Investigations of *in vitro* innate responses to *Schistosoma mansoni*

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ABSTRACT

Schistosomes infect their hosts via a percutaneous route, during which they are likely to come into contact with numerous innate accessory cells, including those with potent antigen presenting cell (APC) function. In this thesis, innate interactions between skin-stage *Schistosoma mansoni* larvae and accessory cells were investigated *in vitro*. Molecules released by the parasite following transformation (0-3hRP) but not soluble preparations of whole larvae stimulated cytokine production by a variety of accessory cells independently of endotoxin. Furthermore, Interleukin (IL)-10 and IL-12p40 but not IL-6 production stimulated by 0-3hRP was partly dependent upon recognition by Toll-like receptor (TLR)4, whereas FcγRs had little clear effect upon the cytokine response. In addition, mannose receptor (MR) ligands were identified within 0-3hRP, suggesting numerous pattern recognition receptors (PRRs) are involved in schistosome recognition.

Investigation of the effects of 0-3hRP upon maturation of 'professional' APC demonstrated that dendritic cells (DC) responded by increasing cytokine production, up-regulating MHC II, CD40, and CD86 expression (but not CD80 or OX40L), and exhibiting an increased capacity to prime proliferation of $CD4^+$ cells *in vitro*. However, 0-3hRP induced an 'intermediate' state of maturation when compared to the microbial products LPS and Zymosan A. Further *in vitro* analysis demonstrated that DC matured with 0-3hRP primed $CD4^+$ cells to secrete a Th2 profile of cytokines. Moreover, inoculation of mice with 0-3hRP-matured DC led to the development of polarised Th2-type responses *in vivo*. Finally, treatment with anti-CD40 antibody reversed the capacity of 0-3hRP-matured DC to prime for Th2 responses instead causing the induction of Th1-type responses.

In conclusion, the innate immune system recognises schistosome material released upon infection, resulting in the stimulation of accessory cells. These putative schistosome 'pathogen-associated molecular patterns' (PAMPs) act to promote induction of Th2 acquired responses via their direct effect upon APC.

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DECLARATION

All of the research described in this thesis is my own with the following exceptions:

Chapter 2: In the later stages of my studies, Mrs Ann Bamford performed many of the parasite cultures.

Chapter 5: One cell-stimulation assay and the associated cytokine ELISA's were carried out by Dr. Karen Hogg.

Chapter 7: Subcutaneous injections were carried out by Dr. Adrian P. Mountford.

CHAPTER ONE

INTRODUCTION

INTRODUCTION OVERVIEW

Schistosomiasis is a serious parasitic disease of humans and has been the focus of intense scientific study. Although much work has centred upon the immune responses following vaccination with radiation-attenuated cercariae and the immune-related pathology induced by schistosome eggs, little is known about the initial innate response to infectious cercariae. Recently, the role of innate responses in the generation of acquired immunity to pathogens has become clearer, providing valuable insights into the mechanisms that may be involved during infection with schistosomes.

In this introduction, I will describe the evidence for innate and acquired immune responses during infection with *Schistosoma mansoni*, primarily in the murine model. This will cover;

- the induction of inflammatory and acquired responses generated by normal larvae during the initial stages of migration from the skin to the lungs,
- the induction of inflammatory and acquired / protective responses generated by radiation-attenuated larvae,
- and finally, the immune responses elicited to adult worms and to schistosome eggs.

I will then provide an overview of the current understanding of innate immune responses, focussing upon;

- the induction of innate responses by infectious agents and disease,
- the role of innate responses in the initiation of acquired immune responses,
- the role of innate responses in the polarisation of acquired immune responses.

PART 1 Parasite / Host interactions during schistosomiasis

1.1 Introduction to the disease

Schistosomiasis is a debilitating parasitic disease of both humans and livestock. It is prevalent in parts of sub-Saharan Africa, south and central America, and eastern Asia. Recent estimates suggest that 200 million people are infected worldwide, with 1 million mortalities per year. The WHO places it second in importance only to malaria in terms of morbidity, suggesting that it contributes considerably to the economic deprivation of many developing countries. Thus, schistosomiasis is a global disease of huge social and economic importance.

The disease is caused by infection with trematode helminth worms of the *Schistosoma* genus. There are three main species of schistosomes that infect man, *S. mansoni*, *S. haematobium*, and *S. japonicum*, although less frequently zoonoses can occur with a variety of other mammalian species. Of these species, most experimental work, including that presented in this thesis, has centred on *S. mansoni*.

Infection of the mammalian host follows exposure to water containing free-swimming cercariae that are released from the intermediate host (freshwater snail). Infective cercariae penetrate the skin of the host and transform into skin-stage schistosomulae. These larvae undergo a complex migration from the skin by an intra-vascular or intra-lymphatic route to the lungs, before reaching the liver where they mature to adults (reviewed by Wilson, 1987). The mature worms then form male and female pairs that migrate to either the hepatic portal / mesenteric veins (*S. mansoni* and *S. japonicum*), or the veins of the bladder (*S. haematobium*). Here, they take up a long-lived residency and female oviposition occurs approximately 5 weeks post-infection. The eggs cross into the lumen of the gut, or the bladder, from which they are excreted in faeces, or urine, respectively. The excreted eggs hatch upon contact with fresh water, releasing miracidia which infect the intermediate molluscan host.

Adult schistosomes live for an average of 5 to 10 years resulting in chronic infection of their hosts. The parasite has co-evolved with its human host to increase host / parasite

survival, thus increasing the chance of successful transmission. In this respect, schistosome infection results in a spectrum of chronic disease that is graded according to severity. Whilst the majority of cases are associated with low-level morbidity, termed "intestinal schistosomiasis", approximately 4% - 12% develop "hepatosplenic disease" characterised by severe fibrosis of the liver. This pathology is mainly due to the immune response to the egg. Unfortunately for the host, not all eggs released by the female exit from the body. Using experimental models of primate infection, it has been estimated that 10% of eggs are washed by the hepatic portal blood-flow to the liver (Farah *et al.*, 1997). Within the liver, eggs become trapped in the sinusoids and the host initiates the formation of inflammatory granulomas around them. These granulomas are associated with fibrotic scarring and destruction of the surrounding tissue. However, it should be noted that these granulomas appear to serve a protective role against hepatocytotoxicity induced by egg molecules, possibly by limiting or preventing toxin release (Dunne *et al.*, 1981; Doenhoff *et al.*, 1981; Dunne and Doenhoff, 1983; Murare *et al.*, 1992). Ultimately, the cumulative effect of fibrosis can lead to an increased portal blood pressure due to blockage of blood-flow, resulting in the development of oesophageal varices and upper gastro-intestinal bleeding, and contributing to splenomegaly. Indeed, pathology directly correlates with egg burden (Cheever *et al.*, 1977). In severe cases of chronic schistosomiasis these varices can rupture leading to death.

Control of schistosomiasis via the use of anti-helminthics, molluscicides, education, and improved sanitation is either too expensive, or has inherent difficulties in effective execution. Therefore, the development of a vaccine is a major goal of much of the current research into schistosomes (reviewed by Bergquist *et al.*, 2002). To be effective, a vaccine would have to limit infection, or limit infection-induced pathology. However, in designing vaccine strategies it is essential to understand the host immune responses that occur during normal infection, and those that may lead to protection. In this respect, study of innate immune responses to schistosomes during the initial stages of infection could provide valuable insights into early host / parasite interactions, and lead to a greater understanding of the events that culminate in the priming of the acquired immune response.

The scope of the work that can be covered studying human infection is limited for ethical and practical reasons. Therefore, experimental models of infection in laboratory animals have been developed. Indeed, the initial host / parasite interaction has often long passed

once a person presents with clinical disease, necessitating the use of animal models in this area of research. *S. mansoni* can infect many animals, including rodents and primates, and therefore numerous models of infection have been developed. Of these, the mouse model has been the most intensely studied due to both its wide availability and because it is fully permissive to infection. Using mice has the added advantage that they are the most widely-studied animals in immunological research. For these reasons, the mouse model forms the focus of my work presented in this thesis.

1.2 Parasite migration and development during infection.

1.2.1 Parasite migration

Cercariae need to successfully infect their mammalian host in order to develop into adults and continue the life-cycle. This requires the parasite to undergo a complex process of skin penetration and internal migration. Penetration is an active process achieved by both physical burrowing of the parasite and the release of proteolytic contents of the pre- and post-acetabular glands which digest a pathway through the stratum corneum and epidermis (reviewed by Salter and Mckerrow, 2002). During this period of penetration the cercariae transform into schistosomulae, lose their tails, and shed much (60%) of their surface glycocalyx (Samuelson and Caulfield, 1985). Within approximately 30 minutes, the larvae breach the stratum corneum and progress through to the basement membrane of the epidermis (Wilson and Lawson, 1980).

The basement membrane appears to impede parasite migration, and after remaining there for between 1 and 3 days the schistosomulae cross into dermis (Wheater and Wilson, 1979). The majority of larvae then exit the dermis via post-capillary venules but approximately 10% - 20% exit via lymph ducts (Wheater and Wilson, 1979; Mountford *et al.*, 1988). Those larvae that exit the skin via the vasculature are carried to the lungs directly by the venous blood flow, whilst lymphatic migration first requires traversal of the local skin draining lymph node (sdLN). The length of time it takes for schistosomulae to migrate from the skin varies considerably, with some larvae detectable in the lungs or sdLN as early as 2 days post-infection. The T½ of parasite migration from the skin varies between reports, and ranges from approximately 4 days (Miller and Wilson, 1978; Georgi,

1982; Mangold and Dean, 1984), to 5.3 days (Wilson *et al.*, 1986), and most likely reflects the technique used to assess migration.

Schistosomulae eventually arrive in the lungs, with peak numbers of parasites detected at approximately day 6 (Miller and Wilson, 1980) to day 7 (Wheater and Wilson, 1979; Mangold and Dean, 1983; Mangold and Dean, 1984; Wilson *et al.*, 1986). The lungs, with their complex narrow capillary network, represent a formidable obstacle to the parasite, apparent by the slow migration of the larvae through this organ (Wilson and Coulson, 1986). Indeed, following arrival at the lungs, larvae undergo considerable elongation, which is thought to be necessary for successful migration through the pulmonary vasculature (Wilson *et al.*, 1978).

It is generally accepted that after leaving the lungs, the parasites travel in the direction of blood flow via the pulmonary vein to the left side of the heart. From here, they are distributed by the systemic blood flow. Those larvae arriving in the splanchic organs negotiate the capillaries and are carried to the liver by the portal blood flow. Most become trapped in the hepatic sinusoids and develop into blood feeding worms (Wilson and Coulson, 1986). Those larvae that arrive in other organs traverse the capillary networks and return to the pulmonary vasculature whereupon the cycle of migration is repeated. Hepatic accumulation of the larvae appears to be complete by day 21 post-infection (Wilson *et al.*, 1986). Following arrival in the liver, larvae initiate blood-feeding and mature to adult worms. What causes maturation is not known. Upon pairing, adult worms migrate back up the veins of the hepatic portal system, where they take up residency in a state of permanent copulation.

1.2.2 Parasite attrition during migration

Parasite migration in naïve mice is associated with a large degree of attrition, with only about 40% - 50% reaching maturation (Mangold and Dean, 1983; Mangold *et al.*, 1986). Autoradiographic tracking of larvae showed that more than 90% of the parasites had exited the skin and were present at other host sites 14 days post-infection, demonstrating that the skin is not the major site of parasite death (Wilson *et al.*, 1986). Lymphatic migration also does not appear to affect parasite viability (Miller and Wilson, 1978; Mangold *et al.*, 1986). Since autoradiographic tracking demonstrates that the hepatic-portal worm

population is complete by day 21 post-infection, it is thought that migration of the remaining parasites must be halted and that their position indicates their place of death (Wilson *et al.*, 1986). Based upon this supposition, the lungs appear to be the predominant site of parasite attrition.

Death in the lungs has been indirectly associated with the entry of parasites into the alveoli (Crabtree and Wilson, 1986). Since, short-term residence in the alveoli does not affect parasite viability (Coulson and Wilson, 1988), it has been suggested that the mechanism of parasite death in the lungs is probably one of starvation through unsuccessful migration (Wilson, 1987).

The fact that less than 50% of larvae reach maturation suggests migration through the host is inherently difficult. However, a role for the immune system in parasite attrition cannot be ruled out. Several studies assessing different immune components have demonstrated that the absence IL-12 (Anderson *et al.*, 1998), IL-4 (King *et al.*, 1996a), IFNγ receptor (Wilson *et al.*, 1996), and TNF receptor I (Street *et al.*, 1999), do not affect the number of larvae reaching maturation. However, the absence of CD28 (King *et al.*, 1996b), or IgE (King *et al.*, 1997) does result in increased worm burdens, suggesting that parasite attrition may represent 'innate resistance' to schistosome infection.

Once larvae have matured to adults there is little evidence in the murine host that parasite attrition occurs. In addition, the longevity of adult survival during human infection also suggests there is little attrition of the parasites once they have reached this stage.

1.3 Early inflammatory responses to normal infection: from penetration to lung stage migration

Skin penetration and migration of larvae are highly invasive and relatively protracted events that involve the traversal of several immuno-competent organs. It would therefore seem likely that the onset of schistosome infection would trigger host innate / inflammatory immune responses. The evidence to support this hypothesis is described below.

1.3.1 Local inflammatory responses

Infection of the skin is accompanied by acute inflammation. Both histological and physiological examination demonstrates that the skin-exposure site approximately doubles in thickness 2 - 3 days after infection, although increased tissue thickness has been reported as early as 6 hr post-infection (Mastin *et al.*, 1983; Incani and McLaren, 1984). Inflammation is protracted, and although peaking 2 - 3 days post-infection (Mastin *et al.*, 1983) the skin stays significantly thickened up to day 14 (Mountford *et al.*, 2001), considerably later than the point when most parasites have left the skin. This inflammation represents thickening of both the epidermal and dermal layers (Incani and McLaren, 1984).

1.3.2 Local cellular responses

Infection-induced inflammation is accompanied by changes in both epidermal and dermal cellular constituents and tissue ultrastructure. Histological studies provide evidence of neutrophilic / granulocytic infiltration in the epidermis, as early as 3 hr post-infection, which form micro-abscesses in the epidermal squames by 6 hr (Incani and McLaren, 1984; Ward and McLaren, 1988; Elsaghier and McLaren, 1989). Some of these neutrophils showed signs of degeneration, whereas others contained phagosomes possibly filled with debris and parasite gland secretions and could be observed adhering to the schistosomulae (Incani and McLaren, 1984). Resolution of abscesses occurred by day 4 post-infection (Incani and McLaren, 1984) by which point most larvae have left the epidermis. This acute neutrophilia can be mimicked using an experimental model of parasite-induced chemotaxis, whereby intra-peritoneal injection of artificially-transformed schistosomulae results in the transient influx of neutrophils (2 - 12 hr) into the body cavity (Chao *et al.*, 1986). In addition, keratinocytes (which comprise the majority of the epidermal cells) in the vicinity of larvae show a considerable amount of hypertrophy (Incani and McLaren; 1984). More recently, immuno-histochemical analysis has demonstrated that epidermal accessory cells, which may be Langerhans cells (LC) up-regulate surface expression of the activation markers MHC II and CD86 from 1 and 12 hr post-infection respectively (Angeli *et al.*, 2001a). Although migration of these cells from the epidermis may be impaired during infection due to parasite-derived Prostaglandin D_2 (PGD_2; Angeli *et al.*, 2001a), this data suggests that infection may stimulate local antigen presenting cells (APC) to capture and present parasitic antigen.

In the dermis, inflammatory responses can be observed by one to two days post-infection and are characterised by oedema, hyperkeratosis, leukocyte and erythrocyte infiltration, and considerable vasodilation (Miller and Wilson, 1978; Wheater and Wilson, 1979; Mastin *et al.*, 1983; Incani and McLaren 1984; Ward and McLaren, 1988; Elsaghier and McLaren, 1989). This corresponds with the gross swelling of the skin described earlier. Specifically, dermal cellular infiltrate is initially diffuse, although some congregation below the basement membrane occurs. The infiltrate is comprised of both granulocytes, which are predominantly neutrophils (but include some mast cells), and mononuclear cells (Wilson and Wheater, 1979; Incani and McLaren, 1984; Ward and McLaren, 1988). Moreover, dermal MHC II^+ cells aggregate beneath epidermal larvae, again suggesting that local APC become activated (Riengrojpitak *et al.*, 1998). By days 3 - 4, the inflammatory infiltrate was mainly mononuclear, and not granulocytic (Elsaghier and McLaren, 1989). Moreover, these cells were organised into distinct foci (Wheater and Wilson, 1979), which suggests chemotaxis towards parasites or tissues damaged / stimulated by their presence. Notably, mast cells appeared to undergo degranulation, and free granules were seen amongst the other cell types (Incani and McLaren, 1984). Importantly, this may be a direct consequence of interaction with parasite molecules in the skin, since material released by cercariae has recently been shown to cause degranulation of a rat basophil cell-line (Machado *et al.*, 1996; Rao *et al.*, 2002). It is unlikely that the inflammatory infiltrate is due to haemorrhage caused by physical trauma of the vasculature, since intra-dermal injection of guinea pigs with cercarial homogenate, or material released by cercariae, resulted in rapid (30 min) oedema with an influx of neutrophils and eosinophils (Teixeira *et al.*, 1993). Notably, some cells in the vicinity of schistosomulae were seen to undergo apoptosis in the skin of infected mice, and it has been suggested that the larvae may actively induce cell death as a method of immune evasion (Chen *et al.*, 2002).

In contrast to the skin, the appearance of schistosomes in the sdLN 2 to 7 days post-infection did not correspond with a visible host leukocyte response using histological techniques, although cellular damage to host leucocytes in the vicinity of the parasite was observed (Wheater and Wilson, 1979). Similarly, with the arrival of schistosomes in the lungs there is initially no recognisable host inflammatory tissue response detectable at the gross level (Wheater and Wilson, 1979; Mastin *et al.*, 1983; von Lichtenberg *et al.*, 1985). Although larvae can adhere to lung microvasculature endothelial cells *in vitro* (via active

cell remodelling), suggesting direct interactions may occur *in vivo*, these cells appear to take on an anti-inflammatory phenotype (Trottein *et al.*, 1999a; 1999b). However, *in vivo* an inflammatory response develops by day 9 post-infection with increasing time post-infection (Menson and Wilson, 1990; Smythies *et al.*, 1992a), and cellular foci can be observed within the vicinity of the lung schistosomulae (von Lichtenberg *et al.*, 1985). In addition, alveolar macrophages increase their expression of MHC II and CR3 but decrease the expression of F4/80 (Menson and Wilson, 1990). It is possible that these inflammatory foci have a role in the parasite attrition that occurs in the lungs, potentially prohibiting the passage of larvae through the vasculature. In this respect, IL-6 $^{-/-}$ mice have greater numbers of leukocytes recruited to the alveoli in response to a secondary infection with schistosomes, and also a much greater level of parasite mortality in the lungs (Angeli *et al.*, 2001b).

1.3.3 Local cytokine production

Cytokines are the hormones of the immune system, allowing cells to communicate in paracrine, endocrine and autocrine fashion during responses to pathogens. The balance of regulatory and inflammatory cytokines is thought to play a key role in controlling immune responses to infection. Relatively few studies have addressed whether cytokines are produced in the skin in response to infection, and which cells are responsible for producing them. Wolowczuk *et al.* (1997) detected increased expression of IL-7 mRNA but not IL-2, IL-5 or IL-10 in the skin infection site from day 1 post-infection. It is noteworthy that increased IL-6 mRNA expression in the skin was also detected but not restricted to the site of infection, suggesting that parasite infection may lead to a general heightened inflammatory status of the host. Transient expression of IFNγ and IL-4 was also detected on day 5 post-infection (Wolowczuk *et al.*, 1997). In addition, Angeli *et al.* (2001a) detected increased TNFα, IL-1β, IL-4, IL-10, and TNFR-II but not inhibitory IL-1Ra mRNA expression in the epidermis from 1 to 120 hr post-infection. More recently, a technique for culturing split pinnae has allowed detailed examination of cytokine production by the infection site as a whole organ (Hogg *et al.*, 2003a). Using this technique, increased IL-1β, IL-6, IL-10, IL-12p40, IL-18, MIP-1α, MIP1β, and eotaxin, can be detected in the culture supernatant from day 1 (earliest time-point examined) to day 8 post-infection. Notably, production of IL-12p40 and IL-18 remained increased

(compared to naïve pinnae) up to day 14 post-infection, albeit at sub-peak quantities. *In vitro* work has demonstrated that schistosomulae and their products stimulate human and murine keratinocytes to produce IL-1Ra, IL-10, and PGE_2, which has been linked to parasite-derived PGE_2 production (Ramaswamy *et al.*, 1995a; 1995b; 2000). These host molecules have anti-inflammatory functions and may regulate the innate response during infection (Ramaswamy *et al.*, 2000). In addition, human dermal microvasculature endothelial cells produce increased levels of IL-7 following *in vitro* culture with schistosomulae, and IL-7 mRNA expression could be detected in the vessels of human skin grafts 6 days post-infection (Roye *et al.*, 1998).

1.3.4 Inflammatory response upon re-infection

Re-infection of mice previously exposed to cercariae results in increased dermal inflammation, characterised by more rapid and greater swelling and cellular influx compared to primary infection, indicative of an immediate hypersensitivity reaction (Incani and McLaren, 1984; Ramaswamy *et al.*, 1997). Indeed, re-infection is also characterised by a much greater eosinophilic influx into the tissue and greater mast cell degranulation in re-infected tissue. Larvae can also be observed invested in a surface coat, with neutrophils and eosinophils attached (Incani and McLaren, 1984). This response may be mediated by parasite-specific IgE which is thought to be both induced and directed, in part, against the proteases released by the parasite (Verwaerde *et al.*, 1986; 1988). Notably, skin from mice infected several times with the related schistosome *Trichobilharzia regenti* displays dramatic signs of type I hypersensitivity upon re-infection, with an abundant mast cell influx not seen in primary infection, and greatly increased levels of histamine, IL-1β, IL-4, IL-6 and IL-10 production by *in vitro*-cultured pinnae (Kourilova *et al.*, 2003).

1.3.5 Human inflammatory responses

The potent inflammatory responses generated in mice following exposure to schistosomes reflect the immediate hypersensitivity reaction observed in the skin (dermatitis) of some humans following infection (Farid *et al.*, 1987). Some patients also develop acute pulmonary pneumonitis. These illnesses are caused by immediate hypersensitivity responses thought to be a consequence of mast cell and basophil activation by skin and

lung-stage schistosomulae (Hofstetter *et al.*, 1982), although the pulmonary disease may also be caused by immune complexes between antibody and antigen from migrating lung schistosomulae (de Jesus *et al.*, 2002).

1.4 Acquired immune responses to normal infection: from penetration to lung-stage migration

1.4.1 Lymphocyte activity

Following infection with schistosomes, an acquired immune response is generated in both the sdLN and the mediastinal (lung-draining) LN (mLN). Cells from both sites are able to proliferate *in vitro* in response to re-stimulation with schistosome antigen, peaking on day 7 post-infection in the sdLN but much later at day 15 in the mLN (Pemberton *et al.*, 1991). Analysis of the cells within these organs demonstrates that both T-cell and B-cell numbers greatly increase *in vivo* following infection (Constant *et al.*, 1990), corresponding with an increase in organ weight (Pemberton and Wilson, 1995). Again, peak cell number in the sdLN occurs 4 - 7 days post-infection, but not until approximately day 28 in the mLN (Constant *et al.*, 1990; Pemberton *et al.*, 1995). Notably, the kinetics of cell proliferation *in vitro* and cell numbers *in vivo* follows the pattern of parasite migration through the skin to the lungs, and probably reflects the levels of parasite antigen presented within these organs.

A study using bronchoalveolar lavage (BAL) to analyse cellular events in lung alveoli demonstrated a transient influx of lymphocytes between days 14 and 21 post-infection (Smythies *et al.*, 1992a). These cells produced cytokines upon *in vitro* culture with soluble schistosomulae antigen (SSP) suggestive of antigen-specific lymphocytes. These studies imply that differentiated effector T-cells migrate to the lung during the latter periods of larval migration through this organ.

1.4.2 Cytokine production

Detailed analyses of the antigen-specific cytokine production by cultured sdLN cells showed that there was production of IL-2, IL-3, IL-4, IL-10, and IFNγ from day 4 post-

infection, suggestive of a mixed Th1 / Th2 or Th0-type response (Pemberton *et al.*, 1991; Hogg *et al.*, 2003a). However, IFNγ production peaked at day 4 post-infection whereas IL-4 production peaked at day 6, suggesting that Th1 responses slightly precede Th2 (Pemberton and Wilson, 1995). However, by day 14 post-infection both IFNγ and IL-4 production returned to near naïve levels (Pemberton and Wilson, 1995; Hogg *et al.*, 2003a). In addition, increased levels of IL-12p40 were produced by *in vitro* cultured sdLN cells from day 4 post-infection, which decreased but remained elevated above naïve levels by day 14 post-infection (Hogg *et al.*, 2003a). This cytokine is of particular interest due to its ability to drive Th1 polarisation.

A similar profile of IL-4 and IFNγ production was also detected upon re-stimulation of mLN cells, although peak production did not occur until day 15 post-infection (Pemberton *et al.*, 1991). As with cell proliferation, the kinetics of T-cell cytokine production probably reflects the levels of parasite antigen being presented within these organs.

Subsequent analysis of cytokine gene expression within the sdLN using semi-quantitative RT-PCR showed a similar cytokine profile to that produced *in vitro*, with Th1-associated IFNγ, and Th2-associated IL-4 transcripts being detected at day 5 post-infection (Betts and Wilson, 1998). However, by days 10 and 15 transcripts of IFNγ had reduced to levels within naïve animals, but IL-4 remained high and a small increase in IL-5 was detected, possibly suggesting the cytokine profile was becoming more Th2 dominated (Betts and Wilson, 1998).

Notably, infection with the related schistosome species *T. regenti* also results in a mixed Th1 / Th2 response, however, in multiply-infected mice re-infection results in an extremely Th2-polarised response, which is associated with Type I immediate hypersensitivity in the skin (Kourilova *et al.*, 2003). Therefore, it is possible that local hypersensitivity reactions will help to drive Th2 cytokine production.

1.5 Inflammatory and acquired immune responses to γ-irradiated cercariae

Although the initial interactions between the normal parasite and the host's immune system have received little attention, responses to vaccination with radiation-attenuated larvae are

relatively well studied, since this regime induces a high level of protective immunity to challenge infection (reviewed by Dunne and Mountford, 2001). A brief review of this work provides greater evidence of host inflammatory responses upon exposure to larvae, and an understanding of events involved in priming for this protective acquired response.

1.5.1 Inflammatory responses and APC stimulation in the skin

The presence of attenuated larvae results in a pro-inflammatory response in the skin exposure site (as determined by pinnae thickness and *in vitro* cytokine production) similar to that seen in normal infection, although some inflammatory parameters appear more protracted (Mastin *et al.*, 1983; Hogg *et al.*, 2003a). Immunohistochemical analysis demonstrates an influx of $7/4^+$ neutrophils into the dermis by day 2 post-vaccination (Kumkate and Mountford, unpublished data). Increased $CD11b^+$ populations were also detected, suggestive of them being macrophages (Mϕ). Furthermore, IL-$12p40^+$ cells were detected within the areas of greatest cellular influx (Hogg *et al.*, 2003a). Analysis of cells detaching from the pinnae during culture shows a dramatic increase in number by day 1 post-vaccination, reaching a peak by day 4 (Mountford *et al.*, 2001). Moreover, some of these cells were IL-$12p40^+$ and expressed CD11b, CD11c, and F4/80, suggestive of them being myeloid dendritic cells (DC) and / or possibly Mϕ (Hogg *et al.*, 2003a). These cells could be taken to be indicators of those contributing to dermal inflammation, but also indicate which types are capable of migrating to the sdLN to drive acquired responses.

Further evidence of APC activation by irradiated parasites is provided by immunohistochemical studies. Larvae in the epidermis were shown to attract and interact with MHC II^+ cells following migration into the dermis (Riengrojptak *et al.*, 1998). There were also increased numbers of $CD11c^+$ cells in the dermis, and as such may be dermal DC (Riengrojptak *et al.*, 1998; Hogg *et al.*, 2003a). Upon migration to the dermis, larvae become surrounded by $CD11c^+$ cells, which could be detected up to 15 days post-exposure (Riengrojptak *et al.*, 1998). In addition, Langerin$^+$ LC decrease in number in the epidermis after vaccination, suggesting maturation and migration of these cells (Kumkate *et al.*, 2003). Indeed, LC have been detected in the dermis below penetrating larvae immediately following vaccination. The apparent emigration of LC corresponds with increased numbers of Langerin$^+$ and MHC II^+ cells detectable in the sdLN (Kumkate *et al.*, 2003).

Furthermore, accessory cells in the periphery of the sdLN interact with migrating larvae, and appear to contain endosomal vesicles comprised of cellular debris (Riengrojptak *et al.*, 1998).

1.5.2 Acquired immune responses

When compared to normal infection, exposure to irradiated larvae results in a markedly different acquired immune response. Firstly, antigen-specific responses are more protracted in the sdLN and mLN from vaccinated mice, as judged by proliferation and cytokine production of *in vitro* re-stimulated LN cells (Pemberton *et al.*, 1991; Hogg *et al.*, 2003a). Moreover, cells from vaccinated mice produce greater levels of IFNγ but not IL-4. One interpretation of this data is that vaccination induces a more Th1 response, however, it could equally be said that normal infection induces a more Th2 response.

Several studies have attempted to determine what factors are involved in the induction of this protective acquired response. T-cell priming appears not to involve antibody or antigen-specific B-cells (Anderson *et al.*, 1999; Jankovic *et al.*, 1999). In contrast, T-cell IFNγ production is highly dependent on IL-12 production, since sdLN cells from IL-12p40-deficient mice produce greatly reduced levels of IFNγ and greatly increased levels of IL-4 both at the protein and mRNA level (Anderson *et al.*, 1998). Indeed, administration of recombinant IL-12 shortly after vaccination boosts IFNγ production (Anderson *et al.*, 1998). However, in situations where IL-12p40 is elevated at the site of infection (*e.g.* IL-4Rα $^{-/-}$ mice), production of IFNγ by sdLN does not increase and IL-4 does not decrease, although levels of IL-5 and IL-13 are lower (Mountford *et al.*, 2001). This suggests that IL-12p40 production at the site of infection may not increase Th1 responses but could decrease Th2 responses, and that T-cell IL-4 production is not dependent upon IL-4 or IL-13 signalling (since these cytokine receptors share the IL-4Rα chain). In addition, IL-10 appears to have a role in regulating the induction of protective pro-inflammatory responses, since IL-10-deficient mice develop more highly polarised and more protective Th1 responses, and exhibit greater inflammation at the skin site of exposure (Hoffman *et al.*, 1999; Hogg *et al.*, 2003b).

