H2 cells provide optimum support for humoral immune responses through the production of cytokines which help B cells to proliferate and differentiate. T_H2 cells typically secrete IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-25,

amphiregulin and low to negligible amounts of IFN-γ (reviewed in Zhu and Paul, 2008). IFN-γ is thought to have a selective inhibitory effect on the proliferative response of T_H2 cells. The overriding factor in determining the likelihood for T_H2 polarization in cultured cells appears to be IL-4. Although both T_H1 and T_H2 cells proliferate in response to IL-2, T_H2 cells are much more responsive to IL-4 than T_H1 cells (reviewed in Romagnani, 1994).

To assess whether stimulating MDDCs with IL-1F8 produced functionally mature DCs, immature day 5 MDDCs were cultured (sections 2.9.0-2.9.2) at 4 x 10^5 cells/ml and stimulated for 48 hours with IL-1F8 (50ng/ml, 200ng/ml, 400 ng/ml, or 500ng/ml), IL-1β (10ng/ml) or *E. coli* 0127:B8 LPS (500 ng/ml). Control cells were cultured in cytokine-free complete RPMI 1640 medium for the same period. At the end of the culture period, supernatants were collected from the wells and IL-12 p70 and IL-18 concentrations were measured in the supernatant of stimulated or unstimulated DCs by specific ELISA methods as previously described (sections 2.9.1-2.9.2). Results are shown in Figures 3.13 and 3.14.

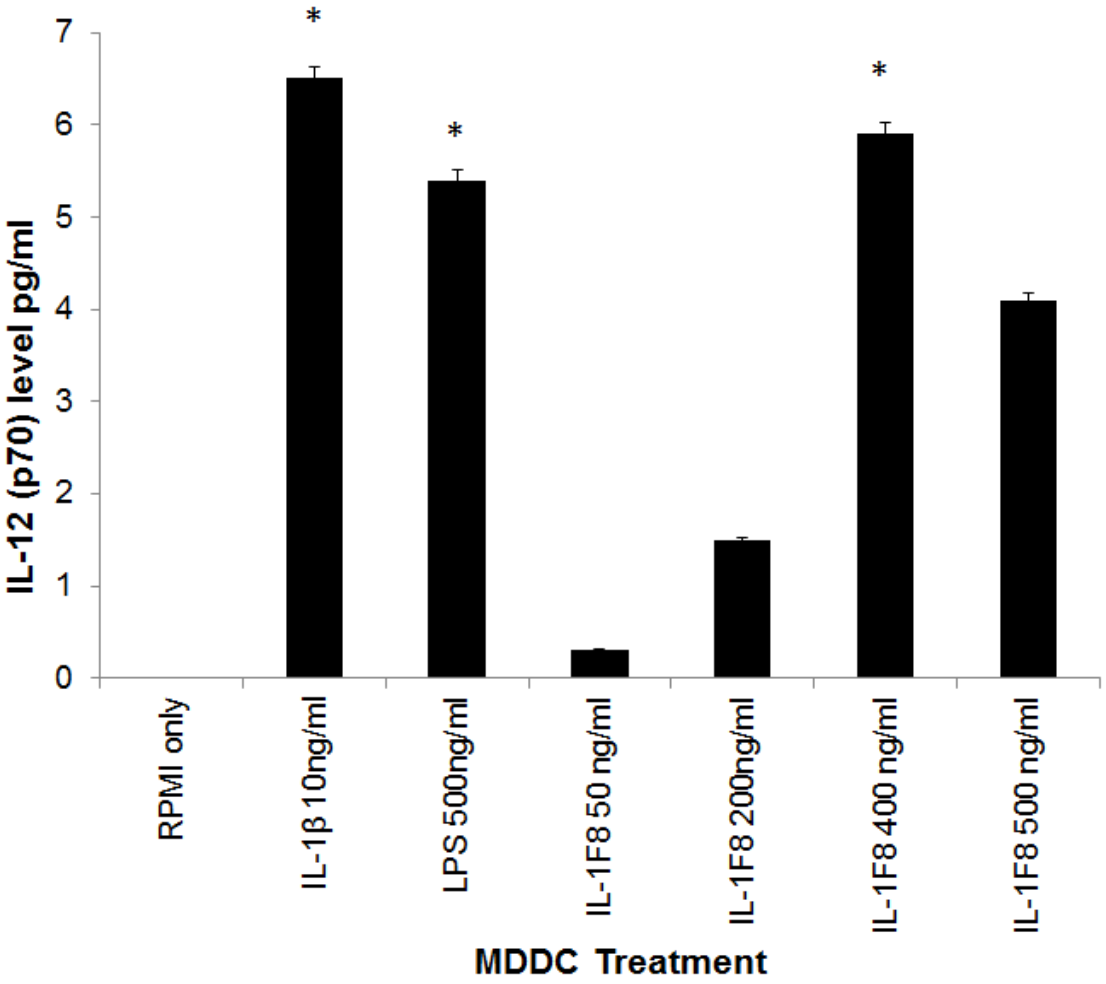


Figure 3.13 IL-1F8 induces IL-12p70 production in day 5 human MDDCs in a dose-dependent manner. An ELISA was done to quantify IL-12p70 levels in the supernatant of immature day 5 MDDCs which had been stimulated for 48 hours with IL-1F8 (50ng/ml, 200ng/ml, 400 ng/ml, or 500ng/ml), IL-1β (10ng/ml) or *E. coli* 0127:B8 LPS (500 ng/ml). Control cells were cultured in cytokine-free complete RPMI 1640 medium only. IL-1F8 induced significant IL-12p70 production (*$p < 0.05$ compared with unstimulated control) at levels higher than 200ng/ml and reached a plateau at around 400ng/ml. On the other hand, IL-1β significantly induced IL-12p70 production at a concentration as low as 10 ng/ml Data shows mean ± SD of IL-12p70 levels obtained from three independent experiments. Each analysis was performed in duplicate.

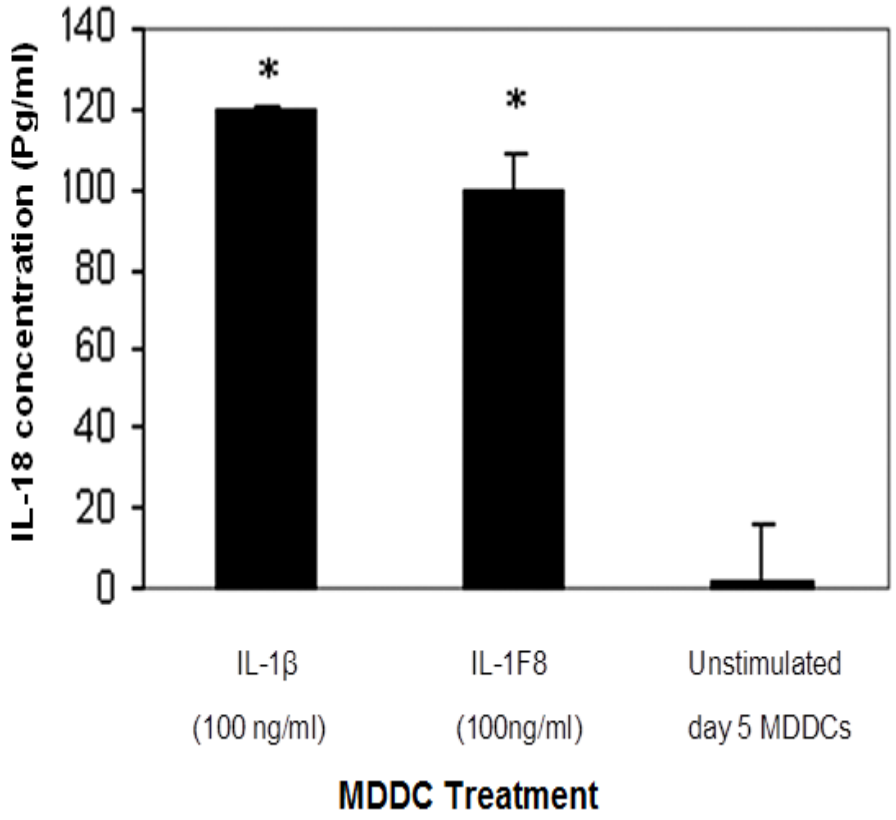


Figure 3.14 IL-1F8 stimulates IL-18 production in human MDDCs. Graph shows IL-18 ELISA levels present in supernatants following 48 hour-culture of day 5 human MDDCs with IL-1β (100ng/ml), IL-1F8 (100 ng/ml) or without cytokines. *$p < 0.05$ = Statistical increase in the concentration of IL-18 in the supernatants of MDDCs stimulated with IL-1β or IL-1F8, compared with unstimulated controls. Data shown are means ± SD obtained from at least three independent experiments performed in triplicate.

IL-1F8 induced significant IL-12p70 production at levels higher than 200ng/ml and reached a plateau at around 400ng/ml. On the other hand, IL-1β significantly induced IL-12p70 production at a much lower concentration; 10 ng/ml (Figure 3.13). Data obtained from the IL-18 (IL-1F4) ELISA analyses shows that IL-1F8 (100 ng/ml) stimulated a very significant increase ($p < 0.05$) in IL-18 production and this was comparable with the effect of IL-1β at the same concentration (Figure 3.14).

3.2.9 Activation of allogeneic T lymphocytes by IL-1F8-matured MDDCs

T-lymphocyte recognition and responsiveness to determinants expressed on genetically non-identical antigen-presenting cells can be studied *in vitro* by means of the allogeneic primary mixed lymphocyte reaction (MLR). Reactivity during a MLR indicates the T-cell proliferative response to differences in donor and recipient major histocompatibility complex (MHC) antigen expression (Bluman *et al.*, 1996).

Upon maturation with an appropriate stimulus, myeloid DCs upregulate surface markers (such as CD40, CD80, CD86) and migration markers such as CCR7, and can efficiently prime naive T cells (reviewed in Banchereau *et al.,* 2000). Dendritic cells are the principal activators of naive T cells *in vivo* (Palucka and Banchereau, 1999). Having established that IL-1F8 can induce maturation of MDDCs, as evidenced by phenotypic changes (CD1a, CD83 and HLA-DR)

(Figure 3.10), the ability of IL-1F8-matured MDDCs to induce T cell proliferation *in vitro* was studied using an allogeneic MLR.

3.2.9.1 IL-1F8-stimulated MDDCs activate T cell Proliferation

1.44×10^6 day-5-MDDCs per well were plated on 12-well cell culture plates and cultured for 48 hours in complete RPMI 1640 culture medium supplemented with one of the following: IL-1F8 (100ng/ml or 500ng/ml), IL-1β (10ng/ml or 100ng/ml), or Escherichia coli 0127:B8 LPS (10ng/ml or 100ng/ml). Control samples were left unstimulated and cultured in complete RPMI 1640 medium only. All cultures were performed in duplicate. At the end of the culture period cells were harvested and viability and maturation were assessed as previously described (Chapter 2) prior to further culture with CD3+ T cells. Prior to assessing the effect of mature MDDCs on T cell populations, the surface expression of CD40 and CD80 on IL-1F8-matured MDDCs was measured by FACS analyses using antibodies listed in Table 2.2 (Chapter 2, section 2.6.1.2) to further confirm the ability of IL-1F8 to induce MDDC maturity and the FACS results are shown below (Figures 3.15 and 3.16).

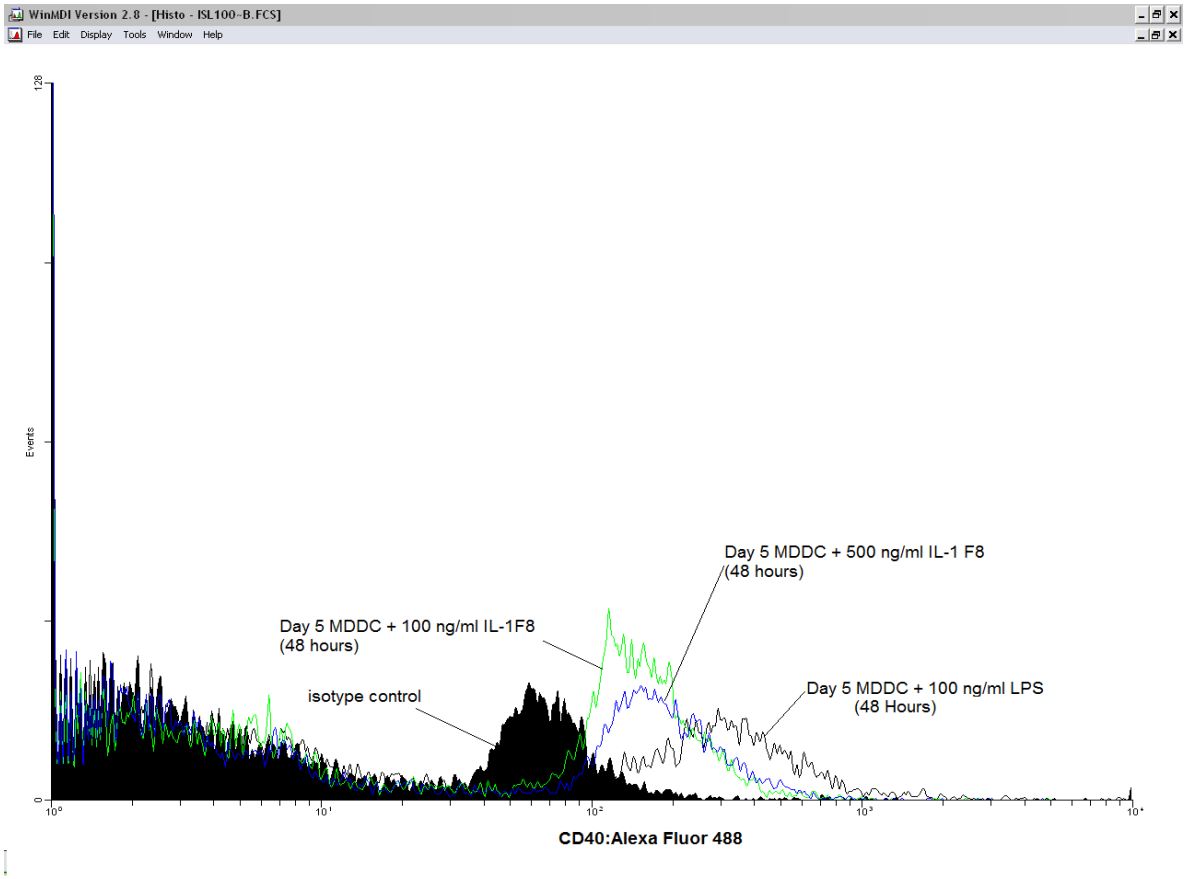


Figure 3.15 IL-1F8-stimulated-MDDCs upregulate CD40 expression. Immature (day 5) MDDCs were cultured with IL-1F8 (100 ng/ml or 500ng/ml) or with positive control (*E. coli* LPS-100 ng/ml) for 48 h prior to co-culture with allogeneic CD3+ T lymphocytes and the level of CD40 on the MDDC surface was determined by flow cytometry. Results show that IL-1F8-matured MDDCs upregulate CD40 expression in a comparable manner to LPS, an established inducer of MDDC maturation. Data are representative of three independent experiments.

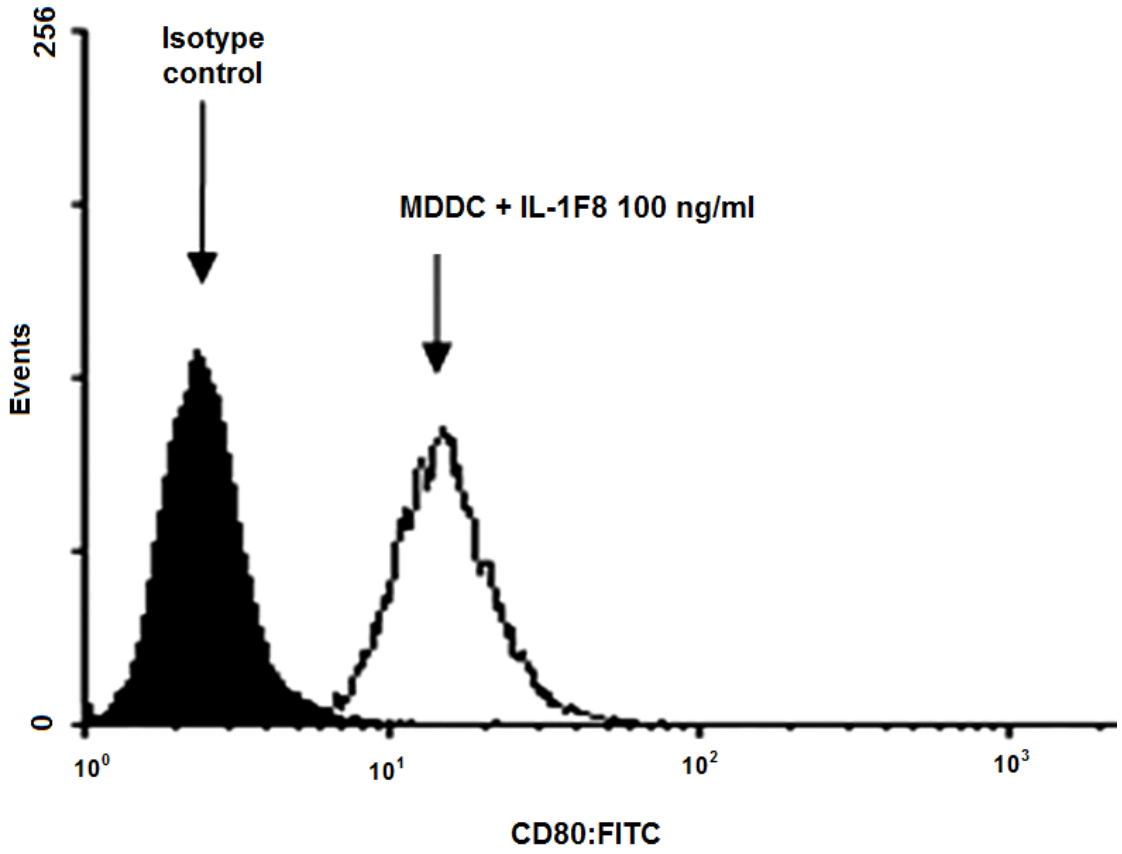


Figure 3.16 IL-1F8-stimulated-MDDCs upregulate CD80 expression.

Immature (day 5) MDDCs were cultured with IL-1F8 (100 ng/mL) for 48 h prior to co-culture with allogeneic CD3+ T lymphocytes and the level of CD80 on the surface was determined by flow cytometry. Data are representative of three independent experiments.

Results showed that IL-1F8 increased CD40 (Figure 3.15) and CD80 (Figure 3.16) expression above the level expressed by isotype controls. After confirming MDDC maturation, the WST-1 cell proliferation assay (Roche Diagnostics Ltd., West Sussex, UK) was performed to assess the effect of mature MDDCs on T cell proliferation. Briefly, mature MDDCs (or unstimulated controls) were co-cultured for 96 hours with allogeneic CD3+ T cells at a stimulator cell (MDDCs) to responder cell (CD3+ T cells) ratio of 1:10 per well as already described (Chapter 2, section 2.8.1). As a positive control, CD3+ T cells were also cultured in medium containing Concanavalin A (Con A) (75 µg/ml) and as a negative control CD3+ T cells were cultured for the same period in medium containing no additional supplements. All cultures were performed in triplicate and repeated on three separate occasions.

After 96 hours, supernatants from the co-cultures were harvested and frozen for cytokine analysis as previously described (Chapter 2, sections 2.8.1 and 2.9.0). The WST-1 cell proliferation assay was then used to measure T cell proliferation in the co-cultures as previously described (Chapter 2, section 2.8.1). Briefly, the measured absorbance of each co-culture (measured at 450nm against a background/blank control) directly correlated to the number of viable cells in each well. The mean absorbance of each co-culture was divided by the mean absorbance of the unstimulated negative control and expressed as a proliferation ratio. The results obtained are summarised in the graph below (Figure 3.17).

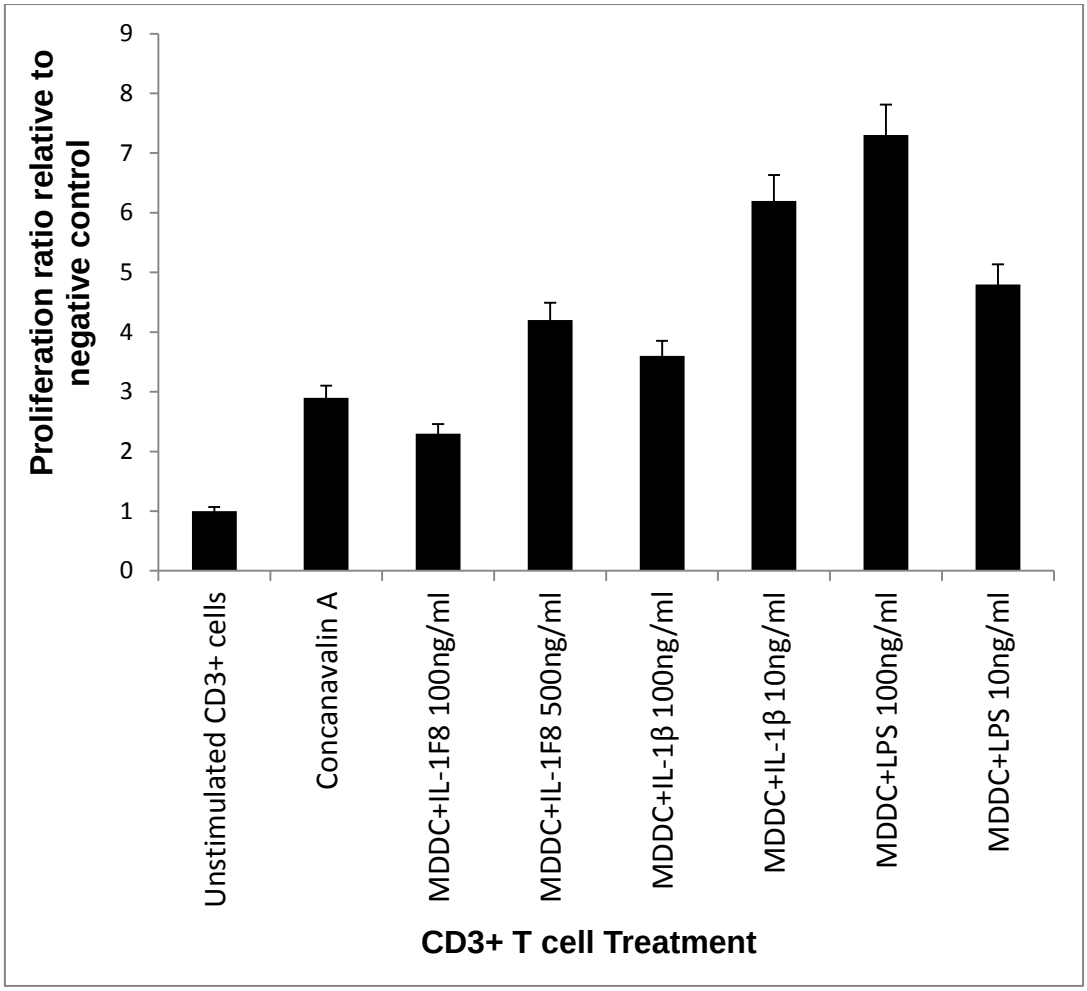


Figure 3.17 IL-1F8-stimulated MDDCs induce T cell Proliferation. CD3+ lymphocytes were cultured for 96 h either alone (unstimulated control cells), in the presence of Concanavalin A (Con A) or with allogeneic MDDCs that had been matured for 48 hours with IL-1F8 (100 or 500 ng/mL), IL-1β (10 or 100 ng/ml) or *E. coli* 0127:B8 LPS (10 or 100 ng/ml). T cell proliferation was determined using the Roche Cell Proliferation Assay (Cell Proliferation Reagent WST-1) and is shown relative to the unstimulated CD3+ T cells. Results show that IL-1F8-stimulated MDDCs activate T cell proliferation in a comparable way to IL-1β- or LPS-stimulated MDDCs. Data shown are means ± SD obtained from three independent experiments. Each experiment was performed in triplicate.