1.5.3 Challenge parasite death

A great deal of controversy surrounds the mechanism of challenge parasite death in vaccinated animals. Following a single vaccination, the role of antibody in schistosome death is highly debatable. Results from several studies of B-cell-deficient mice disagree with each other, suggesting B-cell effector mechanisms either do, or do not, contribute to resistance (Jankovic *et al.*, 1999; Anderson *et al.*, 1999). Moreover, mice deficient in several Fc receptors show no defect in vaccine-induced immunity (Jankovic *et al.*, 1999), but single vaccination of IL-4Rα-deficient mice results in impaired resistance associated with a lack of Th2-type antibodies (Mountford *et al.*, 2001). In contrast, a Th1 $CD4^+$ T-cell-mediated, IFNγ-dependent, mechanism of challenge parasite death is largely undisputed (Smythies *et al.*, 1992b; Wilson *et al.*, 1996; Wynn 1996; Jankovic *et al.*, 1999), and protection can even be boosted by addition of rIL-12 (Wynn *et al.*, 1995; 1996; Anderson *et al.*, 1998). In comparison to single exposure, there is clear evidence that antibodies play a role in the increased level of protection elicited by multiple vaccination (Caulada-Benedetti *et al.*, 1991; Hoffman *et al.*, 1999). Thus, both humoral and cell-mediated mechanisms can operate in challenge parasite death. This prompted Wilson and Coulson (1999) to propose the 'Happy Valley Hypothesis' suggesting that schistosomes can survive if there is a relatively ineffective mixed Th1 / Th2 response, but the environment becomes much more hostile if the response is strongly polarised to either pole (Wilson and Coulson, 1999). However, Wynn and Hoffman (2000) disagree, having recently demonstrated that high-level protective Th1 and Th2 responses can develop simultaneously in multiply-vaccinated mice (Hoffman *et al.*, 1999), and suggest that greatest protection will be afforded by a vaccine that elicits both strong humoral and cellular responses.

1.5.4 What is special about attenuated larvae?

What causes the difference in cytokine profile between normal infection and vaccination is the subject of much debate. Radiation-attenuated parasites undergo retarded migration through the skin and the sdLN with delayed arrival in the lungs. Larvae fail to migrate further than the lungs in which most parasites die within the first 3 weeks post-vaccination

(Mastin *et al.*, 1983; Mangold and Dean, 1984; Mountford *et al.*, 1988). Harrop and Wilson (1993a) observed that irradiated parasites had constrictions on their bodies indicative of contractions of circular muscle, and suggest that delayed migration may be due to a disruption in neuromuscular function.

Alternatively, a recent report suggests that irradiated parasites may stimulate less anti-inflammatory IL-10 production by host keratinocytes (Ramaswamy *et al.*, 2000). This could allow greater inflammatory responses to occur in the skin, which subsequently impair migration. However, Hogg *et al.* (2003a) demonstrate that there is little difference in pro-inflammatory cytokine production following vaccination compared to infection, although vaccination induces transiently lower levels of IL-10 production compared to infection, at early time-points.

Delayed migration may allow for greater deposition of antigens and pathogen-associated molecular patterns (PAMP), and more time for parasite / accessory cell interaction (Mountford *et al.*, 1988; Mountford *et al.*, 1992). In this respect, more parasite-released material was found in the sdLN of vaccinated mice compared to normally infected mice (Mountford *et al.*, 1988). However, it is possible that the process of attenuation causes the larvae to release more antigen material (Mountford *et al.*, 1988). There could also be other differences between attenuated and normal larvae. Chen *et al.* (2002) state that irradiated parasites do not release a T-cell apoptotic factor and thus cause less cellular apoptosis during migration. Alternatively, it has been suggested that irradiation may induce abnormal antigens that make these parasites more immunogenic (Wales *et al.*, 1992). However, a limitation of this theory is that, in order to be effective, a memory T-cell population would need to recognise unaltered parasite epitopes. In addition, the material contained within cercariae is pre-formed, with little *de novo* synthesis occurring until after 24 hr post-transformation (Harrop and Wilson, 1993b), therefore, it is likely that irradiated parasites have the same repertoire of molecules as normal cercariae. Moreover, there is not a great deal of difference in the initial (day 1 - 4) inflammatory immune response induced by vaccination compared to infection (Hogg *et al.*, 2003a), suggesting that there is little 'bio-chemical' difference between these larvae at this early stage. Therefore, if normal larvae express parasite PAMPs, the irradiated larvae should have the same PAMP complement, and thus APC which are stimulated by exposure to irradiated parasites are also likely to be stimulated in a similar fashion / mechanism by exposure to normal larvae.

1.6 Inflammatory and acquired responses to normal infection: adult worms and eggs

Many normal larvae successfully migrate through the lungs and proceed to mature in the liver. The onset of egg production leads to a dramatic increase in the levels of circulating antigen, with large quantities of soluble material released by the ova (Ashton *et al.*, 2001).

1.6.1 Acute infection

The high levels of foreign antigen result in immune hyper-responsiveness, with increased levels of both IFNγ and IL-4 detectable in infected mouse serum by 4 weeks-post infection (Wolowczuk *et al.*, 1997). Prior to the onset of egg production (3 weeks post-infection), splenocytes re-stimulated *in vitro* with SSP, or soluble adult worm antigen (SWAP), produced high levels of IFNγ and low levels of IL-5, and this is thought to be suggestive of a Th1 response (Pearce *et al.*, 1991; Grzych *et al.*, 1991). Following the onset of egg production, a dramatic switch is seen in the profile of cytokines produced by splenocytes upon re-stimulation with parasite antigens. This is characterised by down-regulation of the Th1 response in favour of a Th2-type cytokine profile (Pearce *et al.*, 1991). The role of these Th2 cytokines may be to inhibit, or reduce, the pathogenic effect of inflammatory mediators (Brunet *et al.*, 1997; Fallon *et al.*, 2000).

The ability of schistosome eggs to drive Th2 responses has been thoroughly investigated and the mechanisms leading to this switch are becoming more understood. Artificial 'egg only' infections (in which eggs are injected into recipients) are characterised by transient Th0 followed by Th2 responses (Vella and Pearce, 1992). Moreover, SEA preferentially drives Th2-related responses after administration *in vivo* as judged by cytokine and IgE production, a characteristic dependent on its carbohydrate constituents (Okano, *et al.*, 1999). Specifically, the pentasaccharide lacto-N-fucopentose III (LNFP III) appears to have Th2 adjuvant properties, driving acquired responses as well as polarising towards a Th2 phenotype (Okano *et al.*, 2001), suggesting it has PAMP characteristics. One mechanism by which these carbohydrate moieties could induce Th2 responses is through the induction of IL-10 production, and the expansion of IL-10 producing B-cells (Velupillai and Harn, 1994; Velupillai *et al.*, 1997), since this cytokine can inhibit pro-inflammatory Th1 responses (Section 1.12.3). Egg-induced IL-6 production by LN cells

has also been implicated in IL-10 production and Th2 polarisation (La Flamme *et al.*, 1999; 2000). A glycoprotein from SEA can also stimulate IL-4 production by human basophils *in vitro*, which might potentiate Th2 polarisation (Haisch *et al.*, 2001). Alternatively, it was recently shown that SEA can preferentially induce Th2 polarisation through its direct effect upon DC (MacDonald *et al.*, 2001). In this respect, the role of DC-derived cytokines and co-stimulatory signals in this polarising function has been explored in several recent publications and will be discussed later in chapters 6 and 7.

1.6.2 Chronic infection

The down-regulation of host inflammatory Th1 immune responses is critical for the progression to a chronic state of infection (Brunet *et al.*, 1997; Fallon *et al.*, 2000). The study of egg granulomas, which are mediated by egg-antigen specific $CD4^+$ T-cells (Mathew and Boros, 1986), suggests that in addition to modulation of inflammatory responses, chronic infection is characterised by down-regulation of immune responses *per se*, leading to a state of immune hypo-responsiveness (reviewed by King, 2001; Sadler *et al.*, 2003).

One potential mechanism that could induce immune hypo-responsiveness is the inhibition of T-cell proliferation. In this respect, exposure to glycolipids contained within SEA causes DC to prime for regulatory T-cells that suppress T-cell proliferation through IL-10 production (van de Kleij *et al.*, 2002). The LNFP III sugar from SEA also expands a subset of Mϕ (termed 'suppressors'), which can directly suppress T-cell proliferation and suppress Th1-type inflammatory cytokine production via contact-dependent and cytokine-dependent (IFNγ & IL-10) mechanisms (Atochina *et al.*, 2001; Terrazas *et al.*, 2001). Moreover, IL-10 appears to be critical in the down-regulation of the granulomatous immune response during chronic schistosomiasis (Flores-Villanueva *et al.*, 1996; Sadler *et al.*, 2003).

1.6.3 Human acute and chronic disease

The regulation of acquired immune responses depicted in the mouse model reflects much that is known about pathology observed during human infection. A study of patients with

clinically acute disease showed their peripheral blood mononuclear cells (PBMC) produced numerous pro-inflammatory cytokines, and higher levels of IFNγ but lower IL-5 when stimulated with SEA, or SWAP, compared to cells from patients with chronic schistosomiasis (de Jesus *et al.*, 2002). Immune hypo-responsiveness is also characteristic of the majority of chronic infections in the human population, and has been linked to IL-10 production (Malaquias *et al.*, 1997; King, 2001). Most individuals with chronic but relatively asymptomatic intestinal disease appear to develop Th2 patterns of cytokine production (Williams *et al.*, 1994; Araujo *et al.*, 1996). In contrast, development of more severe hepatic disease in a minority of patients is associated with immune hyper-responsiveness (Dunne and Pearce, 1999), characterised by greater antigen-specific PMBC proliferation (Bahia-Oliveira *et al.*, 1992), and elevated levels of the Th1 cytokines IFNγ and TNFα (Mwatha *et al.*, 1998; Henri *et al.*, 2002).

1.7 Summary

The first section of this introduction has provided the reader with the current body of knowledge surrounding the inflammatory innate responses and acquired immune responses that follow infection with normal schistosomes. This has demonstrated that acute inflammatory responses characterise the immediate events in the skin following infection with schistosomes. It is highly possible that these responses are a result of direct parasite recognition by the innate immune system, although it is equally possible that tissue damage caused by parasite penetration and migration may also stimulate the inflammatory cascade.

Following the innate response, a parasite-specific acquired response is generated in the local lymphoid tissues. This response may contribute to the considerable level of larval attrition that occurs during parasite migration, but may also lead to acute allergic hypersensitivity. However, compared to the relative wealth of information regarding the development and regulation of acquired responses to schistosomal egg antigens, it is not known what innate / inflammatory factors are important in the development of the acquired response during the early stages following infection. It is most likely that the initial inflammatory / innate reaction is critically important in this process. In this respect, the current body of knowledge surrounding the immune response following vaccination with radiation-attenuated larvae has been summarised. This provides a more in-depth

understanding of the orchestration of innate and acquired responses to larvae, and may indicate factors that are involved in the generation of acquired immune responses during normal infection.

Part II Innate immunity and its impact upon the induction of acquired immunity: innate recognition and T-cell polarisation

The innate immune system is the first line of defence against infectious agents. Whilst minor pathogen insults may be dealt with without the requirement of further 'immune help', the outcome of many infections is usually determined by the induction of highly specific adaptive / acquired immune responses. This event involves the recognition of non-self antigen peptides by lymphocytes through diverse, and therefore, highly specific receptors in the form of T-cell receptors (TCR) and B-cell receptors (BCR or soluble antibody). Clonal expansion of the antigen-specific lymphocytes leads to the generation of acquired effector responses, under the direction of cytokines produced by $CD4^+$ T-helper cells (Th). These effector responses can be divided into 'inflammatory' cell-mediated responses and humoral immune responses, and the generation of the correct type is thought to be key to the resolution or outcome of many infections.

Although the basic model of adaptive immunity was proposed many years ago, only relatively recently has it been proposed that the initiation of acquired responses, and more specifically the type of effector mechanisms employed, is dependent upon the initial innate immune response to the pathogen (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997). In this respect, it has been suggested that the functional outcome of the immune response to a pathogen is determined by the innate response (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997).

Therefore, I will briefly introduce the current theories surrounding innate recognition during infection, and the downstream events that culminate in the generation of acquired T-cell help and the polarisation of the effector response.

1.8 Current theories on innate recognition

The innate immune system is thought to be evolutionarily ancient compared to the acquired system. Murine innate immunity also closely resembles what is known about human innate immunity, therefore, both the human and murine systems will be discussed together.

The innate immune system differs fundamentally from the acquired system in the manner in which it recognises potential infections. Whereas the latter can generate a seemingly infinite repertoire of receptors (approximately 10^{11} specificities) by genetic recombination of TCR and BCR genes, the innate immune system relies upon germline-encoded receptors which are limited in number by the size of the genome. In addition, while acquired immunity is primarily concerned with recognition of pathogens, innate recognition has been the subject of much debate. In this respect, innate recognition can be grouped into 2 strategies: recognition of 'pathogen non-self' (or 'pattern recognition'), and recognition of changes in host tissue associated with infection / disease, termed 'danger' or 'extended-self'.

1.8.1 Pathogen 'non-self' recognition or pattern recognition

Janeway (1992) and Medzhitof and Janeway (1997) suggest that the innate immune system has evolved with the fundamental ability to discriminate between host molecules and pathogen molecules, enabling invading organisms to be destroyed without non-specific damage to the host. They propose that the innate immune system evolved a number of non-clonal receptors, termed pattern recognition receptors (PRRs), which recognise molecular motifs, or patterns, that are common to many different pathogens, termed PAMPs. Several evolutionary pressures have determined the nature of these PAMPs. Firstly, genetic limitations on the number of possible innate receptors, means PAMPs must be shared by large groups of pathogens, hence the recognition of patterns or motifs rather than unique / specific sequences. Second, the generally high rate of pathogen mutation has provided a great selection pressure to recognise only conserved PAMPs that are essential to pathogen survival, such that any mutation is lethal, or renders the organism non-pathogenic. Third, Medzhitof and Janeway suggest that PAMPs are absolutely distinct from self-antigens, thus allowing discrimination between self and non-self.

Many PAMPs have so far been identified, the majority of which appear to have glycan or lipid components. These include bacterial cell-wall components, (LPS, lipo-teichoic acid, peptidoglycan), viral double-stranded RNA (dsRNA; Alexopoulou *et al.*, 2001), yeast wall products (mannan, β-glucan), spirochete glycolipids (Schroder *et al.*, 2000) and non-methylated CpG DNA motifs which are characteristic of bacterial but not mammalian

DNA (Sparwasser *et al.*, 1998; Hemmi *et al.*, 2000). In addition, putative protozoan PAMPs have also been identified from Leishmanial species (Borges *et al.*, 2001; Hawn *et al.*, 2002) and Trypanosomes (Camargo *et al.*, 1997) including glycophosphoinsitol (GPI) anchors (Campos *et al.*, 2001). The intense amount of study in this field has meant that the number and types of PAMPs so far identified is still rapidly growing.

1.8.2 Danger theory of innate recognition

Originally proposed by Matzinger (1994), the danger model of innate awareness describes how innate accessory cells can become activated by signs of tissue distress that occur during infection. In this respect, molecules produced by stressed cells, such as heat-shock proteins (HSPs), or molecules released by damaged tissues and necrotic cells, are normally not visible to innate accessory cells and therefore their appearance could signal for 'danger'. Evidence supporting the existence of 'danger signals' is now starting to accumulate. Several groups have demonstrated that necrotic cells but not apoptotic cells stimulate DC (Gallucci *et al.*, 1999; Sauter *et al.*, 2000; Basu *et al.*, 2000). In addition, certain human HSPs, such as HSP60, HSP70, HSP 90, and gp96, activate Mϕ and / or DC (Basu *et al.*, 2000; Ohashi *et al.*, 2000; Kol *et al.*, 2000; reviewed by Wallin and Ljunggren, 2002), as do host molecules generated during tissue damage and inflammation (Okamura *et al.*, 2001; Smiley *et al.*, 2001; Termeer *et al.*, 2002; Johnson *et al.*, 2002), although this data may have been an artefact of endotoxin contaminants (Wallin and Ljunggren, 2002; Akira *et al.*, 2003). Interestingly, many of these host danger signals are highly conserved between species, and related HSP from microbes also stimulate the innate immune system (Kol *et al.*, 2000). Recognition of danger signals may occur through PRRs that also mediate recognition of PAMPs (Ohashi *et al.*, 2000; Kol *et al.*, 2000), indicating that innate discrimination between self- and non-self is not as rigid as current models suggest (Janeway and Medzhitov, 1997; Bendelac and Medzhitov, 2002). In this respect, Gallucci and Matzinger (2001) refer to PAMPs as 'exogenous danger signals'.

Under the blanket term of the 'danger model' could be included the 'induced-self' and 'missing-self' recognition of abnormal cells (reviewed by Diefenbach and Raulet, 2003). Induced-self recognition relies on stressed cells up-regulating surface expression of cellular molecules that are associated with 'abnormal' cells, whereas 'missing-self' recognition

describes the loss of inhibitory signals (supplied by the expression of certain surface molecules on normal cells) that may occur upon cellular damage.

This is a rapidly changing area of immunology and it should be noted that there is still much debate on the finer theoretical points of innate recognition (Medzhitov, 2001; Gallucci and Matzinger, 2001; Johnson *et al.*, 2003). However, for this thesis, the models discussed above represent adequate descriptions of the mechanisms of recognition.

Innate recognition of both exogenous and endogenous 'danger signals' could occur during the initial stages of infection with schistosomes. In this respect, schistosomes may express molecules with PAMP-like activity. In addition, penetration and migration of larvae causes a high degree of cell lysis and necrosis, and might induce cellular stress or changes in surface expression of regulatory molecules.

1.8.3 Pattern recognition receptors

PRRs are either soluble plasma proteins, or are present on the surface of innate accessory cells. These receptors are highly diverse in their nature and function and six distinct families have been identified (Medzitof and Janeway, 1997). These include the C-type lectins, such as the homologues DEC-205 and the mannose receptor (MR), integrins, such as the complement receptor 3 complex (CR3), leucine-rich receptors, scavenger receptors, pentraxins such as Serum amyloid P and C-reactive protein, and lipid transferases, like LPS-binding protein (LBP). Notably, the family of leucine-rich receptors termed Toll-like receptors (TLRs), due to their homology with *Drosophilia* Toll-receptor, appear to play a central role in the recognition and signalling of pathogen products. These have been the focus of much research and so far, 10 mammalian TLRs have been identified. Each of these TLRs appear to be involved in the recognition of distinct ligands, although direct ligand-binding by many of them has not been formally demonstrated.

Another property of some PRRs, is their ability to recognise several structurally related, or even unrelated ligands. This receptor promiscuity is not fully understood, but in the case of TLRs it may occur through the association with distinct accessory proteins / receptors. For example, TLR4 together with MD-2 and the PRRs CD14 and LBP, form the LPS receptor complex (reviewed by Underhill and Ozinsky, 2002). In addition, TLR2 dimerisation with

TLR1 and TLR6 contributes to the diverse array of TLR2 PAMP ligands (Ozinsky *et al.*, 2000). Table 1.1 demonstrates some of the known self and non-self ligands for PRRs.

Receptor Family	Example	Ligands
C-type lectins	MR	Terminal Mannose
	DEC-205	Terminal Mannose
Leucine-rich receptors	TLR2 + (TLR6 or TLRX)*	Bacterial lipoproteins
		Peptidoglycan
		Zymosan
		GPI anchor
		Lipoarabinomannan
	TLR3**	dsRNA
	TLR4	LPS
		HSP 60 (human and chlamydial)
		Fibronectin EDA
		F protein
	TLR5	Flagellin
	TLR9	CpG DNA
Scavenger receptors	Macrophage scavenger receptor	Bacterial cell wall components
Pentraxins	C-reactive protein	Phosphatidyl choline
		LPS
Lipid transferases	LBP	LPS
Intergrins	CD11b,c:CD18	LPS

***Table 1.1* Examples of pattern recognition receptors of the innate immune system and their ligands.** Table adapted from Medzhitov and Janeway (1997) and Underhill and Ozinsky (2002).

* TLRX = TLRs

** Alexopoulou *et al.*, 2001.

PRRs are highly diverse in their function. Soluble receptors are mainly involved in opsonisation of pathogens and activation of the complement effector pathway (Sastry and Ezekowitz, 1993; Mold *et al.*, 2001). In contrast, ligand binding of cellular PRRs can stimulate a variety of 'hard-wired' innate accessory cell functions, detailed below. Importantly, the type of response that is elicited is thought to be dependent upon the receptor (Akira *et al.*, 2003). For example, intracellular signalling pathways utilized by TLR4 have functional distinctions compared to other TLRs, and will be discussed in more detail in Chapter 3. In addition, Underhill and Ozinsky (2002), emphasise that individual pathogens are likely to express a variety of different PAMPs that will be recognised simultaneously. Therefore, the response generated upon PAMP / 'danger' recognition is dependent on both the individual PRRs involved, and the interaction between multiple PRRs. This confers a certain amount of pathogen-specificity upon innate responses, which could be important in determining the types of acquire immune response that follows.

A non-classical mechanism of pathogen (or self) lipid recognition that may also contribute to innate immunity occurs through CD1d-restricted receptors on a specialised T-cell subset termed natural killer T-cells (NKT). These receptors are TCRs but have an invariant TCRα chain, and NKT cells have many properties unlike normal αβ T-cells and more like innate cells, including a high natural frequency and the rapid production of IL-4 and IFNγ following stimulation (reviewed by Kronenberg and Gapin, 2002).

1.9 Innate immune responses

The innate immune system is comprised of numerous immuno-competent accessory cells that can act as sentinels in sites of potential infection, such as the skin. This tissue is populated by a vast array of accessory cells including epidermal LC, Mϕ, DC, natural killer (NK) cells, keratinocytes, and granulocytes, such as mast cells, eosinophils, and neutrophils (Williams and Kupper, 1996). Depending upon the cell type and the PRR engaged, PAMP recognition can cause a plethora of cellular responses, including internalisation of pathogens / pathogen material (via phagocytosis, endocytosis, macropinocytosis), induction of cell cytotoxicity, the release of soluble immune mediators including cytokines and chemokines, and the maturation of APC. These responses may be directly, or indirectly, important in the development of innate immune responses and the

induction of acquired immunity. In addition, innate recognition by accessory cells also contributes to effector functions during acquired immune responses.

1.9.1 Phagocytosis, endocytosis, and macropinocytosis

Phagocytosis, endocytosis, and macropinocytosis are the mechanisms by which leukocytes ingest particulate and soluble pathogens / pathogen material. Numerous PRRs have been implicated in initiation of these mechanisms, such as MR (Sallusto *et al.*, 1995), DEC-205 (Jiang *et al.*, 1995), and scavenger receptors (Peiser *et al.*, 2002). In addition, opsonised material can be ingested via Fc and complement receptors (Mold *et al.*, 2001). Cytokines, such as IL-4 and IL-10, also effect the endocytic activity of Mϕ, providing a mechanism for regulation of antigen uptake during inflammatory responses (Montaner *et al.*, 1999).

Internalisation is involved in several accessory cell functions. Particularly important to the development of acquired responses is the processing and presentation of antigen by the specialised APC subset of accessory cells. Internalisation leads to the delivery of pathogen material to proteolytic endocytic, or phagocytic organelles, within which the antigens are catabolised, and become complexed with MHC II (reviewed by Harding *et al.*, 2003). Although APC constitutively sample the environment by macropinocytosis (independently of PRR-ligation), receptor-mediated uptake dramatically enhances the efficiency of antigen capture and presentation (Jiang *et al.*, 1995; Sallusto *et al.*, 1995; Tan *et al.*, 1997).

In addition to antigen presentation, it has recently been demonstrated that internalisation can have an important role as a precursor event in TLR-mediated intracellular signalling by certain PAMPs. In this respect, TLR9 is restricted to endosomal intracellular compartments (Ahmad-Nejad *et al.*, 2002). In contrast, most TLRs appear to be expressed on the surface of accessory cells, although site-restricted expression within specialised subsets of accessory cells has been reported (Hornef *et al.*, 2002).

1.9.2 Production of cytokines, chemokines, and eicosanoids

Innate recognition also results in the production and release of important inflammatory mediators, such as chemokines, eicosanoids, and pro-inflammatory and regulatory

cytokines, including TNFα, IL-1β, IL-6, IL-10 and IL-12. These molecules act as soluble messengers with paracrine, endocrine, and autocrine functions, allowing the activation of further accessory cells. One consequence of this cross-talk is a series of complex events that results in inflammation, characterised by recruitment of further accessory cells into the site of infection. Another is the regulation of the local APC population in terms of composition and activity. A brief overview of these soluble molecules will be given.

Cytokines

The control of inflammatory cytokine production is thought to be mediated through induction of NF-κβ signalling pathways. However, some accessory cells, such as neutrophils, contain preformed cytokines whose release does not require transcription (Bliss *et al.*, 2000). TLRs are the major players in signal transduction for cytokine production utilising a pathway that shares much in common with IL-1R signalling due to the shared Toll / IL-1R domains (TIR) they contain. Signalling through TIRs ultimately leads to the translocation of NF-κβ transcription factors to the nucleus through the recruitment of an adapter protein MyD88, and activation of IRAK serine / threonine kinases (reviewed by Underhill and Ozinsky, 2002). Alternative pathways that are specific to individual TLR have also recently been identified and will be discussed further in Chapter 3. This may explain the differences in cytokine gene expression induced by different TLR ligands (Re and Strominger, 2001). In addition, non-TLR PRRs, such as the MR and CD11b, may also be involved in signalling for cytokine production (Yamamoto *et al.*, 1997; Perera *et al.*, 2001), possibly through a phagocytic mechanism (Shibata *et al.*, 1997).

Key cytokines in the development and regulation of inflammatory responses are TNFα, IL-1β, IL-6, and IL-10, which are produced upon stimulation by numerous different accessory cells including Mϕ, neutrophils, and DC. Both TNFα and IL-1β are potent pro-inflammatory cytokines sharing a similar spectrum of action that includes the activation of most accessory cells (reviewed by Mantovani, 1999 and Wallach *et al.*, 1999). In addition, they stimulate APC maturation and emigration to local draining LN (Cumberbatch *et al.*, 1997a; 1997b). IL-12 is thought to mainly exert a pro-inflammatory effect by orchestrating IFNγ production (Ma and Trinchieri, 2001), although it can also directly stimulate NF-κβ

translocation in DC (Grohmann *et al.*, 1998). Although the paracrine effect of IL-6 is the up-regulation of acute phase proteins (involved in anti-microbial defence) by the liver, its local effect during acute inflammation appears more regulatory (Xing *et al.*, 1998). Concurring with this, IL-6 can reduce Mϕ and DC production of pro-inflammatory cytokines (Takenaka *et al.*, 1997). IL-6 also stimulates differentiation of human monocyte precursors to Mϕ rather than DC, suggesting this cytokine will affect the constituent APC population (Chomarat *et al.*, 2000). IL-10 is a potent anti-inflammatory cytokine favouring the resolution of immune responses. Its effects include down-regulation of APC activity (Wang *et al.*, 1999; Corinti *et al.*, 2001), down-regulation of pro-inflammatory cytokine (*e.g.* TNFα and IL-12) production and inhibition of cellular cytotoxicity by Mϕ (Kane and Mosser, 2001; Corinti *et al.*, 2001).

Chemokines

The production of chemokines by tissue Mϕ and DC, and other accessory cells, is thought to play a major role in the initiation of inflammatory responses (Luster, 2002). Chemokine production is thought to be under control of a NF-κβ-dependent pathway, and thus principally the domain of TLRs. Chemokine genes expressed following TLR engagement include IL-8, MIP-1α, and MIP-1β, although as for cytokines, these are differently regulated by separate TLRs (Re and Strominger, 2001). Briefly, IL-8 recruits neutrophils (Barker *et al.*, 1991; Kuijpers *et al.*, 1992), whereas MIP-1α and MIP-1β recruits Mϕ, immature DC (Dieu *et al.*, 1998) and NK (Salazar-Mather *et al.*, 2000). Chemokines may also be able to stimulate accessory cells to up-regulate cytokine production, since signalling through CCR5 by *T. gondii* can result in IL-12 production (Aliberti *et al.*, 2000). In this respect, it has been reported that MIP-1α, MIP-1β, and RANTES co-operate with IFNγ to up-regulate CD40, IL-12, and TNFα expression by Mϕ (Dorner *et al.*, 2002).

Eicosanoids

Eicosanoids are small lipid molecules with endocrine and autocrine activity, and most is known about the prostaglandin and leukotriene eicosanoids. Stimulation of accessory cells, such as Mϕ and DC, is thought to result in the phospholipase A_2-dependent release of the membrane-bound progenitor arachidonic acid. This is then converted to different

prostaglandins, via constitutive cyclo-oxygenase (COX)-1 and inflammation-inducible COX-2 enzymes, and various prostaglandin synthases (Harizi *et al.*, 2002), or to leukotrienes by the lipoxygenase pathway. Prostaglandins and leukotrienes have profound effects on innate accessory cells. Of these, PGE_2 stands out as the most studied, and is known to cause decreased production of IL-12, IL-6, and TNFα by Mϕ (Zhong *et al.*, 1995), but increased IL-10 production (Shinomiya *et al.*, 2001). PGE_2 has also been implicated in the inhibition of DC activity, via the up-regulation of IL-10 production (Harizi *et al.*, 2002).

1.10 Innate immune responses: induction of acquired immunity

Effector $CD4^+$ Th cells co-ordinate the acquired immune response. The single crucial event in the activation of naïve Th cells is ligation of their surface TCRs with peptide-MHC II complexes. APC are a specialised subset of accessory cells that can capture exogenous antigen and present it to Th cells in complex with MHC II. In this respect, APCs are the cells that link innate and adaptive immunity. In addition, the initial activation of the innate immune system is critical to the induction of strong effector T-cell responses, including increased T-cell proliferation and the generation of long-lived memory (Reinhardt *et al.*, 2001).

1.10.1 APCs

Several types of accessory cell can express MHC II and can therefore act as APCs. However, DC, and to a lesser extent Mϕ, are the most potent activators of naïve Th cells, and in this respect they have become regarded as 'professional APC'. Their potency is emphasised by work using antigen-loaded DC as 'natural adjuvants' to elicit protective immune responses to pathogens (Flohe *et al.*, 1998). DC can be divided into several subsets that are described in more detail in Chapter 5.

The type of APC that will be involved in T-cell priming depends upon the site of antigen deposition and the inflammatory response that may accompany this. In infections where entry to the host is percutanous, such as schistosomiasis, APC within the skin may capture exogenous antigen and then migrate to local sdLN. The skin is known to contain several

types of APC: a specialised subset of DC termed LC resides in the epidermis, while additional DC and Mϕ also reside in the dermis. Moreover, the innate response that follows infection may critically change the repertoire of APC that are present (see above).

1.10.2 Antigen uptake and processing

As discussed earlier, APC can internalise antigen via a number of constitutive and PRR-mediated mechanisms. Antigen uptake can also be mediated by specific antibodies on B-cells (Rock *et al.*, 1984), and through FcR and complement receptors on phagocytes such as Mϕ. Following degradation of antigen material in the endocytic compartment, peptides are loaded onto freshly synthesised MHC II molecules. The mechanisms of peptide loading are relatively well defined, and involve a sequence of proteolytic events to remove a protein (invariant chain) which blocks the MHC II peptide binding site, allowing antigen peptides to bind (Harding *et al.*, 2003).

1.10.3 Migration to T-cell areas

Priming of naïve T-cells requires APC to be present in the T-cell areas of primary lymphoid tissues, such as spleen or local draining LN. This can occur in two ways: APC at the site of infection can capture antigens and then migrate to the lymphoid tissues. Alternatively, antigen may be carried directly to the LN, or spleen, in draining lymphatic fluid, or blood, where it can be processed by the resident APC. Moreover, migration of the pathogen may also take it through primary lymphoid tissue (as during schistosomiasis), where-upon antigen could be deposited directly.

During percutaneous infections, skin-derived APC are likely to be important for antigen presentation. In this respect, stimulated resident tissue DC are known to down-regulate their expression of chemokine receptors CCR1, CCR5 and CCR6, whilst up-regulating expression of CCR7, allowing them to migrate from the tissue through the lymphatics towards the T-cell rich areas of the local LN (Sozzani *et al.*, 1998; Dieu *et al.*, 1998). Certain cytokines, including IL-1β and TNFα, are essential to the ability of LC to migrate from the skin, and can stimulate the cells to up-regulate expression of MHC II

(Cumberbatch and Kimber, 1995; Wang *et al.*, 1997; Cumberbatch *et al.*, 1997a; 1997b). In fact, in the absence of these cytokines LC migration is greatly impaired (Cumberbatch and Kimber, 1995; Wang *et al.*, 1997; Cumberbatch *et al.*, 1997a). In contrast, IL-10 inhibits LC migration, possibly by down-regulating IL-1β and TNFα production in the epidermis (Wang *et al.*, 1999), and by maintaining the expression of CCR6 (Dieu-Nosjean *et al.*, 2001).

1.10.4 T-cell priming

In order to proliferate and generate effector cells, naïve $CD4^+$ T-cells must receive co-stimulatory signals in addition to those signals received through the TCR. The most important co-stimulatory signal is thought to be received through CD28 but other signals also contribute to T-cell activation, including CD154 and OX40, and will be discussed further in Chapter 6 and 7. These co-stimulatory signals are supplied by membrane surface molecules expressed on APC, such as CD80, CD86, CD40, and OX40L, and are thought to be essential for full activation of T-cells and prevention of T-cell death. In this manner, their absence can lead to reduced T-cell responses or T-cell anergy (unresponsiveness) (Boise *et al.*, 1995; Van Gool *et al.*, 1999). Indeed, T-cell responses to schistosome egg deposition are greatly reduced in $CD28^{-/-}$ mice (King *et al.*, 1996b). In addition, APC-derived cytokines may also contribute to the co-stimulatory signal (Holsti *et al.*, 1994; Vella *et al.*, 1997; Teague *et al.*, 1997). Co-stimulatory signals act to enhance T-cell survival during proliferation by the induction of anti-apoptotic 'intrinsic survival factors', such as Bcl-XL and Bcl-2 (Boise *et al.*, 1995; Vella *et al.*, 1997; Teague *et al.*, 1997; Rogers *et al.*, 2001). Upon activation, T-cells up-regulate their expression of a number of co-stimulatory receptors, such as CD154, OX40, and 4-1BB. Ligation of these molecules by their APC-expressed counterparts (CD40, OX40L and 4-1BBL, respectively), is thought to act as a series of check points in priming, delivering survival and maintenance signals to developing T-cells (Rogers *et al.*, 2001; Cannons *et al.*, 2001).

Immature APC express low levels of co-stimulatory molecules and MHC II, and are inefficient in activating naïve T-cells. However, upon exposure to a variety of stimuli including many pathogens and PAMPs, such as LPS (Whelan *et al.*, 2000), mycobacteria (Schnare *et al.*, 2001), CpG DNA motifs (Sparwasser *et al.*, 1998), yeast cells and hyphae

(d'Ostiani *et al.*, 2000), 'danger signals' such as necrotic cells (Gallucci *et al.*, 1999), and pro-inflammatory cytokines (Brunner *et al.*, 2000; Schnare *et al.*, 2001), DC undergo a maturation program that leads to increased expression of MHC II and co-stimulatory molecules, and an increased ability to prime T-cells.

The mechanisms that control the up-regulation of these molecules are not fully understood. Similar to the control of cytokine production, PAMP-stimulated expression of co-stimulatory factors is also thought to mainly be controlled by signal transduction through the TLRs (reviewed by Medzhitov, 2001). However, the signalling pathways that lead to DC cytokine production can be different to pathways resulting in maturation and co-stimulatory factor expression. For example, DC from mice deficient in the adapter protein MyD88 fail to produce pro-inflammatory cytokines but still up-regulate MHC II and co-stimulatory factor expression upon exposure to LPS (Kaisho *et al.*, 2001) or dsRNA (Alexopoulou *et al.*, 2001). In contrast, both cytokine production and maturation stimulated by CpG motifs, or mycobacteria, rely on MyD88 (Schnare *et al.*, 2000; 2001). Signalling could also be due, in part, to the induction and release of stimulatory autocrines, such as IL-1β and TNFα, the latter of which also signals through a MyD88-independent pathway (Schnare *et al.*, 2001). The role of non-TLR PRRs in PAMP-driven DC maturation remains to be determined.

1.11 Polarisation of acquired immune responses

1.11.1 Polarity of the acquired response: Th1 and Th2 subsets

$CD4^+$ T-cells are responsible for both the type and the co-ordination of the acquired immune response. This is achieved through the repertoire of cytokines they produce upon activation. It has become widely accepted that murine effector $CD4^+$ T-cells can be divided into two subsets based upon their profile of cytokine production (Mosmann *et al.*, 1986). Th1 cells produce IFNγ and are potent drivers of cell-mediated 'inflammatory' effector responses, characterised by delayed-type hypersensitivity (Cher and Mosmann, 1987). These cytokines can induce cellular cytotoxicity in Mϕ, and cause class-switching of antibody production to IgG_{2a}. In contrast, Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which drive antibody-associated humoral and allergic-type responses through enhanced

B-cell help, mast cell and eosinophil proliferation and activation, and switching to IgG_1 and IgE antibody production (Cherwinski, 1987; Fiorentino *et al.*, 1989). A similar Th1 and Th2 paradigm of cytokine production has been described in human effector T-cell responses (Del Prete *et al.*, 1991).

Recent advances in this field demonstrate that are there are a number of other $CD4^+$ T-cell subsets which do not fit into this Th1 / Th2 model. First, Th0 cells have been described which produce both Th1 and Th2 cytokines (Firestein *et al.*, 1989). These may represent an intermediate pluripotent phase of development prior to differentiation to polarised Th1 or Th2 cells (Kamagowa *et al.*, 1993). However, it is also possible that Th0 cells form a stable subset of T helper cells (Miner and Croft, 1998). Second, resurgence in the field of T-cell mediated suppression has led to the identification of several 'suppressor' or 'regulatory' T cell populations (T_{reg}). 'Naturally occurring' populations of T_{reg} cells are present in un-manipulated mice and are enriched within $CD4^+$ $CD25^+$ cells (reviewed by Read and Powrie, 2001). In addition, a number of inducible T_{reg} subsets have been described, including Th3 (Chen *et al.*, 1994), Tr1 (McGuirk *et al.*, 2002), and anergic T-cells (Chai *et al.*, 1999). Again, similar cells types have been described in humans. The relationship between these different cells is unclear, but they appear to act through a diverse variety of cytokine (IL-10 and TGFβ) and cell-contact dependent mechanisms, in order to suppress both innate and T-helper cell responses (Chen *et al.*, 1994; Chai *et al.*, 1999; Read and Powrie, 2001; McGuirk *et al.*, 2002; Maloy *et al.*, 2003).

1.11.2 Th1 / Th2 response in disease

Infection can result in a broad spectrum of acquired Th cell responses, which is important in determining the course of disease. Some disease states are characterised by highly polarised responses, as demonstrated by;

- the extensive work from R. Locksley's and P. Scott's laboratories on healing / non-healing *Leishmania* dependent on the induction of Th1 and Th2 responses, respectively (Heinzel *et al.*, 1989; Scott, 1991)
- the work done on *Trichuris muris* infection in which Th2 responses result in protection and Th1 responses result in susceptibility (Else *et al.*, 1993; Bancroft *et al.*, 1998).

Alternatively, under the correct conditions mixed Th1 / Th2 responses can be highly protective, such as against challenge schistosome infection following vaccination of IL-10 $^{-/-}$ mice with irradiated larvae (discussed above; Hoffmann *et al.*, 1999). Moreover, in malaria infection both Th1 and Th2 responses can contribute to protection against the pathogen but at different stages of its life-cycle. These studies demonstrate that, depending upon the disease, immune responses need not be highly polarised in order to be protective.

1.12 Innate immune responses: factors affecting T-cell polarisation

Many factors have been linked to the development and regulation of Th1 and Th2 subsets. Those derived from the innate immune system are thought to be critical for the initial progression towards a polar response (Fearon and Locksley, 1996). There is also thought to be a significant amount of cross-regulation between the Th subsets through the repertoires of cytokines they produce. However, exogenous innate signals may tip the balance of regulatory ones.

1.12.1 PAMPs and PRRs

A certain degree of specificity in innate responses is provided by differences in the 'hard-wired' responses to ligation of particular PRRs or PRR combinations, allowing the discrimination of diverse PAMPs or pathogens (Underhill and Ozinsky, 2002). Indeed, there is evidence that DC can directly interpret the information encoded in PAMPs, or 'danger signals', and subsequently drive polarised Th responses (Whelan *et al.*, 2000; MacDonald *et al.*, 2001; de Jong *et al.*, 2002; Manickasingham *et al.*, 2003), even to the extent of distinguishing between different cellular stages of the same organism (d'Ostiani *et al.*, 2000). This will be discussed in further detail in chapters 6 and 7.

The flexibility of innate accessory cells probably lies in the down-stream signalling effects of engagement of specific PRRs. The importance of TLR signalling in polarisation of T-cells has been investigated in a number of studies using mice deficient for the signalling adapter protein MyD88. These mice fail to mount Th1 responses but develop Th2-associated responses when exposed to *Toxoplasma gondii* (Jankovic *et al.*, 2002). They also fail to mount a Th1 response when immunised with ovalbumin in complete Freunds adjuvant (Schnare *et al.*, 2001), a mixture of mycobacterial PAMPs that signal through

TLR2 (Means *et al.*, 1999; Underhill *et al.*, 1999a). Although MyD88 is also important in the signalling pathways of the pro-inflammatory cytokines IL-1β and IL-18 (Adachi *et al.*, 1998), mice deficient in bioactive IL-1β and IL-18 (*i.e.* caspase-1 $^{-/-}$) exhibited normal T-cell responses (Schnare *et al.*, 2001). These studies have led to the hypothesis that TLR recognition and signalling pathways may only be relevant for the development of Th1 and not Th2 responses (Schnare *et al.*, 2001), although this requires further work to be verified.

1.12.2 Antigen presentation, co-stimulatory factors, and APCs

In addition to the crucial involvement in T-cell priming, co-stimulatory factors such as CD28, CD154, and OX40 may also have differential roles in Th1 and Th2 development. In this respect, the role of CD80, CD86, CD40, and OX40L expressed upon APC has been the focus of intense study that has lead to contrasting observations, and will be discussed more thoroughly in Chapter 6 and 7.

Many studies show that the certain types of APC preferentially stimulate either Th1 or Th2 responses. In this respect, DC infected with *M. tuberculosis* drive Th1 differentiation, whereas infected Mϕ do not (Hickman *et al.*, 2002). The inherent difference in T-cell polarisation by APC is possibly due to the repertoire of cytokines or co-stimulatory factors that these cells express (Hickman *et al.*, 2002). Different DC subsets have also been implicated in preferentially priming Th1 or Th2 responses. However, despite these studies, it has been suggested that certain APCs, such as DC, are pluripotent in their T-cell polarising capacity, and currently this is an intensely studied area of research.

1.12.3 Cytokines

Of all the factors known to affect the development and polarisation of the Th population, cytokines are thought to play the most critical role. They can act directly upon T-cells, or may affect polarisation indirectly by interfering with innate responses. Since the cytokines of the innate and acquired system overlap considerably, their possible roles as exogenous and endogenous factors will be discussed together.

IL-12: This cytokine is produced by a variety of accessory cells and APC, such as Mϕ, DC and neutrophils, and it is a major link between innate and adaptive immunity (Trinchieri,

2003). It has a direct and critical role in driving Th1 polarisation, which has been extensively studied *in vitro* and *in vivo* (Hsieh *et al.*, 1993). However, although APC-derived IL-12 may be required for optimal IFNγ production, it may not be a necessity for Th1 induction (MacDonald and Pearce, 2002), suggesting that other mechanisms are also involved in this pathway. IL-12 may affect T-cell polarisation in a number of ways. Addition of IL-12 to T-cell cultures can result in induction of Th1 responses when a normal Th2 response would prevail, while ablation of IL-12 leads to the inhibition of a normal Th1 phenotype (Hsieh *et al.*, 1993; Manetti *et al.*, 1993; 1994). Moreover, IL-12p40 $^{-/-}$ mice mount impaired Th1 responses (Anderson *et al.*, 1998; Jankovic *et al.*, 2002). One way in which IL-12 could enhance Th polarisation is by stimulating up-regulation of IL-18 receptor α (IL-18Rα) expression, and IL-12 receptor β2 (IL-12Rβ2) allowing cells to respond to these potent Th1 inducing cytokines (Chang *et al.*, 1999; Smeltz *et al.*, 2002). In addition, IL-12 may potentiate Th1 responses by stimulating IFNγ production by NK cells (Kobayashi *et al.*, 1989) and γδ T-cells (Skeen and Ziegler, 1995). Expression of the IL-12 receptor may be critical in the control of T-cell polarisation, since exposure to IL-4 decreases expression of the IL-12Rβ2 subunit, leading to an increased likelihood of Th2 polarisation (Szabo *et al.*, 1997).

IFNγ: One of the most important cytokines produced by Th1 cells, IFNγ can also be produced by activated NK cells (Kobayashi *et al.*, 1989), $CD8^+$ T-cells, and γδ T-cells (Skeen and Ziegler, 1995). In addition, it has been found to be produced by APC such as DC (Ohteki *et al.*, 1999). The involvement of IFNγ in the direct induction of Th1 differentiation remains unclear. It appears that IFNγ is critical in the development and sustenance of Th1 differentiation, but alone it may not be sufficient to drive polarisation down this route (Scott, 1991; Macatonia *et al.*, 1993). Recently, it was observed that IFNγ appears to limit the negative effects of IL-4 on IL-18Rα and IL-12Rβ2 expression (Nakamura *et al.*, 1997; Smeltz *et al.*, 2002), which could explain the positive feedback mechanism of this cytokine. IFNγ may also indirectly induce Th1 differentiation through the activation of APC, such as Mϕ, which then produce more pro-Th1 IL-12 and less inhibitory IL-10, in response to PAMP recognition (see Chapter 3).

IL-18: This cytokine is produced by a number of accessory cells, including IFNγ-activated Mϕ (Okamura *et al.*, 1995). The observations that IL-18 receptor is only expressed on Th1

cells (Xu *et al.*, 1998) partly explains the effects IL-18 has on T-cells. Firstly, it has little effect on Th cell IL-4 or IL-10 production (Micallef *et al.*, 1996; Robinson *et al.*, 1997). Secondly, on its own, IL-18 is relatively ineffective at driving Th1 polarisation, although it acts in synergy with IL-12 to augment T-cell IFNγ production (Micallef *et al.*, 1996; Kohno *et al.*, 1997; Robinson *et al.*, 1997). Therefore, IL-18 may not be an important cytokine in the initial event involved in polarisation but appears to function by enhancing and sustaining Th1 responses (Okamura *et al.*, 1998).

Other potentially 'Th1-associated' cytokines:

- IL-1β and TNFα have been implicated in Th1 polarisation (D'Andrea *et al.*, 1993). These may act indirectly, as IL-1β synergises with IL-12 to stimulate IFNγ production by NK cells and γδ T-cells (Hunter *et al.*, 1995; Skeen and Ziegler, 1995), and both TNFα and IL-1β stimulate accessory cells to exhibit a pro-inflammatory phenotypes and drive APC maturation. TNFα also enhances T-cell expression of IL-12Rβ2 (reviewed by Trinchieri, 2003).
- IFNα can support Th1 polarisation in the absence of IL-12 in response to certain PAMPs, such as dsRNA (Manetti *et al.*, 1995).
- IL-15 and IL-21 were both recently shown to induce T-cell synthesis of IFNγ, IL-12Rβ2 and IL-18R mRNA (Strengell *et al.*, 2002), although the role of these cytokines in Th1 development remains to be fully explored, and a recent report suggests IL-21 is a Th2 cytokine (Wurster *et al.*, 2002).
- IL-23 and IL-27, also have Th1 polarising activity (Trinchieri, 2003), and both are produced by stimulated APCs (Oppmann *et al.*, 2000; Pflanz *et al.*, 2002). The receptor for IL-23 shares the IL-12Rβ1 chain, and induces similar signalling as IL-12 (Parham *et al.*, 2002), which could explain why these molecules share similar functions. However, IL-23 appears to be less efficient than IL-12 at driving IFNγ production (Oppmann *et al.*, 2000). IL-27 is also a strong inducer of IFNγ production, acting in synergy with IL-12 and IL-18 (Pflanz *et al.*, 2002), and mice deficient for the IL-27 receptor chain TCCR (homologous to the IL-12Rβ2) have impaired Th1 responses (Chen *et al.*, 2000).

IL-4: IL-4 is thought to be one of the major players in differentiation of Th2 cells. Gene deletion of IL-4 significantly impairs the generation of Th2 responses (Kuhn *et al.*, 1991; Kopf *et al.*, 1993), while the addition of IL-4 increased the frequency of IL-4-producing

cells, but decreased that of IFNγ-producing cells (Swain *et al.*, 1990; Le Gros *et al.*, 1990). IL-4 may drive Th2 polarisation in a number of ways. Firstly, it is thought to operate in a positive feedback loop since STAT6, an essential transcription factor for Th2 cytokines and Th2 development (Shimoda *et al.*, 1996; Takeda *et al.*, 1996), is activated through the IL-4 receptor (Hou *et al.*, 1994). IL-4 can also act on APCs, such as Mϕ, to inhibit the production of IL-12p40 in response to PAMP stimuli (Major *et al.*, 2002), with IL-4 signalling thought to result in increased binding of a suppressor site in the p40 promoter (Becker *et al.*, 2001).

IL-4 can be produced by a wide array of cells, including T-cell subsets, and accessory cells such as mast cells and basophils (Machado *et al.*, 1996; Haisch *et al.*, 2001; Rao *et al.*, 2002). IL-4 also acts with IL-3 to expand mast cells, which may result in a preferential environment for Th2 responses in the tissues. Recently DC have been shown to produce IL-4 in response to yeast hyphae, and subsequently drive Th2 polarisation (d'Ostiani *et al.*, 2000). However, DC from IL-4 $^{-/-}$ mice do not loose their ability to prime for Th2 differentiation, although IL-4 production by responder T-cells is crucial (MacDonald and Pearce, 2002). This suggests that exogenous IL-4 is not essential for the induction of Th2 responses, and that IL-4 may not be involved in the initial switch to Th2 cytokine production

Perhaps surprisingly it has been shown that there could be a negative feedback mechanism to regulate IL-4 production and Th2 polarisation. Several studies demonstrate that IL-4 stimulates human DC (Kalinski *et al.*, 2000) and PBMC (D'Andrea *et al.*, 1995) to produce increased levels of IL-12p70. Moreover, culture of DC with Th2 cells leads to IL-4-dependent production of IL-12p70 and the reversal of the Th2 cells to a Th0 / Th1 phenotype (Kalinski *et al.*, 2000). However, this is obviously controversial since it conflicts with the inhibitory effect of IL-4 on IL-12 production, as discussed above.

IL-13: IL-13 has many overlapping biological functions with IL-4 (de Waal Malefyt *et al.*, 1993), including the differentiation of Th2 cells (Bancroft *et al.*, 1998; McKenzie *et al.*, 1998). The similarity between the function of these cytokines reflects the shared IL-4Rα chain used for signalling (Mohrs *et al.*, 1999). Although IL-13 is produced by Th0, Th1, and Th2 activated T-cells (de Waal Malefyt *et al.*, 1995), unlike IL-4 it only exerts its

action upon accessory cells, since T-cells do not express the IL-13 receptor. In this respect, IL-13 inhibits pro-inflammatory cytokine production, including IL-12, by monocytes (de Waal Malefyt *et al.*, 1993). Since IL-13 is only expressed by activated T-cells it is not likely to be an early factor involved in Th2 polarisation, but may be a signal to augment it.

IL-6: IL-6 is produced by both APCs, such as Mϕ, DC and B-cells, but also by Th2 cells, and has been documented to be involved in the induction of Th2 polarisation (Rincon *et al.*, 1997; La Flamme *et al.*, 2000). IL-6-deficient mice infected with *Borrelia burgdorferi* have greater incidence of inflammatory arthritis, and their splenocytes produce less IL-4 upon re-stimulation *in vitro* (Anguita *et al.*, 1998). IL-6 also drives naïve T-cell production of IL-4, although this is dependent upon endogenous IL-4 production (Rincon *et al.*, 1997; Diehl *et al.*, 2002). However, IL-6 can directly inhibit Th1 differentiation through an IL-4-independent mechanism, by up-regulating suppressor of cytokine signalling (SOCS)-1 expression, which interferes with IFNγ signalling (Diehl *et al.*, 2000). In addition, IL-6 may act by inducing IL-10 production that could then inhibit Th1 responses (La Flamme *et al.*, 2000). Therefore, IL-6 may not be a primary stimulator of Th2 differentiation but can augment it upon IL-4 production. Moreover, IL-6 can inhibit Th1 differentiation from an early stage, and therefore might be an important innate cytokine in driving Th2 responses.

IL-10: IL-10 is an immunomodulatory cytokine. It is associated with Th2 polarisation, preferentially suppressing Th1 responses, however, it also down-regulates acquired responses *per se* (de Waal Malefyt *et al.*, 1991; Taga and Tosata, 1992). This affect of IL-10 is thought to be an indirect result of its inhibitory actions upon pro-inflammatory Th1-promoting cytokine production by accessory cells and APC, and its down-regulatory effect on MHC II and co-stimulatory factor expression by APC (Fiorentino *et al.*, 1991a; Hsieh *et al.*, 1993; Corinti *et al.*, 2001; Hickman *et al.*, 2002).

Chemokines: No direct effect of chemokines on T-cell polarisation has been described. However, since the type of APC may bias the outcome of priming, chemokines could indirectly influence polarisation by determining the cellular constituents of the APC population immigrating into a site of inflammation (discussed above), and emigrating to the draining LN. In addition, MIP-1α, MIP-1β, and RANTES (produced by NK and Th1

cells and some accessory cells) have been implicated in Th1 polarisation by driving pro-inflammatory cytokine production by IFNγ-activated Mϕ (Dorner *et al.*, 2002).

Prostaglandins: PGE_2 is an inducer of Th2 responses. It is thought to function through its mainly down-regulatory affects upon APC pro-inflammatory cytokine production (Kuroda *et al.*, 2000; discussed above). In this respect, DC cultured with PGE_2 drive potent Th2 responses (Vieira *et al.*, 2000; de Jong *et al.*, 2002).

1.13 Aims of this study

The overall aims of this project are to;

- determine how schistosomes interact with the innate immune system during the initial stages of infection;
- establish what effect this interaction may have on the development of the acquired immune response to the parasite.

Due to the complexities of immune interactions *in vivo*, this thesis focuses almost entirely on dissecting innate responses to schistosomes *in vitro*. To achieve the above aims, this thesis is structured around two key objectives, as follows.

a) The first objective is to determine if skin-stage schistosomes possess PAMP-like molecules that can directly stimulate innate accessory cells.

   Chapter 2 briefly deals with the production of parasite 'PAMP' preparations from schistosomes transformed and cultured *in vitro* to different developmental states.

   Chapter 3 concerns the screening of these schistosome preparations for their ability to stimulate murine Mϕ (as a representative of the innate immune system).

   The involvement of different cellular receptors in the recognition of parasite material is investigated in chapters 4 & 5, focussing upon the MR, and the FcγR.

b) The second objective is to determine what effect putative parasitic PAMPs have on APCs.

   APCs form the bridge that links innate and acquired responses and are likely to be a critical factor affecting both the scale and the type of acquired immune response generated during infection. Therefore, in Chapter 6 the effect of schistosome PAMPs upon the maturation state of DC (the 'professional' APC) is examined in depth. In order to determine the effect of parasite PAMPs upon the priming and polarisation of acquired immune responses, Chapter 7 deals with the outcome of Th cell priming by schistosome-activated DC, using both *in vitro* and *in vivo* methodologies for analysis.

CHAPTER 2

PRODUCTION OF SCHISTOSOME PAMP PREPARATIONS FROM *IN VITRO* CULTURED LARVAE

2.1 INTRODUCTION

In order to determine whether *S. mansoni* larvae express PAMPs during the initial stages of infection, a number of different larval preparations will be screened for their ability to stimulate innate accessory cells. This parasite material must be, 1) representative of the molecules that come into contact with innate accessory cells during the different stages of skin penetration and migration; and 2) produced in large / sufficient quantities.

Infective cercariae are easily obtainable in bulk from patent snails maintained at the University of York. However, *ex vivo* skin-stage schistosomulae are very difficult to obtain in large numbers, as they have to be extracted from the skin infection site, requiring significant numbers of infected mice. Coupled with this, molecules released by the parasite to facilitate penetration and migration will be lost within the host tissues. Therefore, a method for the high yield *in vitro* culture of artificially-transformed larvae was adopted to provide a viable source of large quantities of parasite material. Briefly, this method involves the mechanical-transformation of infective cercariae into schistosomulae, which can then be cultured *in vitro* to different developmental stages. This method also allows for the collection and analysis of the material released by cercariae during transformation and subsequent migration, such as the contents of the pre- and post-acetabular glands. As these released molecules are some of the first schistosome molecules to which innate accessory cells are exposed, they represent an important source of potentially stimulatory PAMPs.

The aim of this short chapter is to describe the techniques used for the *in vitro* culture of schistosomulae, and to simply compare the different larval preparations that will be used in subsequent experimental chapters to study the effects of schistosome PAMPs upon innate accessory cells.

2.2 MATERIALS AND METHODS

2.2.1 Parasites

A Puerto Rican strain of *Schistosoma mansoni* was maintained by routine passage through out-bred NMR-1 mice and albino *Biomphalaria glabrata* snails.

2.2.1.1 Cercariae

Schistosome cercariae were harvested from snails harbouring patent infections (approximately 260 snails from which 650,000 larvae are shed; pers. com. R. Curwen and A. Wilson). The parasites were induced to shed into clean 'aged tap-water' by exposure of infected snails to intense light (2 x 200W bulbs) for 2 hr. Cercariae were then pooled, ensuring that snails and faeces were excluded, and concentrated by sedimentation on ice for 1 hr. In order to reduce naturally occurring microbial contaminants, cercariae were washed three times with sterile chilled 'aged tap-water'. Cercariae were frozen at -20 °C prior to processing into soluble cercarial preparation (SCP; see section 2.2.1.4).

Alternatively, cercariae were mechanically-transformed by vortexing for 90 sec in 2ml of sterile chilled RPMI 1640 containing 200 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Paisley, UK) (RPMI-0).

2.2.1.2 In vitro culture of schistosomulae

Mechanically-transformed larvae were cultured *in vitro* in 25 ml RPMI-0 for 3 hr at 37 °C and 5% CO_2, in a T25 tissue-culture flask (Nalge Nunc International Corp., Naperville, USA). The absence of foetal calf serum (FCS) from this medium ensured that all protein present in the supernatant (SN) after culture would be parasite-derived. During this period, the now immobile cercarial heads (transformed to schistosomulae), and the cercarial tails, settled on the bottom of the culture flask. The top 20 ml of culture SN, was then removed, leaving 5 ml of larval sediment. A further 20 ml of RPMI-0 was added to resuspend any particulate material released by larvae into the sediment. The heads and tails were allowed to sediment for 10 min, before the removal of the top 20 ml of SN. The pooled SN formed the basis of the 0 - 3 hr released molecule preparation (0-3hRP; Section 2.2.1.3).

Parasite heads, were isolated from tails by centrifugation (250 *g* for 12 min) on a discontinuous (45% / 70%) Percoll (Amersham Scientific, Amersham, UK) gradient, made up in RPMI-0 (Lazdins *et al.*, 1982). The denser larvae accumulated at the interface between the 45% and 70% layers, whereas the less dense tails remained in the upper region of the 40% layer, allowing the heads to be removed. Larvae were washed 7 times with 10 ml RPMI-0 to remove Percoll and the few remaining tails. They were then pelleted, frozen at -20 °C, and used to create the soluble 3 hr schistosomule preparation (3hSSP) (Section 2.2.1.4). Alternatively, schistosomulae were cultured for longer periods of time to allow further development. Consequently, larvae were resuspended in 20 ml Medium 169 (adapted from Basch, 1981), containing 200 U/ml penicillin, 100 µg/ml streptomycin and 5% heat-inactivated, low-endotoxin FCS (Harlan Seralab, Loughborough, UK) (M169-5). Medium 169 comprised BME (EAGLE) medium containing HEPES buffer (20 mM), Schneiders medium (5%, v / v; Invitrogen), lactalbumin hydrosolate (0.1%), glucose (0.1%), hypoxanthine (5 x 10^{-7} M), serotonin (1 x 10^{-6} M), hydrocortisone (1 x 10^{-6} M) triiodothyronine (2 X 10^{-7} M), and MEM vitamins (0.5%, v / v; Sigma-Aldrich, Poole, UK). Schistosomulae were then cultured in M169-5 (approximately 2000 - 3000 parasites / ml) in 24-well plates (Nalge Nunc), and harvested 18 hr, 3 days, 5 days or 8 days later, depending on the stage of development required. The larvae were resuspended and pooled into 50 ml tubes and centrifuged at 45 *g* for 5 min. The SN was discarded and the larvae washed 4 times with RPMI-0, to remove any FCS components. The final larval pellet was frozen at -20 °C. These larvae were then used to create the 18 hr (18hSSP), 3 day (3dSSP), 5 day (5dSSP) or 8 day (8dSSP) soluble schistosomulum preparations (Section 2.2.1.4).

2.2.1.3 Preparation of soluble released larval molecules (0-3hRP)

During the following procedure, parasite material was kept on ice at all times to ensure minimal enzymatic degradation. Pooled SN from the first 3 hr schistosomulae culture was centrifuged (120 *g*), 4 °C, for 8 min to pellet any remaining heads and tails. This process was optimised in order to reduce any contaminating heads or tails, whilst increasing the amount of particulate released material remaining in suspension. The released parasite material within the SN was then concentrated 50-fold using Ultrafree-MC centrifugal filter units with 5 kDa cut-off membranes (Millipore, Watford), and used to produce 0-3hRP. As

a control, an equivalent volume of RPMI-0 culture medium containing no parasite material was concentrated 50-fold using the same method.

2.2.1.4 Production of soluble preparations from whole larvae and released molecules

As above, parasite material was kept on ice at all times to minimise enzymatic degradation of the constituents. The suspensions were then sonicated (21 kHz at 6.5 μm amplitude) for 3 min. Each preparation was then centrifuged (100,000 *g*, for 1 hr) to separate soluble material within the supernatant from the insoluble pellet. These were finally sterilised by irradiation with UV light for 30 min. The resulting soluble preparations are summarised in Table 2.1.

Source of larvae	*Equivalent larval developmental stage in vivo*	*Acronym*
Infective cercariae	Infective cercariae	SCP
Artificial-transformation and in vitro culture of schistosomulae		
Molecules released during first 3 hr of *in vitro* culture	Molecules released @ onset of infection (x50 conc.)	0-3hRP
Medium control [a]	N / A	RPMIc
3 hr *in vitro* larvae	Epidermis	3hSSP
18 hr " "	Epidermal / dermal interface	18hSSP
3 d " "	Dermis	3dSSP
5 d " "	Lymph node / Lung	5dSSP
8 d " "	Lung	8dSSP

***Table 2.1* Summary of the schistosome preparations analysed in this thesis and the source material used to produce them.**

[a] equivalent medium control to 0-3hRP.