Results showed that MDDCs that had been matured with 100 ng/mL IL-1F8 induced a two-fold expansion of the CD3+ T-cell population; however, this was below that observed for Con A-stimulated (positive control) cells (Figure 3.17). On the other hand, MDDCs that had been matured using 500 ng/mL IL-1F8 induced >4-fold expansion of the T-lymphocyte population. This increase in T cell proliferation was greater than that measured in Con A-stimulated cultures and was comparable to that in IL-1β- or LPS-stimulated cultures. Taken together, these results show that IL-1F8-stimulated MDDCs activate T cell proliferation in a comparable way to IL-1β- or LPS-stimulated MDDCs.

3.2.9.2 Induction of interferon gamma (IFN-γ) production by MDDC/CD3+ T cell cultures

Supernatants from the MDDC/T cell and Con A/T cell cultures described in section 3.2.9.1 were harvested to measure IFN-γ and IL-10 concentration. IFN-γ ELISA was performed using the BD OptEIA™ Human IFN-γ ELISA Kit II (BD Biosciences, Oxford, UK) as per manufacturer's instructions as previously described (Chapter 2, section 2.9.0). The graph below (Figure 3.18) shows IFN-γ levels in the different co-cultures.

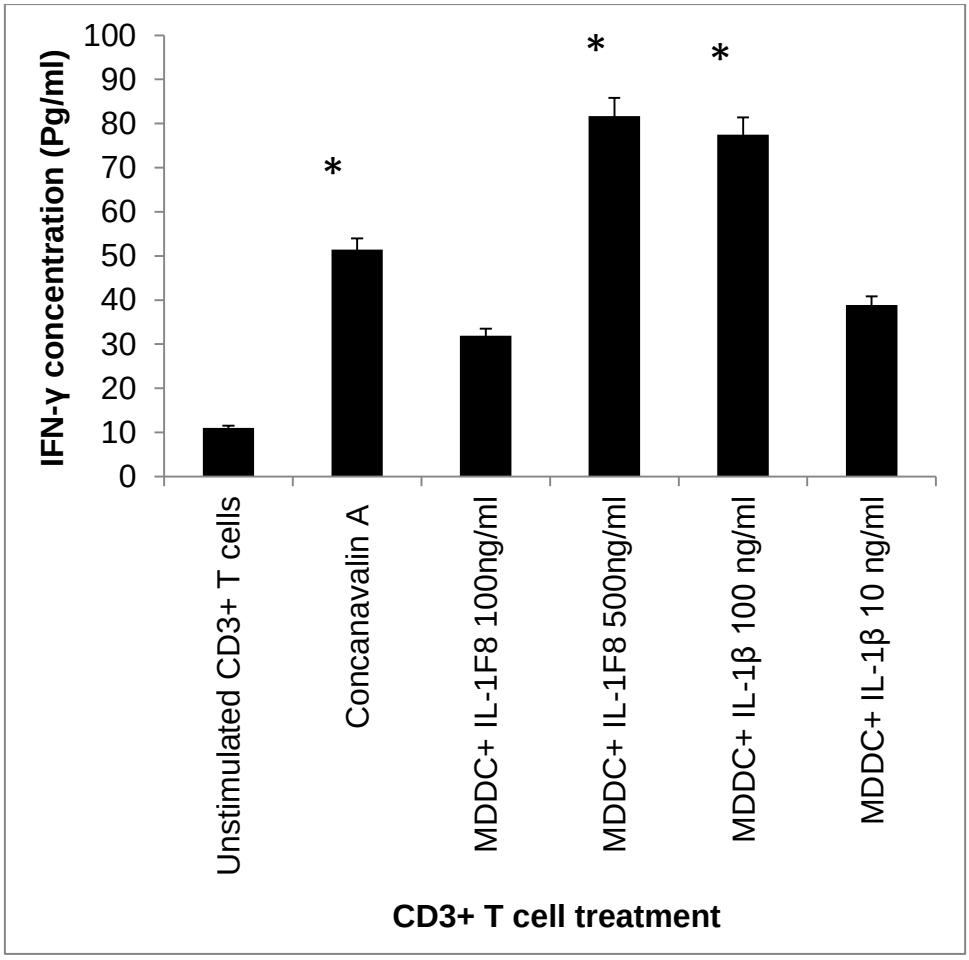


Figure 3.18 IL-1F8-matured MDDCs induce proliferation of allogeneic T_H1 lymphocytes. CD3+ T lymphocytes were cultured for 96 h alone (unstimulated cells), with Con A, or with allogeneic MDDCs that had been matured for 48 hours with IL-1β (10 or 100 ng/mL) or with IL-1F8 (100 or 500 ng/mL) prior to determining IFN-γ concentration in the supernatants by ELISA. *$p < 0.05$ = Statistical increase in the concentration of IFN-γ in the supernatants of T lymphocytes co-cultured with Con A or with MDDCs matured with IL-1β or IL-1F8, compared with unstimulated controls. Data shown are means ± SD obtained from three independent experiments performed in triplicate

Results (Figure 3.18) showed that compared with unstimulated CD3+ T cells, MDDCs matured with IL-1β (100 ng/mL) induced significant ($p < 0.05$) IFN-γ production when cultured with CD3+ T cells. On the other hand, maturation of MDDCs with 100 ng/mL IL-1F8 did not stimulate significant IFN-γ production in allogeneic MDDC/T cell cultures, however, a significant increase ($p < 0.05$) in IFN-γ production was measured in supernatants in which MDDCs had been matured using 500 ng/mL IL-1F8 prior to co-culture with T lymphocytes. The effect, on IFN-γ production in mixed MDDC/T cell cultures, of 500ng/ml IL-1F8 was comparable to that induced by 100 ng/ml IL-1β and higher than that due to Con A (75 µg/ml).

3.2.9.3 IL-10 production by MDDC/ CD3+ T cell co-cultures

IL-10 levels in supernatants from the MDDC/T cell and Con A/T cell cultures described in section 3.2.9.1 were measured using the BD OptEIA™ Human IL-10 ELISA Kit II (BD Biosciences, Oxford, UK) according to the manufacturer's instructions as previously described (Chapter 2, section 2.9.5). Results are summarised in the graph below (Figure 3.19).

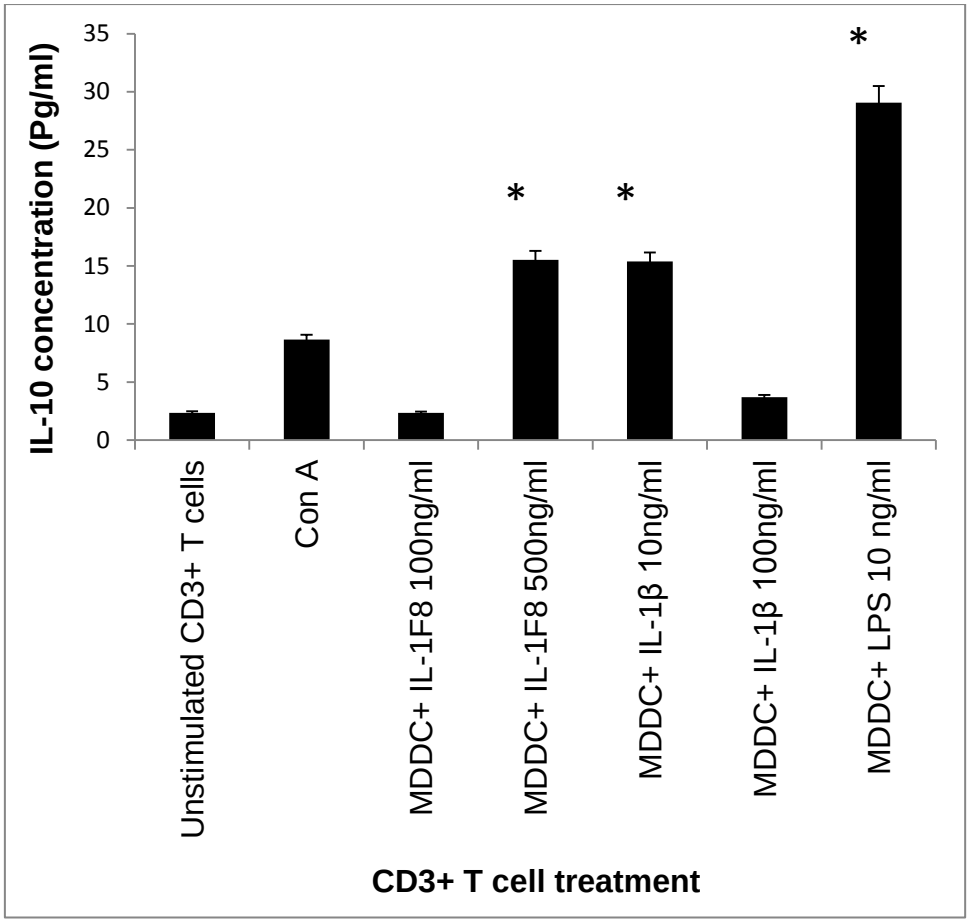


Figure 3.19 IL-10 levels in supernatant of MDDC/T cell co-cultures. CD3+ T lymphocytes were cultured for 96 h alone (unstimulated control cells), with Con A (75 µg/ml), or with allogeneic MDDCs that had been matured for 48 hours with IL-1β (10 or 100 ng/mL), IL-1F8 (100 or 500 ng/mL) or *E. coli* 0127:B8 LPS (10 ng/ml) prior to determining IL-10 concentration in the supernatants by ELISA. *$p < 0.05$ = Statistical increase in the concentration of IL-10 in the supernatants of T lymphocytes co-cultured with Con A or with MDDCs matured with IL-1β, IL-1F8 or LPS, compared with unstimulated controls. Data shown are means ± SD obtained from three independent experiments. Each analysis was performed in triplicate.

Although IL-10 concentration in MDDC/T lymphocyte cultures was generally much lower than IFN-γ concentration, similar trends were observed in that maturation of MDDCs with 100 ng/mL IL-1F8 did not stimulate significant IL-10 production in allogeneic MDDC/T cell cultures where as a significant increase ($p < 0.05$) in IL-10 production was measured in supernatants in which MDDCs had been matured using 500 ng/mL IL-1F8 prior to co-culture with T lymphocytes (Figure 3.19). Furthermore, the effect of 500 ng/ml IL-1F8 on IL-10 production by the MDDC/T cell cultures was also comparable to that induced by 100 ng/ml IL-1β and higher than that induced by a well-established inducer of T cell proliferation, Con A. On the other hand, a much lower concentration of *E. coli* 0127:B8 LPS (10 ng/ml) induced the highest IL-10 production relative to the negative control in MDDC/T cell cultures.

3.3 IL-1Rrp2 expression by blood DC Subsets

Peripheral blood DCs comprise a heterogeneous population of cells that is divided into two main subsets, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), with distinct and complementary roles in the induction of immune responses. Myeloid DCs are further sub-divided into Type 1 myeloid DCs (mDC1) and Type 2 myeloid DCs (mDC2) (Dzionek *et al.,* 2000). The diversity of DC subsets and lineages is thought to be responsible for the diverse functions of DCs in immune regulation. Different DC subsets are thought to recognize and respond to different pathogens and to differ in terms of cytokine production profile and capacity to direct T_H Cell differentiation (reviewed in Liu, 2001). An

imbalance in DC subset levels is thought to play a role in diseases such as primary Sjögren's syndrome (Vogelsang *et al.,* 2010).

3.3.1 Human plasmacytoid DCs (pDCs) but not myeloid DCs (mDCs) constitutively express IL-1Rrp2

Having established that human MDDCs expressed both IL-1Rrp2 mRNA and IL-1Rrp2 protein, the next step was to investigate whether human peripheral blood DC subsets express IL-1Rrp2. Human buffy coat products were purchased from Sheffield National Blood Services and mDC1, mDC2 and pDCs were isolated using magnetic microbead kits purchased from Miltenyi Biotec Ltd (Surrey, UK) as previously described. Quantitative RT-PCR for IL-1Rrp2 mRNA was performed on the DC peripheral blood subsets and results obtained are shown in the graph below (Figure 3.20).

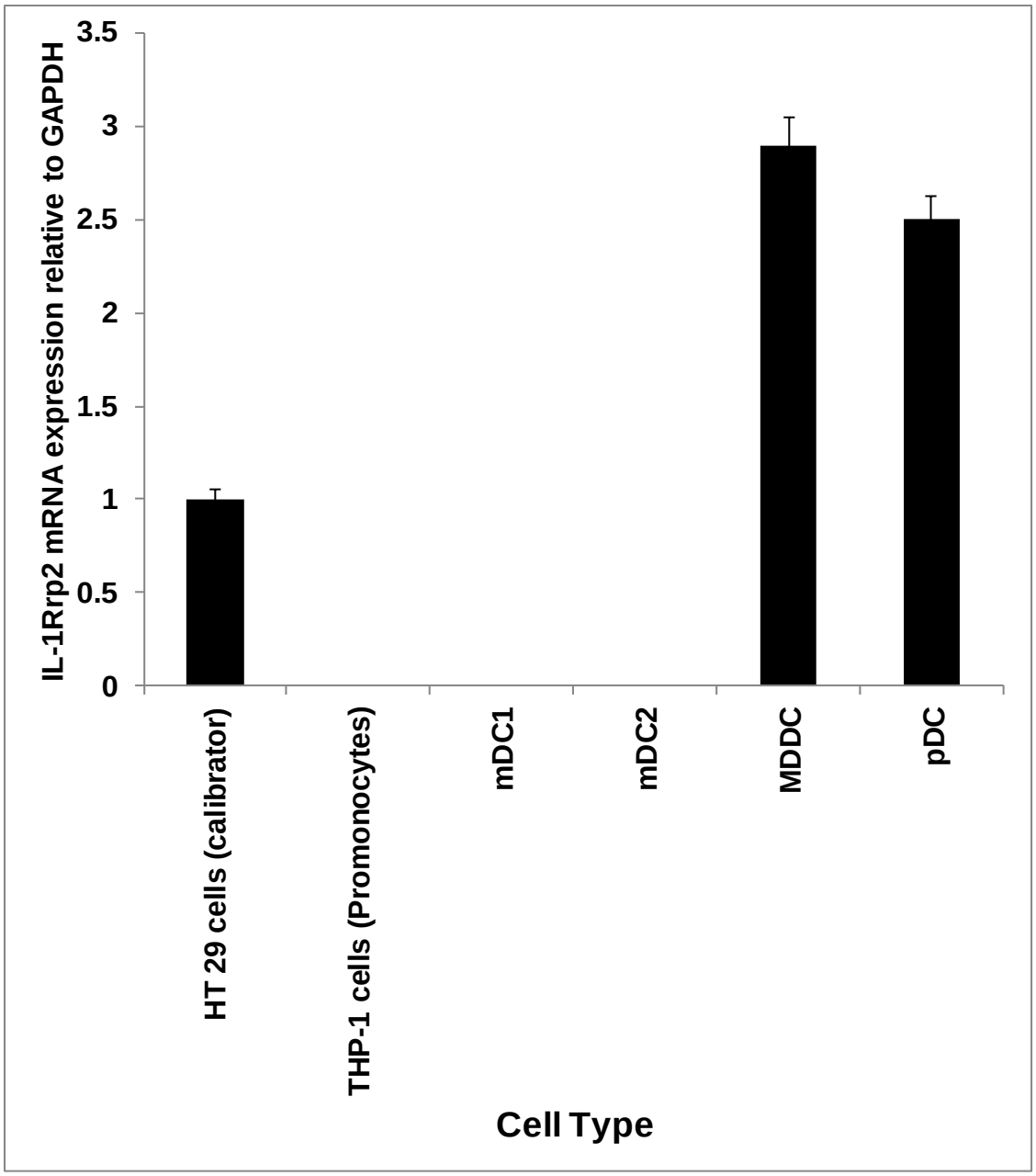


Figure 3.20 Human plasmacytoid DCs (pDCs) but not myeloid DCs (mDCs) constitutively express IL-1Rrp2. Quantitative RT-PCR was used to determine the level of IL-1Rrp2 mRNA expression in promonocytic THP-1 cells, mDC1, mDC2, MDDCs and pDCs isolated from human blood as compared with levels in calibrator HT29 cells. Data shown are means ± SD of at least three independent experiments. Each analysis was performed in triplicate.

Our results (Figure 3.20) showed that neither mDC1 nor mDC2 cells expressed IL-1Rrp2 mRNA at levels above those measured for non-expressing cells (promonocytic THP-1 cells) or the calibrator (HT 29 cells). On the other hand, pDCs expressed IL-1Rrp2 mRNA at levels comparable to those observed in MDDCs which had been generated using 50ng/ml GM-CSF and 10ng/ml IL-4.

3.3.2 Relative expression of IL-1Rrp2 mRNA in human pDCs stimulated with IL-1F8 or IL-1β

Quantitative RT-PCR was performed to investigate whether IL-1Rrp2 mRNA expression is regulated in pDCs stimulated with rhIL-1F8, rhIL-1β, *E. coli* 0127:B8 LPS or imiquimod. Plasmacytoid DCs isolated from peripheral blood using the Diamond Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec Ltd., Bisley, UK) as previously described (Chapter 2, section 2.3.6.4) were plated at a density of 4×10^4 cells/well on 6 well plates and cultured for 24 hours in complete medium supplemented with IL-1F8 (100ng/ml), IL-1β (100ng/ml), *E. coli* 0127:B8 LPS (100ng/ml) or imiquimod (50µg/ml). Negative control cells were left unstimulated and were cultured for 24 hours in cytokine-free medium. Cells were harvested and processed for qRT-PCR to assess IL-1Rrp2 mRNA expression as previously described (Chapter 2, section 2.7.0) and the results are shown below.

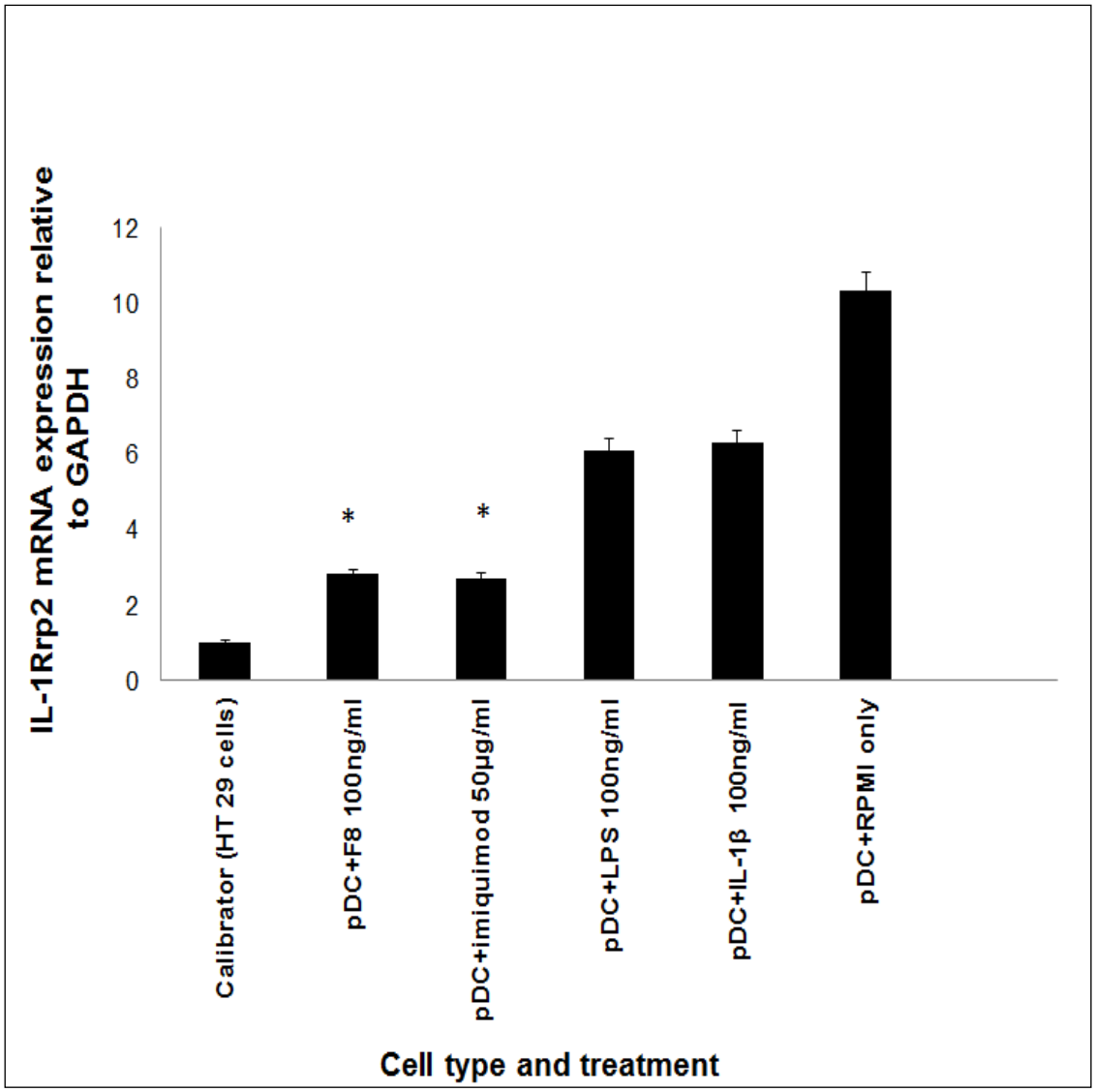


Figure 3.21 Regulation of IL-1Rrp2 mRNA expression in human pDCs stimulated with IL-1F8. Figure shows that qRT-PCR IL-1Rrp2 mRNA expression is significantly down-regulated (*$p < 0.05$ relative to unstimulated pDCs) in pDCs stimulated with 100ng/ml IL-1F8 or 50µg/ml imiquimod (positive control). There is no significant down-regulation of IL-1Rrp2 expression (compared with unstimulated controls) in pDCs stimulated with either IL-1β (100ng/ml) or *E. coli* 0127:B8 LPS (100ng/ml). Results shown are means ± SD of three independent experiments. Each analysis was performed in triplicate.

Unstimulated pDCs (cultured in complete RPMI 1640 medium only) showed higher IL-1Rrp2 mRNA levels than stimulated pDCs, suggesting a down-regulation of IL-1Rrp2 in stimulated pDCs (Figure 3.21). Stimulating pDCs with either IL-1F8 (100ng/ml) or imiquimod (50µg/ml) resulted in a significant down-regulation in IL-1Rrp2 mRNA levels compared with unstimulated controls. Although stimulating pDCs with either IL-1β (100ng/ml) or 100ng/ml *E. coli* 0127:B8 LPS resulted in a down-regulation of IL-1Rrp2 mRNA synthesis, this was not statistically significant.

3.3.3 Measurement of IFN-α production in human pDCs stimulated with IL-1F8 or IL-1β

Plasmacytoid DCs (pDCs) are the principal IFN -producing cells in the blood and are capable of producing vast amounts of IFN-α in response to various stimuli (Cella *et al.,* 1999; Siegal *et al.,* 1999; Kadowaki *et al.,* 2000; Foster *et al.,* 2000; Gibson *et al.,* 2002). Imiquimod, a Toll-like Receptor 7 (TLR7) agonist and immune response modifier, induces INF-α secretion in human pDCs (Gibson *et al.,* 2002). IFN-α has immunoregulatory activities and may be important in linking innate and adaptive immunity (reviewed in Brassard *et al.,* 2002). It inhibits viral replication and can also act to stimulate other immune competent cells (McKenna, Beignon and Bhardwaj, 2005; Colonna, Trinchieri and Liu, 2004).

To assess whether or not pDCs used in experiments were fully functional, their ability to secrete IFN-α in response to various stimuli was examined.

Plasmacytoid DCs isolated from peripheral blood using the Diamond Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec Ltd., Bisley, UK) as previously described (Chapter 2, section 2.3.6.4) were plated at a density of 1.16 x 10^5 cells/well on 6 well plates and cultured for 48 hours in complete RPMI 1640 medium supplemented with IL-1F8 (100 ng/mL), IL-1β (100 ng/mL) or imiquimod (50 µg/mL; positive control). Unstimulated pDCs were cultured for the same period without stimulants and were used as negative controls. At the end of the culture period, supernatants were collected from the wells and the IFN-α concentration was measured in the supernatant of stimulated or unstimulated pDCs using a sandwich ELISA immunoassay (*VeriKine*^{TM} Human IFN-α ELISA Kit, supplied by R & D Systems, Abington, UK) performed according to the manufacturers' instructions (Chapter 2, section 2.9.3). The results are shown in the graph below (Figure 3.22).

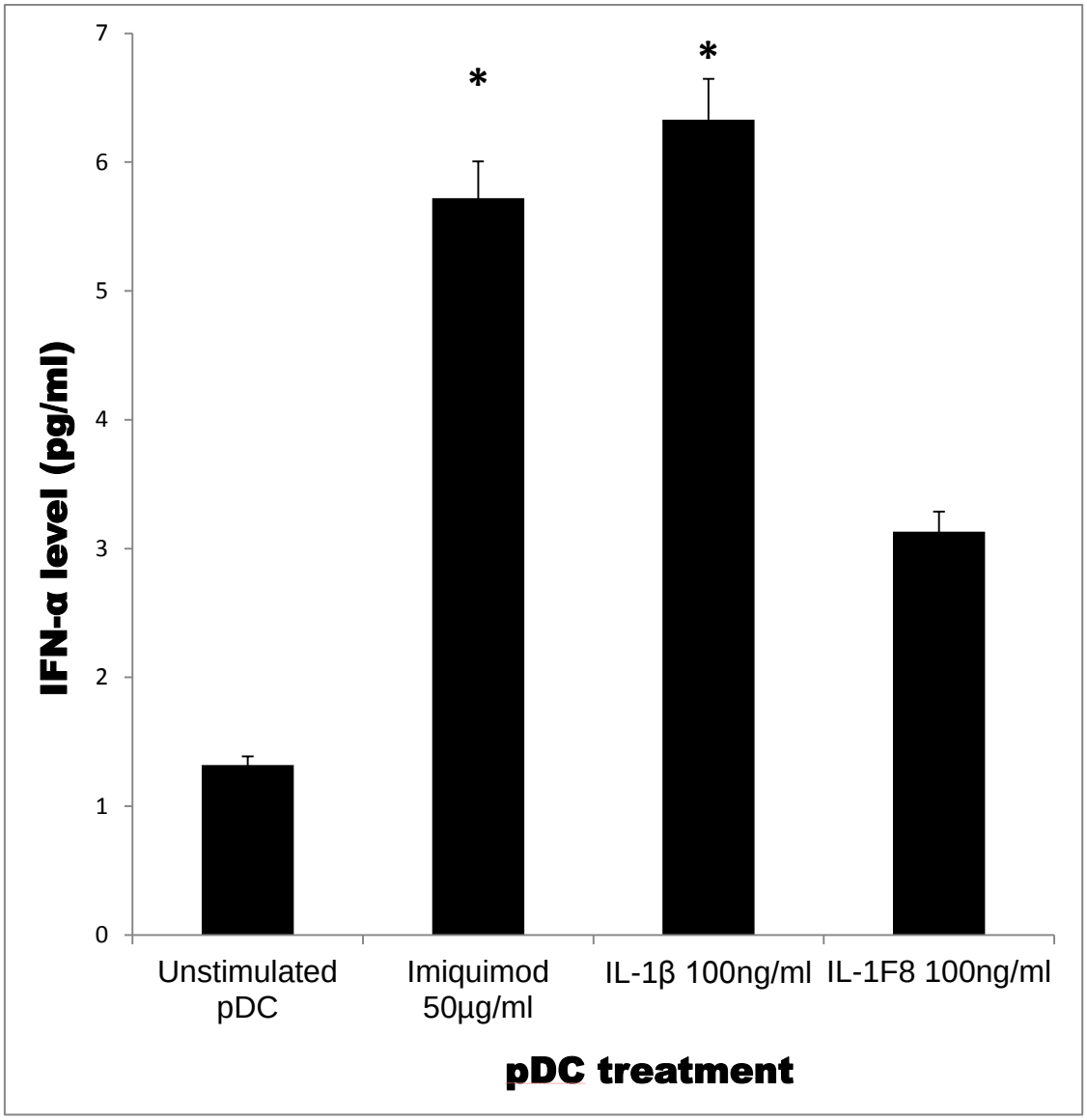


Figure 3.22 IL-1F8-stimulated plasmacytoid DCs (pDCs) induce IFN-α synthesis. Graph shows ELISA analysis of IFN-α concentrations in the culture supernatants of pDCs stimulated with IL-1F8 (100 ng/mL), IL-1β (100 ng/mL), imiquimod (50 µg/mL) or unstimulated controls. *$p < 0.05$ = Statistical increase in the concentration of IFN-α in the supernatants of pDCs stimulated with IL-1β or imiquimod, compared with unstimulated controls. Data shown are means ± SD obtained from five independent experiments performed in triplicate.

Results (Figure 3.22) show that isolated pDCs used in the study were fully functional as they produced significant IFN-α levels in response to imiquimod compared to unstimulated cells. IL-1β (100 ng/ml) induced secretion of IFN-α levels that were comparable to those induced by imiquimod. On the other hand, IL-1F8 (100ng/ml) induced IFN-α secretion but the level of IFN-α was not significantly higher than that in the supernatant isolated from unstimulated controls.

3.3.4 IL-1F8-stimulated pDCs are weak inducers of T cell proliferation

To assess the effect of IL-1F8-matured pDCs on T cell proliferation, pDCs isolated from peripheral blood using the Diamond Plasmacytoid Dendritic Cell Isolation Kit as previously described were stimulated for 24 hours with one of the following: IL-1F8 (500 ng/mL), *E. coli* 0127:B8 LPS (100 ng/ml) or imiquimod (50 µg/ml) as described previously (Chapter 2, section 2.8.2). The WST-1 cell proliferation assay was then used to measure T cell proliferation induced by the stimulated pDCs. Briefly, mature pDCs (or unstimulated controls) were co-cultured for 48 hours with allogeneic CD3+ T cells at a stimulator cell (pDCs) to responder cell (CD3+ T cells) ratio of 1:10 per well as already described (Chapter 2, section 2.8.2). As a positive control, CD3+ T cells were also cultured in medium containing Concanavalin A (Con A) (75 µg/ml) and as a negative control CD3+ T cells were cultured for the same period in medium containing no additional supplements. All cultures were performed in triplicate and repeated on three separate occasions.

After 48 hours, T cell proliferation in the co-cultures was assessed using the WST-1 cell proliferation assay as previously described (Chapter 2, section 2.8.2). Briefly, the measured absorbance of each co-culture (measured at 450nm against a background/blank control) directly correlated to the number of viable cells in each well. The mean absorbance of each co-culture was divided by the mean absorbance of the unstimulated negative control and expressed as a proliferation ratio. The results obtained are summarised in the graph below (Figure 3.23).

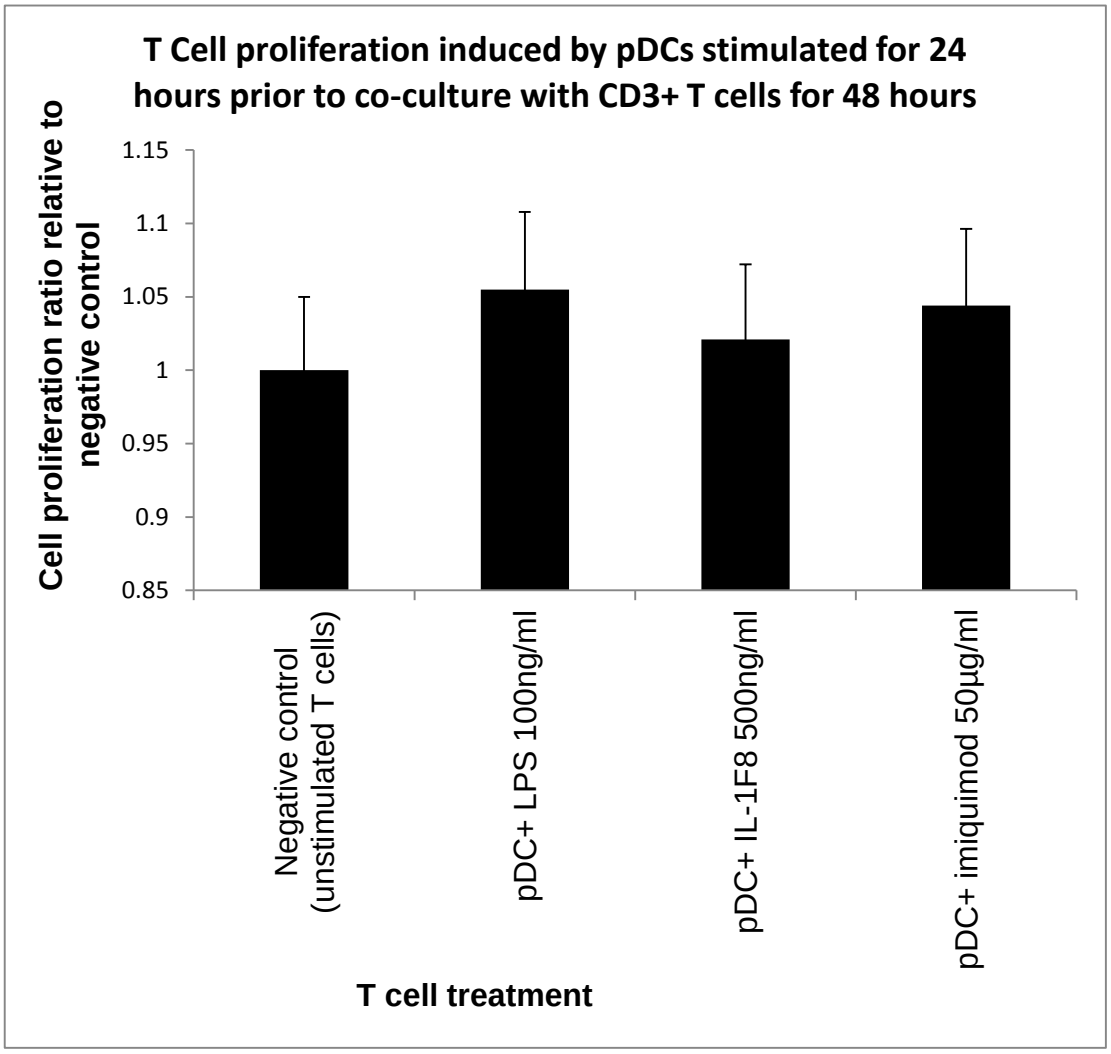


Figure 3.23 IL-1F8-matured pDCs are weak inducers of T cell proliferation. pDCs were stimulated for 24 hours with IL-1F8 (500 ng/mL), *E. coli* 0127:B8 LPS (100 ng/mL) or imiquimod (50 µg/mL) and then cultured for 48 hours with CD3+ T cells. Graph shows that pDCs matured with IL-1F8, LPS or imiquimod did not induce significant cell proliferation above the negative control (unstimulated T cells). Data shown are means ± SD obtained from five independent experiments performed in triplicate.

Results obtained showed that IL-1F8-matured pDCs do not induce significant T cell proliferation relative to unstimulated CD3+ T cells. Plasmacytoid DCs matured with IL-1F8, LPS or imiquimod did not induce significant cell proliferation above the negative control (unstimulated T cells) (Figure 3.23).

3.4 Discussion

Results presented in this chapter show for the first time that within the human myelomonocytic lineage, constitutive expression of IL-1Rrp2 is unique to DCs, however, IL-1Rrp2 expression differs between monocyte-derived and blood isolated DC subsets (Mutamba *et al.,* 2012). RT-PCR results show that monocyte derived DCs (MDDCs) express IL-1Rrp2 mRNA. Flow cytometry and fluorescent microscopy results further confirm that MDDCs express IL-1Rrp2 protein on the cell surface. THP-1 cells (a promonocytic cell line) (Tsuchiya *et al.,* 1980) or peripheral blood monocytes (PBMs) did not express IL-1Rrp2 mRNA. Magne *et al.* (2006) also did not observe IL-1Rrp2 expression in THP-1 cell lines, while work by Towne *et al.* (2004) has suggested that only Colo205, SW48, HT29, HBT75, HaCAT and NCI/ADR-RES cell lines express IL-1Rrp2.

Our results from qRT-PCR analyses also showed that IL-1Rrp2 was not expressed by peripheral blood monocytes, isolated from PBMC using standard density gradient techniques (Mutamba *et al.,* 2012). On the other hand, Debets *et al.* (2001) observed IL-1Rrp2 (then known as IL-1R6) mRNA expression in monocytes isolated from Phytohaemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC). Since Debets *et al.* were working with activated PBMC; it is possible that the IL-1Rrp2 expression that they attributed to monocytes was actually due to immature pre-DCs which were differentiating from the activated monocytes. The fact that they did not observe IL-1Rrp2 expression in unstimulated PBMC (Debets *et al.,* 2001) further supports this

point. PHA is known to induce T cell proliferation in the presence of monocytes (Ceuppens *et al.,* 1988) and in turn, activated T cells induce differentiation of monocytes to DCs (Wirths *et al.,* 2002).

In our study, monocyte-derived macrophages (MDMs) only expressed IL-1Rrp2 mRNA on addition of IL-4 to the culture media. However, IL-1Rrp2 expression in these IL-4-stimulated MDM cultures was associated with a simultaneous increase in the numbers of MDDCs. IL-4 is known to convert macrophages to DCs (Sallusto and Lanzavecchia, 1994). IL-4 also caused a dose-dependent increase in IL-1Rrp2 expression in MDDC cultures but this was also associated with increased MDDC differentiation, evidenced by characteristic dendritic morphology and detachment from plastic culture flasks. However, the effect of IL-4 on IL-1Rrp2 expression by individual MDDCs was not investigated. There is also a possibility that IL-4 may be a trigger for the expression of IL-1Rrp2 by MDDCs. Previous studies have shown the importance of IL-4 in DC biology. For example, IL-4 is known to be the main driving force behind MDDC differentiation (Roy *et al.,* 2004) and is also known to induce expression of DC specific intercellular adhesion molecule grabbing non-integrin (DC-SIGN) (Relloso *et al.,* 2002; Li *et al.,* 2006).

The finding, in the present study, that IL-1F8 significantly increased IL-1Rrp2 mRNA expression above unstimulated controls, is consistent with previous findings regarding the relationship between IL-1 cytokines and their receptors. For example, IL-1β, a classical IL-1 member, stimulates upregulation of the

synthesis of IL-1R1 (Zhang *et al.,* 2007; Docagne *et al.,* 2005). Furthermore, IL-1F6, one of the novel IL-1 family members, has been shown to upregulate IL-1Rrp2 expression in mouse keratinocytes and in human psoriatic skin lesions (Blumberg *et al.,* 2007). A correlation between IL-1Rrp2 expression and cellular responsiveness to IL-1F8 has also been reported by other researchers (Towne *et al.,* 2004; Magne *et al.,* 2006). Regulation of receptor expression must be important in host defence responses to disease and injury. Upregulation of IL-1Rrp2 expression on cells is likely to make the cells more sensitive and more responsive to the corresponding novel IL-1 cytokines thereby further enhancing any subsequent effects. However, this kind of response may not always be beneficial to the host.

Human peripheral blood is thought to contain two major DC populations, $CD11c^+$ myeloid DC (mDC) and $CD123^+$ plasmacytoid DC (pDC) (Robinson *et al.,* 1999). Both populations are immature precursor DC derived from bone marrow stem cells that are on migration to their target sites. Myeloid DCs are further subdivided into CD1c+ myeloid DCs (type 1 myeloid DCs or mDC1) and CD141+ myeloid DCs (type 2 myeloid DCs or mDC2) (Dzionek *et al.,* 2000). Myeloid DC preferentially home to non-lymphoid tissues, where they specialise in uptake of invading pathogens. They rapidly secrete proinflammatory cytokines following engagement of Toll-like receptor 2 (TLR2) and TLR4 (Liu, 2001). On the other hand, pDC are preferentially located in the T cell areas of lymphoid tissues and express TLR-7 and TLR-9. They produce large amounts of interferon (IFN)-α following TLR ligation. Myeloid DCs are thought to preferentially evoke T cell

responses against invading pathogens while pDCs are the principal IFN producing cells in the blood in response to viral infections (Gibson *et al.,* 2002) and are thought to serve as a link to innate immunity and to help in the maintenance of tolerance against self-antigens (Liu, 2001; Shortman and Liu, 2002; Palucka and Banchereau, 2002; Penna *et al*., 2002; Liu, 2005).

Dendritic cells generated *in vitro* from peripheral blood monocytes (MDDCs), are the best-studied human DCs as they are much more readily available than DCs isolated *ex vivo.* The current study adds to previous reported differences between mDCs and MDDCs as we show that IL-1Rrp2 was not expressed by either mDC1 or mDC2 subsets while immature MDDCs showed significant IL-1Rrp2 expression. Unlike mDC1 cells, mDC2 cells lack expression of CD1a and do not produce IL-12 (Chang *et al.,* 2000). In this respect, MDDCs are more closely related to mDC1 (Herbst *et al.,* 1998; Grassi *et al.,* 1998). Other researchers have reported phenotypic and functional differences between the MDDCs and mDCs. Horlock *et al.* (2007) reported that immature MDDCs have a more mature phenotype than unstimulated mDCs with significantly greater expression of CD11c, CD83 and increased expression of HLA-DR. However, in their study, mDCs were not separated into mDC1 and mDC2 populations. In their study, the immature MDDCs also had greater phagocytic capacity and induced greater T-cell proliferation than unstimulated mDCs, although the phenotypic and functional disparity between mDCs and MDDCs was balanced by MDDC maturation.

As previously mentioned, mature DCs are the most potent antigen-presenting cells (APCs), and the only ones capable of activating naive T cells and of initiating adaptive immune responses (reviewed in Steinman and Hemmi, 2006; reviewed in Adema, 2009). Not only are mature DCs able to present antigen, activate naïve T cells and initiate adaptive immune responses, but they can also selectively determine the type of immune response induced by lymphocytes via the dynamic expression of various signalling molecules (reviewed in Banchereau and Steinman, 1998; reviewed in Banchereau *et al.,* 2000).

We investigated (Mutamba *et al.,* 2012) the effect of IL-1F8 and IL-1F9 on maturation of MDDCs using increased HLA-DR (Verhasselt *et al.,* 1997) and CD83 (Zhou and Tedder, 1995) and decreased CD1a (Romani *et al.,* 1996) expression as maturation markers. Consistent with previous findings, our results showed that IL-1β and IFN-γ stimulated MDDC maturation (Guo *et al.,* 2003; Wesa and Galy, 2002) but in addition we showed that IL-1F8 and IL-1F9 also have a potent effect on MDDC maturation.

Further markers of MDDC maturation were examined using IL-1F8 as a model novel IL-1 cytokine. *In vivo*, after receiving a maturation signal, DCs migrate to the T-lymphocyte areas of lymphoid tissue and acquire a phenotype which relates to their stimulatory properties. Among other factors, mature DCs upregulate expression of co-stimulatory molecules such as CD40, CD80 and CD86 (reviewed in Shortman and Liu, 2002; reviewed in Steinman and Hemmi, 2006), which provide co-stimulatory signals that are essential for T cell activation

and survival (reviewed in Banchereau and Steinman, 1998; reviewed in Steinman and Hemmi, 2006). In the present study, IL-1F8 (100 ng/ml) induced CD40 and CD80 expression, suggesting that IL-1F8-matured DCs are capable of activating T lymphocytes, thus pointing to a potential role for novel IL-1 cytokines in DC-mediated induction of T cell (adaptive) immune responses.

IL-1F8, however, proved to be less potent than the classical IL-1, IL-1β in many aspects. Although IL-1F8 and IL-1β had equipotent effects on IL-18 production by MDDCs, IL-1F8 had to be used at a concentration (400 or 500 ng/ml) that was at least 40 times that of IL-1β (10 ng/ml) to induce IL-12 p70 levels similar to those induced by IL-1β. Furthermore, while MDDCs matured using 100ng/ml IL-1F8 induced a two-fold increase in T-cell proliferation, this level was doubled by MDDCs, which had been matured with 500ng/ml IL-1F8.

Previous studies have also found IL-1F8 to be less potent than IL-1β. Towne *et al.* (2004) reported that IL-1F6, IL-F8, and IL-1F9 activate the pathway leading to NF-kB in multiple cell types transfected with IL-1Rrp2 but much higher doses of IL-1F6, IL-1F8, and IL-1F9 are necessary to achieve significant activation of the pathway leading to NF-kB than was the case for IL-1β. They observed that IL-1F8 significantly activated the pathway leading to NF-kB at concentrations above 100ng/ml; reaching a plateau at around 1 µg/ml. On the other hand, IL-1β significantly activated the pathway leading to NF-kB at a concentration as low as 0.02ng/ml (Towne *et al.,* 2004). Magne *et al.* (2006) observed that primary human synovial fibroblasts (hSFs) and human articular chondrocytes (hACs)

express IL-1Rrp2 mRNA and produce inflammatory cytokines (IL-6 and IL-8) in response to IL-1F8, however, the concentration of recombinant IL-1F8 required to stimulate hSFs and hACs was also higher than that of IL-1β. Significant stimulatory effects were observed at IL-1F8 concentrations from 500–5,000 ng/ml, compared with 0.1-10ng/ml for IL-1β (Magne *et al.,* 2006).

It was previously proposed that IL-1F8 and other new members of the IL-1 cytokine family only exert significant biological effects at such high concentrations because the recombinant IL-1 cytokines used in studies probably lack the post-translational modifications which might be vital for their biological activity (Towne *et al.,* 2004; Magne *et al.,* 2006). These speculations were recently confirmed by a study which showed that truncation of IL-1F6, IL-1F8, IL-1F9 and IL-1F5 (recently renamed IL-36α, IL-36β, IL-36γ and IL-36Ra, respectively) enhances their potency (Towne *et al.,* 2011). In this recent study, it was shown that removal, from these IL-1 cytokines, of N-terminal amino acids at specific points increased their specific activity 1000-10000 times (Towne *et al.,* 2011). Whereas previously other researchers (Towne *et al.,* 2004; Magne *et al.,* 2006) had failed to demonstrate the ability of IL-1F5 to antagonise IL-1F9 (Debets *et al.,* 2001), the recent work by Towne et al. (2011) helped resolve this controversy by showing that IL-1F5 is indeed an IL-1F9 antagonist but that antagonist activity requires removal of the N-terminal methionine which is present in the primary translation product. Furthermore, they extended previous findings by showing that truncated IL-F5 is also an antagonist for IL-1F6 and IL-1F8 in a similar manner to the way IL-1Ra inhibits IL-1α and IL-1β (Towne *et al.,*

2011). Although these findings suggest that post-translational modification might be vital for the biological activity of the novel IL-1 cytokines, this is yet to be confirmed *in vivo*. It can be hypothesised that had truncated IL-1F8 been used in our study, its potency might have been more comparable to that of IL-1β, as a recent study in which truncated IL-1F6, IL-F8 and IL-1F9 were used suggests (Vigne *et al.,* 2011).