2.2.2 Characterisation of soluble schistosome preparations

2.2.2.1 Quantification of material within soluble preparations

Protein concentration was used as the basis for quantitative comparison of the different soluble schistosome preparations. This was determined using the Coomasie Plus-200 assay (Perbio Science UK Ltd, Tattenhall, UK). Samples were referenced against a bovine serum albumin (BSA; Sigma-Aldrich) standard curve. The preparations were tested neat, or at dilutions of 1:2, 1:5, or 1:10. The soluble preparations were then stored at -20 °C until further use.

2.2.2.2 SDS-PAGE analysis

Samples (10 μg) of the schistosome preparations were denatured by boiling in LDS sample buffer and reducing agent for 5 min, before being separated on pre-cast 4% – 12% gradient acrylamide gels at 200 V for 40 min, in MES SDS running buffer containing 0.1% antioxidant (all reagents from Invitrogen). Gels were stained with Brilliant Blue G-Colloidal Concentrate™ (BBGC; Sigma-Aldrich), a Coomasie based stain, according to manufacturer's instructions. Specifically, gels were fixed in 7% glacial acetic acid in 40% (v / v) methanol for 1 hr, and then stained in BBGC overnight. Gels were then de-stained with 10% acetic acid in 25% (v / v) methanol for 30 sec and then up to 24 hr in 25% methanol.

2.2.2.3 Endotoxin content

Endotoxin content of the preparations and of LPS from *Escherichia coli* strain 0111:B4 (Sigma-Aldrich) was determined using the Pyrogent Plus® Limulus Amoebocye Lysate (LAL) test kit (BioWhittaker, Wokingham, UK), according to manufacturer's instructions. Specifically, the presence of endotoxin in a sample was detected by the formation of a gel-clot after the lysate was incubated with the sample for 1 hr at 37 °C. The sensitivity of the lysate was first confirmed using an endotoxin standard of known potency. The endotoxin content of the preparations was then determined, in duplicate by performing a limiting dilution assay.

2.3 RESULTS

2.3.1 Analysis of protein content of schistosome preparations

Comparisons of total protein content of the preparations showed that the released larval molecules (0-3hRP) were less abundant (295 ± 39 μg / 650,000 parasites; n = 3) compared to the content of the equivalent 3hSSP preparations (1851 ± 460 μg / 650,000 parasites) from whole larvae. SCP and the later stage schistosomulae preparations also contained higher levels of protein than 0-3hRP.

Analysis by one-dimension (1-D) gel electrophoresis demonstrated that 0-3hRP differs considerably in protein content to SCP and larval SSPs (Figure 2.1). 0-3hRP contained 4 dominant protein bands, at approximately 60, 50, 26 and 12 kDa, plus a large number of minor bands. 0-3hRP also contained a complex of large proteins (75 → 250 kDa) that did not resolve clearly as discrete bands.

SCP contained a larger array of discrete protein bands compared to 0-3hRP. This protein complement included all the components of 0-3hRP, except for the 75 → 250 kDa complex and the 50 kDa band (Figure 2.1). Indeed, the protein complex appeared unique to 0-3hRP, and the 50 kDa protein was much more abundant in the released material compared to all of the soluble whole larval preparations (Figure 2.1 and Figure 2.2).

The 60, 26 and 12 kDa bands present in 0-3hRP were also present in, SCP, 3hSSP and 18hSSP, but were less abundant (Figure 2.1). However, analysis of later stage preparations showed these bands to disappear, or be greatly reduced in concentration (Figure 2.2).

Compared to each other, the preparations of whole larvae were more alike in content, with most bands expressed at similar levels within all preparations (Figure 2.1 and Figure 2.2). SCP contains two bands that were not detectable in other preparations, one at approximately 100 kDa and one at just under 50 kDa, whereas there was comparatively little difference in 3hSSP and 18hSSP (Figure 2.1). A greater variation in protein content was apparent when all the whole larval preparations (3hSSP to 8dSSP) were compared (Figure 2.2).

2.3.2 Low levels of naturally occurring endotoxin are present in soluble preparations

The gel-clot based LAL assay was used to detect and quantify the presence of endotoxin within the preparations. Data is presented as endotoxin in 50 μg of schistosome preparation, because in subsequent chapters up to 50 μg / ml was used in cell stimulation assays. Of all the preparations tested, 0-3hRP contained the highest levels of endotoxin, ranging from 0.5 - 2 Endotoxin Units (EU) per 50 μg of protein (Table 2.2). However, in subsequent screening of all batches of 0-3hRP, the average endotoxin content (n = 16) was 6.17 ± 1.1 (mean ± SEM) EU per 50 μg protein (data not shown). In contrast, a volume of RPMIc that was equivalent to 50 μg 0-3hRP contained less than 0.03 EU.

Compared to 0-3hRP, soluble whole larval preparations contained very low levels of endotoxin (Table 2.2). Of these preparations, SCP had the highest endotoxin content level, containing between 0.06 - 0.2 EU / 50 μg protein, whereas 3hSSP, 18hSSP and 8dSSP all contained less than 0.02 EU / 50 μg protein.

Analysis of the EU content of purified LPS from *E. coli*, demonstrated that it had an endotoxin activity of 4 EU / ng (data not shown). Therefore, an estimate of LPS levels within the preparations could be calculated (Table 2.2).

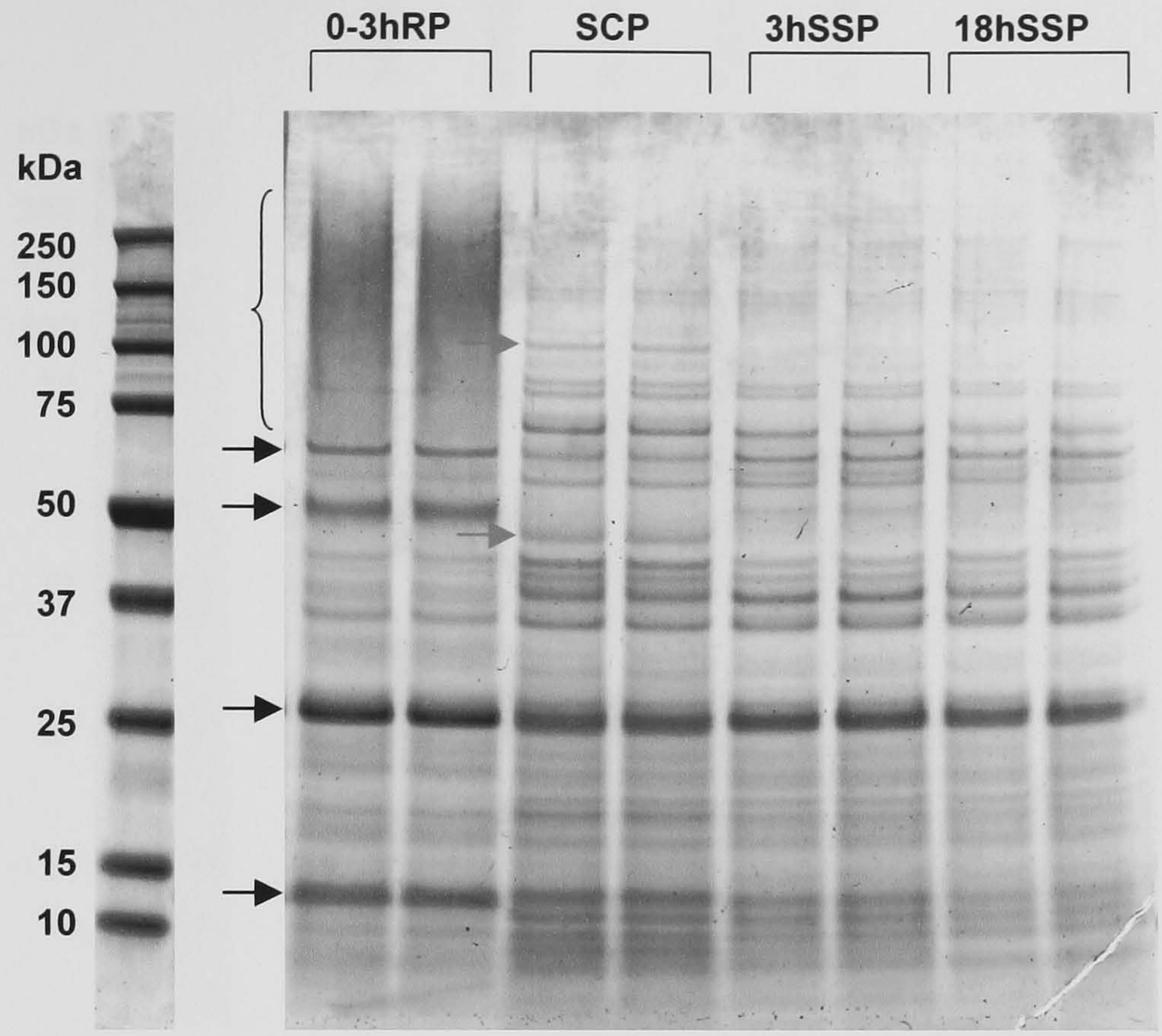


Figure 2.1 **0-3hRP differs in protein composition compared to whole larval SSPs.** Soluble schistosome preparations (10 μg / lane) were separated on a 4% - 12% gradient bis-tris acrylamide gel. Samples were denatured by boiling for 5 min in sample buffer, and run for 40 min at 200 V. The gel was then stained with colloidal Coomasie overnight. Black arrows indicate the 4 dominant protein bands within 0-3hRP. The brace indicates a protein complex within 0-3hRP. Grey arrows indicate two protein bands which are visible only within SCP.

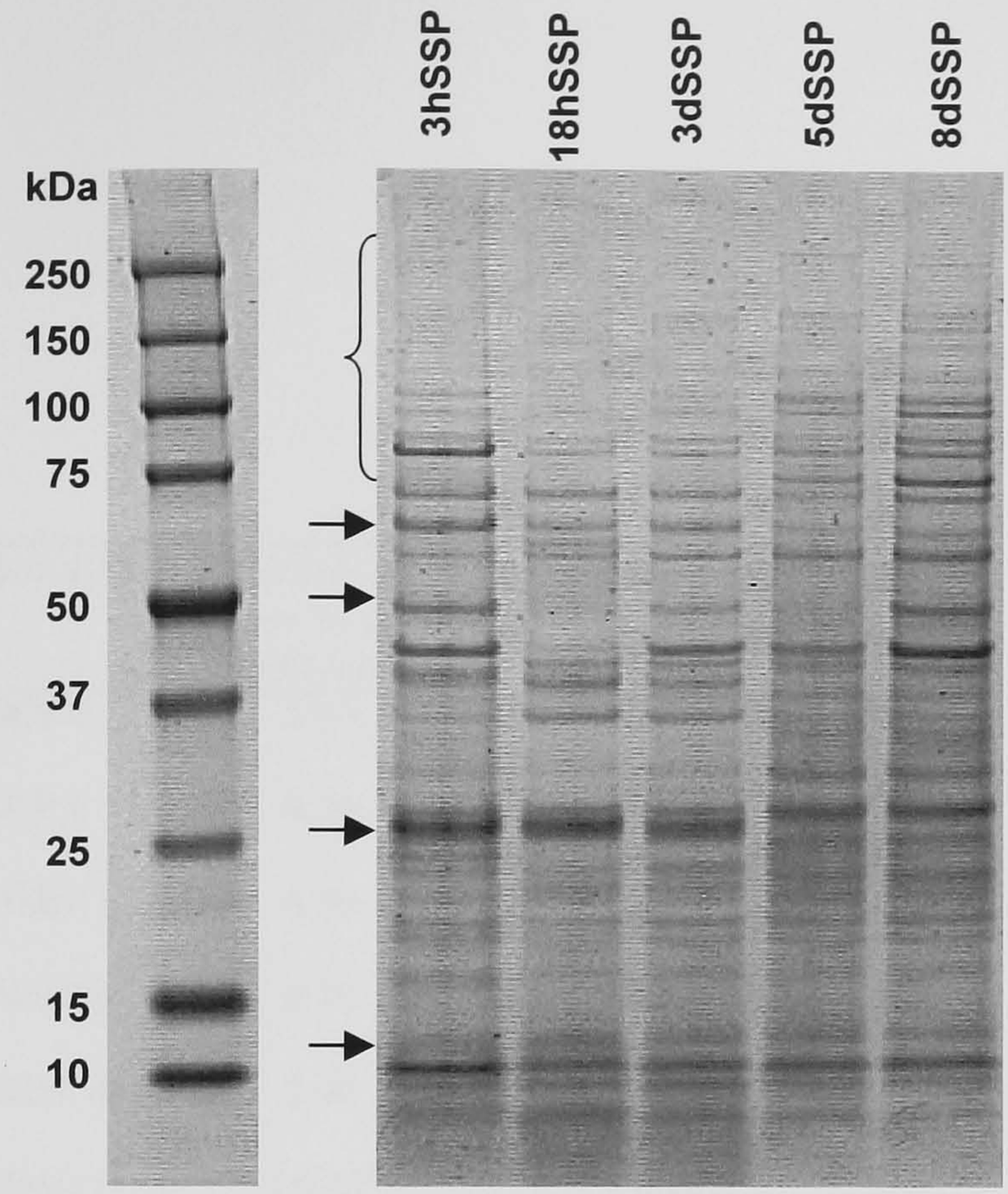


Figure 2.2 **Soluble preparations of schistosomulae cultured *in vitro* to different developmental stages vary little in protein composition.** Soluble schistosome preparations (10 μg / lane) were separated on a 4% - 12% gradient bis-tris acrylamide gel. Samples were denatured by boiling for 5 min in sample buffer, and run for 40 min at 200 V. The gel was then stained with colloidal Coomasie overnight. Arrows indicate the positions of the 4 dominant protein bands within 0-3hRP. The brace indicates the position of the protein complex within 0-3hRP.

Preparation	*Endotoxin (EU) per 50 μg preparation*	*Equivalent LPS*[a] *(ng) per preparation (50 μg)*
SCP i	0.06	0.015
SCP ii	0.20	0.050
0-3hRP i	0.50	0.125
0-3hRP ii	0.70	0.175
0-3hRP iii	2.00	0.5
RPMIc i	< 0.03[b]	< 0.007[b]
3hSSP i	0.02	0.005
18hSSP i	0.01	0.003
8dSSP i	0.003	0.001

Table 2.2 **Endotoxin content of schistosome preparations, as determined by the LAL assay.**

[a] Endotoxin content of LPS from *E. coli* strain 0111:B4.

[b] RPMIc has no protein content, so value displayed represents amount of endotoxin in a volume equivalent to a 50 μg / ml aliquot of 0-3hRP.

2.4 DISCUSSION

In this chapter, I demonstrate that soluble parasite preparations can be produced from schistosomulae cultured *in vitro* to different developmental stages. In addition, 1-D gel electrophoresis reveals that the released molecule preparation, 0-3hRP, differs considerably in protein composition compared to the soluble preparations of whole parasites. In contrast, preparations of whole parasites show considerable similarity between different larval developmental stages. It is important to note that the goal of this chapter was not to provide a detailed analysis of specific proteins or components, but to simply identify whether there were major differences in the preparations, which will be used in subsequent experimental chapters for investigation of their immunological properties. Moreover, Rachel Curwen has undertaken an in-depth two-dimension (2-D) proteomic analysis of these skin-stage larval preparations (with the aim of identifying vaccine candidates) as part of her PhD studies under the supervision of Prof. R. A. Wilson.

In this study, I used a method of mechanical-transformation of cercariae originally documented by Ramalho-Pinto *et al.* (1974). *In vitro* culture of larvae transformed using this technique results in schistosomulae that are viable, as reintroduction of both *in vitro* and *ex vivo* parasites results in similar levels of maturation (Harrop and Wilson, 1993b). In addition, several other methods of artificial-transformation have been documented. These can be grouped into 'penetration through skin membranes', using excised mouse or human skin, or 'chemical' transformation of cercariae, such as incubation with skin-derived products, like linoleate or linoleic acid. Although 'penetration of skin membranes' is the most natural method, it produces very low numbers of schistosomulae. Also, much of the material released during transformation is lost in the membrane, rendering this a poor method against the original criteria (Section 2.1). In a comparison of mechanical (vortex) *versus* chemical (linoleate) transformation, both methods resulted in larvae with surface characteristics of schistosomulae (Salafsky *et al.*, 1988). However, significant differences in the biochemical characteristics of these schistosomulae were observed, with chemical transformation resulting in dramatic increases in the detectable levels of eicosanoids, such as PGE_2, in the larval culture SN. In an immunological context, eicosanoids can exert both stimulatory and inhibitory effects on a variety of innate accessory cells (Section 1.9.2). Moreover, fatty acids, such as linoelic acid, are potentially toxic and are known to have

effects on immune cell function, including inhibition of Mϕ cytokine production (Tappia *et al.*, 1995). Therefore, the method of mechanical-transformation was chosen since it allows for the collection of material released by cultured schistosomulae that will lack potentially toxic / inhibitory fatty acids and contain only low levels of eicosanoids, allowing screening for stimulatory characteristics without interference from the presence of inhibitory prostaglandins.

The parasite preparations used in my studies will be composed of protein, lipid, and carbohydrate moieties. All of these molecules are represented within the repertoire pathogen PAMPs that have been identified to date (Section 1.8.1 & 1.8.3). However, protein is likely to be the major constituent of my preparations, since others report that similar released preparations consist of protein and carbohydrate in ratios of 5:1 - 7:1 (Vieira *et al.*, 1986). Therefore, the quantitative and qualitative analysis of the different preparations in this study was based upon protein content. Moreover, methods for detection and quantification of protein are also far more reliable than for the other molecular species.

My studies demonstrate that the mean (n = 3) amount of soluble material released by the parasite during the first 3 hr of culture (0-3hRP) represents approximately one sixth of the protein contained within the 3 hr soluble schistosomula preparation (3hSSP). This compares with values of one third (Harrop and Wilson, 1993b) and one twentieth (Ramaswamy *et al.*, 1995b), reported in previous studies. The variation in these estimates is probably due to differences in either quantification techniques, or in the methods of production. Therefore, since 0-3hRP is less abundant than soluble material from whole larvae, the availability of 0-3hRP will be the limiting factor for use in subsequent cell-based stimulation assays.

In addition to the material released during the first 3 hr post-transformation, migrating schistosomulae are also known to release material up to 14 days post-infection (Mountford *et al.*, 1988). However, the amount of material released rapidly decreases with time post-infection. The profile of antigen release by schistosomulae *in vivo* is similar during *in vitro* culture, with three times as much material being released during the first 3 hr post-transformation, than released during the subsequent 24 hr of culture (Harrop and Wilson, 1993b). Although the material released by the larvae from 3 hr to 8 days of culture may

contain parasite PAMPs, the low levels at which it is released make it impossible to collect enough for subsequent *in vitro* analysis of the stimulatory properties.

1-D gel-electrophoresis showed the released molecule preparation, 0-3hRP, to have a more simple protein composition than the soluble whole larvae preparations. However, in-depth analysis of the 0-3hRP proteome using 2-D gel-electrophoresis has shown that it is comprised of over 100 components (pers. com. R. Curwen). As there is little protein synthesis by schistosomulae during the first 24 hr post-transformation (Harrop and Wilson, 1993b), the molecules released by the larvae should be a constituent of the whole cercariae. Indeed, the majority of proteins within 0-3hRP were also present in SCP. However, most of these were less abundant, effectively diluted by the many additional protein species contained within SCP. Conversely, some of the shared components were more concentrated in SCP. This suggests that these represent largely non-secreted molecules, or somatic constituents. There are also bands within 0-3hRP that are not present in SCP. These may either represent the products of enzymatic degradation that could occur during the 3 hr period, or could be very dilute within SCP.

Much work has been done to characterise the material that is released by cercariae as they undergo transformation. Up to 60% of the cercarial surface glycocalyx is shed into the culture SN during the first 3 hr post- *in vitro* transformation (Samuelson and Caulfield, 1985; Samuelson and Caulfield, 1986; Marikovsky *et al.*, 1986). The glycocalyx is highly glycosylated (Xu *et al.*, 1994; Cummings and Nyame, 1999). For example, mucins are present within the cercarial glycocalyx and are secreted into the skin by the parasite and are thought to aid adherence and penetration of the host (Cummings and Nayame, 1999; Theodoropoulos *et al.*, 2001). Much of this glycosylated material will be of high molecular weight, yet unlikely to resolve as a discrete protein bands under SDS-PAGE electrophoresis. Therefore, the complex of large proteins (75 → 200 kDa) observed within 0-3hRP, may potentially be comprised of the soluble fraction of these molecules. However, due to the membranous nature of the glycocalyx, the majority should be removed in the insoluble fraction of the concentrated SN, when centrifuged at 100,000 *g* for 1 hr. Indeed, analysis of crude non-centrifuged 0-3hRP shows it contains a much greater quantity of this high molecular weight protein complex (R. Curwen), suggesting that most glycocalyx material is absent in 0-3hRP, and consequently from SCP as well.

A large proportion of the material released by the parasite during transformation originates from the pre- and post-acetabular glands. This material is packaged into distinct vesicles, which are deposited as the parasite penetrates the host (Fishelson *et al.*, 1992). Much controversy has surrounded the abundant protease component of this material, which is primarily thought to facilitate the degradation and penetration of host skin. Indeed, many skin structural molecules, such as elastin and collagen, are known to be substrates (Mckerrow *et al.*, 1985a), and protease inhibitors can block cercarial penetration (Lim *et al.*, 1999). Moreover, cercarial proteases have also been implicated in immune evasion (Pleass *et al.*, 2000) and the shedding of the cercarial glycocalyx (Marikovsky *et al.*, 1988a). The main proteolytic component released is a serine-protease termed elastase. Historically, there have been 4 cercarial elastase genes registered (Salter *et al.*, 2002), and numerous serine proteases of 25 kDa (Landsperger *et al.*, 1982), 27 kDa (Darani *et al.*, 1997), 28 kDa (Marikovsky *et al.*, 1988b), 30 kDa (McKerrow *et al.*, 1985b), 47 kDa (Chavez-Olortegui *et al.*, 1992) and 60 kDa (Marikovsky *et al.*, 1988b) identified from cercariae. It has been suggested that some of these are products of the same gene, but with different post-translational modifications (McKerrow *et al.*, 1991). However, a more simple picture of released cercarial proteases is emerging, with a single chymo-trypsin serine protease composed of multiple 25 kDa iso-forms proposed to be solely responsible for the observed elastase activity. Elastase has also been identified on the surface membrane of transformed schistosomulae (Ghendler *et al.*, 1996), and therefore, may be present in the SSPs used in my study. Indeed, in the 1-D gel shown, the most dominant band observed within 0-3hRP, also present in the preparations of whole larvae up to 3dSSP, was approximately 26 kDa. Other typsin-like proteases that had previously been identified in released cercarial material are thought to be contaminants derived from the intermediate snail host (Salter *et al.*, 2000; Salter *et al.*, 2002). With the method described in this chapter for the extensive washing of cercariae, snail protease contaminants should not be present in the schistosome preparations used in this thesis. Immuno-histochemical techniques have also identified cysteine-proteases, including cathespin-L, within the vesicles of the post-acetabular gland, suggesting these will be present in the released material (Dalton *et al.*, 1997). Therefore, the proteases contained within the pre- and post-acetabular glands are likely to constitute a large proportion of 0-3hRP.

Other molecules known to be released during transformation include two 23 kDa proteins, Translationally Controlled Tumour Protein (TCTP; Rao *et al.*, 2002) and *S. mansoni*-derived apoptosis inducing factor (SMAF; Chen *et al.*, 2002). TCTP is also expressed in all other life-cycle stages, as a variety of differently sized but related molecules. Oddly, TCTP and SMAF were described by the same group, however appear to be the same molecule, as a BLAST search on the N-terminal sequence of SMAF perfectly matches part of TCTP, yet the authors make no mention of this (R. Curwen). The same group has also described a 16.8 kDa protein with anti-inflammatory properties within the released material and soluble preparations of schistosomulae (Ramaswamy *et al.*, 1995a; Rao and Ramaswamy, 2000). 2D-gel separation followed by mass-spectrometry has confirmed that Sm 16.8 is abundant within 0-3hRP (R. Curwen). Eicosanoids, such as PGE_2, and leukotriene B_4 (LTB_4), are synthesised upon transformation (Fusco *et al.*, 1985) but are only detected at low levels in the released material from mechanically transformed parasites (Salafsky *et al.*, 1988). Moreover, the protein thought to control eicosanoid synthesis, a 28 kDa glutathione-s-transferase (GST 28; Herve *et al.*, 2003) is known to be abundant in 0-3hRP, as well as SSPs (R. Curwen). Some of the pre- and post-acetabular gland contents are also glycosylated, binding to numerous different lectins (Linder, 1985). Indeed, glycans containing the Lewis X (Le^x) epitope, which is also carried by the immuno-regulatory pentasaccharide LNFP III from schistosome eggs (Section 1.6.1 - 1.6.2), are found within the released material (Koster and Strand, 1994), and are likely to be present in 0-3hRP. Therefore, the contents of 0-3hRP have been well characterised by others, demonstrating that it contains a complex mixture of potentially stimulatory molecules.

In addition to molecules shared with 0-3hRP, the soluble schistosomulae preparations (SSPs) also contain many additional protein bands. Indeed, a 60 / 66 kDa doublet, potentially present on the surface of cercariae and schistosomulae, was recently described to stimulate neutrophil migration (Coelho-Castelo *et al.*, 2002). However, as with the cercarial glycocalyx, the majority of the membrane-bound surface molecules are likely to have been removed from the preparations by centrifugation at 100,000 *g*. Therefore, the SSPs will largely represent somatic molecules, such as Heat Shock Proteins (HSPs), actin, and paramyosin.

A major consideration from the start of this project, has been the potential for exogenous endotoxin contamination within the schistosome preparations. The infective cercariae used

for the *in vitro* culture of schistosomulae originate from a non-sterile environment. Therefore, the method used to create the preparations was developed at the beginning of my PhD in order to greatly reduce the potential for contamination. This involved washing cercariae three times with sterile water prior to transformation, and the use of low endotoxin culture components and equipment. In addition, in all culture steps, the larvae were incubated with penicillin and streptomycin to inhibit bacterial growth. However, I identified that a limited amount of endotoxin is present in the different preparations (0.003 - 6.17 EU per 50 μg / protein). It must be emphasised that these levels of endotoxin are classed as low (*e.g.* commercially available FCS marketed specifically as 'low endotoxin' [Invitrogen] is certified as containing not more than 10 EU / ml). 0-3hRP consistently contained higher levels of endotoxin than the whole larval preparations. This is most probably due to the process of concentrating culture SN 50-fold, effectively concentrating endotoxin as well. Moreover, the protein concentration of 0-3hRP is much lower than the other preparations, in effect increasing the relative protein to endotoxin ratio.

The stimulatory effect of endotoxin has been well documented, and therefore, could potentially affect the stimulatory properties of the schistosome preparations. In this respect, the bacterial PAMP lipopolysaccharide (LPS) is the major active constituent of endotoxin. Several steps were taken in subsequent chapters to minimise the effect that this endotoxin contamination would have on the stimulatory properties of the schistosome preparations. The cyclic peptide polymyxin B (PMB) binds to the stimulatory lipid A region of LPS. Therefore, Detoxi-gel^{TM} (Perbio Science UK Ltd), a PMB gel matrix, was used to remove the LPS. Unfortunately, much of the schistosome preparations were lost on these gel columns (data not shown), and therefore, this technique was abandoned. As an alternative, PMB is also known to inactivate the stimulatory properties of LPS when used directly in culture (Duff and Atkins, 1982). Indeed, it has become widely used to determine immunological properties of experimental agents, in the absence of endotoxin signalling (*e.g.* Yoshida and Koide, 1997).

In summary, I have produced several soluble preparations of released and somatic molecules from *in vitro* cultured schistosomulae. These preparations have been produced in sufficient quantities for use in *in vitro* cell-based stimulation assays. The released molecule preparation (0-3hRP) is considerably different to the whole larvae preparations,

which on the other hand are broadly similar. Further to this, endotoxin contamination is detected at low levels in all preparations but is greatest in 0-3hRP.

CHAPTER 3

Mϕ CYTOKINE PRODUCTION IN RESPONSE TO SCHISTOSOME PAMPS

3.1 INTRODUCTION

The schistosome preparations produced in Chapter 2 are likely to have stimulatory effects on innate accessory cells. Screening for these stimulatory properties requires an experimental *in vitro* cell-based assay using a defined source of cells, which should be available in large quantities and have measurable function as a readout of stimulation.

The innate accessory cells present in the skin which may respond to invading schistosomes include epidermal LC, Mϕ, DC, granulocytes, NK, and keratinocytes (Williams and Kupper, 1996). In depth analysis of events following parasite penetration and migration through the skin, demonstrate acute local inflammation at the infection site, during which large numbers of mononuclear and granulocytic cells immigrate into the dermis and epidermis (Section 1.3.2). This cellular influx is likely to be caused by soluble factors, such as cytokines and chemokines, released by resident innate accessory cells stimulated by the presence of invading larvae. Recruited inflammatory accessory cells may respond in a similar way, and there is both *in vivo* and *ex vivo* evidence of pro-inflammatory and regulatory cytokine production at the site of infection (Section 1.3.3). Many of these innate accessory cells are rare or difficult to obtain *ex vivo* in sufficient quantities to screen the soluble schistosome preparations for their stimulatory properties. However, there are several techniques for the isolation of large numbers of Mϕ from murine tissues. Mϕ are also known to release large quantities of many pro-inflammatory and regulatory cytokines upon *in vitro* recognition of numerous pathogen PAMPs (Stein and Gordon, 1991; Moors *et al.*, 2001; Nau *et al.*, 2002) giving a quantifiable readout of stimulation.

Mϕ, or their tissue-specific subsets, can be found in the majority of organs within the body and there are a variety of isolation techniques to enable their study *in vitro*. Mϕ may be resident within close cellular matrices, such as in the skin, but their isolation involves the use of traumatic enzymatic digestion techniques to dissociate them from connective tissues, and usually only a few cells are recovered. One alternative is to use Mϕ that exist in tissues lacking this close cellular restraint, such as the lung or the peritoneal cavity, from which they can be extracted by simple lavage. Using the peritoneal cavity as the Mϕ source has the additional benefit that relatively large quantities of cells are obtainable. For these

reasons, most *in vitro* studies on Mϕ have been performed using cells obtained from the peritoneal cavity.

From a single mouse, approximately 2 - 3 x 10^6 peritoneal exudate cells (PEC) can be obtained, of which 50% - 70% are "resident" Mϕ (reviewed by Fortier and Falk, 1994). However, injection of sterile inflammatory mediators 3 to 7 days prior to lavage causes the elicitation of "inflammatory" Mϕ (iMϕ) into the cavity, which dramatically increases the numbers of cells that can be harvested. A variety of different inflammatory irritants can be used, such as proteose peptone, thioglycollate, casein (Fortier and Falk, 1994), or Bio-gel™ polyacrylamide beads (Stein and Gordon, 1991). Of these, thioglycollate increases the number of peritoneal Mϕ to the greatest extent, resulting in approximately 6-fold more cells (Fortier and Falk, 1994). Therefore, this method was chosen to produce the large number of iMϕ needed to screen the parasite preparations.