Having established that human MDDCs express IL-1Rrp2, mature in response to IL-1F8 and subsequently acquire the ability to induce T cell proliferation, the next step was to determine the T helper ($T_{H)}$ cell phenotype within the proliferating CD3+ T-cell population. Results from the present study show that IL-1F8-matured MDDCs were functionally mature as they were able to secrete IL-12p70 and IL-18 (Mutamba *et al.,* 2012). As previously discussed, these two cytokines synergistically induce interferon-γ production, which in turn enhances the production of IL-12p70 and thus favours the development of T_H1 cells (reviewed in Okamura *et al.,* 1998; Kaliński *et al.,* 1999; Kaliński *et al.,* 2000; Sabatté *et al.,* 2007). That IL-1F8-matured MDDCs stimulate differentiation of T_H1 phenotypes was further evidenced by a much greater concentration of IFN-γ compared with IL-10 in the supernatant of allogeneic MDDC/CD3+ cultures (Mutamba *et al.,* 2012). IL-10 generally contains and suppresses inflammatory responses and hence down-modulates effector adaptive immune responses so as to minimize tissue damage in response to microbial challenges. It is known to suppress the differentiation and effector functions of T_H1 cells through down-regulation or complete inhibition of IL-12 expression (reviewed in Maynard and Weaver,

2008). The present study, therefore, shows that IL-1F8 interaction with IL-1Rrp2 induces T_H1 polarization and hence production of proinflammatory cytokines in human MDDCs. However, a significant effect on T_H1 cytokine production was only observed when MDDCs were matured using an IL-1F8 concentration that was five times that of IL-1β, suggesting that the IL-1F8 used in this study was less potent than IL-1β. Alternatively, the result may also suggest that the IL-1F8 receptor (IL-1Rrp2) may not be as strongly expressed as the IL-1β receptor (IL-1R1) on human MDDCs.

IL-12 levels secreted by human MDDCs in the current study were generally lower than those observed in other studies (Ebner *et al.,* 2001; Nakahara *et al.,* 2005). This discrepancy might be due to differences in the maturation stage of MDDCs used in the studies, differences in the numbers of MDDCs used, differences in analytical methodologies, differences in duration of stimulation and differences in maturation stimuli. IL-12 production is influenced by the environmental conditions at the site of induction of DC maturation (reviewed in Kalinski *et al.,* 1999; Ebner *et al.,* 2001; Nakahara *et al.,* 2005).

Some common features between pDCs and MDDCs (for example, IL-1Rrp2 expression) were observed in the present study. IL-1Rrp2 was expressed by pDCs at levels comparable to IL-1Rrp2 expression measured in MDDCs generated from peripheral blood monocytes. This is a very interesting result because MDDCs are considered to be more similar to myeloid DCs (mDCs) than pDCs; for example, MDDCs and mDCs express the same TLRs (reviewed in

Iwasaki and Medzhitov, 2004). Furthermore, neither mDCs nor MDDCs are recognized as primary type I interferon producers whereas pDCs constitutively express large amounts of type I interferons (Grouard *et al.,* 1997; Siegal *et al.,* 1999). It has been suggested that the difference between mDCs and pDCs is such that they even originate from different haematopoietic lineages, with mDCs arising from myeloid precursors in the bone marrow and pDCs arising from lymphoid precursors (Galy *et al.,* 1995). More recently, this has been questioned since it has been found that pDCs or mDCs can be generated from either pDC or mDC precursors (Ishikawa *et al.,* 2007; Zuniga *et al.,* 2004), suggesting that there may not be a strict lineage specific development of mDCs or pDCs. On the other hand, human pDCs express pre T-cell receptor α mRNA (Res *et al.,* 1999) and murine pDCs have IgH gene D-J gene rearrangements, (Corcoran *et al.,* 2003) neither of which are found in mDCs nor MDDCs. Two possible models for the generation of functionally distinct (DC) subtypes have been proposed; these are the functional plasticity model and the specialized lineage model. The functional plasticity model proposes that all DCs belong to a single haematopoietic lineage and that the different subtypes of DCs arise due to local environmental influences on a relatively mature but “plastic” end-product cell. The specialised lineage model, on the other hand, proposes that the different DC subtypes are the products of entirely separate developmental lineages (reviewed in Shortman and Liu, 2002). In reality, both mDCs and pDCs show functional plasticity, thus, the origin and developmental pathways of DC subtypes is an area that still needs clarification.

Not only do human MDDCs and pDCs constitutively express IL-1Rrp2 and respond to IL-1F8, they both regulate IL-1Rrp2 expression in response to IL-1F8. It was interesting to note the differences in the way MDDCs and pDCs responded to IL-1F8 with regards to IL-1Rrp2 expression. MDDCs up-regulated IL-1Rrp2 expression in response to IL-1F8, on the other hand, pDCs responded to IL-1F8 by down-regulating IL-1Rrp2 expression. This difference in terms of response to stimulation suggests that different sets of DCs perform different functions as previously suggested by others (reviewed in Shortman and Liu, 2002).

Berglof *et al.* (2003) investigated the expression of IL-1Rrp2 and the effect of IL-1F9 on known IL-1 signaling pathways in the different cell types of the mouse brain in culture. They observed that LPS strongly reduced expression of IL-1Rrp2 mRNA in both mixed glia and microglia, suggesting that during inflammatory situations, possible IL-1F9 actions might be suppressed by decreased expression of IL-1Rrp2 mRNA (Berglof *et al.,* 2003), however, in our study, MDDCs responded to both LPS and IL-1F8 stimulation by up-regulating IL-1Rrp2 expression, suggesting that novel IL-1 cytokines have different effects on different cells.

While the finding that pDCs down-regulate IL-1Rrp2 expression in response to stimulation with IL-1F8 was an unexpected finding since MDDCs had shown IL-1Rrp2 up-regulation in response to IL-1F8, it is easy to see why this could be an appropriate or desirable response in certain diseases. For example, it is now

known that pDCs are activated in psoriasis and that pDCs initiate psoriasis through interferon-α production (Nestle *et al.,* 2005). The contribution of IL-1Rrp2 and its ligands to the cytokine network in psoriasis is beginning to be appreciated. Using a mouse model of human psoriasis, Blumberg *et al.* (2010) found that TNF-α, IL-17A, and IL-23, inflammatory cytokines thought to contribute to the development of psoriasis, were upregulated in the mouse skin. They also observed that these cytokines were induced by and could induce IL-1F6 and related IL-1 family cytokines. Inhibition of TNF-α or IL-23 inhibited the increased epidermal thickness, inflammation, and cytokine production. Blockade of IL-1Rrp2 (which they called IL-1RL2) also resolved the inflammatory changes in human psoriatic lesional skin transplanted onto immunodeficient mice. Therefore, this latter study may suggest that employing strategies which reduce or block IL-1Rrp2 expression could be beneficial in the treatment of psoriasis. The down-regulation of IL-1Rrp2 expression in pDCs observed in the present study may suggest that in an effort to limit the potentially harmful inflammatory effects of IL-1F8, pDCs respond by down-regulating the expression of the IL-1F8 receptor.

Another area in which IL-1F8-matured MDDCs and pDCs responded differently in the present study was with regards to their interaction with CD3+ T cells. While IL-1F8-stimulated MDDCs strongly and significantly induced T cell proliferation, IL-1F8-stimulated pDCs were very weak inducers of T cell proliferation. The ability of pDC to present antigen to naïve T cells is still somewhat controversial. In a previous study, although CpG-activated pDC

induced higher proliferation of T cells than pDC activated by influenza virus, this proliferation was observed to be considerably weaker compared with T cells stimulated by mDC (Iparraguirre *et al.,* 2007). Whether or not direct antigen presentation is the main function of pDCs during immune responses is yet to be determined, however, pDCs are the major type I IFN-producing cells in response to virus infection (Siegal *et al.,* 1999). The *in vitro* and *in vivo* use of model ligands for the two main TLRs expressed by pDC, TLR7 and TLR9 has facilitated understanding of the interaction between pDC and pathogens (or pathogen components). Imiquimod, an imidazoquinoline and synthetic TLR7agonist, is a stronger inducer of IFN-α production in pDCs (Gibson *et al.,* 2002; Iparraguirre *et al.,* 2007) and was therefore used as a positive control marker/indicator of pDC maturation/stimulation in the present study. Although pDCs express IL-1Rrp2 and mature in response to IL-1F8 with subsequent secretion of IFN-α, results suggest that IL-1F8 is not as potent as IL-1β in stimulating IFN-α production in pDCs.

Although the *in vivo* significance of the maturation of human DC subsets by novel IL-1 cytokines (acting via the IL-1Rrp2 receptor) is yet to be demonstrated, a lot can be gleaned from previous studies. As already mentioned, a role for IL-1Rrp2 ligands (IL-1F5, IL-1F6, IL-1F8- and IL-1F9) in the induction of proinflammatory cytokines (Debets *et al,* 2001; Towne *et al.,* 2004; Magne *et al.,* 2006, Blumberg *et al.,* 2010) and in the pathogenesis of psoriasis (Debets *et al.,* 2001; Blumberg *et al.,* 2007; Blumberg *et al.,* 2010) has already been suggested.

Results presented in this chapter extend current understanding regarding the interaction between myelomonocytic cells and IL-1F8/IL-1F9 via the IL-1Rrp2 receptor. In view of previous studies and results from the current study, there is a possibility that IL-1Rrp2 and its ligands act differently on different cells. The effect of the newer IL-1 cytokine family members on Langerhans cells or epidermal DCs in the skin, which are phenotypically similar to MDDCs, is not known (Galy *et al.,* 1995; Ishikawa *et al.,* 2007). One study has shown that Langerhans cells act to dampen skin inflammation (Kaplan *et al.,* 2005) and another study has shown that Langerhans cell recruitment is increased in the skin of psoriatic patients successfully treated with propylthiouracil, which has anti-inflammatory properties (Elias *et al.,* 2003).

The potential of IL-1 cytokines for immunomodulatory therapies has previously been reviewed (reviewed in Dumont, 2006). Added to that, the recent finding that IL-1Rrp2 (IL-1RL2) and its ligands contribute to the cytokine network in human psoriasis (Blumberg *et al.,* 2010) raises interesting questions about the potential of therapeutic strategies targeting IL-1Rrp2. If IL-1F8 and IL-1F9 function as maturation signals for other cells similar to MDDCs, such as Langerhans cells, then it is possible that IL-1F8 and IL-1F9 may have potential in Langerhans cell activation and immune modulation *in vivo*.

CHAPTER 4: IL-1RRP2 EXPRESSION IN VARIOUS HUMAN CELLS

4.1 Introduction

At the commencement of the current study, the function of IL-1Rrp2 was only starting to be clarified. It had just been identified as being part of the receptor complex through which IL-1F6, IL-1F8 and IL-1F9 signal. It had also been shown to mediate inflammatory responses triggered by these newer members of the IL-1 cytokine family (Towne *et al.,* 2004; Magne *et al.,* 2006). IL-1Rrp2 expression had been reported in rat meninges, choroid plexus and cerebral cortex (Lovenberg *et al.,* 1996). In humans, high IL-1Rrp2 expression had been observed in skin while lower levels were found in prostate, ovary, thyroid, uterus, liver, kidney, lung, and trachea. Extremely low levels were found in human testis, heart, spleen, and small intestine. IL-1Rrp2 expression was also observed in some human cell lines (Towne *et al.,* 2004). Recent studies are suggesting the therapeutic potential of targeting IL-1Rrp2 in chronic inflammatory conditions such as psoriasis, which are mediated by IL-1Rrp2 and its ligands (Blumberg *et al.,* 2010). The present study has extended previous findings, not only by showing differential IL-1Rrp2 expression in human myelomonocytic cells, but also by showing differential biological response of these cells to IL-1Rrp2 ligands (Chapter 3; quoted in Mutamba *et al.,* 2012).

After showing that human MDDCs and pDCs express IL-1Rrp2 and respond to IL-1F8 and IL-1F9 (Chapter 3, partially quoted in Mutamba *et al.,* 2012) the next step was to investigate IL-1Rrp2 expression in other human cells and cell lines so as to identify other potential targets for the novel IL-1 ligands. Cells in which IL-1Rrp2 expression had not yet been extensively studied were chosen. The specific aim of the work described in this chapter was to investigate IL-1Rrp2 expression in human immature blood CD209+/CD14+ Langerhans cells, human lymphocytes, granulocytes, human lamina propria cells and NCI/ADR-RES cells (OVCAR-8 ovarian adenocarcinoma cell line previously thought to be a breast cancer cell line) (Towne *et al.,* 2004; Liscovitch and Ravid, 2007).

Phenotypically, MDDCs share some characteristics with Langerhans cells and it has been hypothesised that these cells may be the blood-borne precursors of these skin DCs (Geissmann *et al.,* 1998; Grassi *et al.,* 1998; Herbst *et al.,* 1998). Langerhans cells (LCs), first described by Paul Langerhans in 1868, are DCs found in an immature state mainly within epidermal and mucosal stratified squamous epithelia and constituting approximately 2-4% of all epidermal cells (reviewed in Stingl and Shevach, 1991). They are the first antigen presenting cells that come into contact with pathogens at the skin surface (reviewed in Banchereau and Steinman, 1998). After encountering antigen, LCs migrate from the skin to T cell areas in the regional lymph node where they present processed antigen to T cells (reviewed in Banchereau and Steinman, 1998).

Previously, LCs were thought to initiate adaptive immune responses but this view is currently being debated based on more recent *in vivo* studies using LC-deficient mice which suggest that LCs actually dampen skin inflammation (Kaplan *et al.,* 2005; reviewed in Romani *et al.,* 2006).

Based on the findings presented in Chapter 3 of this thesis (also reported in Mutamba *et al.,* 2012), it was hypothesised that maturation of MDDCs by novel IL-1 cytokines *ex vivo* may mirror the effect of these cytokines on immature Langerhans cells *in vivo*. Such a scenario could have important effects during human disease considering that over-expression of IL-1F6 in murine basal keratinocytes with co-existing IL-1F5 deficiency was found to increase skin inflammation in a human psoriasis mouse model (Blumberg *et al.,* 2007). Since Langerhans cells have been shown to dampen skin inflammation (Kaplan *et al.,* 2005) and to be recruited into the skin of psoriatic patients successfully treated with propylthiouracil (an antithyroid thioureylene drug with immunomodulatory effects) (Elias *et al.,* 2003), there is a chance that the production of IL-1F6/IL-1F8/IL-1F9 during skin pathology may induce maturation of skin DCs which would in turn down-regulate the inflammatory response. As such, ascertaining whether immature human Langerhans cells express IL-1Rrp2 and whether they are matured by novel IL-1 cytokines was considered an important step in the present study.

Having investigated IL-1Rrp2 expression in human MDDCs and human blood DCs and having demonstrated the ability of IL-1Rrp2 ligands to induce inflammatory T_H1 subsets (Chapter 3, Mutamba *et al.,* 2012), and also, in view of published literature suggesting a role for IL-1Rrp2 and its ligands in inflammation-mediated diseases (Blumberg *et al.,* 2007; Blumberg *et al.,* 2010), attention was turned to studying IL-1Rrp2 expression in other human immune cells. Human lymphocytes and granulocytic neutrophils were considered to be of interest because of their role in immune responses, including inflammation. The inter-dependent relationship that exists between DCs and other immune cells has been highlighted in preceding chapters of this thesis (Chapters 1 and 3).

A common feature of the innate immune response to an immunological challenge is the localized production of proinflammatory cytokines (such as TNF-α, IFN-α/β and IL-1β), leading to the influx and activation of neutrophils and macrophages at the site of infection, resulting in inflammation, thus, neutrophils play a vital role in the innate arm of the immune response that rapidly develops at sites of injury and infection (Nathan, 2006). Although primarily recognized for the destruction of invading pathogens, neutrophils can also shape immune responses (Nathan, 2006). Neutrophils deliver activation signals and antigenic molecules to DCs, and in turn, activated DCs mature and migrate to secondary lymphoid organs, where they initiate activation of naive T lymphocytes and elicit antigen-specific responses (Megiovanni *et al.* 2006).

Using human live blood neutrophils and immature monocyte-derived DCs, Megiovanni *et al.* (2006) demonstrated that co-culture of immature MDDCs with live neutrophils led to DC maturation as evidenced by up-regulation of CD40, CD86, and HLA-DR on DCs and that neutrophil-DC cell contact was essential for CD86 and HLA-DR up-regulation. They also reported that DCs acquired *Candida albicans*-derived antigens from neutrophils via a cell-contact-dependent mechanism (Megiovanni *et al.* 2006), suggesting that cross-talk between DCs and neutrophils could be more intimate than previously appreciated (Megiovanni *et al.* 2006). Furthermore, in different models and animal species, it has been shown that the classical IL-1, IL-1β, causes both dose-and time-dependent neutrophil migration (Faccioli *et al.,* 1990; Perretti *et al.,* 1993) through the release of neutrophil chemotactic mediators (Simbirtsev *et al.,* 2001; Oliveira *et al.,* 2008).

In inflammatory diseases like Rheumatoid Arthritis (RA), neutrophils play a huge part in the inflammation and tissue destruction associated with the diseases (Jonsson, Allen and Peng, 2005). In a more recent study, a mouse model was used to show that IL-1F11 (IL-33) orchestrates neutrophil migration in arthritis (Verri *et al.,* 2010). We therefore speculated that IL-1Rrp2 ligands might also induce neutrophil migration in a similar manner to the other IL-1 family members, IL-1β and IL-1F11, thereby enhancing inflammatory processes. Thus, the decision to investigate IL-1Rrp2 expression in human granulocytic neutrophils was based on the mentioned published reports showing the involvement of other

IL-1 family members (IL-1α, IL-1β and IL-1F11) in neutrophil migration culminating in inflammation (Faccioli *et al.,* 1990; Perretti *et al.,* 1993; Simbirtsev *et al.,* 2001; Jonsson, Allen and Peng, 2005; Oliveira *et al.,* 2008) and also because there is cross-talk between neutrophils and DCs (Megiovanni *et al.* 2006).

Interest in IL-1Rrp2 expression in intestinal lamina propria (LP) cells was based on reports that IL-1Rrp2 ligands were highly expressed in tissues containing epithelial cells (stomach, skin, lung and oesophagus) (Debets *et al.,* 2001; Towne *et al.,* 2004) and also because of reports (based on *in vitro* analysis of human gut inflammatory cells and experimental colitis models) suggesting a role for IL-18 (IL-1F4) in the pathogenesis of Crohn's disease (a major form of inflammatory bowel disease (IBD)) (Maerten *et al.,* 2004). Furthermore, IL-1α and IL-1β are known to mediate inflammation in inflammatory bowel diseases like Crohn's disease and ulcerative colitis (Youngman *et al.,* 1993; Ludwiczek *et al.,* 2004). We speculated that in a similar way, novel IL-1 cytokines, acting via IL-1Rrp2-expressing cells in intestinal LP, induce inflammatory T cell responses in the gut. In addition, studies involving both mouse models of ulcerative colitis (UC) (one of the major types of IBD) as well as patients with UC suggest that DCs play a role in the pathogenesis of IBD (reviewed in Rutella and Locatelli, 2011). Because our study had found IL-1Rrp2 expression in some human DC subsets (MDDCs and pDCs), we speculated that human ileal LP might contain IL-1Rrp2-expressing DCs.

The gastrointestinal tract is constantly exposed to a diverse array of commensal bacterial as well as food and potentially pathogenic microorganisms. The intestinal immune system has the ability to maintain tolerance to harmless food antigens and commensal microorganisms on one hand while mounting appropriate protective immune responses to harmful pathogens (and avoiding the development of destructive inflammatory responses) on the other hand. When this balance is upset, chronic inflammatory disorders, such as IBD occur in humans (reviewed in Rutella and Locatelli, 2011). In Crohn's disease, for example, an imbalance of immunoregulatory factors and/or cells is thought to contribute to uncontrolled mucosal T cell activation and inflammation (Maerten *et al.,* 2004).

The gastric mucosa is lined by a monolayer of columnar epithelial cells which are separated from the lamina propria by a basement membrane. The deepest layers of the gastric mucosa comprise of the muscularis mucosae (consisting of a layer of smooth muscle cells) and the submucosa which lies below it. The submucosa consists almost exclusively of connective tissue (Castro and Arntzen, 1993; Wu *et al.,* 1999). Immune responses to antigen in the intestinal lumen are initiated at three sites; the lamina propria (LP), Peyer's patches (PP) in gut-associated lymphoid tissue (GALT) and mesenteric lymph nodes (MLN), all of which are rich in intestinal mononuclear phagocytic cells (Mahida *et al.,* 1997; Neiss *et al.,* 2005). The normal lamina propria consists of connective tissue matrix within which lie many different cell types, T and B lymphocytes,

plasma cells, macrophages, myofibroblasts, and cells that make up blood and lymphatic vessels. The intestinal LP, gut-associated lymphoid tissue (GALT) and epithelium are abundant in T cells; in fact, there are more T cells in these sites than in the rest of the body (reviewed in Maynard and Weaver, 2009). There is interaction between the epithelial monolayer and cellular components of the lamina propria (Wu *et al.,* 1999). This was first shown using an *in vitro* model whereby normal human mucosal samples whose surface epithelium had been denuded were maintained in culture (Yahida *et al.,* 1997). That study demonstrated that there was considerable movement of mononuclear cells (mostly T cells, macrophages and eosinophils) into and out of the intestinal LP, via basement membrane pores (Yahida *et al.,* 1997).

In a follow up study using intestinal mucosal samples from patients with active IBD, McAlindon *et al.* (1998) observed that in mucosal samples that had lost their epithelium, the basement membrane was either destroyed or contained numerous large pores through which polymorphonuclear cells (PMCs), lymphocytes, eosinophils and macrophages migrated into the lumen (McAlindon *et al.,* (1998). Further phenotypic and functional characterisation of the emigrating cells showed that emigrating lymphocytes were predominantly

CD45RO+ and CD69+ T cells (activated T cells) while emigrating macrophages expressed cyclooxygenase (COX) 1 and 2 enzymes. Furthermore, in addition to

PMCs and eosinophils, myofibroblasts expressing COX 1 and 2 enzymes and prostaglandin E2, were also observed emigrating (Wu *et al.,* 1999).

It is now known that during inflammation, polymorphonuclear cells, lymphocytes, and monocytes are recruited into the lamina propria from the systemic circulation (reviewed in Maynard and Weaver, 2009). Lamina propria T cells receive signals from epithelial cells, stromal cells, and the matrix itself via integrin receptors, and are closely associated with dendritic cells and macrophages (reviewed in Maynard and Weaver, 2009). CD4+ T cells predominate in the lamina propria and CD8+ T cells in the gut epithelium. A number of studies involving humans have shown that the CD4+ T cells in normal gut lamina propria have the phenotype of activated effector T cells (Wu *et al.,* 1999; reviewed in Maynard and Weaver, 2009).

Cells with antigen-presenting function within the intestine and associated lymphoid tissue include macrophages (the most abundant) and DCs (including conventional CD11c-expressing DCs and plasmacytoid DCs) (Varol *et al.,* 2009; reviewed in Rutella and Locatelli, 2011). In addition to their established role as efficient antigen-presenting cells (APCs), human LP DCs ($CD11b^+$ $CD14^+$ CX_3CR1^+ DCs, $CD103^+$ CX_3CR1^- DCs and CD1c+ DCs) (Varol *et al.,* 2009;

Dillon *et al.,* 2010) as well as DCs in the draining MLN and PP, have been shown to be instrumental in the maintenance of immune homeostasis and tolerance in the gut (reviewed in Coombes and Powrie, 2008; reviewed in Rutella and Locatelli, 2011). Interest in LP DCs was first aroused when it was shown that in mice and humans, LP DCs penetrate epithelial tight junctions (by projecting dendrites through the epithelial cell layer) to directly sense and sample antigen in the gut lumen (Rescigno *et al.,* 2001; Niess *et al.,* 2005).

Mouse and human studies suggest that tolerogenic DCs act by promoting the differentiation and expansion of intestinal FoxP3^{+} regulatory T cells (FoxP3^{+} Tregs) which then efficiently modulate gut inflammation. On the other hand, migrating DC subsets which are recruited to the gut in response to pathogenic insults are thought to initiate immune responses (reviewed in Rutella and Locatelli, 2011). The unique ability of DCs to sense the local environment and to shape the ensuing immune response accordingly is thought to be the reason behind the functional diversity and plasticity seen in gut DCs (reviewed in Coombes and Powrie, 2008).

Based on reports that IL-1Rrp2 ligands were highly expressed in tissues containing epithelial cells (Debets *et al.,* 2001; Towne *et al.,* 2004) and that IL-1Rrp2 was expressed in very low amounts in human small intestine (Towne *et al.,* 2004), as well as on studies showing migration of cells in and out of human

intestinal LP (Mahida *et al.,* 1997; McAlindon *et al.,* 1998; Wu *et al.,* 1999), and after demonstrating IL-1Rrp2 expression in human MDDCs and pDCs (Chapter 3 of this thesis; Mutamba *et al.,* 2012), we speculated that human LP contains IL-1Rrp2-expressing cells which can move in and out of the LP (based on Mahida *et al.,* 1997), making it a target for potentially inflammatory novel IL-1 cytokines.

The present study also assessed IL-1Rrp2 expression in the OVCAR-8 ovarian adenocarcinoma cell line; NCI/ADR-RES. When Towne *et al.* (2004) looked for a cell line which naturally expressed IL-1Rrp2; they observed that out of all cell lines tested, NCI/ADR-RES cells, which they called a breast cancer cell line, showed the highest IL-1Rrp2 expression, suggesting that this cell line could be useful in studies involving IL-1Rrp2 ligands. NCI/ADR-RES cells, previously known as MCF-7/AdrR cells, are widely used as a multidrug-resistant model in cancer research (Liscovitch and Ravid, 2007). They were previously thought to be a breast cancer cell line (MCF-7); however, single-nucleotide polymorphism (SNP) and karyotypic analyses have since revealed that NCI/ADR-RES cells are derived from OVCAR-8 ovarian adenocarcinoma cells (Liscovitch and Ravid, 2007; Ke *et al.,* 2011). In view of reports suggesting that NCI/ADR-RES cells are definitely not breast cancer cells (Liscovitch and Ravid, 2007; Ke *et al.,* 2011) and the reported high expression of IL-1Rrp2 in these cells (Towne *et al.,* 2004), we decided to investigate IL-1Rrp2 expression in NCI/ADR-RES cells to ascertain if we could replicate previous findings by Towne *et al.* (2004).

4.2. Results

4.2.1 Flow cytometry results showing that IL-1Rrp2 is not expressed by CD209+/CD14+ (immature blood) Langerhans cells

CD209+(DC-SIGN+)/CD14+ blood cells were freshly isolated from PBMC using MACS beads (Miltenyi Biotec Ltd., Surrey, UK) as already described (Chapter 2, section 2.3.6.5). The population of cells was then stained for IL-1Rrp2 expression prior to FACS analysis as previously described (Chapter 2, section 2.6.1.1). Results showed that neither $CD209^{+}/CD14^{+}$ (immature blood Langerhans cells) (Figure 4.1) nor $CD209^{-}/CD14^{+}$ cells (monocytes used as a negative control) (Figure 4.2) expressed IL-1Rrp2 above that measured for isotype controls.

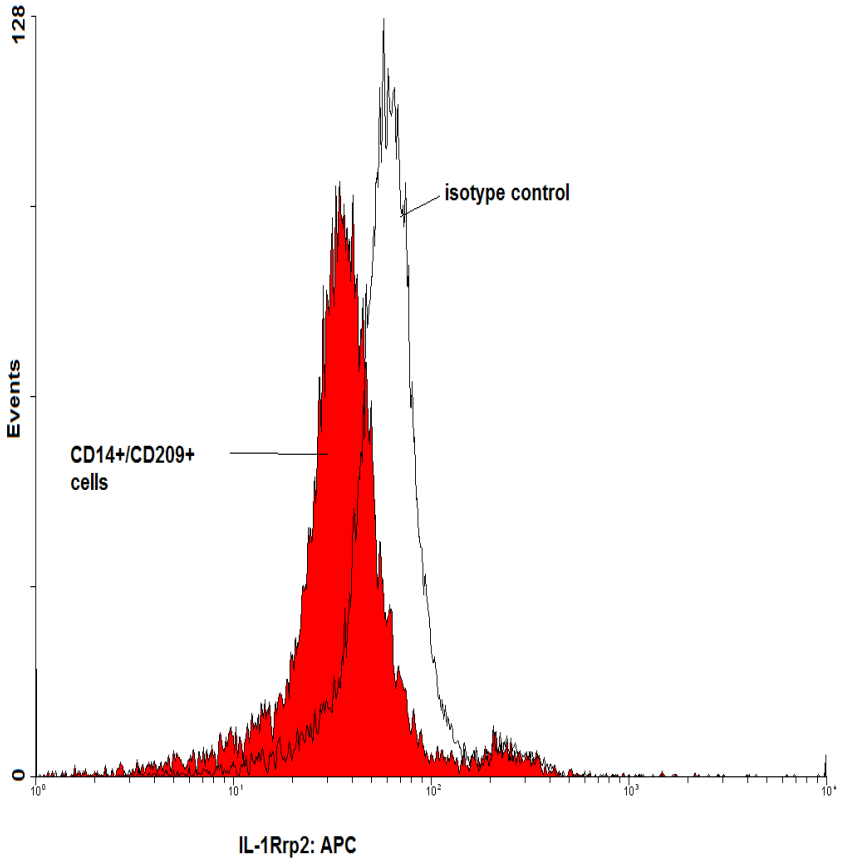


Figure 4.1 Immature blood CD209+/CD14+ Langerhans cells do not express IL-1Rrp2. Peripheral blood CD209+(DC-SIGN+)/CD14+ cells were stained for IL-1Rrp2 expression using an appropriate monoclonal antibody and isotype control prior to FACS analysis. Results show that CD209+/CD14+ cells (immature blood Langerhans cells) did not express IL-1Rrp2 above that measured for the isotype control.

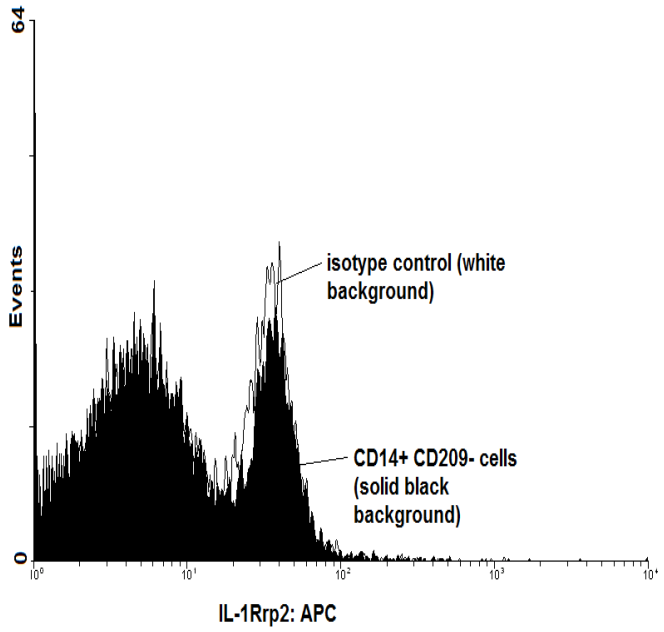


Figure 4.2 Immature blood CD209$^-$/CD14$^+$ cells do not express IL-1Rrp2. Peripheral blood CD209$^-$/CD14$^+$ cells were stained for IL-1Rrp2 expression using an appropriate monoclonal antibody and isotype control prior to FACS analysis. Results show that CD209$^-$/CD14$^+$ - cells did not express IL-1Rrp2 above that measured for the isotype control.

4.2.1.1 Quantitative RT-PCR results showing that CD209+/CD14+ (immature blood) Langerhans cells do not express IL-1Rrp2 mRNA

To further investigate lack of surface expression of IL-1Rrp2 by CD209+/CD14+ blood cells, qRT-PCR was used to measure IL-1Rrp2 mRNA expression in human peripheral blood CD14+/CD209+ cells, THP-1 promonocytes (negative control), HT29 cells (positive control/calibrator), MDDCs stimulated with IL-1F8 (500ng/ml) for 48 hours and MDDCs stimulated with IL-1β (100ng/ml). Results again showed that IL-1Rrp2 was not expressed by immature blood Langerhans cells (CD14+/CD209+) (Figure 4.3).

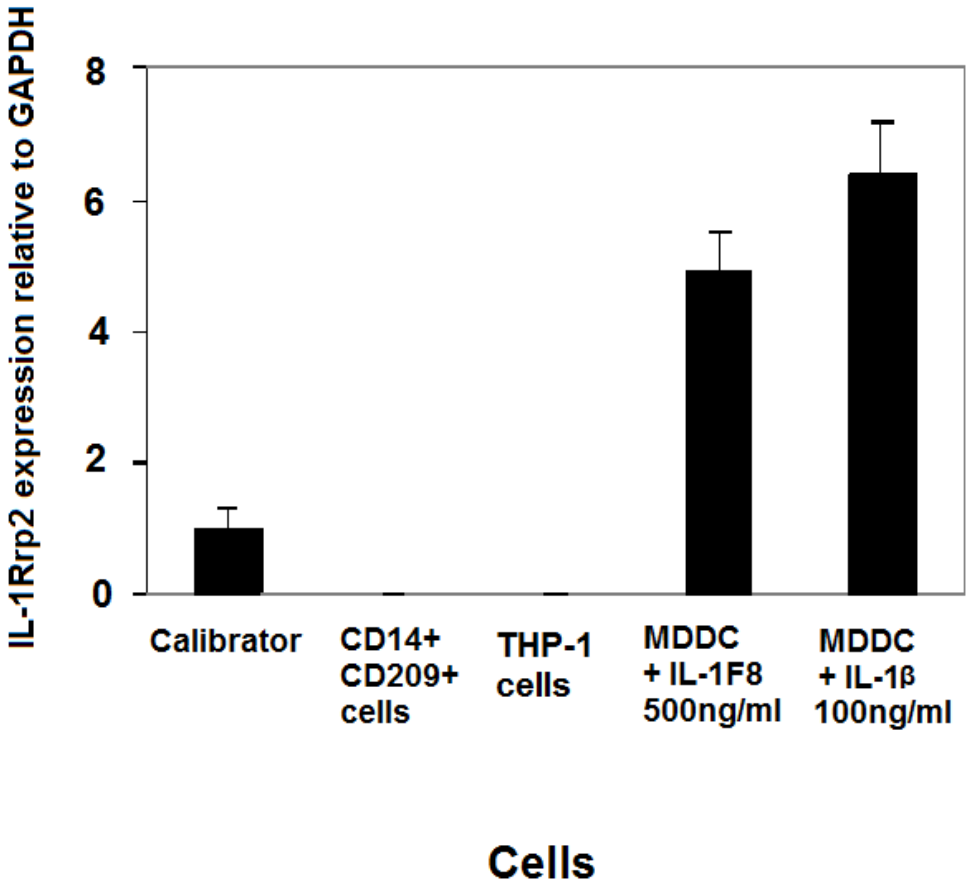


Figure 4.3 Quantitative RT-PCR results showing that immature human blood Langerhans cells (CD14+/CD209+ cells) do not express IL-1Rrp2 mRNA. qRT-PCR was used to measure IL-1Rrp2 mRNA expression in peripheral blood CD14+/CD209+ cells, THP-1 promonocytes (negative control), HT29 cells (positive control/calibrator), MDDCs stimulated with IL-1F8 (500ng/ml) for 48 hours and MDDCs stimulated with IL-1β (100ng/ml) for 48 hours. Results show that MDDC stimulated with IL-1β or IL-1F8 express IL-1Rrp2 mRNA but immature peripheral blood Langerhans cells (CD14+/CD209+) and THP-1 cells do not.

4.2.2 Human peripheral blood lymphocytes and CD3+ T cells express IL-1Rrp2 mRNA

Total lymphocytes were isolated from PBMCs by density gradient centrifugation using sterile- filtered ACCUSPIN™ Histopaque®-1077 centrifuge tubes as per manufacturer's instructions (Sigma-Aldrich, Poole, UK) as previously described (Chapter 2, section 2.3.4.2). In separate experiments, human CD3+ T cells were isolated from PBMC by positive selection using CD3+ microbeads (human) followed by magnetic separation on an AutoMacs™ Separator column according to the supplier's instructions (Miltenyi Biotec Ltd., Surrey, UK). Analysis of purity was performed by flow cytometry with appropriate fluorochrome-labeled antibodies (Abd Serotec, Oxford, UK) (Chapter 2, section 2.3.6.6.) Total RNA was extracted from both human peripheral blood lymphocytes and CD3+ T cells and 1µg of total RNA from each sample was reverse-transcribed into cDNA using random hexamer primers prior to performing qRT-PCR for IL-1Rrp2 gene as previously described (Chapter 2, section 2.7.0). GAPDH was used as the reference gene. HT 29 cells and THP-1 cells were used as positive and negative controls respectively. As Figure 4.4 shows, qRT-PCR analysis indicated that IL-1Rrp2 mRNA was expressed by human lymphocytes (non-adherent fraction of PBMCs) and by CD3+ T cells.

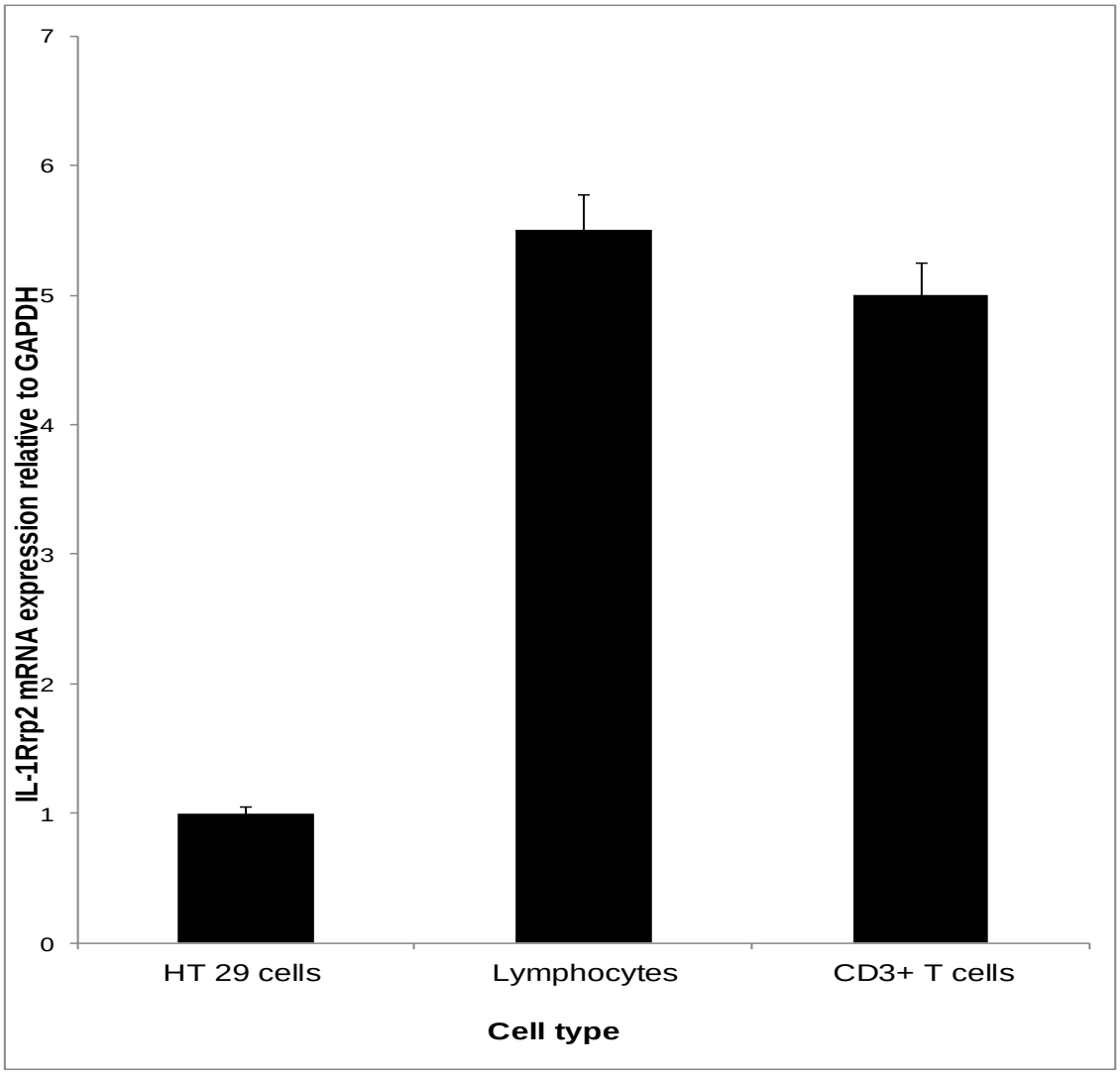


Figure 4.4 Quantitative RT-PCR results showing that lymphocytes (non-adherent PBMC fraction) and CD3+ T cells express IL-1Rrp2 mRNA. Non-adherent lymphocytes were isolated from blood using ACCUSPIN™ Histopaque®-1077. In separate experiments, CD3+ T cells were positively selected from PBMCs using CD3 magnetic beads on an AutoMacs™ Separator column. Isolated cells were subsequently assessed for IL-1Rrp2 mRNA expression relative to GAPDH. HT29 cells were used as a positive control/calibrator and THP-1 cells were used as a negative control. Results shown are means ± SD obtained from three independent experiments. Each analysis was performed in triplicate.

4.2.2.1 Immunofluoresence analysis of IL-1Rrp2 protein expression by human peripheral blood lymphocytes

After showing that human total blood lymphocyte populations express IL-1Rrp2 mRNA, fluorescence microscopy was used to further study IL-1Rrp2 protein expression by these cells. Briefly, non-adherent lymphocytes, isolated from PBMCs, were resuspended in PBS at a concentration of 1 x 10^5 cells/ml prior to being fixed with 4% v/v paraformaldehyde (PFA) in PBS as previously described (Chapter 2, sections 2.3.4.2 and 2.6.2). Cytospin preparations were made and cells were then stained with a primary IL-1Rrp2 antibody (M145, Amgen Corporation, Seattle, WA) followed by staining with an appropriate FITC-conjugated, secondary/detection antibody and DAPI nuclear stain. Isotype control cells were incubated with secondary antibody only. Unstained cells were also included as controls. Slides were then analysed using a Leica fluorescence microscope (Leica Microsystems, UK) as previously described (Chapter 2, section 2.6.2). Results are shown in Figure 4.5.

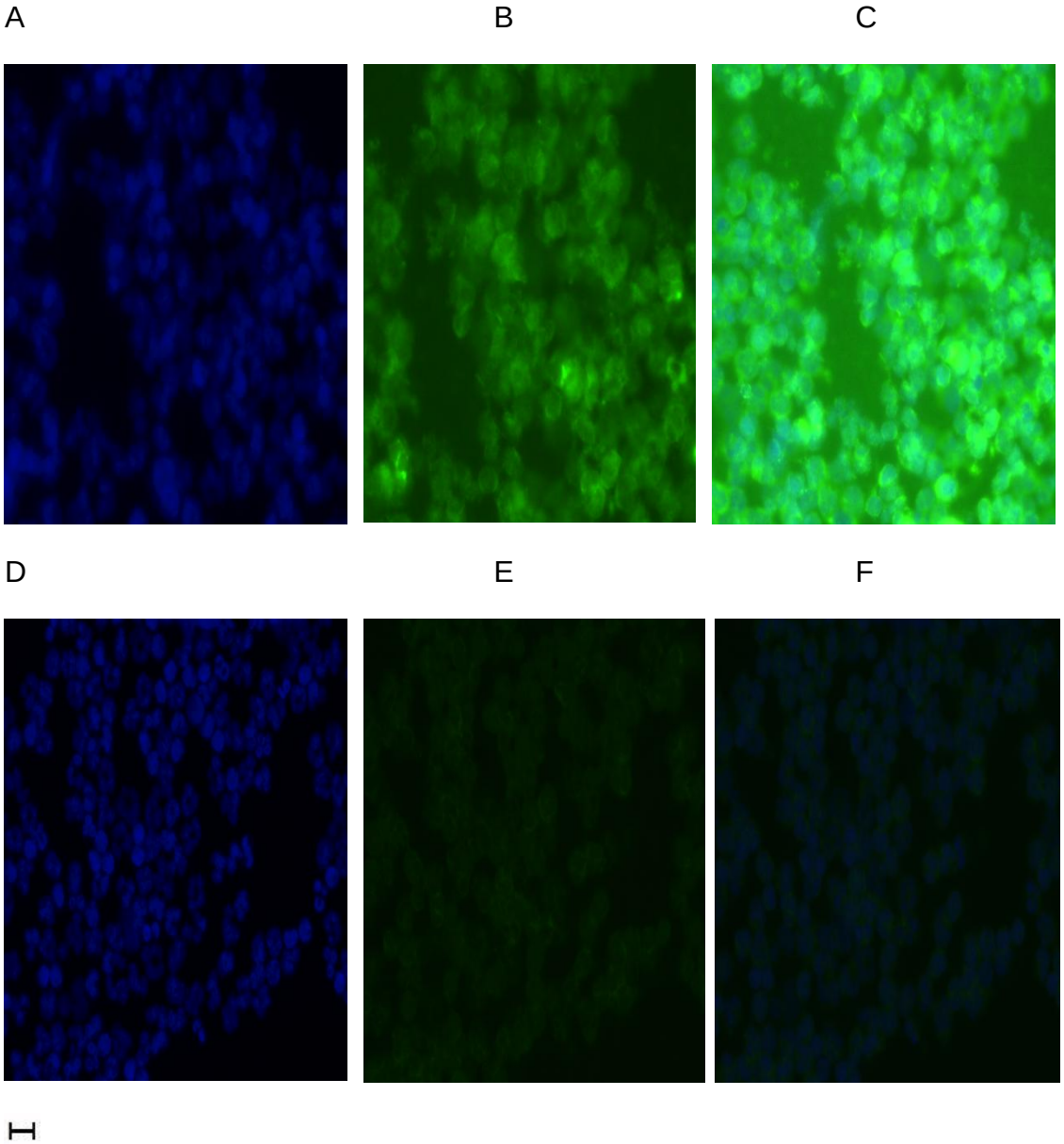


Figure 4.5 Human peripheral blood lymphocytes express IL-1Rrp2 on their cell membrane. Lymphocytes were incubated with an IL-1Rrp2 monoclonal antibody ((M145, Amgen Corporation, Seattle, WA) followed by staining with a FITC-conjugated secondary antibody and DAPI nuclear stain (images A-C). The negative control sample (images D-F) was incubated with secondary antibody and DAPI only but not with the primary IL-1Rrp2 mAb. C and F are the overlay images for the test and negative control samples respectively. Images are representative of three independent experiments with similar results. (Scale bar represents 20µm)

Compared with the negative control (Figure 4.5, images D-F), results confirm that human lymphocytes (Figure 4.5, images A-C) express IL-1Rrp2 protein on the cell surface. The blue images (Figure 4.5, A and D) show nuclear staining with DAPI while Figure 4.5, images B and E show presence/absence of IL-1Rrp2 protein (green-staining due to the fluorescein isothiocyanate (FITC)-labelled secondary antibody binding to the IL-1Rrp2 monoclonal antibody (mAb) which is in turn bound to IL-1Rrp2 in the cells). Figure 4.5, images C and F are the overlay images showing co-localisation (or lack of) of IL-1Rrp2 and DAPI. IL-1Rrp2 (FITC) staining was not observed when the primary antibody was omitted (negative control cells; Figure 4.5, images E and F).

4.2.3 qRT-PCR analysis of blood granulocyte showing lack of expression of IL-1Rrp2 mRNA

Granulocytes were isolated from PBMCs as already described (Chapter 2, section 2.3.5). Contaminating RBCs were lysed and removed before granulocytes were assessed for viability and purity of isolation as already described (Chapter 2). Total RNA was extracted from the granulocytes and reverse-transcribed into cDNA and quantitative RT-PCR for IL-1Rrp2 gene was performed on a LightCycler® 480 analyser as previously described (Chapter 2, section 2.7.0). GAPDH was used as the reference gene. HT 29 cells and THP-1 cells were used as positive and negative controls respectively. Results are shown in the graph below (Figure 4.6).