Peritoneal lavage results in a mixed population of peritoneal exudate cells (PEC), which in addition to resident and iMϕ, includes neutrophils and B lymphocytes. There are several methods to purify Mϕ, based on cell size, density, or adherent function (reviewed by Gessani *et al.*, 2000). Of these, the latter technique is the simplest and most widely used, relying upon the strongly adherent properties of Mϕ while less / non-adherent PEC (*e.g.* granulocytes and lymphocytes) are removed by washing.

Mϕ can exist in a variety of maturation and activation states, resulting in a heterogeneous population within the tissues (Gordon *et al.*, 1986; Rutherford *et al.*, 1993). A two-step model of activation has been proposed, whereby Mϕ are 'primed' by cytokines but need a secondary signal, such as PAMPs, to 'trigger' or 'stimulate' a fully activated functional phenotype (Pace *et al*, 1985). Numerous cytokines are known to prime for Mϕ activation, such as IFNα, IFNβ (Pace *et al.*, 1985), IL-4 (Crawford *et al.*, 1987) and GM-CSF (Reed *et al.*, 1987). However, one of most potent and intensely studied is IFNγ (Pace *et al.*, 1985), known to dramatically increase levels of some cytokines produced by iMϕ in response to stimulation with PAMPs, such as LPS (Rutherford *et al.*, 1993) and glycoproteins from *Typanosoma cruzi* (Camargo *et al.*, 1997). Thus, it may be necessary to prime the iMϕ with IFNγ to achieve detectable cytokine production in response to the schistosome PAMP preparations.

In addition to primary cultures, Mϕ cell-lines have been used to study the stimulatory properties of pathogen PAMPs (Kirikae *et al.*, 1993; Yoshida and Koide, 1997). The use of cell-lines is beneficial because the population is completely homogeneous in maturation and activation status, and large numbers can be produced. There are a variety of murine Mϕ-derived cell-lines that could offer an insight into the stimulatory properties of the parasite preparations. These include the BALB/c-derived J774 and RAW 264.7 lines. Furthermore, some clones have been created to lack certain PRRs, such as the CD14-deficient J3.DEF.3 line (Kirikae *et al.*, 1993).

The aim of this chapter is to determine whether the schistosome preparations created in Chapter 2 are capable of stimulating the production of cytokines by primary iMϕ and IFNγ-primed Mϕ (IFNγ-Mϕ), and by Mϕ cell-lines. The profile of cytokine production in response to schistosome preparations will be compared to that of other well-documented PAMPs, such as LPS and Zymosan A. Finally, the response of Mϕ to live schistosomes will be examined.

3.2 MATERIALS AND METHODS

3.2.1 Mice

All mice were maintained in open housing at the University of York animal unit. C57BL/6 mice were bred in house, while C3H/HeN and C3H/HeJ mice were obtained from Harlan UK. Mice were age and / or sex matched in individual experiments.

3.2.2 Production of inflammatory Mϕ (iMϕ)

Peritoneal exudate cells (PEC) were extracted from mice by peritoneal lavage 5 days post-injection with 0.5 ml sterile 0.09% thioglycollate medium FTG (Sigma-Aldrich). Where stated, in later experiments 0.5 ml 3% Brewers thioglycollate medium (Sigma-Aldrich) was used as an alternative inflammatory agent. The medium used during collection and culture of iMϕ consisted of RPMI 1640, containing 200 U / ml penicillin, 100 μg / ml streptomycin, 2mM L-glutamine (Invitrogen), and 10% heat-inactivated low-endotoxin

FCS (Harlan-Seralab; RPMI-10). Due to the highly adherent nature of Mϕ, all medium and plastic-ware was kept chilled throughout the cell extraction procedure. Mice were killed by cervical dislocation, and chilled RPMI-10 (10 ml) plus air (1 ml) was then injected into the peritoneal cavity using a 25 gauge needle. The cavity was then palpated before removal of the injected medium using a 23 gauge needle, attached to a 10 ml syringe. The PEC-containing medium was immediately decanted into a chilled 50 ml polypropylene tube (Falcon), and pooled from several mice if required. PEC were centrifuged at 350 *g* for 5 min to remove remnants of thioglycollate and resuspended in fresh chilled medium (1 ml / mouse). An aliquot of the cell suspension was diluted 1:1 with diluting fluid (0.5 % Malachite green solution in 1.5 % acetic acid) and the cells counted using a haemocytometer. Cells were adjusted to 1 x 10^6 cells / ml, and plated out in either 24-well plates (1 x 10^6 cells / well), or 96-well plates (2 x 10^5 cells / well; Nalge Nunc).

iMϕ were purified from the PEC population by adherence to plastic. Specifically, after culture for 2 hours at 37 °C, 5% CO_2, in a humidified incubator, the non-adherent cells were discarded from the adherent iMϕ monolayer. Semi-adherent cells were then removed and discarded by gently washing the monolayer 3 times with pre-warmed medium.

3.2.3 Phenotypic characterisation of the iMϕ population

PEC and adherent Mϕ populations were analysed for the expression of myeloid differentiation markers using flow cytometry.

3.2.3.1 Preparation of cells for phenotyping

To remove adherent iMϕ from the cell-culture wells, the culture media was discarded and the cell monolayer rinsed with phosphate buffered saline (PBS; pH 7.2; 137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4; 2.6 mM KCL). Following incubation with Trypsin-EDTA (0.25%, 1 mM, respectively; Invitrogen) for 10 min at 37 °C, to loosen attachment, the cells were then removed using the bung of a 1 ml pipette as a rubber policeman. RPMI-10 was immediately added to the cell suspension to quench the trypsin and limit the cleavage of cell surface markers. Cells were then washed with chilled medium and all subsequent

steps carried out on ice to minimise iMϕ adherence. Alternatively, the whole PEC population prior to adherence-selection was analysed.

3.2.3.2 Staining of cells with specific antibodies

PEC or iMϕ were re-suspended in cold phenobuffer (PB; PBS containing 0.01% $CaCl_2$, 0.01% $MgCl_2$ and 0.1% BSA; Sigma-Aldrich) and all subsequent steps carried out on ice to minimise non-specific staining. Aliquots of cells (up to 5 x 10^5) were incubated with 2 µl Rabbit IgG (Sigma-Aldrich) for 10 min, to block Fcγ receptors and any non-specific antibody binding sites. Cells were then incubated for 30 min with optimum concentrations (100 µl) of purified, biotin-conjugated, or fluorochrome-conjugated antibodies against specific surface markers. Fluorochromes used in this and subsequent chapters were; Fluorescein isothiocyanate (FITC), R-Phycoerythrin (PE), Quantum Red™ (QR™) or Cy-Chrome™. Antibodies against surface markers were as follows (clone; supplier): Gr-1 (RB6-8C5; Caltag-MedSystems Ltd, Towcester, UK), F4/80 (CI:A3-1; Caltag), B220 (RA3-6B2; BD PharMingen, Oxford, UK), CD11b (Mac-1, M1/70; BD PharMingen), or CD45 (30-F11; BD PharMingen). Alternatively, corresponding cell aliquots were incubated with isotype-matched antibodies of irrelevant specificity, in order to control for non-specific binding and to establish background levels of cellular auto-fluorescence. After staining, cells were washed in 1.5 ml PB. Cells stained with fluorochrome-conjugated antibodies were re-suspended in 100 ml PB and analysed. Cells stained with purified antibodies, were incubated for 30 min with optimal concentrations (100 µl) of FITC-conjugated antibody to rat IgG γ-chain (STAR 69; Serotec, Kidlington, UK), whereas cells stained with biotinylated antibody were incubated with streptavidin-conjugated QR™ (Sigma-Aldrich). Cells were washed, as previously, and resuspended in 100 µl PB for analysis.

3.2.3.3 Differentiation of live from dead cells

To determine the percentage of dead cells, 10 µl of Propidium Iodide (PI; 200 mg / ml in PBS; BD PharMingen) was added to relevant cell aliquots and incubated for 20 sec before flow-cytometric analysis. Those cells staining positive for PI were considered dead.

3.2.3.4 Flow-cytometry

Flow-cytometry was performed using an EPICS XL (Coulter) equipped with a 15 mW argon ion laser of 488nm wavelength. This machine contained three different filters, allowing for analysis of three different 'colour' flourochromes. FITC (green; 530 nm peak fluorescence) was measured using the FL-1 detector, PE (orange; 575 nm) and PI were measured using the FL-2 detector, while QR (violet; 670 nm) and Cy-Chrome (violet; 670 nm) were measured using the FL-3 detector. In addition, size and granularity of the cells was determined using forward and side light scatter (90^{o} to the laser source), respectively.

3.2.3.5 Analysis of cell phenotype

One-colour flow-cytometry was performed on the cells. On histograms depicting fluorescence intensity, a minimum-fluorescence cursor was set at an arbitrary value, typically on the 1% to 5% most fluorescent cells stained with the appropriate isotype control antibody. When cells stained with the specific antibody were analysed, the percentage of cells within this cursor was taken as the positive cell value, and the value adjusted to account for the arbitrary background staining. To determine the physical size and granularity of the positive or negative cells, this cursor was used to 'gate', or limit, analysis on the desired population. In addition, on the size *versus* granularity scatterplots, 'regions' were set around particular cell populations. Using these regions, the population could then be excluded from, or focused upon, in subsequent analysis. Details of the 'gates' and 'regions' used in analysis, are included in figure legends. In all experiments a 'discriminator' was set on the forward light scatter parameter. Any events below this setting were subsequently excluded from all analyses, thus ignoring very small events, such as cell debris. Equally, all oversized events (*i.e.* clumped cells) were removed from subsequent analysis using a cell 'region'.

3.2.4 iMϕ stimulation assays

Adherent iMϕ were used to determine the stimulatory properties of different pathogen PAMPs or parasite preparations. In addition, responses by IFNγ-Mϕ were also analysed. This was achieved by co-culturing iMϕ with IFNγ (5 U / ml equivalent to 0.5 ng / ml;

purified from the culture supernatant of 211A CHO cell-line) during stimulation assays. All assays were carried out within 96-well plates.

3.2.4.1 Stimulation of Mϕ with parasite preparations and pathogen PAMPs

The adherent monolayer of Mϕ was cultured for defined periods of time between 30 min - 48 hr with soluble parasite preparations (10 - 200 μg / ml; produced as described in 2.2.1), LPS (0.1 - 10 ng / ml; from *E. coli*, strain 0111:B4; Sigma-Aldrich), or Zymosan A (0.2 - 5 μg / ml; Sigma-Aldrich). Stimulation assays were carried out in the presence, or absence, of polymyxin B (PMB; 0.01 - 100 μg / ml; Sigma-Aldrich). Culture supernatants were then removed and frozen at -20 °C, to await the detection of cytokines released by Mϕ, using ELISA.

3.2.4.2 Stimulation of iMϕ with live parasites, or neat parasite culture supernatant

In order to determine the stimulatory properties of live parasites, iMϕ were cultured for 24 hr with larvae (50 - 200 parasites / well) and PMB (3 μg / ml). Three different parasite groups were analysed, and consisted of cercariae (CercN), mechanically-transformed cercariae (CercT; vortexed for 90 sec), or mechanically-transformed larvae that had been cultured *in vitro* for 3 hr followed by removal of the culture supernatant (3hSom). This culture supernatant, termed 0-3hSN, was also assayed for its stimulatory properties. Cell-culture supernatants were then removed and frozen at -20 °C, to await the detection of cytokines using ELISA.

3.2.5 Culture of murine Mϕ cell-lines

3.2.5.1 Maintenance of cell-lines

The BALB/c derived Mϕ cell-line J774A.1 was a gift from Dr. M. Taylor, University of Liverpool, and the J774A.1 derived CD14-deficient J7.DEF.3 line was a gift from Dr. F. Kirikae, International Medical Centre of Japan. Both lines were grown as monolayer cultures in RPMI-10 in the absence of penicillin and streptomycin. Cells were cultured in 25 cm^2 (10 ml media), or 75 cm^2 (50 ml media) tissue-culture flasks (Nalge Nunc). Cells

were sub-cultured when grown to confluence, typically every 2 - 3 days. To harvest, adherent cells were detached into the culture media using a cell scraper (Nalge Nunc). Cells were then seeded into new flasks at 1:5 - 1:10 split ratio. Both cell-lines tested negative for mycoplasma contamination using the Mycoplasma Plus^{TM} PCR primer set (Stratagene, La Jolla, CA, USA).

3.2.5.2 Mϕ cell-line stimulation assays

J774A.1 or J7.DEF.3 cells were harvested, transferred into fresh RPMI-10, and seeded into 96-well plates at 1 x 10^5 cells / well. This quantity of cells was shown to be optimal for IL-6 and IL-12p40 production in response to stimulation with LPS (data not shown). Cells were then cultured for 24 hr alone, in the presence of LPS (0.3 - 2000 ng / ml), or with different soluble schistosome preparations (50 μg / ml), and with or without the addition of PMB (10 μg / ml). In some experiments, an optimal concentration of IFNγ (1 U / ml) was used to prime the cells simultaneous to culture with pathogen stimuli. Cell-culture supernatants were then removed and frozen at -20 °C, to await the detection of cytokines using ELISA.

3.2.6 Detection of Mϕ – released cytokines by ELISA.

3.2.6.1 ELISA procedure

Paired antibody capture ELISAs, utilising a biotinylated detection step, were used to detect and quantify the levels of cytokines within the Mϕ culture supernatants. At all stages, incubations were carried out in a total volume of 50 μl unless otherwise stated. Maxi-sorb^{TM} 96-well plates (Nalge Nunc) were coated with an optimal concentration of capture antibody (see Section 3.2.6.2) diluted in PBS, and incubated in a humidified chamber overnight at 4 °C. Plates were subsequently washed 3 times with 150 μl PBS, before blocking for 6 hr at room temperature (RT) with 150 μl of 10% FCS in PBS (block). Following removal of blocking agent, cell-culture supernatants were added to duplicate wells, either neat, or diluted in block at 1:2, 1:5, or 1:10. In addition, duplicate serial dilutions of known recombinant cytokine standards were performed. Plates were then incubated overnight at 4 °C, following which they were washed 5 times with 200 μl of PBS

containing 0.5% Tween 20 (Sigma-Aldrich) (PBS-T). Wash was discarded and plates incubated with optimal dilutions of biotinylated detecting antibody (Section 3.2.6.2) in PBS-T, for 1.5 hr at RT. Plates were washed as before, and incubated with streptavidin-conjugated horseradish peroxidase (Amersham Scientific), at a dilution of 1:5000 in PBS-T, for 1 hr at RT. Following a final wash, 50 µl of SureBlue^{TM} tetra-methylbenzindine substrate (TMB; Kirkgaard and Perry Ltd., via Insight Biotechnologies Ltd., Wembly, UK) was added to each well and allowed to develop before reading at 630 nm, at 10 min intervals, using a MRX microplate reader (Dynex Technologies, Billingshurst, UK). Concentrations of cytokine within samples was determined against the recombinant standard curve.

3.2.6.2 Antibodies and cytokine standards

All antibodies were obtained from BD PharMingen, unless other wise stated. The presence of the following cytokines were analysed in the Mϕ culture supernatants (coating antibody clone; detection clone); IL-1β (30311; polyclonal; both from R&D Systems, Abingdon, UK), IL-6 (MP5-20F3; MP5-32C11), IL-10 (JES5 2A5; SXC-1), IL-12p40 (C15-6; C17.8), and TNF-α (G281-2626; MP6-XT3). Recombinant standards were as follows: IL-1β and IL-10 (R&D Systems), IL-6 (BD PharMingen), IL-12p40 (Dr S. Wolf, Genetics Institute, Cambridge, MA, USA), and TNFα (Genzyme).

3.3 RESULTS

3.3.1 Purification and characterisation of peritoneal iMϕ.

Injection of inflammatory mediators into the peritoneal cavity results in the immigration of numerous 'inflammatory' accessory cells. Purification of inflammatory macrophages (iMϕ) from these peritoneal exudate cells (PEC) was achieved by adherence of iMϕ to plastic, followed by removal of non-adherent cells. In order to confirm this technique, the PEC population both before and after adherence were analysed.

The cellular composition of the PEC population was notably different to the population of adherent iMϕ (Figure 3.1a). Moreover, within the PEC population, only 50% were positive

for the pan-leucocyte marker CD45, compared to 93% of the adherent iMϕ (Figure 3.1b). Analysis of the size and granularity of $CD45^+$ cells within the PEC, demonstrated that there were 6 main cellular subsets (Figure 3.1c). However, selection by adherence resulted in the dramatic decrease or complete removal of three of these subsets, leaving three major cell groups. Further analysis demonstrated that one of the adherent subsets was comprised of dead cells (Figure 3.1c region B), leaving two major cell populations. However, in many repeats of this experiment, these two populations were shown to merge, with proportional loss of the smaller less granular population (Figure 3.2a). Subsequent analysis of the live adherent population (approximately 95% of all cells) showed that 98% were $CD45^+$, 79% were $F4/80^+$, and 90% were $CD11b^+$ (Figure 3.2 b, c, d). The large granular population was shown to consist entirely of $F4/80^+$ and $CD11b^+$cells, whereas the small, less granular population was entirely $F4/80^-$, and contained all the $CD11b^-$ cells (Figure 3.2c & d).

3.3.2 Defining the optimum conditions for Mϕ stimulation by schistosome PAMPs.

Cytokine production by Mϕ was used to determine the stimulatory properties of the different schistosome preparations. Prior to screening all of the preparations, the soluble cercarial antigen preparation (SCP) and the classic PAMP LPS were used to optimise the culture conditions for *in vitro* stimulation of iMϕ cytokine production. These studies were based upon the detection of IL-12p40 and IL-6 in the supernatant of the cultured cells, following overnight stimulation with SCP or LPS.

3.3.2.1 Mϕ concentration, schistosome PAMP concentration, and IFNγ concentration

From initial studies, it was shown that the optimal iMϕ concentration was 1×10^6 cells / ml for IL-12p40 production but 2×10^6 cells / ml for IL-6 production (data not shown). Since limited numbers of cells were obtainable from a single mouse, 1×10^6 cells / ml were used in further experiments. Titration of IFNγ demonstrated that 5 U / ml primed Mϕ for optimum IL-12p40 production in response to PAMP stimulation, and the optimal concentration of SCP using both iMϕ and IFNγ-primed Mϕ (IFNγ-Mϕ) was 50 μg / ml (data not shown). Therefore, these concentrations were used when comparing the stimulatory properties of the different schistosome preparations.

3.3.2.2 Kinetics of cytokine production in response to schistosome PAMPs

Kinetics of cytokine production by both iMϕ and IFNγ-Mϕ were studied in response to stimulation with the cercarial preparation, SCP, the released preparation, 0-3hRP, and the concentrated-media control for 0-3hRP (RPMIc). IL-12p40 was detectable in the supernatant of iMϕ as early as 3 hr post-stimulation with 0-3hRP, and 6 hr post-stimulation with SCP (Figure 3.3a). Moreover, the levels of IL-12p40 detected in iMϕ supernatants in response to stimulation with either parasite preparation appeared to reach a plateau by 6 hr. In contrast, IL-12p40 production by IFNγ-Mϕ continued to increase with time up to the final time-point (Figure 3.3b). Dramatically greater levels of IL-12p40 were produced by IFNγ-Mϕ, than by iMϕ, in response to stimulation with SCP or 0-3hRP (Figure 3.3a & b; note difference in y-axis scale).

IL-6 production by iMϕ showed a similar pattern to that of IL-12p40, reaching a plateau by 6 hr post-stimulation (Figure 3.3c). However, an increase in IL-6 production over background levels was detectable by 1 hr post-stimulation. IFNγ-priming of Mϕ had little effect on the kinetics of IL-6 production following stimulation with either parasite preparation (Figure 3.3d). Moreover, in contrast to IL-12p40 production, priming of iMϕ with IFNγ had a little effect on the levels of IL-6 produced. Indeed, when the responses of Mϕ from individual mice (n = 3) were compared, priming with IFNγ did not significantly ($p > 0.05$) effect the levels of IL-6, but significantly increased the levels of IL-12p40 ($p < 0.05$) produced in response to SCP (data not shown).

IL-10 was also detectable in the supernatants of iMϕ stimulated with 0-3hRP, although not in response to SCP (Figure 3.3e). The production of IL-10 was detectable by 3 hr post-stimulation, from which point the levels increased, reaching a plateau by 6 hr. In contrast to its effects upon Mϕ production of IL-12p40, IFNγ-priming abolished IL-10 in response to 0-3hRP (Figure 3.3f).

It is important to note that compared to the stimulatory properties of 0-3hRP, the concentrated medium control (RPMIc) had no stimulatory effect upon IL-12p40, IL-6, or IL-10 production by iMϕ or IFNγ-Mϕ (Figure 3.3).

Analysis of later time points showed that detectable IL-12p40 from iMϕ stayed at a plateau up to 48 hr post-stimulation with either preparation (Figure 3.4a). In contrast, the levels of IL-12p40 detected in IFNγ-Mϕ supernatants continued to rise with time, failing to reach a plateau by 48 hr post-stimulation (Figure 3.4b). The levels of IL-6 detected in response to either parasite preparation, in both iMϕ and IFNγ-Mϕ supernatants, remained static between 6 to 48 hr post-stimulation (Figure 3.4c & d). Detectable IL-10 in the supernatants of iMϕ stayed at a plateau until 24 hr, after which the level decreased (Figure 3.6e). The dramatic inhibitory effects of IFNγ-priming upon IL-10 production by Mϕ appeared to be transient, since 48 hr after stimulation with 0-3hRP, IL-10 could be detected in the supernatants (Figure 3.4f). In addition, low levels of TNFα from iMϕ stimulated with 0-3hRP only, could be detected at the earlier time points (6 and 12 hr) (data not shown). In contrast, IL-1β was only detectable at 24 hr and 48 hr post-stimulation with 0-3hRP (data not shown). IFNγ-activation of Mϕ dramatically increased production of TNFα in response to 0-3hRP, peaking later at 12 hr, but still detectable up to 48 hr, whereas IFNγ had little effect on IL-1β production (data not shown). Therefore, all subsequent analyses were confined to detection of cytokine production at 18 hr, and restricted to IL-12p40, IL-6 and IL-10.

3.3.3 Material released by schistosomes stimulates Mϕ cytokine production independently of naturally-occurring endotoxin

Analysis of the kinetics of iMϕ and IFNγ-Mϕ cytokine production showed that for all cytokines tested, 0-3hRP consistently stimulated much higher levels of production than SCP (Figure 3.3 and 3.4). Analysis of these parasite preparations using the LAL assay showed that they contained low levels of naturally-occurring endotoxin (Section 2.3.2). In addition, there was considerably more endotoxin within 0-3hRP, than in SCP. Therefore, this leads to the hypothesis that the stimulatory properties of these preparations may be due to levels of endotoxin contained within them. Thus, two approaches to determine the stimulatory effects of the parasite preparations independently of endotoxin were explored. Firstly, the major active component of endotoxin is lipopolysaccharide (LPS), the stimulatory properties of which are well known to be inhibited by the antibiotic Polymyxin B (PMB) (Haranaka *et al.*, 1984; Iwagaki *et al.*, 2000). Therefore, PMB was used to determine the stimulatory properties of the parasite preparations in the absence of LPS

signalling. Secondly, the most potent types / strains of LPS are known to stimulate innate cells through TLR4 (reviewed by Netea *et al.*, 2002). Therefore, the stimulatory properties of the different parasite preparations were determined in the absence of signalling by endogenous LPS using Mϕ from C3H/HeJ mice that lack the functional *TLR4* gene (Poltorak *et al.*, 1998).

3.3.3.1 PMB inhibits the stimulatory properties of LPS in a dose-dependent manner, whilst not affecting cell function, or viability.

Dose-response assays were performed in order to determine the most effective concentration of PMB to block the stimulatory properties of quantities of LPS similar to that within 0-3hRP. Using the LAL assay, it was determined that LPS from *E. coli* strain 0111:B4 had an activity of 4 EU / ng (Section 2.3.2). Thus, 0.1 and 1 ng / ml of LPS (0.4 and 4 EU / ml, respectively), were used to test the effectiveness of PMB to block the stimulatory properties of endotoxin.

LPS stimulated low levels and high levels of dose-dependent IL-12p40 production by iMϕ and IFNγ-primed Mϕ, respectively (Figure 3.5a & b). Addition of PMB inhibited the stimulatory properties of LPS, also in a dose-dependent manner. Indeed, 0.1 μg / ml and greater levels of PMB completely blocked IL-12p40 production stimulated by 0.1 ng / ml LPS, whereas 1 - 10 μg / ml PMB completely blocked IL-12p40 production stimulated by 1 ng / ml LPS. Addition of PMB had no effect on IL-12p40 production by resting cells, with no cytokine detected at any of the PMB concentrations.

A similar trend in IL-6 production by iMϕ and IFNγ-Mϕ in response to LPS and PMB was observed (Figure 3.5c & d). Again, 0.1 μg / ml of PMB was enough to block cytokine production in response to the lowest concentration of LPS. However, 10 μg / ml was needed to completely block the higher concentration of LPS. From this data, it was decided that in all subsequent experiments PMB would be used at concentrations between 1 and 10 μg / ml in order to block the stimulatory properties of endotoxin present within the schistosome preparations.

It has been shown that PMB can stimulate innate accessory cells, such as human monocytes (Hogasen and Abrahamsen, 1995), to produce cytokines. However, in my study this effect was not evident using iMϕ or IFNγ-Mϕ cultured with 0.01 - 10 μg / ml of PMB (Figure 3.5a - d). In addition, low levels of PMB (0.2 - 20 μg / ml) had no effect on cell viability (data not shown), demonstrating that the concentrations of PMB to be used in further assays (1 - 10 μg / ml) are not cytotoxic to Mϕ.

It has also been shown that high levels of PMB can synergise with pro-inflammatory stimuli to further stimulate monocyte production of cytokines (Cavaillon and Haeffner-Cavaillon, 1986). Therefore, PMB could potentially synergise with schistosome PAMPs within the parasite preparations if PMB was used to block the stimulatory properties of the endogenous endotoxin. Therefore, it was important to determine if low levels of PMB interfere with the stimulatory properties of PAMPs other than LPS. In this respect, Zymosan A (a yeast PAMP preparation) stimulated dose-dependent IL-12p40 production by iMϕ, which remained unaffected by the presence of PMB (Figure 3.6).

3.3.3.2 0-3hRP retains its stimulatory properties in the presence of PMB.

The two strategies to determine the stimulatory properties of schistosome preparations in the absence of endotoxin signalling were combined. Thus, cytokine production by Mϕ from 'LPS-responsive' C3H/HeN mice was compared to that of Mϕ from 'LPS-unresponsive' C3H/HeJ mice, in response to stimulation with schistosome preparations in the presence of increasing concentrations of PMB.

The stimulatory properties of 0-3hRP were compared to a concentration of LPS (1 ng / ml) shown to have twice the quantity of endotoxin activity, as that calculated to be in 0-3hRP. Neither LPS, nor 0-3hRP, stimulated iMϕ from 'LPS-responsive' C3H/HeN mice to produce detectable levels of IL-12p40 (Figure 3.7a). Moreover, neither LPS, nor 0-3hRP stimulated IL-12p40 production by iMϕ 'LPS-unresponsive' C3H/HeJ (Figure 3.7b). In contrast, LPS stimulated high levels of IL-6 production by C3H/HeN iMϕ (Figure 3.7c). However, this was dramatically reduced by the addition of PMB, with 1 μg / ml PMB causing a 10-fold decrease and 10 μg / ml causing a 90-fold decrease in the levels of IL-6 detected. In comparison, 0-3hRP stimulated lower levels of IL-6 than LPS but these

properties were only slightly reduced (1.6 fold) by the addition of 1 μg / ml PMB. Moreover, a higher concentration of PMB (10 μg / ml) had no further effect on IL-6-inducing capacity of 0-3hRP.

LPS stimulated 10-fold less IL-6 from C3H/HeJ iMϕ, compared to C3H/HeN iMϕ (Figure 3.7d). This low level of IL-6 could be further reduced up to 5-fold by addition of PMB. In contrast, responses to 0-3hRP were only slightly reduced (1.5 fold) in iMϕ from C3H/HeJ compared to C3H/HeN, and subsequent addition of PMB had no effect upon these stimulatory properties (Figure 3.7d). It is important to note that in the presence of PMB, 0-3hRP appeared to stimulate the same amount of IL-6 from both C3H/HeN and C3H/HeJ iMϕ (Figure 3.7c & d).

When IL-10 production from C3H/HeN iMϕ was analysed, the highest concentration of PMB completely inhibited the stimulatory properties of LPS, whereas 0-3hRP remained unaffected (Figure 3.7e). However, in contrast to IL-6 production, 0-3hRP stimulated higher levels of IL-10 than LPS, in the absence of PMB. Moreover, the production of IL-10 in response to 0-3hRP remained unaffected by addition of PMB. No IL-10 production by C3H/HeJ iMϕ was detected in response to LPS, whereas 0-3hRP stimulated a low but detectable amount of IL-10, which was not inhibited by the addition of PMB (Figure 3.7f). However, the levels of IL-10 production by C3H/HeJ iMϕ in response to 0-3hRP were more than 4-fold less than that produced by C3H/HeN iMϕ (Figure 3.7e & f).

In a parallel experiment was performed using IFNγ-Mϕ from each mouse strain, elevated levels of IL-12p40 were detected after stimulation with LPS and 0-3hRP (Figure 3.8a & b). However, the stimulatory properties of LPS but not 0-3hRP, were ablated by addition of PMB (Figure 3.8a & b). The stimulatory properties of LPS were greater than that of 0-3hRP, when using IFNγ-Mϕ from C3H/HeN mice (Figure 3.8a). However, the response to LPS was dramatically lower (13-fold), using IFNγ-Mϕ from C3H/HeJ mice (Figure 3.8b). The response to 0-3hRP was also considerably reduced for IFNγ-Mϕ from C3H/HeJ mice (5-fold), but the fold decrease was not as great as for LPS.

The pattern of IL-6 production by IFNγ-Mϕ was very similar to that of iMϕ (Figures 3.7 c & d and 3.8c & d). However, the amount of IL-6 produced in response to 0-3hRP in the

presence of PMB was lower in C3H/HeJ cell-cultures compared to C3H/HeN cell-cultures, although this was less than a 2-fold reduction (Figure 3.8c & d). In addition, priming with IFNγ had a different effect upon the levels of IL-6 produced in response to both 0-3hRP and LPS between the two cell types. In this respect, IFNγ-Mϕ from C3H/HeN mice produced up to 4-fold more IL-6 than their iMϕ counterparts, whereas IFNγ-Mϕ from C3H/HeJ mice produced only 2-fold more IL-6 than iMϕ (Figures 3.7 c & d and 3.8c & d). In contrast to iMϕ, no detectable IL-10 was produced by IFNγ-Mϕ from either C3H/HeN or C3H/HeJ mice in response to any stimuli (Figure 3.8e & f).

Cytokine responses by iMϕ and IFNγ-Mϕ from C57Bl/6 mice followed a similar pattern to that produced by the respective C3H/HeN Mϕ (Figure 3.9).

3.3.4 Molecules released by schistosomes but not soluble preparations of whole larvae, are stimulatory for cytokine production by Mϕ.

Having determined the stimulatory properties of 0-3hRP in the absence of signalling by contaminating endotoxin, the stimulatory properties of all early skin-stage schistosome preparations were compared in a set of parallel experiments.