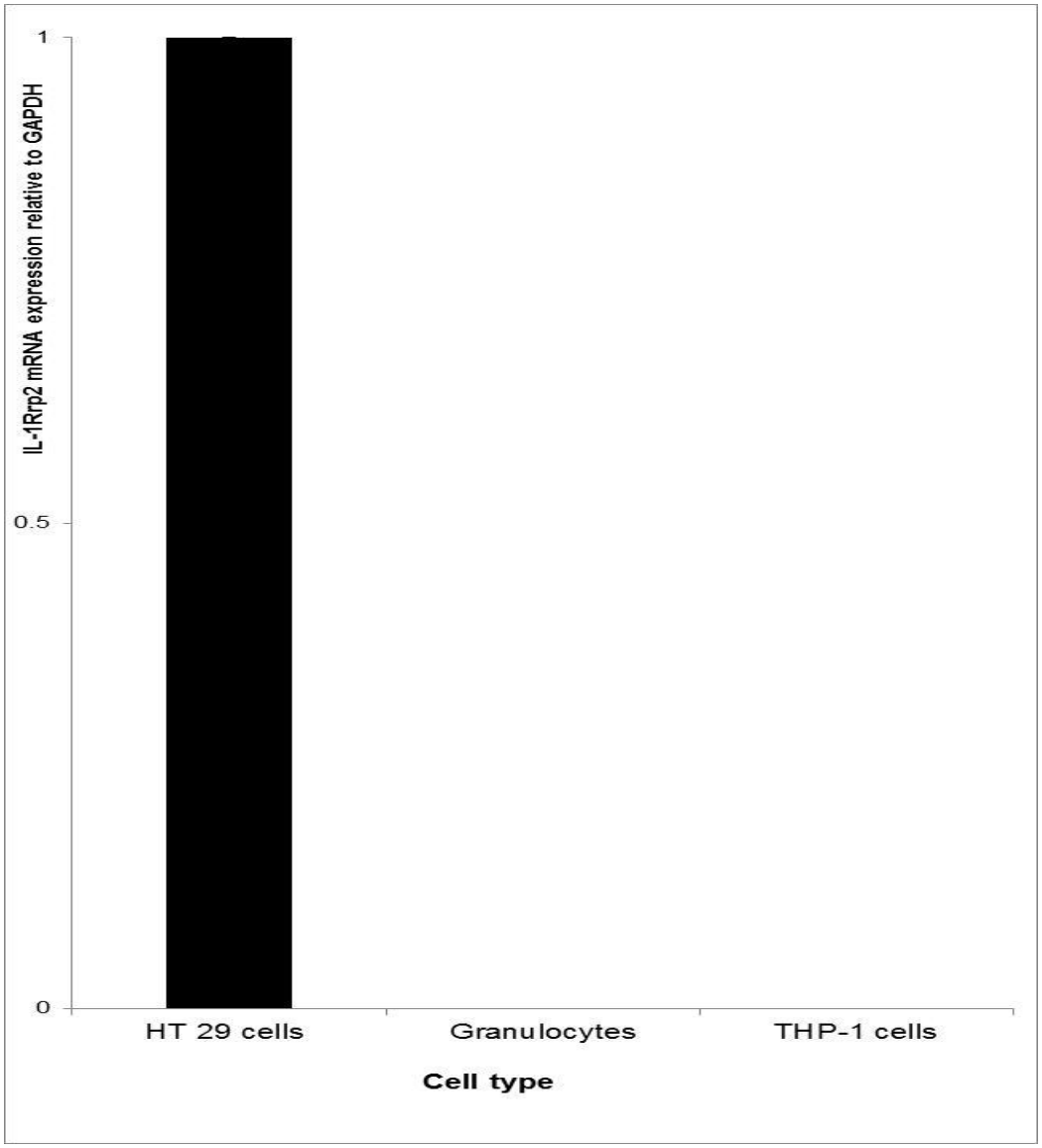
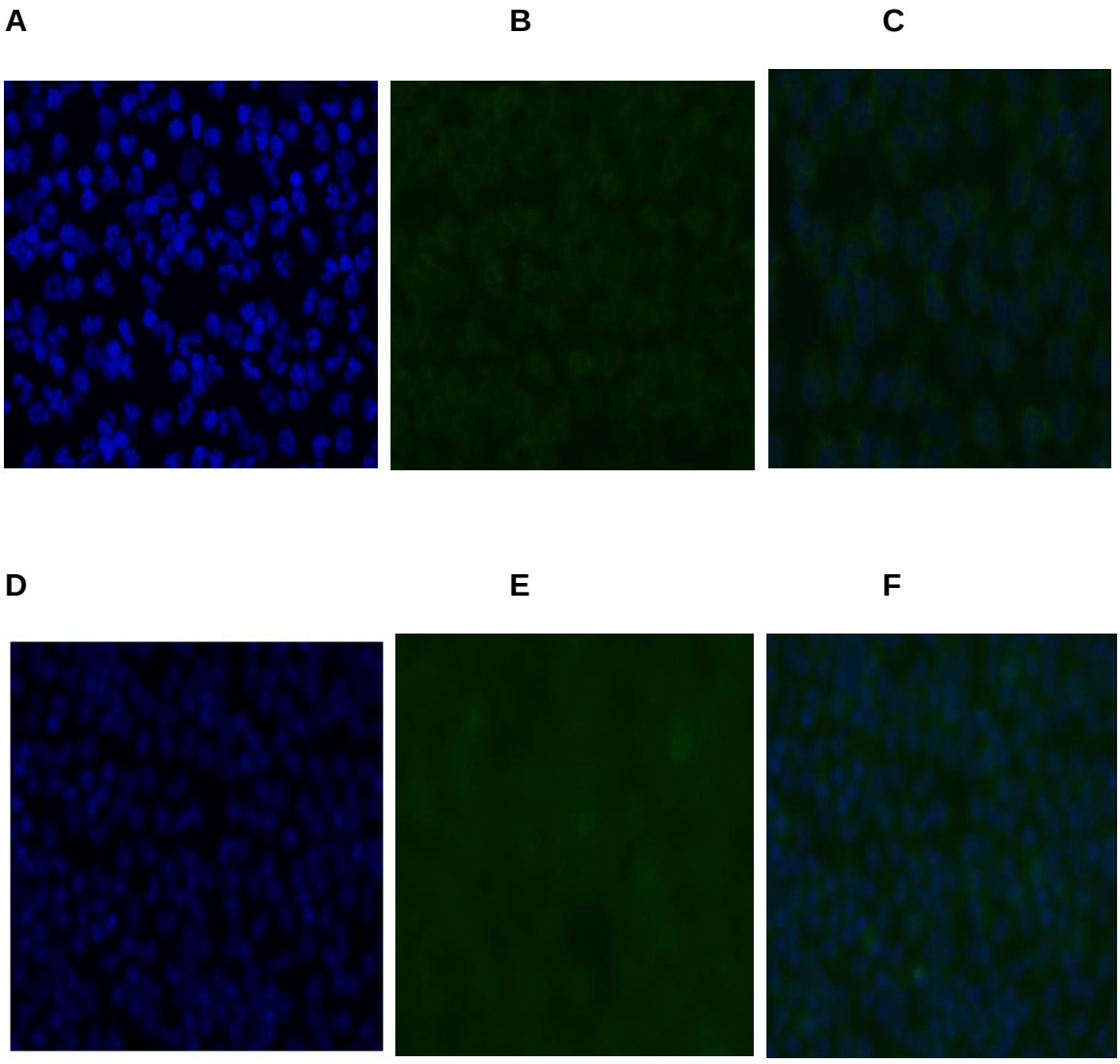


Figure 4.6 Quantitative RT-PCR results showing that human granulocytes do not express IL-1Rrp2 mRNA. Granulocytes were isolated from PBMC using Histopaque®-1077 density gradient centrifugation. Isolated cells were subsequently assessed for IL-1Rrp2 mRNA expression relative to GAPDH. HT29 cells were used as a positive control/calibrator and THP-1 cells were used as a negative control. Results shown are means ± SD obtained from three independent experiments. Each analysis was performed in triplicate.

As the graph in Figure 4.6 shows, granulocytes do not express IL-1Rrp2 mRNA. The graph shows mean ± SD of data from three independent experiments. Each analysis was performed in triplicate.

4.2.3.1 Immunofluoresence analysis of IL-1Rrp2 protein expression by human peripheral blood granulocytes

Fluorescence microscopy was used to further investigate IL-1Rrp2 expression in human granulocytes. Granulocytes were isolated from PBMCs as already described (Chapter 2, section 2.3.5). Briefly, after removing any contaminating RBCs as already described (Chapter 2, section 2.3.5), granulocytes were assessed for viability and purity using previously described methods (Chapter 2) prior to being resuspended in PBS at a concentration of 1×10^5 cells/ml. Cytospin preparations were made from the cell suspensions as previously described (Chapter 2, section 2.6.2) and cells were stained for fluorescence microscopy using a monoclonal primary antibody specific for IL-1Rrp2 (M145, Amgen Corporation, Seattle, WA) followed by staining with a secondary detection antibody (FITC-conjugated Goat anti-mouse IgG). Cells were counterstained with DAPI nuclear stain prior to being analysed using a Leica fluorescence microscope (Leica Microsystems, UK) as described in Chapter 2, Section 2.6.2. The images are shown below (Figure 4.7). Images are representative of at least three independent experiments.



DAPI Stain | mouse anti-human IL-1Rrp2 (FITC-conjugated) | Overlay image

Figure 4.7 Immunofluorescence images showing that human peripheral blood granulocytes do not express IL-1Rrp2 on their cell membrane. Granulocytes were incubated with an IL-1Rrp2 monoclonal antibody (M145) followed by staining with a FITC-conjugated secondary antibody and DAPI nuclear stain (A-C). The negative control sample (D-F) was incubated with secondary antibody and DAPI only but not with the primary IL-1Rrp2 mAb. C and F are the overlay images for the test and negative control samples respectively. Images are representative of three independent experiments with similar results. Scale bar represents 20µm.

Fluorescence microscopy images (Figure 4.7) showed no significant difference between test images (Figure 4.7, A-C) and negative control images (Figure 4.7, D-F). The blue-staining images (Figure 4.7, A and D) show nuclear staining with DAPI while B and E show presence/absence of IL-1Rrp2 protein (green-staining due to the fluorescein isothiocyanate (FITC)-labelled secondary antibody binding to the IL-1Rrp2 monoclonal antibody (mAb) which is in turn bound to IL-1Rrp2 in the cells). Figure 4.7, C and F are the overlay images showing no co-localisation of IL-1Rrp2 and DAPI due to weak IL-1Rrp2/FITC immunoreactivity. IL-1Rrp2 (FITC) immunoreactivity was not observed when the primary antibody was omitted (negative control cells; Figure 4.7, E and F). The faint green staining shows non-specific binding.

4.2.4 Human lamina propria cells express IL-1Rrp2 mRNA (qRT-PCR)

Lamina propria cells were isolated from histologically normal human ileal mucosal samples as described in Chapter 2 (section 2.2.4). To stabilize RNA prior to total RNA extraction, the isolated lamina propria cells were immediately pelleted and suspended in RNA*later* RNA Stabilization Reagent according to the manufacturer's recommendations (QIAGEN, Crawley UK). Total RNA was extracted from $1x10^6$ cells, reverse-transcribed into cDNA and amplified in a typical real time PCR reaction as described previously (Chapter 2, section 2.7.0). A relative quantification analysis using GAPDH as the reference gene was performed on a LightCycler® 480 analyser as previously described (Chapter 2,

section 2.7.0). HT 29 cells and THP-1 cells were used as positive and negative controls respectively. Each analysis was performed in triplicate on samples from three different patients. Results are shown in the graph below (Figure 4.8).

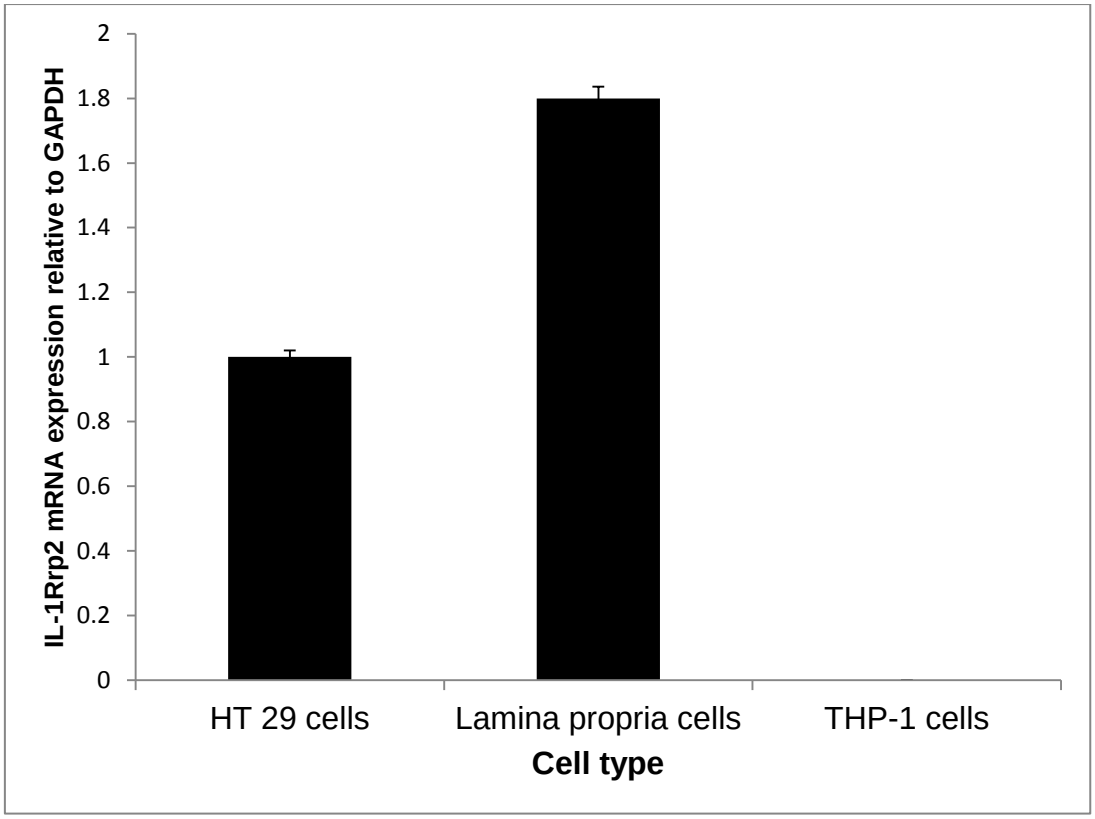


Figure 4.8 Quantitative RT-PCR results showing that human ileal lamina propria (LP) cells express IL-1Rrp2 mRNA. LP cells were isolated from histologically normal human ileal mucosal samples. Isolated cells were subsequently assessed for IL-1Rrp2 mRNA expression relative to GAPDH. HT29 cells were used as a positive control/calibrator and THP-1 cells were used as a negative control. Results show that compared with the negative control (THP-1 cells), human ileal lamina propria cells express IL-1Rrp2 mRNA. The level of IL-1Rrp2 mRNA expression in human ileal lamina propria cells is nearly twice that of the positive control (HT-29 cells). Results shown are means ± SD obtained from three different patients. Each analysis was performed in triplicate.

Results show that compared with the negative control (THP-1 cells), human ileal lamina propria cells express IL-1Rrp2 mRNA. The level of IL-1Rrp2 mRNA expression in human ileal lamina propria cells is nearly twice that of the positive control (HT-29 cells) (Figure 4.8).

4.2.5 qRT-PCR analysis showing IL-1Rrp2 mRNA expression in NCI/ADR-RES cells

NCI/ADR-RES cells, a kind gift from Professor Susan Watson (Division of Pre-Clinical Oncology, University of Nottingham), were maintained in culture as previously described (Chapter 2, section 2.2.2). Total RNA was extracted from $1x10^6$ cells using the RNeasy Mini Kit and qRT-PCR for IL-1Rrp2 gene relative to GAPDH was performed as previously described (Chapter 2, section 2.7.0). HT 29 cells and THP-1 cells were used as positive and negative controls respectively. Each analysis was performed in triplicate and the experiment was repeated on three separate occasions. Results are shown below (Figure 4.9).

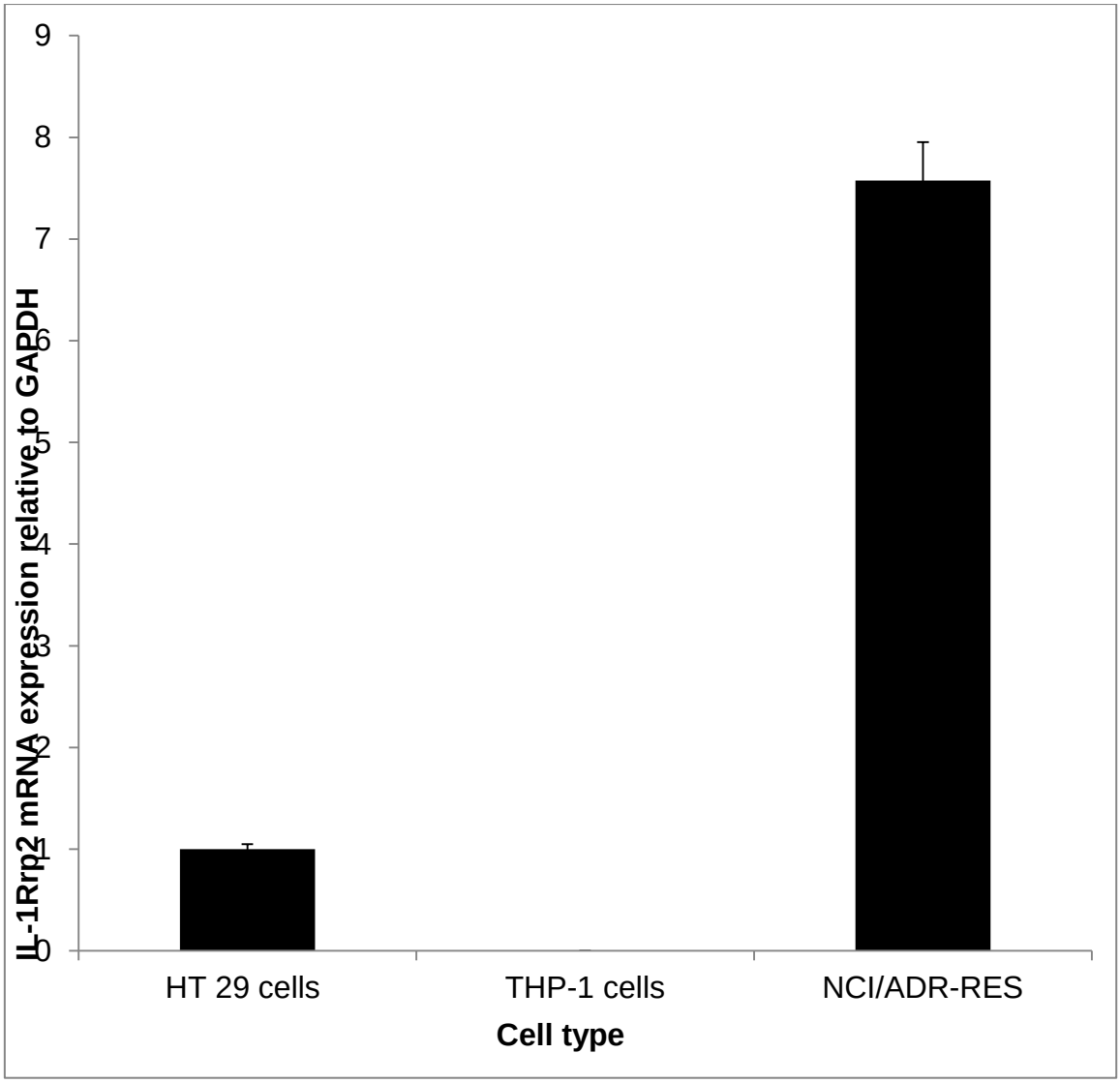


Figure 4.9 Quantitative RT-PCR results showing that NCI/ADR-RES cells express IL-1Rrp2 mRNA. NCI/ADR-RES cells were assessed for IL-1Rrp2 mRNA expression relative to GAPDH (reference gene). HT29 cells were used as a positive control/calibrator and THP-1 cells were used as a negative control. Results show that compared with the negative control (THP-1 cells), NCI/ADR-RES cells express IL-1Rrp2 mRNA. The level of IL-1Rrp2 mRNA expression in NCI/ADR-RES cells is more than seven times that of the positive control (HT-29 cells). Results shown are means ± SD obtained from three independent experiments. Each analysis was performed in triplicate.

Results (Figure 4.9) show that compared with the negative control (THP-1 cells), NCI/ADR-RES cells strongly express IL-1Rrp2 mRNA. The level of IL-1Rrp2 mRNA expression in NCI/ADR-RES cells is more than seven times that of the positive control (HT-29 cells) (Figure).

4.3 Discussion

After showing that IL-1F8-stimulated human MDDCs have the ability to activate CD3+ T lymphocytes, induce their proliferation and stimulate T_H1 responses *in vitro* and that the only other DCs we found to express IL-1Rrp2 were pDCs (Chapter 3, partially quoted in Mutamba *et al.,* 2012) which may be lymphoid in origin (Galy *et al.,* 1995), the next step was to investigate IL-1Rrp2 expression in other human cells and cell lines in an effort to identify other potential targets for the novel IL-1 cytokines. As already stated in the introduction (section 4.1), cells in which IL-1Rrp2 expression had not yet been extensively studied were chosen for reasons that have already been stated (section 4.1). The aim of the work described in this chapter was to investigate IL-1Rrp2 expression in human immature peripheral blood CD209+/CD14+ Langerhans cells, human lymphocytes, granulocytes, human lamina propria cells and NCI/ADR-RES cells.

The role of MDDC *in vivo* is controversial; it is not known whether monocytes stimulated with IL-4 and GM-CSF *in vivo* serve as a DC pool, replenishing activated DCs during infection. However, MDDCs generated *in vitro* by culture with IL-4 and GM-CSF have phenotypic characteristics similar to Langerhans cells (Geissmann *et al.,* 1998; Grassi *et al.,* 1998; Herbst *et al.,* 1998), lacking CD14 expression and gaining CD209 (DC-SIGN) expression (Relloso *et al.,* 2002). CD209 has important functions *in vivo* since it initiates T cell contact via ICAM (Geijtenbeek *et al.,* 2000a) and also functions during DC trafficking from

blood to peripheral tissues (Geijtenbeek *et al.,* 2000b) and is now known as a pathogen recognition receptor for some microbial pathogens, particularly viruses (Collin *et al.*, 2011).

Although the conversion of monocytes to DCs is associated with an epigenetic switch from CD14 to CD209 (Bullwinkel *et al.,* 2011), a rare sub-population of CD14+/CD16+ blood monocytes also co-express CD209 (Merino *et al.,* 2011). CD209 has also previously been shown to be expressed by immature pDCs (which do not express CD14) (Masten *et al.,* 2006) and macrophages in peripheral tissue (Geijtenbeek *et al.,* 2000b; Soilleux *et al.,* 2002). It is also expressed in high concentration by intradermal DCs (Turville *et al.,* 2002) and by macrophage-like intradermal APCs (Angel *et al.,* 2007) which may differentiate into Langerhans cells (Larregina *et al.,* 2001).

In the current study, CD14+/CD209+ cells were isolated from blood and results show that these cells, in contrast to MDDC, do not express IL-1Rrp2. Whether this CD209+/CD14+ population represents a rare monocyte population or is indeed blood-borne precursors of dermal DCs, as previously reported (Larregina *et al.,* 2001; Turville *et al.,* 2002; Angel *et al.,* 2007) is a matter that still needs elucidation, however, results presented in this chapter clearly show that this population is not comparable to MDDC with regards to IL-1Rrp2 expression and, hence, activation via IL-1F6, IL-1F8 or IL-1F9. As such, this study suggests that

MDDCs are functionally different from blood borne immature skin DCs (or $CD209^{+}/CD14^{+}$ blood monocytes).

In the current study, IL-1Rrp2 expression was observed in total lymphocytes and in CD3+ T lymphocytes. This suggests that IL-1Rrp2 ligands may exert direct stimulatory effects on lymphocytes, although time did not allow further studies regarding this possibility in this thesis. Using a mouse model, one study found that by acting directly on CD4+ T cells, IL-1β enhances antigen-driven expansion and differentiation *in vivo and in vitro* (Ben-Sasson *et al.,* 2009). Results observed in the present study show that human T cells express IL-1Rrp2, suggesting that they could be a direct target for IL-1Rrp2 ligands such as IL-1F8.

In previous initial studies involving the newer IL-1 family members, Debets *et al.,* (2001) demonstrated that IL-1F9 activates NF-κB in IL-1Rrp2-transfected Jurkat T cells (a T lymphocyte cell line). Towne *et al.,* (2004) extended this finding by showing that IL-1F6 and IL-1F8, in addition to IL-1F9, activate the pathway leading to NF-κB in an IL-1Rrp2/IL-1RAcP-dependent manner in Jurkat cells as well as in multiple other human and mouse cell lines. However, when Magne *et al.,* (2006) investigated endogenous IL-1Rrp2 expression in human cell lines they did not observe constitutive IL-1Rrp2 expression in Jurkat cell lines, which explains why previous researchers had to transfect (Debets *et al.,* 2001).

Although Jurkat T cells are a T cell leukaemia cell line (Gillis and Watson, 1980), there are a number of reasons why they may have failed to show constitutive IL-1Rrp2 expression in Magne *et al.* (2006)'s study even though total lymphocytes and CD3+ T lymphocytes in the present study showed IL-1Rrp2 expression. Being a tumour cell line, Jurkat T cells probably have aberrant expression or totally lack expression of the IL-1Rrp2 protein. It is not unusual for tumour cells to lack expression of some surface molecules (reviewed in Yokota, 2000). A number of studies have found that Jurkat T cells either do not express certain molecules that are normally found on T cells or they show reduced expression levels. One study found that Jurkat cells lack surface expression of Fas/CD95; a type-I membrane glycoprotein which induces apoptotic cell death (Monleón *et al.,* 2002). Another study which used Jurkat cells to investigate glucocorticoid resistance in lymphoid malignancies found that deficient Glucocorticoid Receptor (GR) expression in Jurkat cells was due to a mutated GR allele (Riml *et al.,* 2004). In view of these previous findings, it can be hypothesised that Jurkat T cells either lack the IL-1Rrp2 protein or they have a mutated version; hence the reported absence of constitutive IL-1Rrp2 expression (Magne *et al.,* 2006). Further studies are needed to confirm this hypothesis.

The ability of IL-1F8-stimulated MDDCs to activate CD3+ T cells and to induce their proliferation has already been demonstrated in the current study. It has already been shown that IL-1F8-matured MDDCs secrete cytokines which

induce T_H1 polarization leading to secretion of inflammatory cytokines (Chapter 3, partially quoted in Mutamba *et al.,* 2012). If indeed human CD3+ T cells express IL-1Rrp2 as results presented in this chapter suggest, it can be extrapolated that IL-1 cytokines which signal via IL-1Rrp2 can also stimulate T lymphocytes directly. Further studies are needed to investigate the effect of such direct stimulation and also to establish IL-1Rrp2 expression in all human T cell subsets as well as human B cells and B cell subsets.

The present study also investigated IL-1Rrp2 expression in human granulocytes. Granulocytes are polymorphonuclear cells (PMCs) characterized by the presence of differently staining granules in their cytoplasm when viewed under light microscopy. They can be divided into three subtypes; neutrophils, eosinophils, and basophils. Eosinophils make up about 3% of total circulating white blood cells and basophils constitute less than 1%. Collectively, granulocytes phagocytose pathogens, secrete toxic granules or histamines, contribute to inflammatory responses and regulate innate immune responses. Neutrophils are the most abundant type of phagocyte and make up 50% to 60% of the total circulating white blood cell population. They also form the major proportion of granulocytes in mammalian peripheral blood (Hoffbrand *et al.,* 2001).

Recruitment of neutrophils to sites of inflammation is promoted by adhesion molecules and chemokines expressed on endothelium following its activation by IL-1β and tumour necrosis factor (TNF)-α (Furie and McHugh, 1989). Previous studies have shown that IL-1α/β can also affect human neutrophils directly through interaction with IL-1 receptors which are present on human neutrophils (Rhyne *et al.,* 1988; Parker *et al.,* 1989). Other studies have shown that human neutrophils can regulate their expression of IL-1R2 in response to IL-1β or other stimulants both *in vivo* and *in vitro*. Increased expression of the IL-1R2 receptor was observed on blood neutrophils of humans with the sepsis syndrome (Fasano *et al.,* 1991).

In another study, when human neutrophils obtained from healthy donors were incubated with various doses of IL-1β, IL-1β specifically down-modulated IL-1 binding to neutrophils by reducing IL-1R2 expression. On the other hand, when IL-1β was intravenously administered into healthy donors followed by assessment of peripheral blood neutrophils, up-regulation of IL-1R2 expression and up-modulation of IL-1β binding to neutrophils was observed (Shieh *et al.,* 1993). This difference between *in vitro* and *in vivo* effects of IL-1β on human neutrophils could be because there may be an indirect effect *in vivo* owing to the involvement of other biological molecules induced by IL-1β. It was recently shown that human neutrophils also express IL-1R1 and respond to *in-vitro* IL-1α/β stimulation by up-regulation of chemokines, both at gene and protein levels,

and the effect is blocked by pretreatment with IL-1Ra (IL-1F3) (Paulsson *et al.,* 2012). During initial stages of the inflammatory reaction, neutrophils are the dominant mediating cells. Reports that neutrophils respond to *in-vitro* IL-1α/β stimulation by up-regulation of chemokines show that neutrophils are important modulators of the proinflammatory environment (Nathan, 2006; Megiovanni *et al.* 2006). It remains to be established whether neutrophils respond to stimulation by the newer IL-1 family members.

To date, the expression of IL-1Rrp2 (or its ligands) by human neutrophils has not been extensively studied. The present study sought to address this by studying IL-1Rrp2 expression in human granulocytes since neutrophils make up more than 95% of the total granulocyte population in normal patients (Hoffbrand *et al.,* 2001). Both quantitative RT-PCR and fluorescence microscopy methods did not detect IL-1Rrp2 expression in human granulocytes in this study. This suggests that IL-1Rrp2 ligands do not have a direct effect on human neutrophils. Another possibility is that neutrophils normally express IL-1Rrp2 at levels that are below the detection level of the methods used in this study. This is certainly the case with regards to IL-1R1/R2 expression on resting (unstimulated) neutrophils where the number of IL-1R has been estimated previously as 500–900 receptors per cell (Rhyne *et al.,* 1988; Parker *et al.,* 1989). Low numbers of receptors (less than 2000 molecules of target antigen per cell) may be difficult to detect by conventional qRT-PCR or immunofluorescence techniques (Zola *et al.,* 1990). Since low receptor expression may still be biologically significant, further work

needs to be done to establish whether stimulated neutrophilic granulocytes express IL-1Rrp2.

The present study found IL-1Rrp2 expression in lamina propria (LP) cells isolated from histologically normal human small intestine (ileum) mucosa following removal of the epithelium. This finding is consistent with the report by Towne *et al.* (2004) which shows that IL-1Rrp2 is expressed in very low amounts in the human small intestine, however, in their study; Towne *et al.* (2004) used whole tissue extract rather than LP cells.

Results described in this chapter, which show that LP cells express IL-1Rrp2, therefore add a new dimension to studies which investigate IL-1Rrp2 expression in humans. Furthermore, these results indicate that lamina propria mononuclear cells (and not epithelial cells) may be the source of the IL-1Rrp2 expression found in the small intestine (Towne *et al.,* 2004). However, although histologically normal human small intestine mucosae were used as a source of the LP cells used in our study, the patients from whom the samples were obtained were resected for cancer or IBD and these underlying conditions could have had an effect on IL-1Rrp2 expression.

Using an *in vitro* model in which normal human intestinal mucosal samples from which the surface epithelium had been removed were kept in culture, Yahida *et al.,* (1997) demonstrated the migration of large numbers of cells (mostly T cells; 63.4-73.6% and macrophages; 9.2-11.8%) out of the lamina propria of the normal human small and large intestine via basement membrane pores following loss of the surface epithelium. They speculated that this *in vitro* model reflected the *in vivo* host defence mechanism following loss of surface epithelial cells as a result of injury.

The intestinal mucosa is a battlefield between the immune system, pathogens, and dietary and environmental antigens. It is now known that more T cells are found in the gut-associated lymphoid tissue (GALT), intestinal lamina propria and epithelium than in the rest of the body (reviewed in Maynard and Weaver, 2009). T cells in the lamina propria receive signals from epithelial cells, stromal cells, and the matrix itself via integrin receptors (reviewed in Maynard and Weaver, 2009).They are also closely associated with dendritic cells and macrophages. Many studies in humans have shown that the CD4+ cells in normal gut lamina propria have the phenotype of activated effector T cells, probably in response to diet, or intestinal microorganisms (reviewed in Maynard and Weaver, 2009).

IL-1Rrp2 ligands have been implicated in the pathogenesis of cytokine-mediated, inflammatory diseases such as psoriasis (Blumberg *et al.,* 2007; Blumberg *et al.,*

2010). An imbalance between pro- and anti-inflammatory cytokines produced within the intestinal mucosa is also thought to be behind the initiation and perpetuation of inflammation in intestinal diseases such as Crohn's disease (Colpaert *et al.,* 2002). The finding that lamina propria cells express IL-1Rrp2 is interesting since it suggests that proinflammatory IL-1Rrp2 ligands (IL-1F6, IL-1F8 or IL-1F9) may act on these cells. Results in this thesis, therefore, may indicate that treatment strategies which target IL-1Rrp2, or its ligands, may have future therapeutic value in conditions such as IBD, although much greater investigation regarding the biology of IL-1Rrp2 in lamina propria cells needs to be performed.

Further studies are needed to identify the IL-1Rrp2-expressing cells within the lamina propria cell population; time constraints hindered this pursuit in the present study. However, based on published reports and findings from the current research project, the most likely source would have been T cells and/or DCs. In the previously mentioned *in vitro* model which showed migration of cells into and out of the lamina propria, T cells were the most abundant emigrating population (Mahida *et al.,* 1997). Although migration of macrophages and eosinophils was also observed in that study, the possibility of either of these two cell populations being the IL-1Rrp2-expressing cells in the lamina propria cells used in the current study can be ruled out since IL-1Rrp2 expression was not observed in macrophages (Chapter 3, Mutamba *et al.,* 2012) or granulocytic eosinophils in the current study.

Speculation that T cells and/or DCs were the IL-1Rrp2-expressing LP cells in the current study is based on the observation that CD3+ T cells (Chapter 4), MDDCs and pDCS (Chapter 3) showed strong IL-1Rrp2 expression in the present study. Furthermore, it is now known that human intestinal LP contains an abundance of DC subtypes (including pDCs) and T cells which play a vital role in intestinal immune defence and gut tolerance but can also play a role in the pathogenesis of chronic inflammatory disorders such as IBD (Wu et al., 1999; Rescigno *et al.,* 2001; Maerten *et al.,* 2004; Neiss *et al.,* 2005; Sun *et al.,* 2007; reviewed in Coombes and Powrie, 2008; reviewed in Maynard and Weaver, 2009; Varol *et al.,* 2009; Dillon *et al.,* 2010). Clearly, further work is needed to take the current findings forward.

The present study confirmed that NCI/ADR-RES cells show very strong IL-1Rrp2 mRNA expression. Towne *et al.,* (2004) reported that out of all the human cell lines that they tested, NCI/ADR-RES cells showed the highest level of IL-1Rrp2 expression. In the current study, NCI/ADR-RES cells showed the highest constitutive IL-1Rrp2 mRNA expression out of all primary cells and cell lines we tested. The significance of this finding needs to be investigated further. However, identifying a cell line which strongly expresses IL-1Rrp2 constitutively is invaluable given the attention that IL-1Rrp2 and its ligands are currently getting. NCI/ADR-RES could prove to be just as useful in research involving the newer IL-1 family members as they have been in cancer research.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

IL-1Rrp2 and the signal-transducing sub-unit, IL-1RAcP, form the receptor complex through which the newer IL-1 family members, IL-1F6, IL-1F8 and IL-1F9 signal (Debets *et al.,* 2001; Towne *et al.,* 2004). Highest expression of IL-1Rrp2 ligands has been reported in skin and epithelial tissue of internal organs that are exposed to pathogens, possibly suggesting that these ligands play a role in inflammatory immune responses in these organs (Kumar *et al.,* 2000; Debets *et al.,* 2001; Towne *et al.,* 2004). It has been shown that like other IL-1 family members (e.g. IL-1α and lL-1β), lL-1F6, IL-1F8 and IL-1F9 stimulate signalling pathways which result in activation of NFκB and MAPKs (Towne *et al.,* 2004) and that IL-1F5 acts as an antagonist for these ligands in a similar manner to the way IL-1Ra antagonises IL-1β activity (Debets *et al.,* 2001; Blumberg *et al.,* 2007; Towne *et al.,* 2011).

Classical IL-1 family members, such as IL-1α and lL-1β, are important not only in the initiation of protective immunity but also in the development of inflammatory pathology (reviewed in Dinarello, 1984; 1993; 1996; 1997; 2000; 2002). The role of IL-1F6, IL-1F8 and IL-1F9 in activating responses that augment immunity and promote inflammation is becoming elucidated, thanks to recent studies (Blumberg *et al.,* 2007; Blumberg *et al.,* 2010; Johnston *et al.,* 2011; Carrier *et al.,* 2011). However, to date, very little has been reported regarding the role of novel IL-1 cytokines in human immune cells which are known to be activated by

and/or produce classical members of the IL-1 family.

The overall objective of the work presented in this thesis was to investigate the expression and biological response of the IL-1Rrp2 receptor in various human immune cells. Interest in this receptor is growing, not only because it is the receptor through which potentially inflammatory cytokines (IL-1F6, IL-1F8 and IL-1F9) signal and hence its expression profile points to the cells/tissues which are potential targets for its ligands, but also because of its potential role as a therapeutic target in certain inflammatory diseases. Recently, IL-1Rrp2 blockade was observed to resolve the inflammatory changes in human psoriatic lesional skin that had been transplanted onto immunodeficient mice (Blumberg *et al.,* 2010). Furthermore, IL-1F5, which antagonises the inflammatory effect of IL-1F6 (and of IL-1F8 and IL-1F9) *in vitro* (Debets *et al.,* 2001; Towne *et al.,* 2011) and *in vivo* (Blumberg *et al.,* 2007) also signals via IL-1Rrp2 and may possibly be used therapeutically as an anti-inflammatory in future.

A number of previously unreported findings are presented in this thesis. Expression of IL-1Rrp2 in human immune cells had not been extensively studied previously. The current thesis reports the first extensive study of IL-1Rrp2 expression in human myeloid and non-myeloid immune cells. It is the first study focusing on investigating IL-1Rrp2 expression in human DC subsets, monocytes, macrophages, lymphocytes and granulocytes in addition to other cells and cell

lines. The current study reports for the first time that within the myelomonocytic lineage, IL-1Rrp2 expression is unique to DCs. MDDCs and pDCs showed constitutive IL-1Rrp2 expression while mDC1, mDC2, monocytes and macrophages did not. By showing differences in IL-1Rrp2 expression, not only between MDDCs and peripheral blood DCs, but indeed among peripheral blood DC subsets, this study also provides further evidence of the complexity of DC biology, plasticity and crosstalk (reviewed in Liu *et al.,* 2001; Liu, 2001).

A number of reports have highlighted the differences between myeloid DCs and pDCs and even suggested that they originate from different haematopoietic lineages (Galy *et al.,* 1995; Liu et al., 2001; reviewed in Shortman and Liu, 2002). The idea that pDCs are of lymphoid origin was initially supported by findings that genes originally found to be expressed only in developing T and B cells, such as pre-TCR-α, λ5, and Spi-B, are also expressed in pDCs, but not in myeloid cells (Res *et al.,* 1999; Bendriss-Vermare et al., 2001. Furthermore, the inhibition of Id2/Id3 expression was shown to prevent the development of pDCs, but not that of myeloid DCs, hence suggesting a lymphoid origin for pDCs (Spits *et al.,* 2000). However, other studies have proposed a myeloid origin for pDCs based on the finding that in humans, IL-3R^{high} pDCs were derived from CD34^{+} M-CSFR^{+} progenitors (Olweus *et al.,* 1997) and also because mice deficient in interferon (IFN) consensus sequence-binding protein (ICSBP), a critical transcription factor in myeloid cell differentiation, display loss of pDCs (Schiavoni *et al.,* 2002), suggesting that ICSBP may also be essential in pDC development.

Another proposal is the view that pDCs can originate from both lymphoid progenitors and myeloid progenitors based on mouse studies showing that both lymphoid and myeloid progenitors have the potential to generate pDCs (Zuniga *et al.,* 2004; Yang *et al.,* 2005; Karsunky *et al.,* 2005; Ishikawa *et al.,* 2007), bringing into question the view that there is a distinct and separate lineage specific development for mDCs and pDCs as previously proposed (Galy *et al.,* 1995; Liu et al., 2001; reviewed in Shortman and Liu, 2002). Nevertheless, it is not yet clear whether there is a phenotypic or indeed functional difference between pDCs of myeloid origin and those of lymphoid origin.

The current study provides an interesting dimension by showing some common features between pDCs and MDDCs, as well as differences. As an example, both MDDCs and pDCs constitutively express IL-1Rrp2. Furthermore, they both regulate IL-1Rrp2 expression in response to stimulation with IL-1F8 and IL-1β, suggesting that IL-1Rrp2 and its ligands may have a role in mediating immune processes involving these cells. However, there is a difference in the way they respond to stimulation. While MDDCs respond to stimulation by up-regulating IL-1Rrp2 expression, pDCS respond by down-regulating IL-1Rrp2 expression, suggesting functional differences between these two DC types. The phenotypic and functional differences between mDCs and pDCs have already been highlighted (Chapter 1 and Chapter 3 of this thesis). Myeloid DCs and pDCs show differences not only in terms of phenotypic markers but also with regards to cytokine secretion pattern, chemokine receptor expression and pathogen

recognition receptor (PRR) expression, suggesting differences in functional properties among these DC subsets (Sallusto and Lanzavecchia, 2002; reviewed in Banchereau *et al*., 2003).

The differences between MDDCs and pDCs in terms of regulation of IL-1Rrp2 expression as observed in this study suggests that regulation of IL-1Rrp2 expression varies depending on cell/ tissue type, nature of immunological challenge, concentration of stimulant and duration of stimulation. Clearly, DC biology is more intricate than previously appreciated. The functional plasticity model for DC origin and development proposes that DCs arise from a single haematopoietic lineage and that local environmental influences determine subtype development (reviewed in Shortman and Liu, 2002), thus, whether or not myeloid DCs and pDCs are different activation states of a single lineage is yet to be revealed.

Another interesting finding from the current study is the observation that in a similar manner to IL-1β (IL-1F2) and IFN-γ, IL-1Rrp2 ligands (IL-1F8 and IL-1F9) induce maturation of human MDDCs as measured by increased expression of HLA-DR and CD83 and decreased expression of CD1a. Further evidence that IL-1Rrp2 ligands induce MDDC maturation is shown by upregulation of accessory/co-stimulatory molecules (CD40 and CD80) which are critical for T cell activation. *In vivo*, DCs are the principal activators of naive T cells (Palucka

and Banchereau, 1999). Following maturation with an appropriate stimulus, myeloid DCs upregulate surface markers (such as CD40, CD80, CD86) and migration markers such as CCR7, and can efficiently prime naive T cells (reviewed in Banchereau *et al.,* 2000). Co-administration of costimulatory molecules (CD40, CD80 and CD86) with a protective antigen target has been shown to enhance immune responses in vaccination studies using bovine (Maue *et al.,* 2004) and murine models (Blackstock, 2003). Thus, the ability of IL-1Rrp2 ligands used in this study to induce upregulation of CD40 and CD80 in MDDCs suggests that these cytokines could play an important role in augmenting vaccine-induced protection.

This study also showed that stimulation of MDDCs by IL-1Rrp2 ligands (represented by IL-1F8) leads to the production of cytokines (IL-12p70 and IL-18) by MDDCs, which favour T_H1 polarization, as evidenced by increased IFN-γ concentrations in allogeneic MDDC/T-cell co-cultures (quoted in Mutamba *et al.,* 2012). In view of the crucial role that DCs play in bridging innate and adaptive immunity, this finding, coupled with the observation that CD3+ T cells express IL-1Rrp2, provides new evidence that IL-1Rrp2 and its ligands play an important role in innate and adaptive immunity in humans. Of particular importance is the observation that IL-1F8 stimulation in MDDCS induces T_H1 polarization and hence inflammatory cytokine production. This provides further evidence of the potential role of IL-1Rrp2 and its ligands in inflammatory responses and suggests that they may also have a role as therapeutic targets.

The finding that CD3+ T cells express IL-1Rrp2, suggesting that IL-1Rrp2 ligands can stimulate them directly, is interesting in that it may suggest a role for IL-1Rrp2 ligands in antigen-presenting-cell-independent T cell-mediated adaptive immune responses. Further studies will help clarify whether IL-1Rrp2 ligands behave like IL-1β in terms of being able to directly act on T cells resulting in enhancement of antigen-driven expansion and differentiation as observed in mouse models (Ben-Sasson *et al.,* 2009). However, results from the current study which show that human CD3+ T cells express IL-1Rrp2 (suggesting that they can respond to stimulation by IL-1Rrp2 ligands) suggest that this could be the case. Our results are also consistent with a recent published report which shows that mouse DCs and CD4+ T lymphocytes express IL-1Rrp2 (which they referred to as IL-36R) and that both cell types respond to IL-1Rrp2 ligands (Vigne *et al.* 2011).

The current study also showed that MDDCs are functionally distinct from blood borne immature skin DCs (or $CD209^{+}/CD14^{+}$ blood monocytes). In line with reports that IL-1Rrp2 ligands are present in skin DCs (Langerhans cells) (Dunn *et al.,* 2001; Debets *et al.,* 2001; Johnston *et al.,* 2011) and also in view of emerging data showing a role for IL-1Rrp2 ligands in skin disorders such as psoriasis (Blumberg *et al.,* 2007; Blumberg *et al.,* 2010) plus findings from the current study which showed IL-1Rrp2 expression in human MDDCs, it was speculated that immature peripheral blood CD209+/CD14+ Langerhans cells would express IL-1Rrp2 and be matured by novel IL-1 cytokines. However,

under the experimental conditions used in this study, immature peripheral blood CD209+/CD14+ Langerhans cells proved to be functionally different from MDDCs with regards to IL-1Rrp2 expression, raising questions about previously suggested similarities between MDDCs and skin Langerhans cells (Geissmann *et al.,* 1998; Grassi *et al.,* 1998; Herbst *et al.,* 1998). Alternatively, it could well be that the CD209+/CD14+ population considered to be immature Langerhans cells in the current study is actually a rare monocyte population and not blood-borne precursors of dermal DCs, as previously reported (Larregina *et al.,* 2001; Turville *et al.,* 2002; Angel *et al.,* 2007). In addition, although MDDCs have served as a convenient *in vitro* model for DCs for a long time, their existence *in vivo* is questionable considering that there are differences in the levels of expression of CD14, CD1a, and DC-SIGN between *in vitro* derived MDDC and *in vivo* dermal DCs (which MDDCs are thought to resemble) (Turville *et al.,* 2002). There is a possibility that either MDDCs are just a unique *in vitro* DC phenotype which does not exist *in vivo* or alternatively, they exist *in vivo* as a completely separate DC population that is used to replenish exhausted peripheral DCs during infection (reviewed in Donaghy, Wilkinson and Cunningham, 2006). Further work would shed more light.