None of the soluble schistosome preparations stimulated detectable production of IL-12p40 by iMϕ from either C3H/HeN or C3H/HeJ mice (Figure 3.10a & b). However, 0-3hRP stimulated much higher levels of IL-6 and IL-10 production by iMϕ from either mouse strain than any of the other schistosome preparations (Figure 3.10c - f). Indeed, of all the soluble whole parasite preparations (SCP, 3hSSP, and 18hSSP), only the cercarial preparation SCP stimulated an increase in IL-6 production by C3H/HeN iMϕ, which was partially inhibited by addition of PMB (Figure 3.10c). Moreover, SCP stimulated only very low levels of IL-6 production by C3H/HeJ (Figure 3.10d). None of the soluble whole parasite preparations stimulated any detectable IL-10 production (Figure 3.10e & f).

The response of IFNγ-Mϕ to the different preparations was similar to the response by iMϕ (Figure 3.11a - f), with 0-3hRP being the only preparation to stimulate IL-12p40 and IL-6

production by cells from either mouse strain in the presence of PMB (Figure 3.11a - d). No IL-10 was detectable in any of the supernatants of the IFNγ-Mϕ (Figure 3.11e - f).

This pattern of cytokine production was mimicked when iMϕ and IFNγ-Mϕ from C57Bl/6 mice were used in this assay with 0-3hRP but not soluble whole parasite preparations stimulating high levels of IL-12p40, IL-6, and IL-10 in the presence of PMB (Figure 3.12).

3.3.5 0-3hRP stimulates a different cytokine profile to other pathogen PAMPs

From this point onwards, a different source of thioglycollate (Brewers Thioglycollate; Sigma-Aldrich; 3% final concentration), was used to elicit iMϕ into the peritoneal cavity. This resulted in greater numbers of PEC obtainable from individual mice ($18 \times 10^6 \pm 2.1 \times 10^6$; n = 3) compared to the original thioglycollate ($7.1 \times 10^6 \pm 0.4 \times 10^6$; n = 3). The adherent iMϕ elicited by 3% Brewers thioglycollate were slightly purer for the Mϕ markers F4/80 (87%) and CD11b (92%; data not shown) when compared to the iMϕ elicited by the original thioglycollate (Figure 3.2). Functionally, these iMϕ produced lower levels of IL-10 and IL-6 in response to a range of stimuli (data not shown).

0-3hRP is unique amongst the parasite preparations in its ability to stimulate Mϕ production of IL-10, IL-12p40, and high levels of IL-6. In order to more fully characterise the response to 0-3hRP, its stimulatory properties were compared to those of other pathogen PAMPs, representing bacteria (LPS), and yeast (Zymosan A). The two pathogen PAMPs were chosen due to their recognition by innate cells through different receptors, with LPS signalling largely through the TLR4 (Poltorak *et al.*, 1998), and Zymosan A signalling through a TLR2-dependent pathway (Underhill *et al.*, 1999b).

Zymosan A appeared to stimulate production of the highest levels of IL-12p40 of all the PAMPs (Figure 3.13a). LPS also stimulated considerable amounts of IL-12p40, whereas 0-3hRP + PMB stimulated only a little. In contrast, LPS stimulated the highest levels of IL-6 (Figure 3.13b). 0-3hRP also stimulated high-level IL-6 production, which was much greater than the very low levels stimulated by Zymosan A. Similar to IL-6, LPS stimulated the highest levels of IL-10 (Figure 3.13c). 0-3hRP also stimulated IL-10, but this was low,

and like the production of IL-12p40, it bordered on the lower limit of detection of the ELISA. In contrast, Zymosan A did not stimulate production of detectable IL-10.

The batches of 0-3hRP tested here contained between 0.5 - 17.7 EU per 50 μg of protein, equivalent to 0.125 - 4.4 ng of LPS. To control for endotoxin, parallel cultures of iMϕ stimulated with LPS and PMB were performed. In all experiments, 1, 3 and 10 ng / ml LPS stimulated higher levels of IL-12p40, IL-6 and IL-10 than 0-3hRP + PMB (data not shown). However, detectable cytokine production in response to all concentrations of LPS was completely inhibited by addition of PMB at 3 μg / ml (data not shown).

3.3.6 The J774A.1 and J7.DEF.3 Mϕ cell-lines do not respond to stimulation with schistosome PAMPs.

The J774A.1 Mϕ cell-line and the derivative J7.DEF.3 line that lacks expression of surface CD14 were used to further study Mϕ responses to schistosome PAMPs and analyse the role of CD14 in parasite recognition. Since CD14 is a PRR known to be involved in the recognition of LPS (Perera *et al.*, 2001), the response of these cell-lines to LPS was initially characterised. LPS (24 - 2000 ng / ml) stimulated dose-dependent production of IL-12p40 by J774A.1 cells (Figure 3.14a). However, at the lower levels of LPS (< 24 ng / ml) production of IL-12p40 was not detected. A non-toxic and non-stimulatory quantity of PMB (10 μg / ml; data not shown) completely inhibited the production IL-12p40 by J774A.1 cells in response to up to 74 ng / ml LPS, and considerably reduced the stimulatory properties of concentrations of LPS higher than this. J7.DEF.3 cells also produced IL-12p40 in an LPS dose-dependent manner (Figure 3.14a). However, in comparison to J774A.1 cells, J7.DEF.3 cells produced much less IL-12p40 in response to 74 - 666 ng / ml LPS, but similar quantities in response to 24 and 2000 ng / ml LPS. Moreover, in the presence of PMB, only the highest concentration of LPS (2000 ng / ml), could stimulate detectable production of IL-12p40 from J7.DEF.3 cells compared to 222 ng / ml LPS when using J774A.1 cells.

Similar to the pattern of IL-12p40 production, both J774A.1 and J7.DEF.3 cells produced IL-6 in a LPS dose-dependent manner (Figure 3.14b), although J7.DEF.3 cells were less sensitive, producing lower levels of IL-6 than J774A.1 cells, and only to ≥ 74 ng / ml LPS

compared to ≥ 24 ng / ml for J774A.1 cells. Again, PMB inhibited production of IL-6 in response to up to 74 ng / ml LPS for J774A.1 cells and 666 ng / ml LPS for J7.DEF.3 cells, and reduced levels of IL-6 production in response to higher levels of LPS (Figure 3.14b).

In comparison to LPS, none of the schistosome preparations, whether in the presence or absence of PMB, stimulated J774A.1 cells to produce detectable levels of IL-12p40 or IL-6 (Figure 3.14c & d). For this reason, the effect of schistosome preparations on J7.DEF.3 cells was not examined.

iMϕ primed with IFNγ produced greatly increased levels of IL-12p40 when stimulated with schistosome PAMPs (Section 3.3.1 - 3.3.4). Therefore, an optimal concentration of IFNγ was used to prime J774A.1 and J7.DEF.3 cells during stimulation with LPS, or schistosome preparations. IFNγ-primed J774A.1 cells were more sensitive to stimulation with LPS, producing detectable levels of IL-12p40 and IL-6 in response to as little as 0.9 ng / ml LPS (Figure 3.15 a & b), when compared to non-primed cells (Figure 3.14a & b). Moreover, IFNγ-primed J7.DEF.3 cells were also more sensitive to stimulation with LPS, producing detectable IL-12p40 in response to as little as 0.9 ng / ml LPS (Figure 3.15a) and detectable IL-6 in response to as little as 2.7 ng / ml LPS (Figure 3.15b). Indeed, IFNγ-primed J7.DEF.3 cells produced more IL-12p40 in response to 0.9 - 2000 ng / ml LPS than the respective J774A.1 cells, whereas in the presence of PMB the IL-12p40 production was similar for the different cell types across the range of LPS concentrations (Figure 3.15a). In contrast to IL-12p40 production, both cell types when primed with IFNγ produced similar levels of IL-6 in response to a range of LPS concentrations, whereas in the presence of PMB, IFNγ-primed J774A.1 cells produced more IL-6 than the respective J7.DEF.3 cells (Figure 3.15b).

0-3hRP stimulated detectable production of IL-12p40 by IFNγ-primed J774A.1 cells, whereas the soluble whole larval preparations SCP, 3hSSP and 18hSSP remained non-stimulatory (Figure 3.15c). Similarly, 0-3hRP, but not the soluble whole larval preparations, stimulated detectable production of IL-12p40 by IFNγ-primed J7.DEF.3 cells (Figure 3.15c). Compared to IFNγ-primed J774A.1 cells, IFNγ-primed J7.DEF.3 produced more IL-12p40 in response to stimulation with 0-3hRP, as observed in response to LPS. However, in the presence of PMB, 0-3hRP failed to stimulate detectable production of IL-

12p40 by J774A.1 or J7.DEF.1 cells. It is important to note that the 0-3hRP introduced approximately 0.5 ng / ml LPS into the cell culture, and in the absence of PMB 0-3hRP stimulated approximately the same amount of IL12p40 as 0.9 ng / ml LPS, by either J774A.1 or J7.DEF.3 cells (Figure 3.15a & c). 0-3hRP also stimulated PMB-sensitive IL-6 production by IFNγ-primed J774A.1 cells, whereas the whole larval preparations remained non-stimulatory (Figure 3.15d). In contrast, none of the schistosome preparations stimulated detectable IL-6 production by IFNγ-primed J7.DEF.3 cells. Again, this pattern of cytokine production was similar to that seen in response to an equivalent concentration of LPS (0.9 ng / ml) to that contained within the 0-3hRP (Figure 3.15b & d).

3.3.7 Live schistosomes stimulate iMϕ cytokine production

As soluble preparations of cercariae and *in vitro* cultured schistosomulae do not stimulate high or detectable levels of cytokine production by iMϕ, this raises the question of whether live larvae can stimulate cellular responses, and if so, whether this is dependent upon the PAMPs released by the parasite. Infective cercariae (CercN) stimulated a dose-dependent increase in IL-6 production by iMϕ (Figure 3.16). Indeed, as few as 50 cercariae / well stimulated an increase in detectable IL-6 over background. However, cercariae that had been transformed immediately prior to co-culture with the iMϕ (CercT) stimulated between 1.3- and 2-fold more IL-6 compared to CercN (Figure 3.16). No IL-12p40 or IL-10 was detected in any of the wells (data not shown).

The stimulatory properties of the transformed cercariae were then compared to those of its components parts (*i.e.* the material released by the parasite during the first 3 hr post-transformation [0-3hSN] and the 3 hr larval heads and tails, devoid of this released material, 3hSom). The 3hSom stimulated increased IL-6 production over background, at 100 and 200 but not 50 parasites / well (Figure 3.17). However, the levels of IL-6 produced were considerably less than detected in response to freshly transformed cercariae. In order to determine if the difference in stimulatory properties of 3hSom and CercT was due to the absence of the released molecules, 0-3hSN was assayed for its stimulatory capacity. 0-3hSN also stimulated an increase in detectable IL-6 over background levels at quantities equivalent to 100 or 200 but not 50 parasites / well (Figure 3.17). Again, these stimulatory properties were less than that of transformed cercariae but similar to 3 hr larvae and tails.

Therefore, the amount of IL-6 production stimulated by both 0-3hSN combined with the 3hSom, approximately equalled that stimulated by freshly transformed cercariae (CercT). Notably, the levels of IL-6 produced in response to 0-3hSN were much lower than produced in response to the concentrated soluble preparation 0-3hRP (see Figure 3.13b).

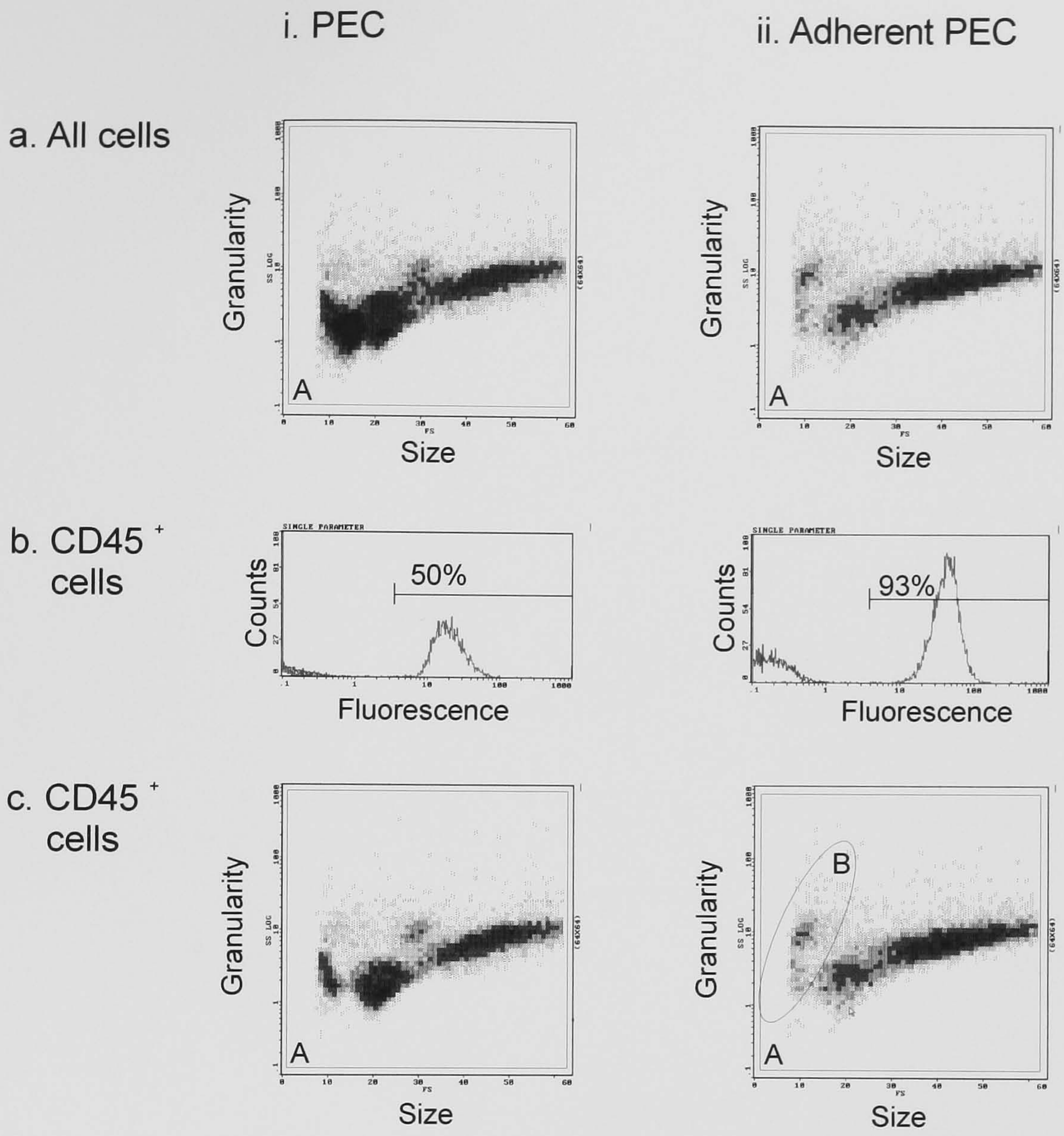
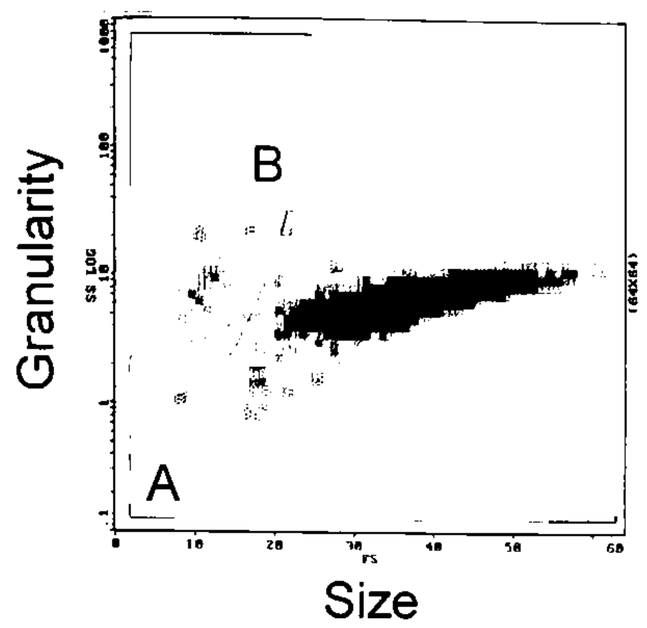


Figure 3.1 **PEC and adherent PEC populations differ in cellular composition.** PEC were extracted from mice 5 days post-injection with 0.09% thioglycollate, and analysed as the whole population (i). Alternatively, PEC were incubated in tissue culture grade plastic for 2 hr, at 37 °C. The non-adherent population was subsequently discarded and the 'adherent' population resuspended and analysed (ii). Flow-cytometry was used to analyse the size and granularity of all cells (a). Cells were stained with CD45-specific Cy-Chrome™-conjugated antibody (red line) or an appropriate isotype control (blue line), and the percentage positive for CD45 determined (b). Alternatively, the size and granularity of $CD45^+$ cells was determined (c). Analysis was based on 10,000 cells (b) or 10,000 $CD45^+$ cells (a & c).The population (B) was comprised of dead cells as determined by PI staining.

Figure 3.2 **The adherent PEC population is highly enriched for Mϕ.** PEC were cultured for 2 hr in 24-well culture plates (1 x 10^6), after-which non-adherent cells were washed off and discarded. The remaining adherent population (A) was removed and the size and granularity of the cells determined (a). Alternatively, cells were stained with specific antibodies (red line) to CD45 (b), F4/80 (c) or CD11b (d), or with appropriate isotype control antibodies (blue line). The dead cell population (B) was determined by PI staining, and gated-out of surface-marker analysis (A-B). Data is representative of a minimum of 3 experiments.

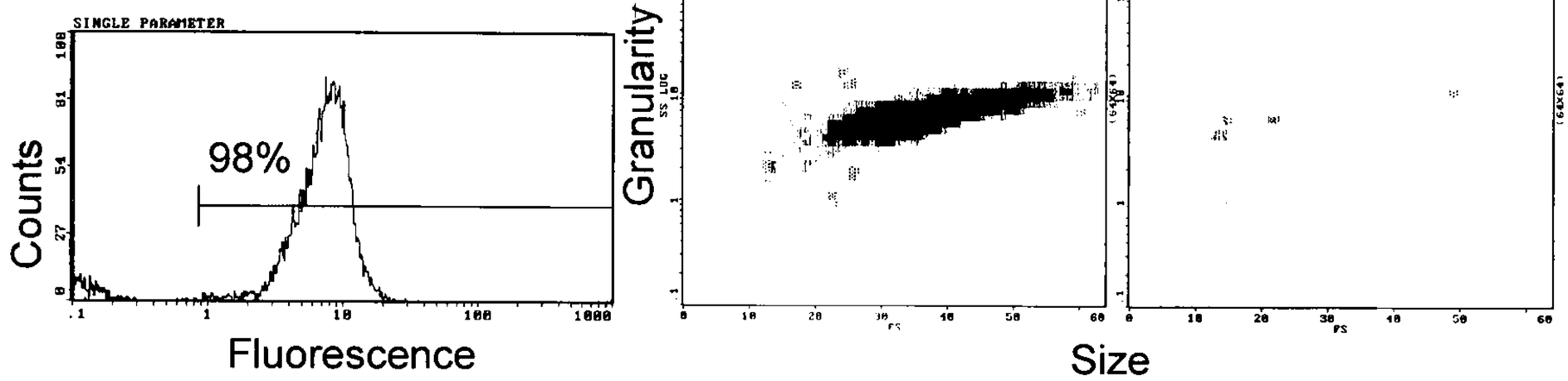
a. All cells



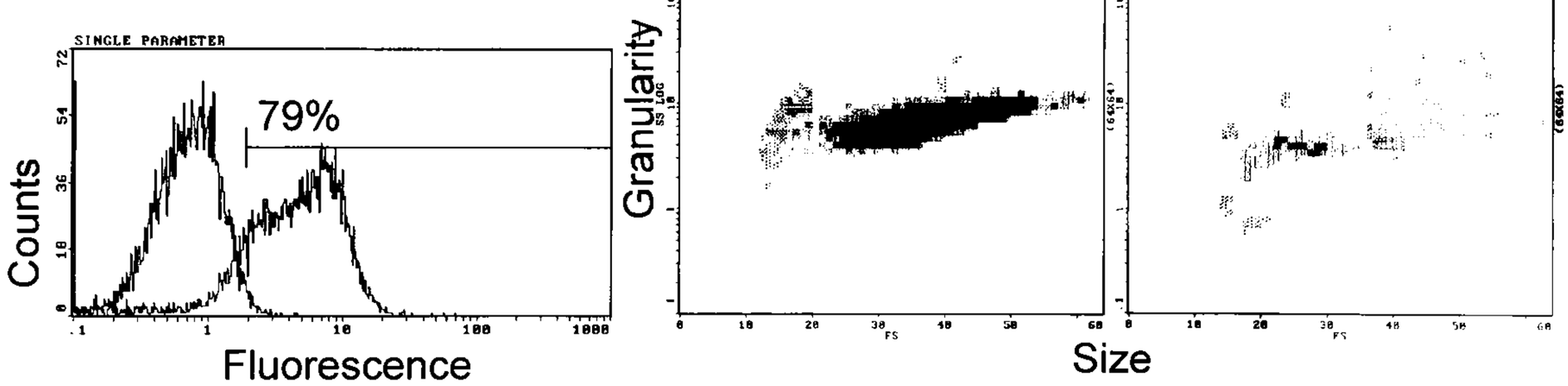
All Cells (A) - Dead Cells (B)

+ ve cells - ve cells

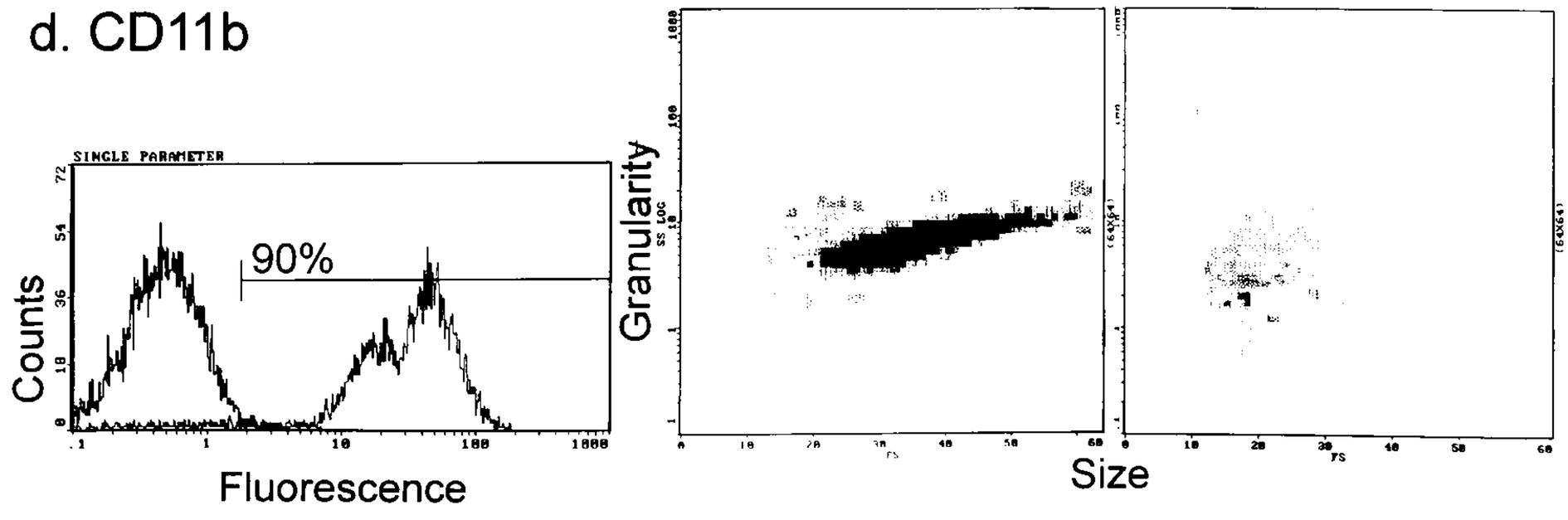
b. CD45



c. F4/80



d. CD11b



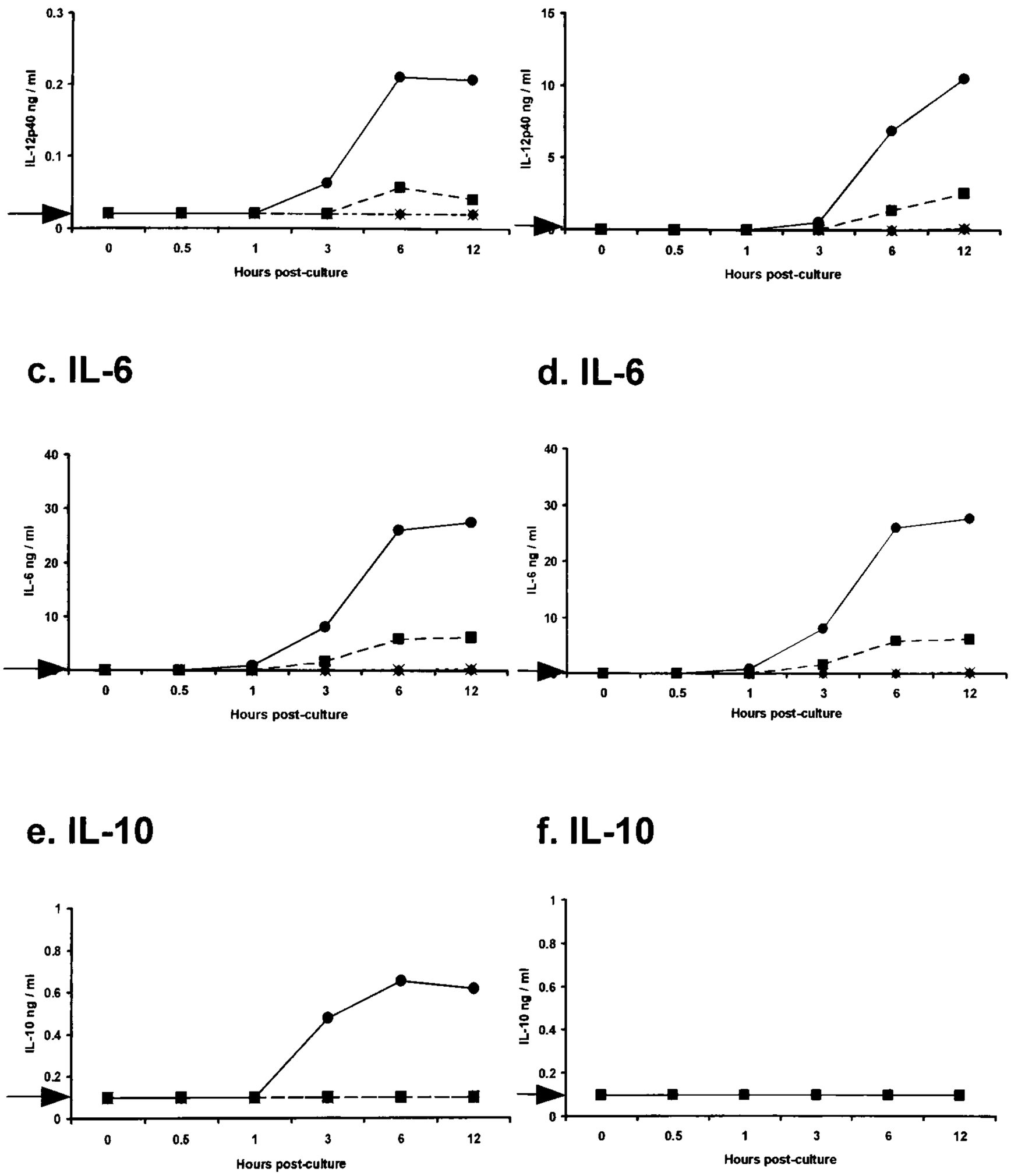


Figure 3.3 **Kinetics of cytokine production by Mϕ stimulated with different schistosome PAMPs.** iMϕ (a, c, e), or IFNγ-Mϕ (b, d, f), were cultured with SCP (■; 50 μg / ml), 0-3hRP (●; 50 μg / ml), RPMIc (**x**), or alone (◆), for varying lengths of time (0 - 12 hr). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), or IL-10 (e & f). Arrows indicate the lower detection limit of the ELISA.

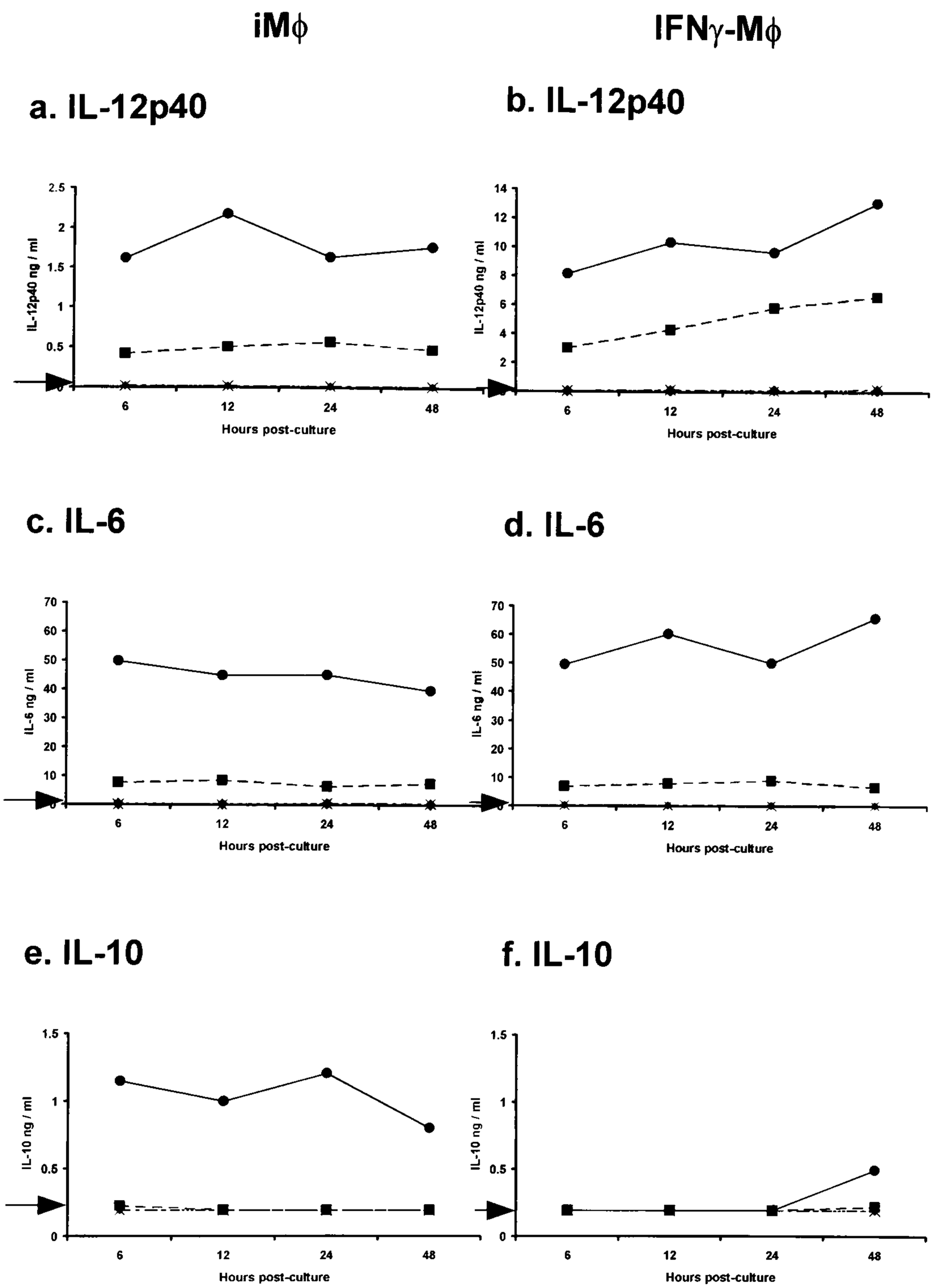


Figure 3.4 **Kinetics of cytokine production by Mϕ stimulated with different schistosome PAMPs.** iMϕ (a, c, e), or IFNγ-Mϕ (b, d, f), were cultured with SCP (■; 50 μg / ml), 0-3hRP (●; 50 μg / ml), RPMIc (**x**), or alone (◆), for varying lengths of time (6 - 48 hr). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), or IL-10 (e & f). Arrows indicate the lower detection limit of the ELISA.

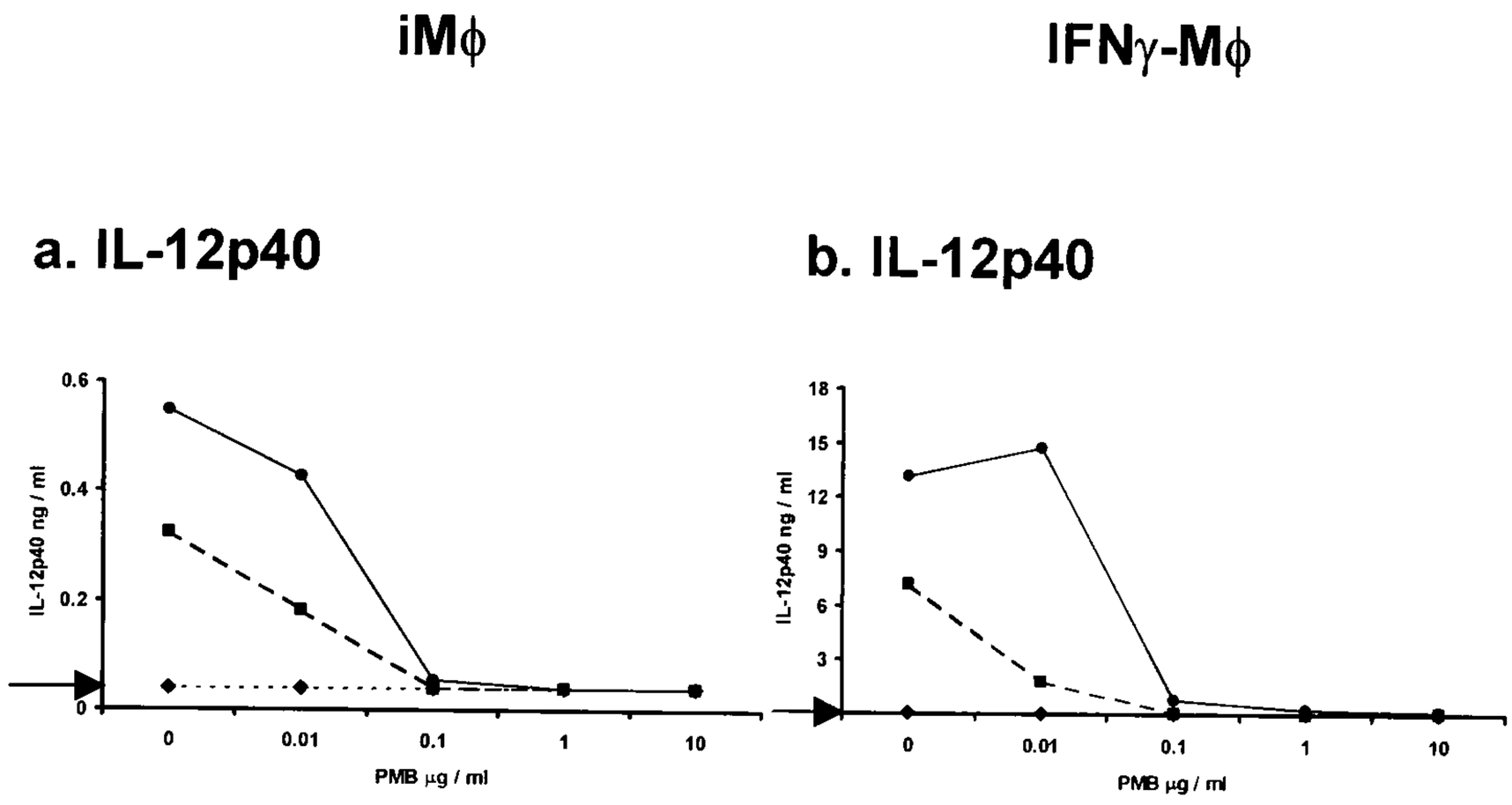


Figure 3.5 **PMB blocks the stimulatory properties of LPS.** iMϕ (a & c), or IFNγ-Mϕ (b & d), were cultured overnight with 0 (◆), 0.1 (■), or 1 (●), ng / ml LPS and the indicated concentrations of PMB (0 - 10 µg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), or IL-6 (c & d). Arrows denotes the lower detection limit of ELISA.

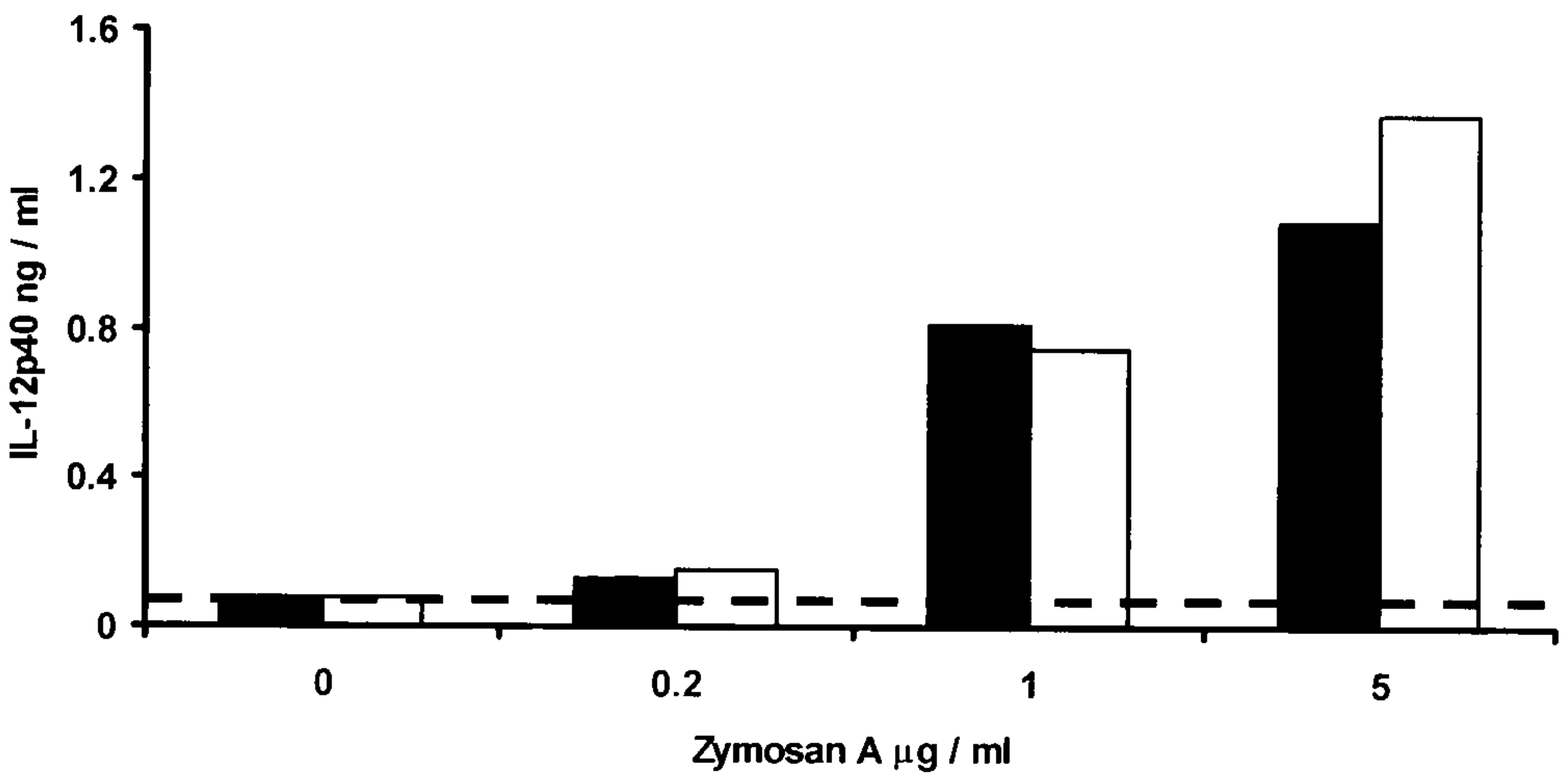


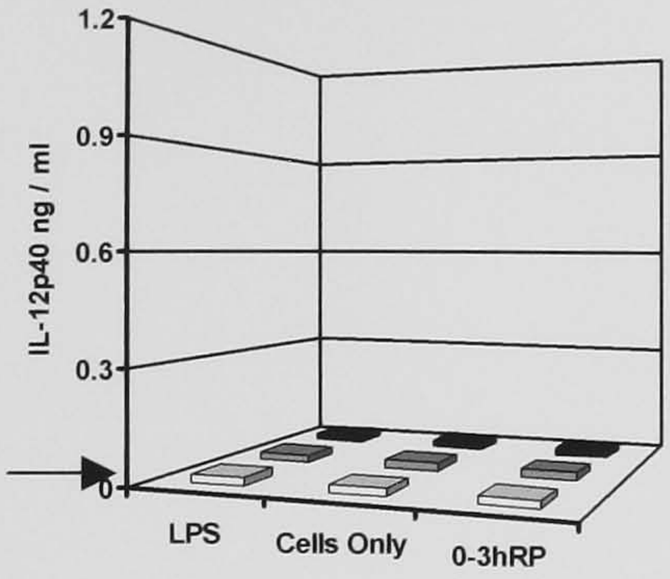
Figure 3.6 **Effect of PMB on cytokine production by iMϕ after culture with Zymosan A.** iMϕ were cultured overnight with different concentrations of Zymosan A (0 - 5 μg / ml) with (clear bars), or without (solid bars), PMB (3 μg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40. Dashed line denotes the lower detection limit of ELISA. Data is representative of 2 experiments.

Figure 3.7 **Mutation in *TLR-4*, or co-culture with PMB, have different effects on iMϕ cytokine production when stimulation with schistosome PAMPs is compared to LPS.** iMϕ from C3H/HeN 'LPS-responsive' (a, c, e), or C3H/HeJ 'LPS-unresponsive mice' (b, d, f), were cultured overnight alone, or with LPS (1 ng / ml), or 0-3hRP (50 μg / ml), in the presence of 0 (black bars), 1 (grey bars), or 10 (white bars), μg / ml PMB. Endotoxin content of 0-3hRP ≤ endotoxin content of 1 ng / ml LPS, as judged by LAL assay. Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), and IL-10 (e & f). Data is representative of 2 experiments. Arrows indicate the lower limit of detection of the ELISA.

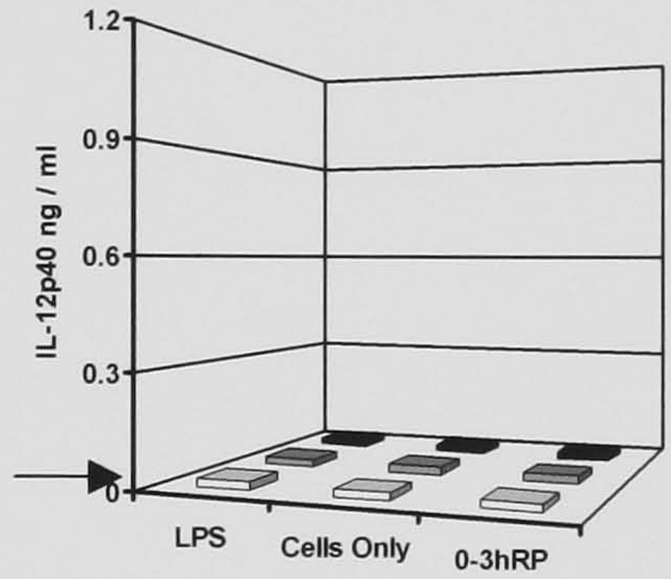
C3H/HeN

C3H/HeJ

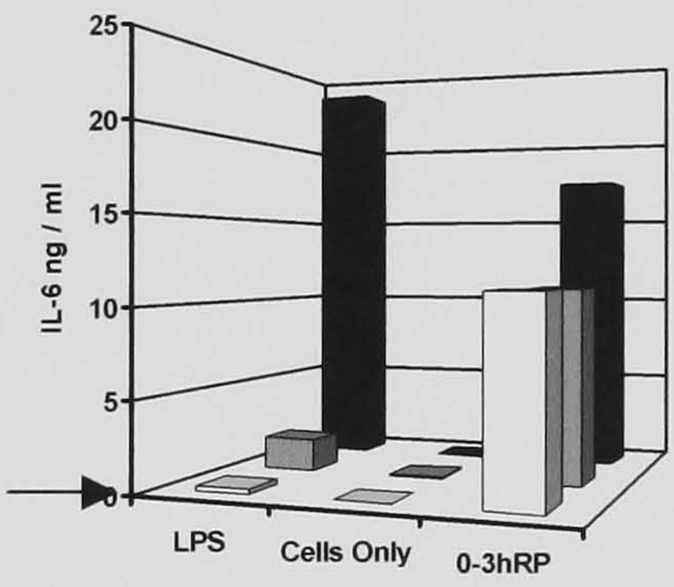
a. IL-12p40



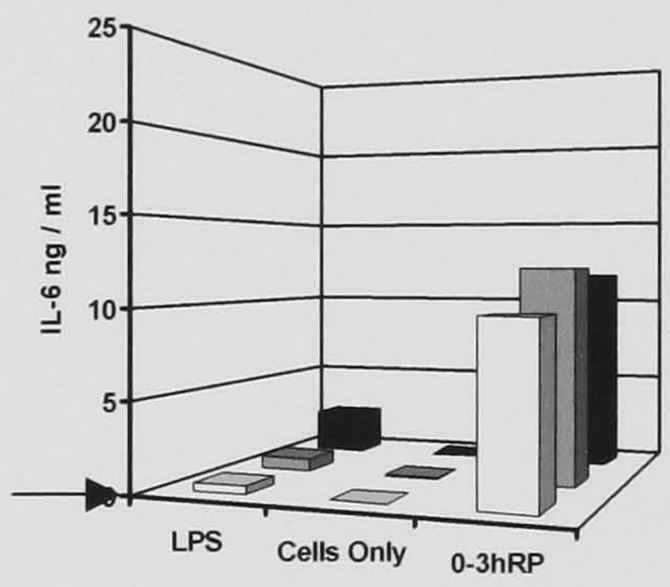
b. IL-12p40



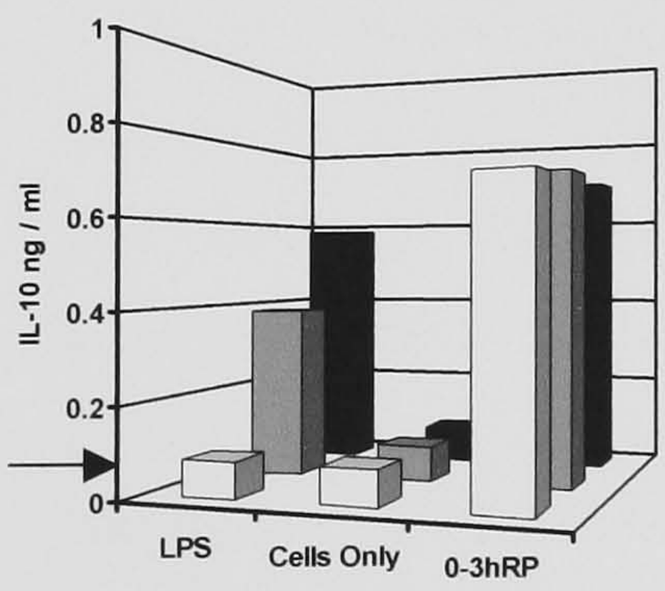
c. IL-6



d. IL-6



e. IL-10



f. IL-10

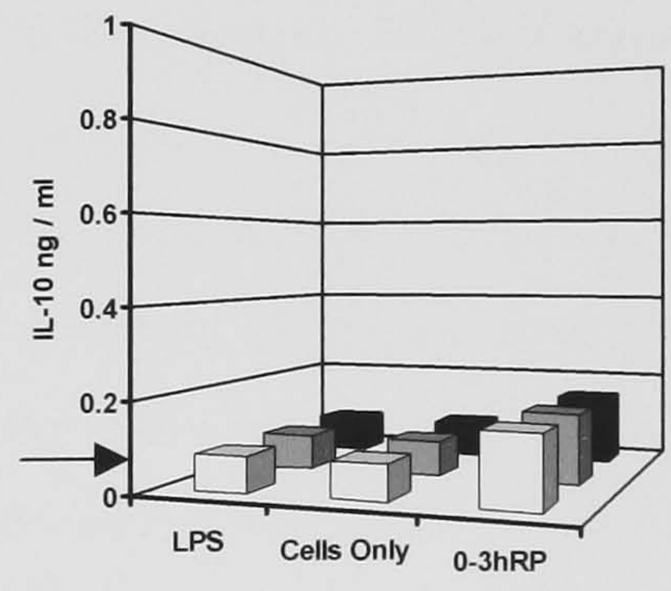
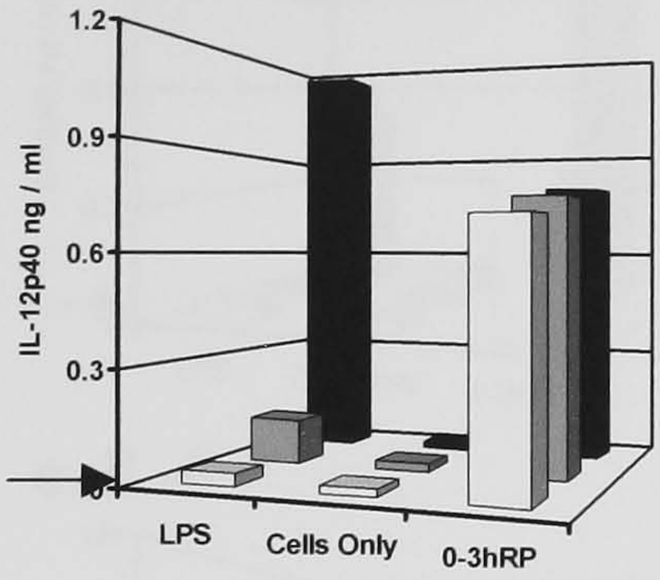


Figure 3.8 **Mutation in *TLR-4*, or co-culture with PMB, have different effects on IFNγ-Mϕ cytokine production when stimulation with schistosome PAMPs is compared to LPS.** IFNγ-Mϕ from C3H/HeN 'LPS-responsive' (a, c, e), or C3H/HeJ 'LPS-unresponsive mice' (b, d, f), were cultured overnight alone, or with LPS (1 ng / ml), or 0-3hRP (50 μg / ml), in the presence of 0 (black bars), 1 (grey bars), or 10 (white bars), μg / ml PMB. Endotoxin content of 0-3hRP ≤ endotoxin content of 1 ng / ml LPS, as judged by LAL assay. Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), or IL-10 (e & f). Data is representative of 2 experiments. Arrows indicate the lower limit of detection of the ELISA.

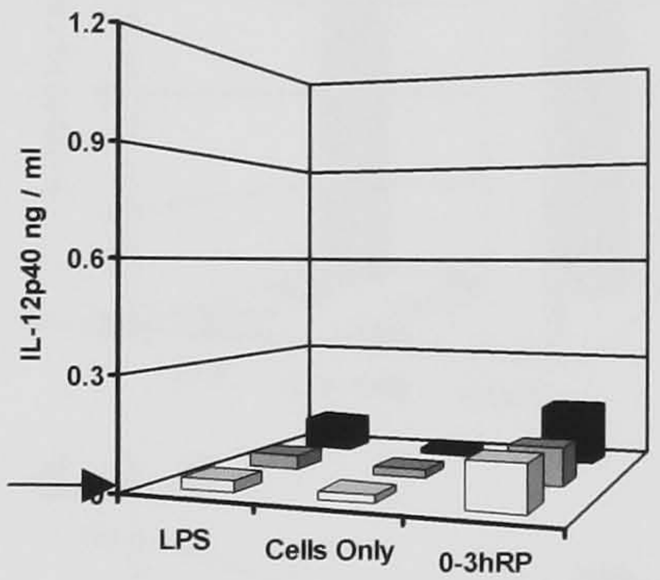
C3H/HeN

C3H/HeJ

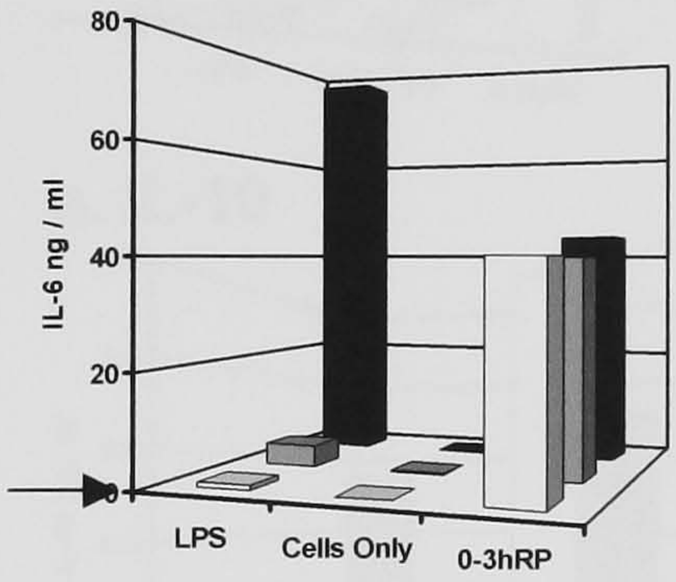
a. IL-12p40



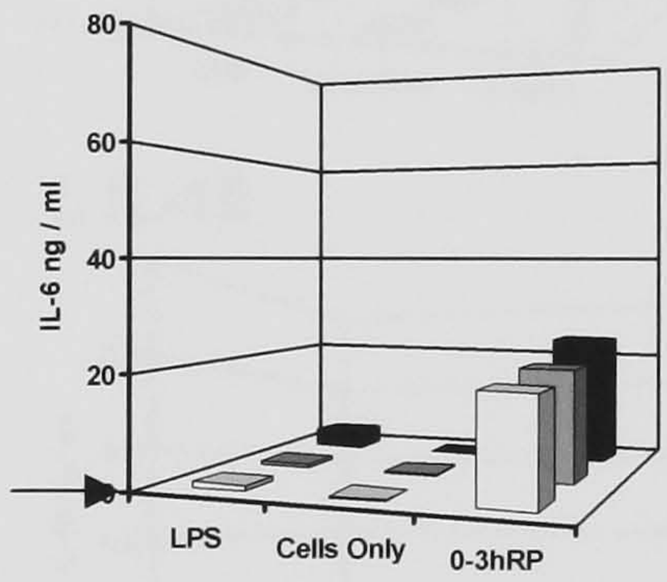
b. IL-12p40



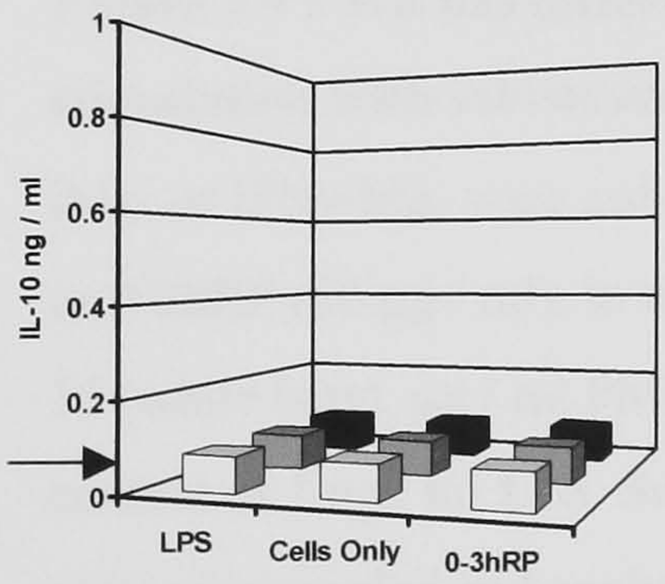
c. IL-6



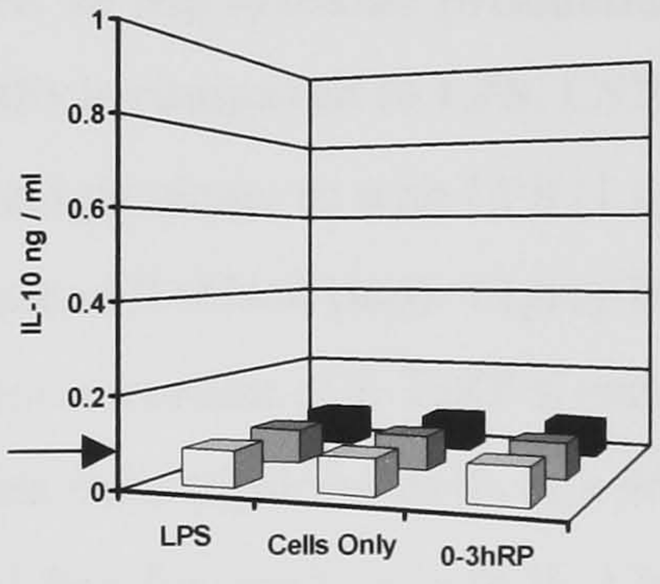
d. IL-6



e. IL-10



f. IL-10



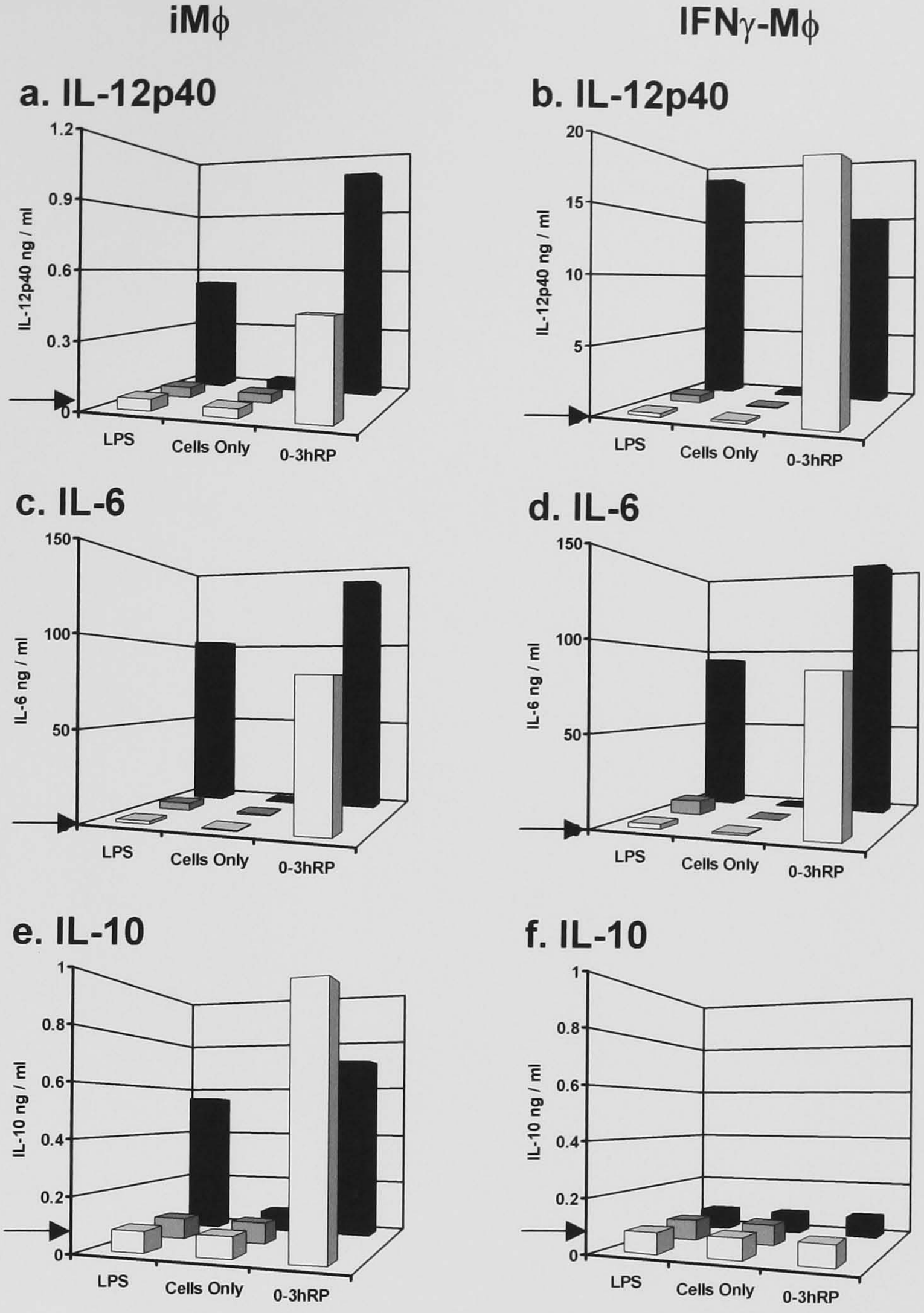


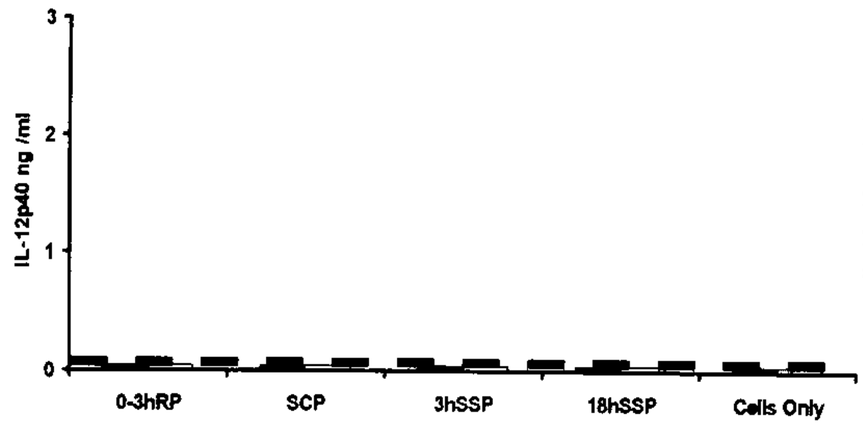
Figure 3.9 **PMB has different effects on Mϕ cytokine production when stimulation with schistosome PAMPs is compared to LPS.** C57Bl/6 iMϕ, or IFNγ-Mϕ, were cultured overnight alone, or with LPS (1 ng / ml), or 0-3hRP (50 μg / ml), in the presence of 0 (black bars), 1 (grey bars), or 10 (white bars), μg / ml PMB. Endotoxin content of 0-3hRP ≤ endotoxin content of 1 ng / ml LPS. Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), and IL-10 (e & f). Arrows indicate the lower limit of detection of the ELISA. No bar means data point was not performed.