Classical IL-1 family members, IL-1α and IL-1β, as well as IL-F11 (a newer IL-1 family member), play a huge role in neutrophil migration which results in inflammation in diseases like RA (Faccioli *et al.,* 1990; Perretti *et al.,* 1993; Simbirtsev *et al.,* 2001; Jonsson, Allen and Peng, 2005; Oliveira *et al.,* 2008).

Furthermore, it is now appreciated that neutrophils can indirectly shape immune responses, for example, by delivering activation signals and antigenic molecules to DCs, resulting in the activation and maturation of DCs and subsequent activation of naive T lymphocytes and induction of antigen-specific responses (Megiovanni *et al.* 2006). In our study, granulocytic neutrophils did not express IL-1Rrp2, suggesting that IL-1Rrp2 ligands do not act on these cells, and hence IL-1Rrp2 ligands cannot cause neutrophil migration.

When Towne *et al.* (2004) searched for a cell line that naturally expressed IL-1Rrp2 and which could respond to IL-1F6, IL-1F8 and IL-1F9 without the need for further transfection, they only found IL-1Rrp2 expression in the following transformed cell lines: Colo205, SW48, HT29, HBT75, HaCAT and NCI/ADR-RES. The current study has confirmed that HT29 and NCI/ADR-RES cells express IL-1Rrp2 (Towne *et al.,* 2004) while THP-1 cells do not (Magne *et al.,* 2006). The current study also went a step further to test the functional status of THP-1 cells since they were going to be used as negative controls in qRT-PCR experiments investigating IL-1Rrp2 expression in our study (Appendix 1). The ability of THP-1 cells to induce TNF-α expression in response to LPS is evidence that they were functional. On the other hand, their inability to respond to IL-1F8 further confirms lack of IL-1Rrp2 expression as previously reported (Magne *et al.,* 2006) and as shown in the current study.

IL-1Rrp2 expression in NCI/ADR-RES (OVCAR-8) cells was also investigated in the present study. NCI/ADR-RES cells are a human ovarian cancer cell line but were previously thought to be a breast cancer cell line (Towne *et al.,* 2004; (Liscovitch and Ravid, 2007; Ke *et al.,* 2011). They are widely used as a multidrug-resistant model in cancer research (Liscovitch and Ravid, 2007). In their study, Towne *et al.,* reported that NCI/ADR-RES cells showed the highest IL-1Rrp2 expression among all cell lines tested, however, they referred to these cells as a breast cancer cell line. Realising that this cell line could be useful in studies involving IL-1Rrp2 ligands (based on reports that they highly expressed IL-1Rrp2) (Towne *et al.,* 2004), we set out to re-investigate IL-1Rrp2 in NCI/ADR-RES cells. Consistent with previous findings (Towne *et al.,* 2004) the present study found highest IL-1Rrp2 expression in NCI/ADR-RES cells, not just among the cell lines studied, but among all cells studied, suggesting that these cells could be useful in studying the biology of IL-1Rrp2 and its ligands . Furthermore, the finding is consistent with their new identity as human ovarian cancer cells since IL-1Rrp2 expression was reported in human ovaries (Towne *et al.,* 2004). Further studies would help establish the full significance of this finding.

The finding that human ileal lamina propria cells express IL-1Rrp2 mRNA is interesting as it suggests that IL-1Rrp2 ligands may play a part in inflammatory responses in the intestine. Classical IL-1 family members, IL-1α and IL-1β, are key mediators of inflammation in inflammatory bowel diseases like Crohn's

disease and ulcerative colitis (Youngman *et al.,* 1993; Ludwiczek *et al.,* 2004). It has been shown that in both of these diseases, the inflammatory IL-1 cytokines are generated by lamina propria cells and not by epithelial cells (Youngman *et al.,* 1993). Other studies have shown that IL-18 (IL-1F4) is expressed in the affected intestinal tissues of patients with Crohn's disease and that the cytokine is found in both intestinal epithelial cells as well as in lamina propria mononuclear cells (reviewed in Dinarello, 2002). Further studies using animal models would help clarify the role of IL-1Rrp2 ligands in intestinal inflammation.

Evidence from the present study suggests that IL-1Rrp2 ligands are functionally similar to IL-1β, albeit with lower levels of potency which other researchers have also previously noted (Towne *et al., 2004;* Magne *et al.,* 2006). A very recent study (Towne *et al.,* 2011) provided possible reasons for the lower levels of potency of IL-1Rrp2 ligands (compared with IL-1β) which we and others (Towne *et al.,* 2004; Magne *et al.,* 2006) noted. Previously, it had been speculated that, in a similar manner to the classical IL-1 cytokines (IL-1β and IL-18), IL-1Rrp2 ligands might require proteolytic cleavage in order to become fully active (Towne *et al.,* 2004; Magne *et al.,* 2006). Indeed, Towne *et al.* (2011) showed that truncation (removal of specific N-terminal amino acid(s) at a specific site) of IL-1F5, IL-F6, IL-1F8 and IL-1F9 (which they called IL-36Ra, IL-36α, IL-36β, and IL-36γ respectively) dramatically enhances their activity, suggesting that post-translational processing is required for full activity. We hypothesise that if truncated IL-1Rrp2 ligands had been used in our study, increased effects

(probably matching IL-1β effects) would have been observed. If that is true, then there is a real possibility that IL-1Rrp2 ligands have similar or even higher biological activity to the other highly inflammatory IL-1 cytokines like IL-1β as suggested by a recent study which showed that truncated IL-1F6, IL-1F8 and IL-1F9 (all used at 100 ng/ml) were more potent than IL-1β (100 ng/ml) at inducing maturation of murine bone marrow derived DCs and subsequent inflammatory cytokine production (Vigne *et al.,* 2011). Further studies are needed to characterize the *in vivo* effects of these IL-1Rrp2 ligands and to identify the proteases that are responsible for their processing *in vivo*.

In conclusion, by investigating IL-1Rrp2 expression and its regulation, findings from the current study will help shed more light into the interaction between IL-1Rrp2 ligands and human immune and non-immune cells. By showing human dendritic cell IL-1Rrp2 expression and biological response, evidence from this study suggests that IL-1Rrp2 ligands may play a role in innate and adaptive immune responses. Classical IL-1 family members and receptors are established therapeutic targets for inflammatory diseases such as rheumatoid arthritis (RA) (Fleischmann, 2002; Cohen *et al.,* 2003). Recent data (Blumberg *et al.,* 2007; Blumberg *et al.,* 2010) and results from the present study suggest that IL-1Rrp2 and its ligands participate in inflammatory responses and could, therefore, be potential therapeutic targets for inflammatory diseases. Alternatively, these inflammatory IL-1Rrp2 ligands could also make good candidates as adjuvants. Although a great deal has been learnt about IL-1Rrp2

expression and biological response in human cells from the *in vitro* models used in this study, full understanding will only come from *in vivo* studies in the presence of disease.

CHAPTER 6: SCOPE FOR FURTHER RESEARCH

The work outlined in this thesis could be expanded by investigating the production and release of IL-1F6, IL-1F8 and IL-1F9 by human DC subsets and T cells, investigating the possibility of autocrine stimulation by IL-1Rrp2 ligands in human DCs subsets and T cells, investigating IL-1Rrp2 expression and biological response in human CD4+ T cells and CD8+ T cells, identifying the particular mononuclear cells expressing IL-1Rrp2 in lamina propria cells, assessing the *in vivo* effects of IL-1Rrp2 and its ligands by studying different disease models (e.g. colitis), studying *in vivo* expression of IL-1Rrp2 among human DC subsets and T cells, investigating whether a soluble form of IL-1Rrp2 which could serve as a decoy receptor exists, studying the *in vivo* effects of IL-1F6, IL-1F8 and IL-1F9, studying the effects of IL-1Rrp2 and IL-1Rrp2-ligand blockade in disease models and studying the effect of IL-1Rrp2 gene knock-out.

CHAPTER 7: BIBLIOGRAPHY

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APPENDIX 1: INVESTIGATION OF THE FUNCTIONAL STATUS OF IL-1RRP2-NEGATIVE THP-1 CELLS

Introduction

Based on published reports which stated that THP-1 cells did not express IL-1Rrp2 (Magne *et al,* 2006) and on results from our own preliminary experiments, THP-1 cells were used as a negative control in all qRT-PCR experiments investigating IL-1Rrp2 mRNA expression (Chapters 3 and 4). However, prior to using THP-1 cells as negative controls for IL-1Rrp2 expression in qRT-PCR studies presented in this thesis, their functional status was tested using an established system; measuring TNF-α mRNA expression by THP-1 cells in response to bacterial (*E. coli* 0127:B8 LPS) stimulation (Essner *et al*, 1990; Agarwal *et al*., 1995; Asakura *et al*., 1996; Dedrick and Conlon, 1995; Jones *et al*., 2003; Foster *et al*., 2005; Lackman and Cresswell, 2006).

THP-1 is a human monocytic cell line originally derived from the peripheral blood of a 1 year old boy with acute monocytic leukaemia by Tsuchiya and co-workers (1980). This cell line expresses Fc and C3b receptors but lacks surface or cytoplasmic immunoglobulins. THP-1 cells also lack Epstein-Barr virus-associated nuclear antigen. Furthermore, they show alpha-naphthyl butyrate esterase activities which can be inhibited by sodium fluoride. They also produce

lysozyme and are able to phagocytose latex particles and sensitized sheep red blood cells. In addition, they can restore T lymphocyte response to Concavalin A (a lectin protein which is also a lymphocyte mitogen) (Tsuchiya *et al*., 1980).

THP-1 cells are widely used as a substitute for human blood monocyte/macrophage models because they easily divide and can be maintained in cell culture for long periods (Auwerx, 1991). In the original culture, THP-1 cells maintained monocytic characteristics for over 14 months (Tsuchiya *et al*., 1980). THP-1 cells can be easily stimulated to differentiate along the monocytic pathway into human monocyte-derived macrophages (MDMs) by treating them with phorbol esters (Tsuchiya et al., 1982) or with 1,25-dihydroxyvitamin D3 (McCarthy *et al.,* 1983; Schwende *et al.*, 1996). Differentiation of THP-1 cells into mature THP-1 monocytes is accompanied by increased expression of CD14 (which is an LPS receptor) by the cells (Foster *et al*., 2005). Vitamin D_3 (1,25-dihydroxyvitamin $D_{3)}$ induces CD14 expression on THP-1 cells which in turn facilitates interaction of LPS with CD14 culminating in induction of cytokine expression. The binding of LPS to CD14 is facilitated by an LPS-binding protein (LBP) which is present in plasma (Gegner *et al.,* 1995; Galdiero *et al.,* 2001).

THP-1 cells can be further differentiated into immature human monocyte-derived dendritic cells (MDDCs) by culturing them with granulocyte-macrophage colony

stimulating factor (GM-CSF) plus interleukin-4 (IL-4) (Sallusto and Lanzavecchia, 1994; Berges *et al*., 2005). In this study, the response of THP-1 cells to LPS and IL-1F8 stimulation was used to confirm their functional status (Essner *et al*, 1990; Agarwal *et al*., 1995; Asakura *et al*., 1996; Dedrick and Conlon, 1995; Jones *et al*., 2003; Foster *et al*., 2005; Lackman and Cresswell, 2006).

Materials and Methods

THP-1 cells, obtained and cultured as previously stated (Chapter 2, Section 2.2.1), were plated at a density of 9 x10^5 cells per well on 24 well plates. Cells were stimulated for 6 or 24 hours with cell culture tested, γ-irradiated *E. coli* 0127:B8 LPS (100ng/ml) (Sigma-Aldrich, Poole, UK) or recombinant human IL-1F8 (100 ng/ml) (Amgen Corporation, Seattle, WA, USA) in RPMI 1640 supplemented with FCS (10% v/v) and L-Glutamine (2mM). Control samples were left unstimulated and were cultured in cytokine-free, RPMI 1640 medium only. Cells were kept in a humidified incubator at 37^{o}C and 5% CO_2 for the specified incubation times. At the end of the incubation period, cells were harvested and preserved in RNAlater Stabilization reagent (Qiagen, Crawley, UK) prior to total RNA extraction using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. 1µg of total RNA was reverse-transcribed into cDNA using random hexamer primers according to the manufacturer's recommendations (Transcriptor First Strand cDNA Synthesis Kit (Roche, West Sussex, UK). After primer optimization, gene-specific primers and

probes for TNF-α and the house-keeping gene (GAPDH), template cDNAs were amplified in a typical PCR reaction on a LightCycler® 480 Real-Time PCR analyser (Roche, West Sussex, UK) and ratios of TNF-α : GAPDH gene expression levels in each sample were automatically generated from crossing point values.

Results

TNF-α mRNA expression in THP-1 cells stimulated with *E. coli* 0127:B8 LPS or recombinant IL-1F8

THP-1 cells, obtained and cultured as already stated (Chapter 2, Section 2.2.1), were plated at a density of 9 x10^5 cells per well on 24 well plates. Cells were stimulated for 6 or 24 hours with cell culture tested, γ-irradiated *E. coli* 0127:B8 LPS (100ng/ml) (Sigma-Aldrich, Poole, UK) or recombinant IL-1F8 (100 ng/ml). Control samples were left unstimulated and were cultured in cytokine-free, RPMI 1640 medium only. Cells were kept in a humidified incubator at 37^{o}C and 5% CO_2 for the specified incubation times. At the end of the incubation period, RNA was extracted from the cells and qRT-PCR for TNF-α gene expression performed as previously stated (Chapter 2). GAPDH was used as the reference gene. Peripheral blood monocytes stimulated with LPS for 24 hours were used as the positive control. Unstimulated THP-1 cells were used as a negative control. Results are shown in the graph below (Figure A.1)

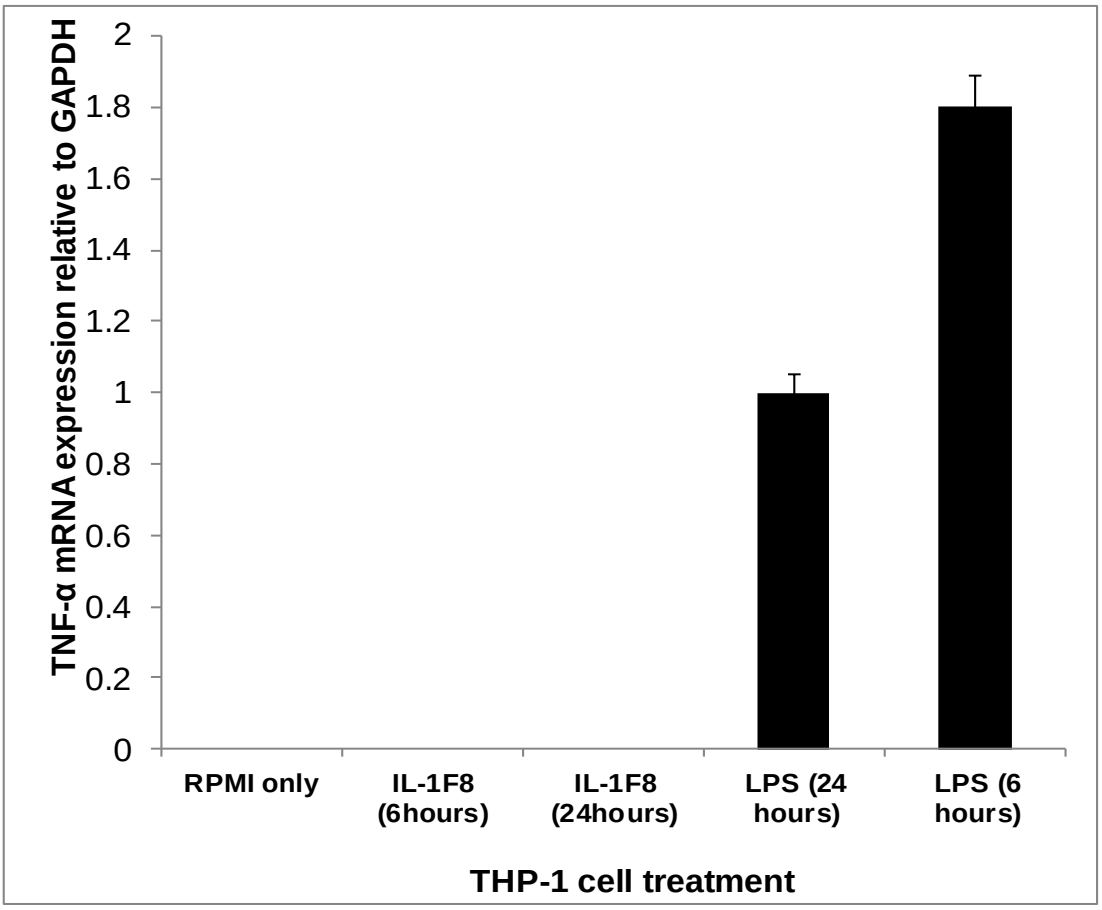

Figure A.1 TNF-α mRNA expression in LPS-stimulated THP-1 cells. Graph shows TNF-α mRNA expression results (measured by qRT-PCR relative to GAPDH) following culture of THP-1 cells with *E. coli* 0127:B8 LPS (100ng/ml), IL-1F8 (100ng/ml) or without stimulant (negative control) for 6 or 24 hours. Results show that unstimulated THP-1 cells (cultured in RPMI 1640 only) or THP-1 cells stimulated with IL-1F8 do not express TNF-α mRNA. On the other hand, E. *coli* 0127:B8 LPS stimulates TNF-α mRNA expression in THP-1 cells. Peak Levels of TNF-α mRNA expression are reached within the first 6 hours of encounter with LPS and are halved by 24 hours post-exposure. Data shown are mean± SD of at least three independent experiments. All analyses were performed in triplicate.

Results (Figure A.1) show that unstimulated THP-1 cells and THP-1 cells stimulated with IL-1F8 do not show TNF-α mRNA expression. On the other hand, THP-1 cells stimulated with *E. coli* 0127:B8 LPS show TNF-α mRNA expression. Peak Levels of TNF-α mRNA expression are reached within the first 6 hours of encounter with LPS and are halved by 24 hours post-exposure (Figure).

Discussion

The purpose of this experiment was to use a well-established method to confirm the functional status of THP-1 cells used as negative controls for IL-1Rrp2 expression. Human monocytes are known to secrete TNF-α following stimulation with bacterial LPS (Henricson *et al*., 1990; Agarwal *et al*., 1995; Dedrick and Conlon, 1995). As a monocytic cell line, functional THP-1 cells should respond in the same way. In this experiment, THP-1 cells were used as a substitute for human blood monocytes or macrophages. Results show that LPS-stimulated THP-1 samples express TNF-α mRNA. This finding is consistent with published literature pertaining to expression of TNF-α mRNA by human monocytes in response to LPS (Henricson *et al*., 1990; Agarwal *et al*., 1995; Dedrick and Conlon, 1995) and confirms that the THP-1 cells used in the present study were functional. The current experiment also showed that LPS-induced TNF-α mRNA expression levels are time-dependant. TNF-α mRNA expression in THP-1 samples stimulated with LPS for 6 hours was nearly twice as high as TNF-α mRNA expression in samples stimulated with LPS for 24 hours.

A number of other researchers have also observed a similar time-dependant trend in TNF-α mRNA expression by activated THP-1 cells (Baqui *et al*., 1998; Jones *et al*., 2003; Foster *et al*., 2005; Lackman and Cresswell, 2006). When Agarwal *et al.* (1995) activated healthy human monocytes with 100ng/ml LPS from *E. coli* strain B:0111 prior to measuring mRNA expression for TNF-α, IL-1β, IL-6 and IL-8, they observed that *E. coli* LPS induced mRNA for TNF-α, IL-1β and IL-8 within 30 minutes of monocyte activation. For IL-6, mRNA expression following LPS-activation occurred nearly an hour later. Generally, expression of mRNA for all cytokines continued to increase for an additional 2.5 hours. After 3 hours, expression of mRNA continued to increase for only IL-6 and IL-8 but not for TNF-α or IL-1β (Agarwal *et al*., 1995).

The time-dependant trend in TNF-α expression by THP-1 cells observed in the current and previous studies ties in perfectly with the biological role of TNF-α *in vivo.* Just like IL-1β, TNF-α is a proinflammatory cytokine with a broad range of effects on host immune responses. Some of the effects of TNF-α are induction of fever, activation of neutrophils, T cells and macrophages, anti-tumour activities, weight loss, lipolysis, loss of muscle, loss of visceral protein, anorexia, chronic nausea, weakness, suppression of lipoprotein lipase and induction of other mediators such as IL-1 cytokines (reviewed in Dinarello, 1993; 1996; 2000). As is the case with IL-1 cytokines, TNF-α synthesis and biological activity is normally tightly regulated. Administration of LPS *in vivo* is known to rapidly increase circulating levels of TNF-α. Under normal circumstances, levels of TNF-α tail off as in-built regulatory mechanisms kick in. Suppression of TNF-α

expression is accomplished through a number of strategies, including synthesis of potent anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (reviewed in Dinarello, 1993; 1996; 2000; Bruera, 1997).

The purpose of this study was to confirm that THP-1 cells which were used as negative controls in experiments investigating IL-1Rrp2 expression (Chapters 3, 4, and 5) were functional. The ability of *E.coli* LPS to induce TNF-α expression in monocytes is an established finding. Results from the current experiment confirm that THP-1 cells used in this study were functional as they showed TNF-α mRNA expression in response to stimulation with *E. coli* 0127:B8 LPS. By showing time-dependent TNF-α mRNA expression, it also successfully reflected the *in vivo* scenario whereby TNF-α mRNA production is rapidly switched on and off following encounter of monocytes/macrophages with pathogen (Dinarello, 2000; Taffet *et al*., 1989; Beutler and Cerami, 1989; Yu *et al*., 1990; Henricson *et al*., 1990; Zhong *et al*, 1993; Agarwal *et al*., 1995; Dedrick and Conlon, 1995).

Under the conditions used in the current experiment, THP-1 cells stimulated with IL-1F8 (used at a concentration of 100ng/ml) showed similar TNF-α expression levels to unstimulated negative controls (Figure A.1) suggesting that IL-1F8 does not induce TNF-α expression in THP-1 cells. The failure of IL-1F8 to induce TNF-α expression in monocytic THP-1 cells in the present experiment is an interesting finding. In previous studies, IL-1F6, IL-1F8 and IL-1F9 stimulated production of TNF-α and other cytokines in Jurkat cells (Debets *et al.,* 2001; Towne *et al.,* 2004) and epithelial cells (Towne *et al.,* 2004). The present study has shown that

IL-1F8 does not have a similar effect on THP-1 cells. THP-1 cells used in this study were clearly functionally viable as they were responsive to LPS. Failure of IL-1F8 to induce TNF-α in the THP-1 cells suggests that these cells either lack the IL-1F8 receptor (IL-1Rrp2) or they express it at very low levels. Throughout the preliminary and current research project, THP-1 cells consistently showed lack of IL-1Rrp2 expression and were used as negative controls in all studies investigating IL-1Rrp2 expression. Absence of IL-1Rrp2 expression in THP-1 cells has also been reported previously (Magne *et al.,* 2006). In a way, by showing that THP-1 cells are unresponsive to IL-1F8, the current study confirms that THP-1 cells do not express the IL-1Rrp2 receptor and is consistent with our report that human promonocytes do not express IL-1Rrp2. Considering that THP-1 cells served as a negative control in all qRT-PCR experiments investigating IL-1Rrp2 expression in the current research project (Chapters 3 and 4), establishing their functional status was an invaluable venture.