Figure 3.10 **In the presence of PMB, 0-3hRP but not other soluble schistosome preparations, stimulates cytokine production by iMϕ from both LPS-responsive and LPS-unresponsive mice.** Peritoneal Mϕ from C3H/HeN (a, c, e), or C3H/HeJ (b, d, e), were cultured overnight alone, or with the different parasite preparations indicated (50 μg /ml), and in the presence (clear bars), or absence (black bars) of PMB (10 μg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), and IL-10 (e & f). Dashed line denotes the lower level of detection of ELISA. Data is representative of 2 experiments.

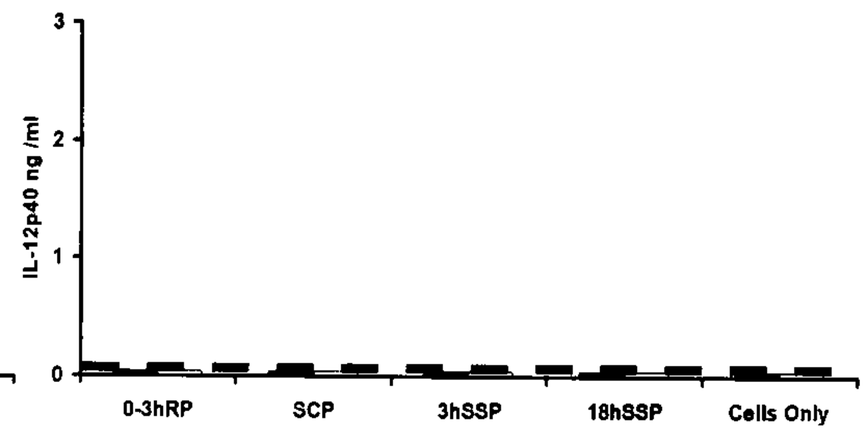
C3H/HeN

C3H/HeJ

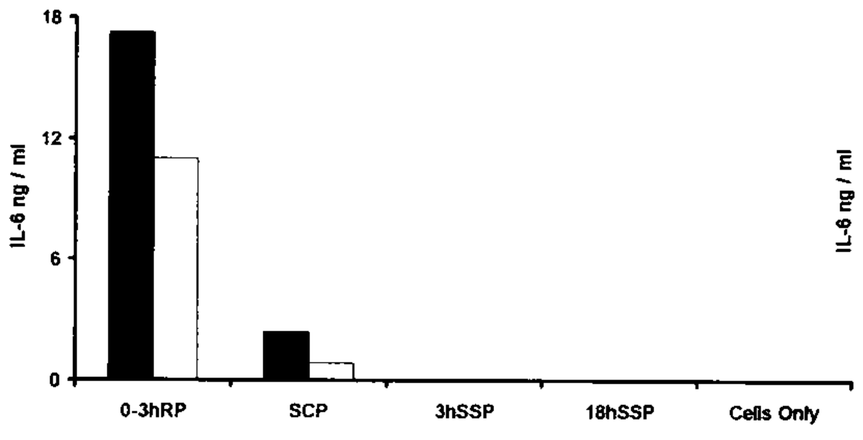
a. IL-12p40



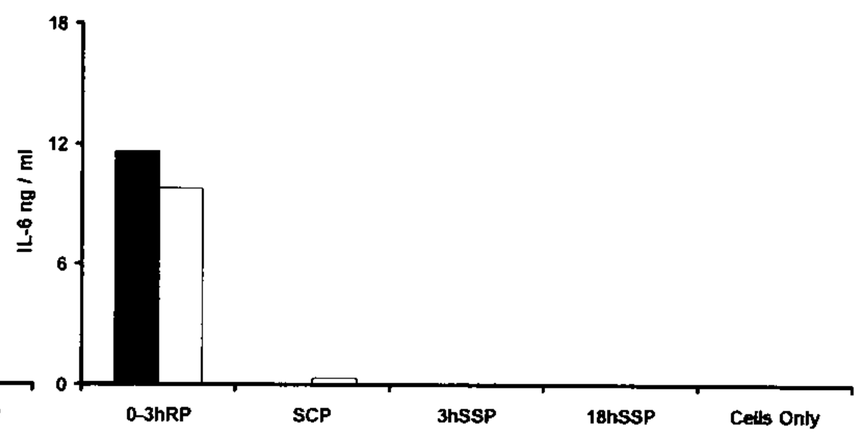
b. IL-12p40



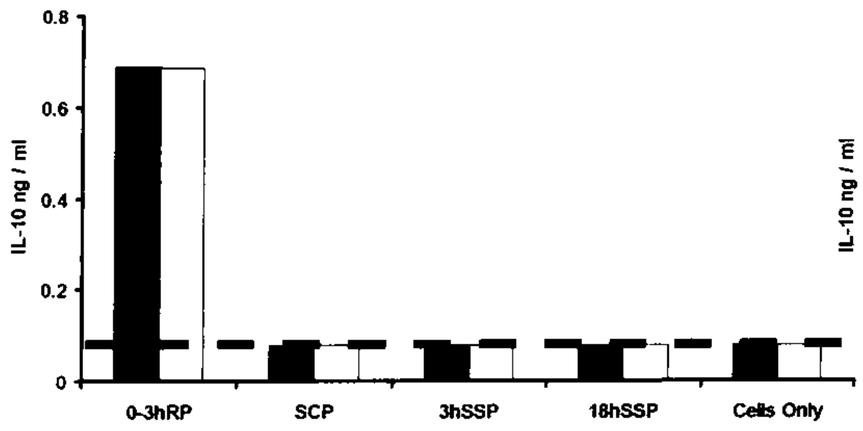
c. IL-6



d. IL-6



e. IL-10



f. IL-10

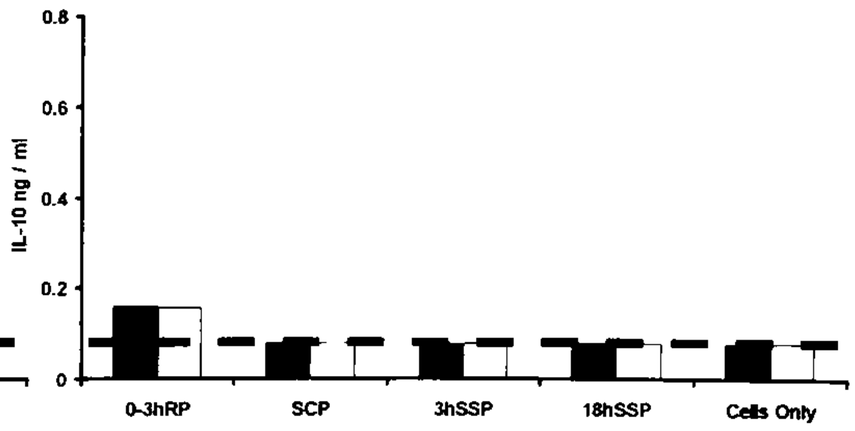
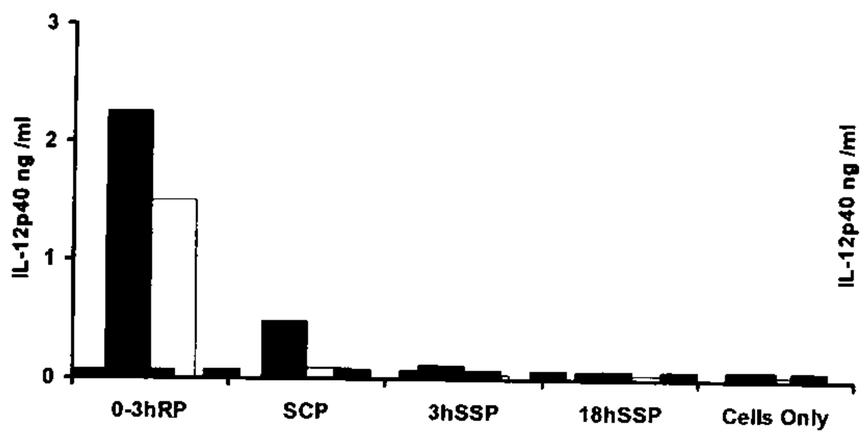


Figure 3.11 **In the presence of PMB, 0-3hRP but not other soluble schistosome preparations, stimulates cytokine production by IFNγ-Mϕ from both LPS-responsive and LPS-unresponsive mice.** IFNγ-Mϕ from C3H/HeN (a, c, e), or C3H/HeJ (b, d, f), were cultured overnight alone, or with the different parasite preparations indicated (50 μg /ml), and in the presence (clear bars), or absence (black bars) of PMB (10 μg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), or IL-10 (e & f). Dashed line denotes the lower level of detection of ELISA. Data is representative of 2 experiments.

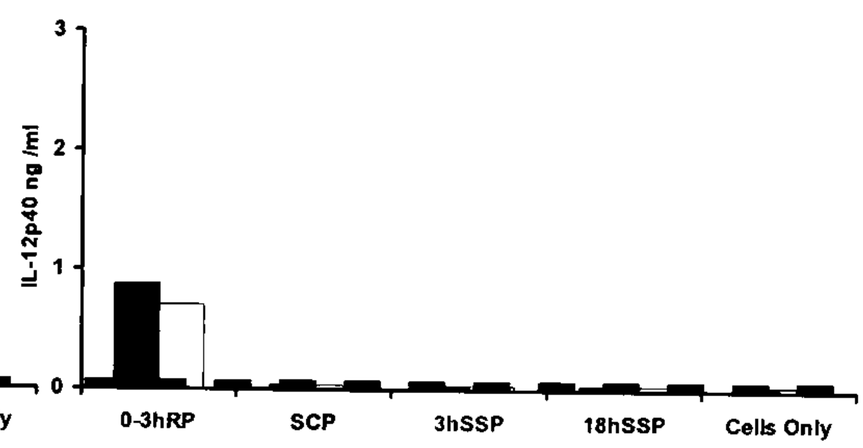
C3H/HeN

C3H/HeJ

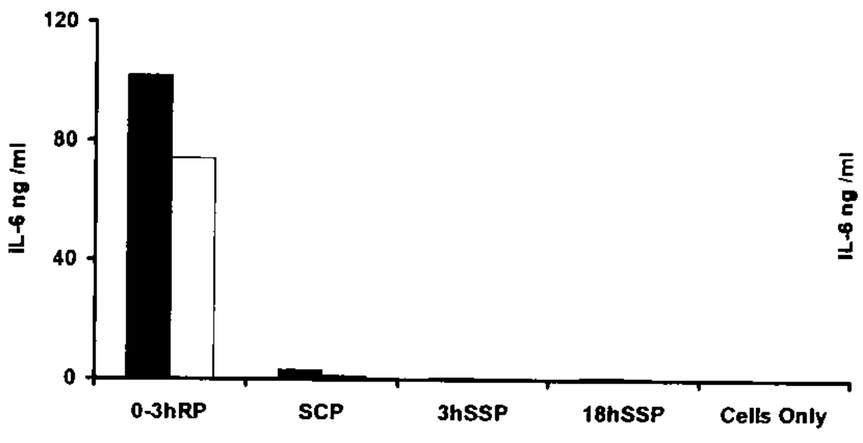
a. IL-12p40



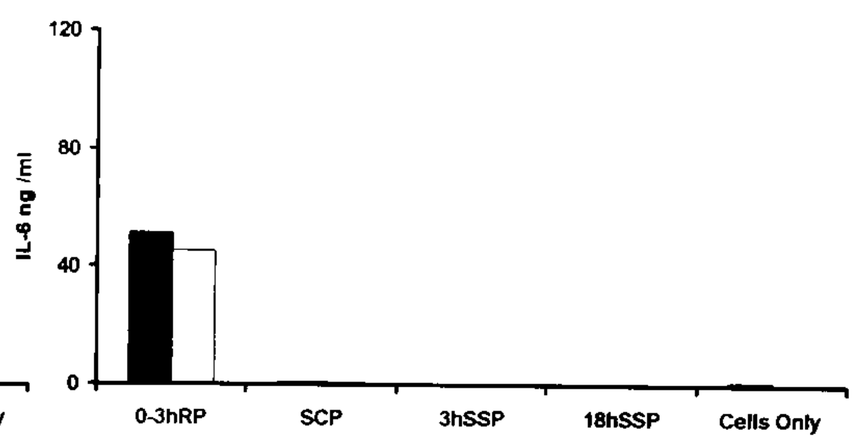
b. IL-12p40



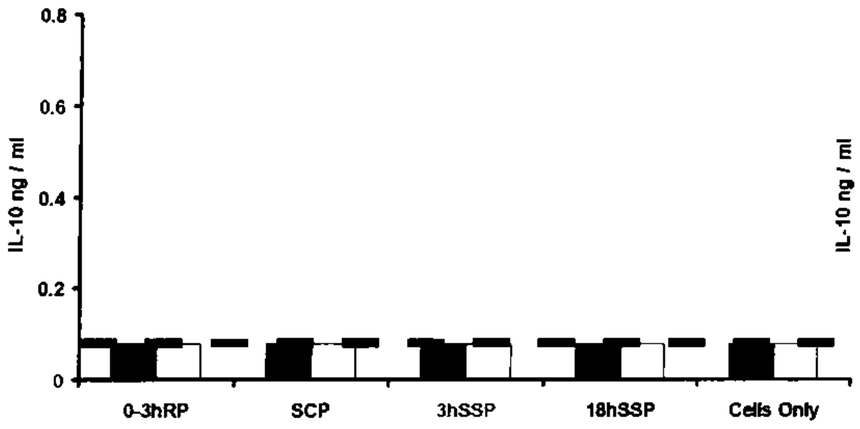
c. IL-6



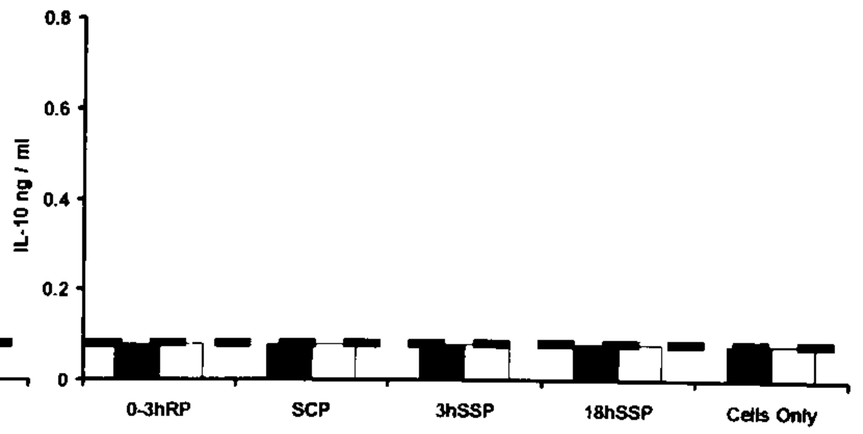
d. IL-6



e. IL-10



f. IL-10



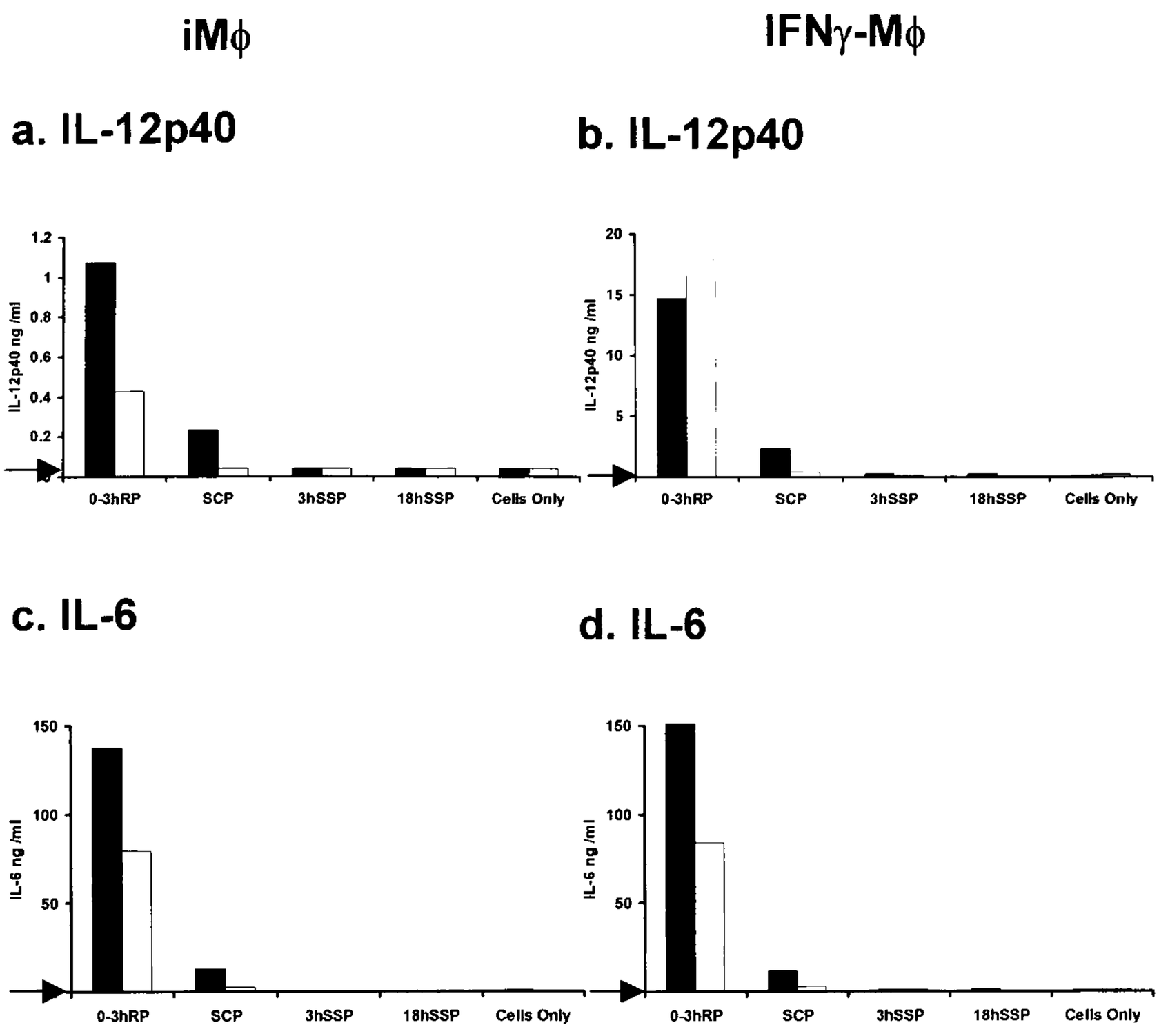


Figure 3.12 **In the presence of PMB, 0-3hRP but not other soluble schistosome preparations, stimulates cytokine production by both iMϕ and IFNγ-Mϕ from C57Bl/6 mice.** iMϕ (a, c, e), or IFNγ-Mϕ (b, d, f), were cultured overnight alone, or with the different parasite preparations indicated (50 μg / ml), in the presence (clear bars), or absence (black bars) of PMB (10 μg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & b), IL-6 (c & d), or IL-10 (e & f). Arrows denote the lower level of detection of ELISA.

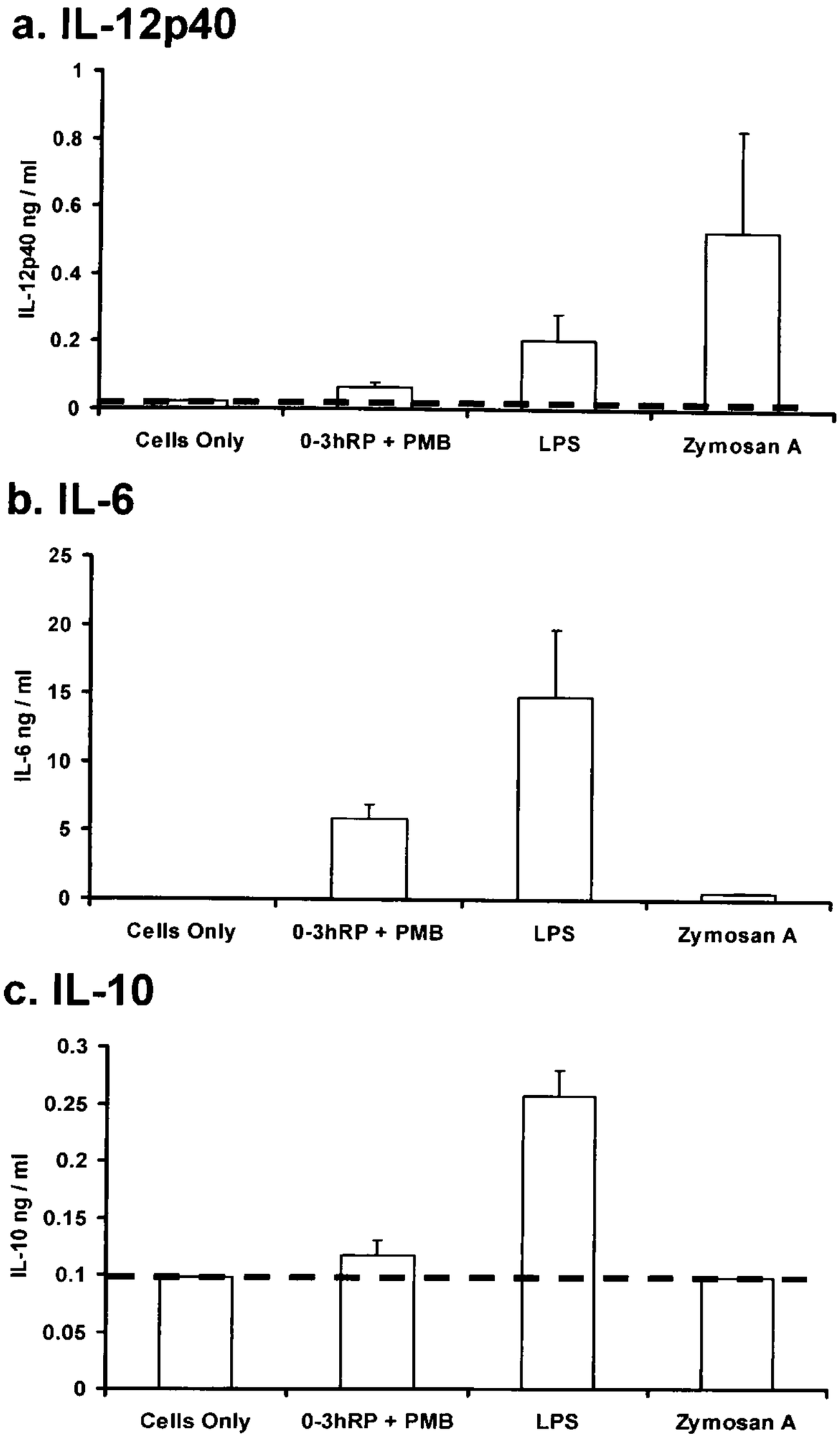


Figure 3.13 **0-3hRP stimulates a different profile of iMϕ cytokine production compared to other pathogen PAMPs.** iMϕ from C57Bl/6 mice were cultured overnight alone, or with 0-3hRP (50 μg /ml) plus PMB (3 μg / ml) (n = 11), LPS (1 ng / ml) (n = 3), or Zymosan A (1.6 μg / ml) (n = 2). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a), IL-6 (b), or IL-10 (c). Data is presented as mean ± SEM of three experiments, except for Zymosan A where n = 2. Within each individual experiment up to 6 batches of 0-3hRP were tested, and contribute to the mean. Dashed line denotes the lower limit of detection of ELISA.

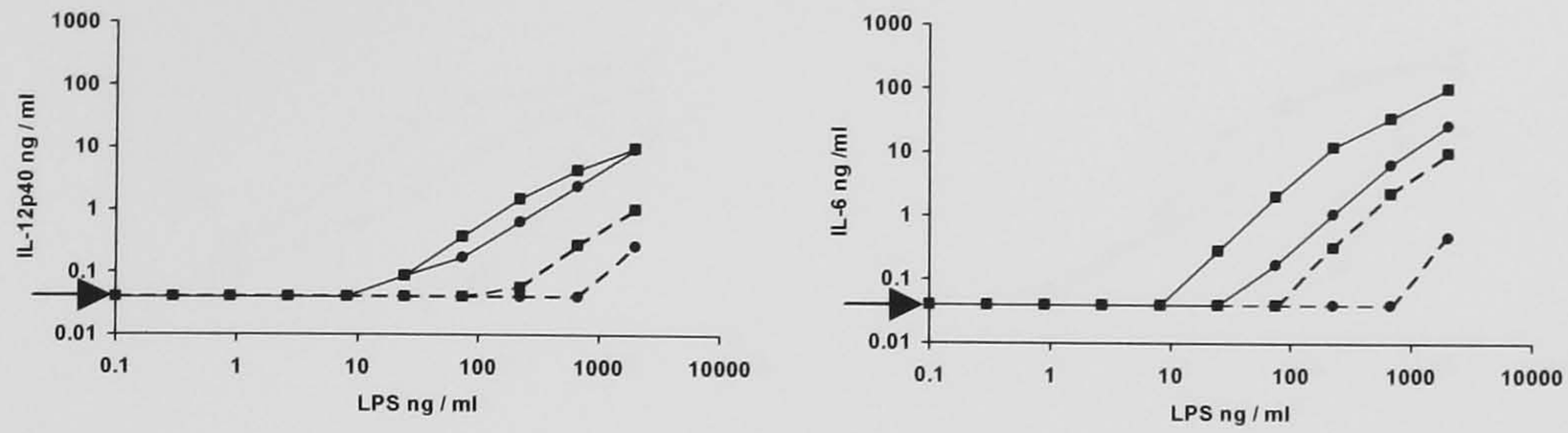
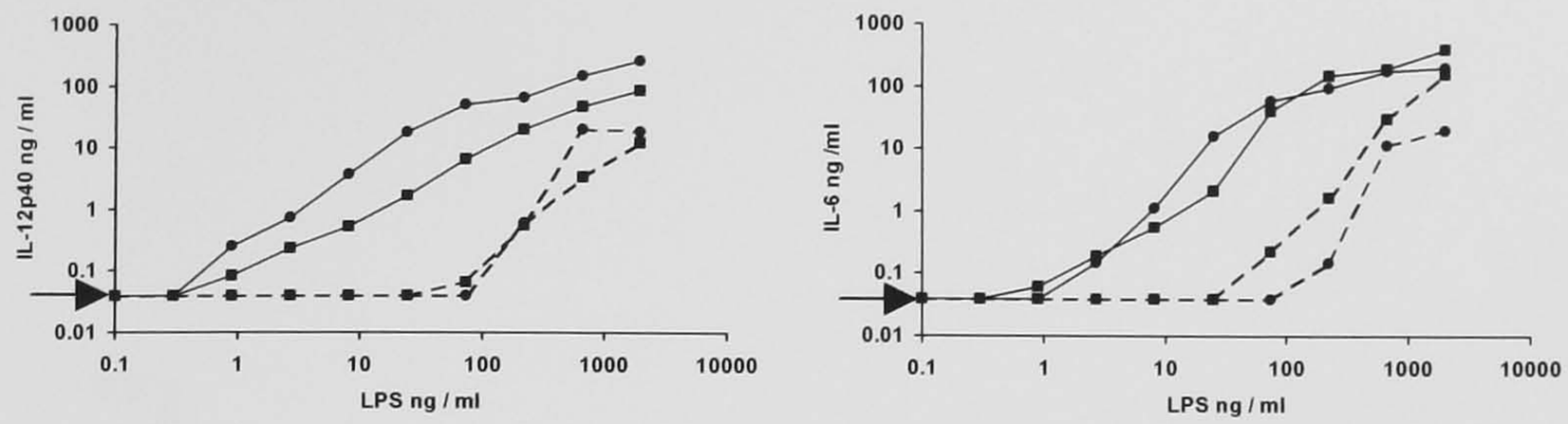


Figure 3.14 **LPS but not schistosome PAMPs, stimulates cytokine production by the J774 Mϕ cell-line, a property which is semi-dependent on expression of CD14.** J774 cells (■, a & b; white bars, c & d), or the equivalent CD14-deficient J7.DEF.3 cells (●, a & b; grey bars, c & d), were cultured overnight with LPS (0.1 - 2000 ng / ml), or different schistosome preparations (50 μg / ml), in the presence (dashed lines, a & b; hatched bars, c & d), or absence (solid lines, a & b; solid bars, c & d), of PMB (10 μg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & c), or IL-6 (b & d). Arrows denote the lower limit of detection of ELISA. Data is representative of up to 3 experiments. n.d. = not done.



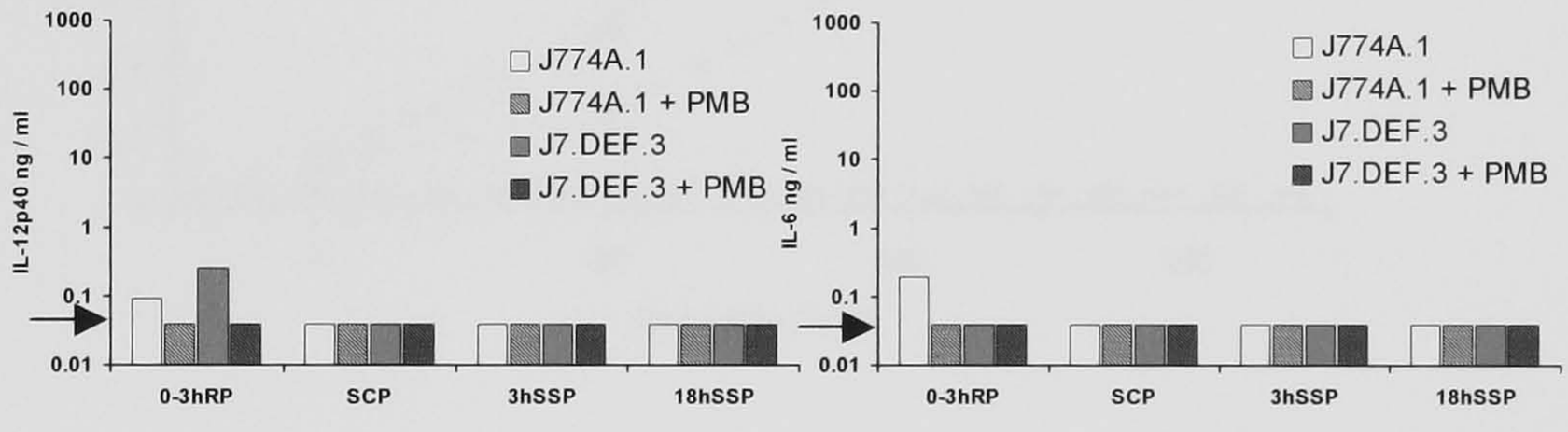


Figure 3.15 **LPS but not schistosome PAMPs, stimulates cytokine production by IFNγ-primed J774 Mϕ cell-line, a property which is not dependent on expression of CD14.** J774 cells (■, a & b; white bars, c & d), or the equivalent CD14-deficient J7.DEF.3 cells (●, a & b; grey bars, c & d), were cultured overnight with IFNγ (1 U / ml), and LPS (0.1 - 2000 ng / ml) or different schistosome preparations (50 μg / ml), in the presence (dashed lines, a & b; hatched bars c & d), or absence (solid lines, a & b; solid bars, c & d), of PMB (10 μg / ml). Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-12p40 (a & c), or IL-6 (b & d). Arrows denote the lower limit of detection of ELISA. Data is representative of up to 3 experiments.

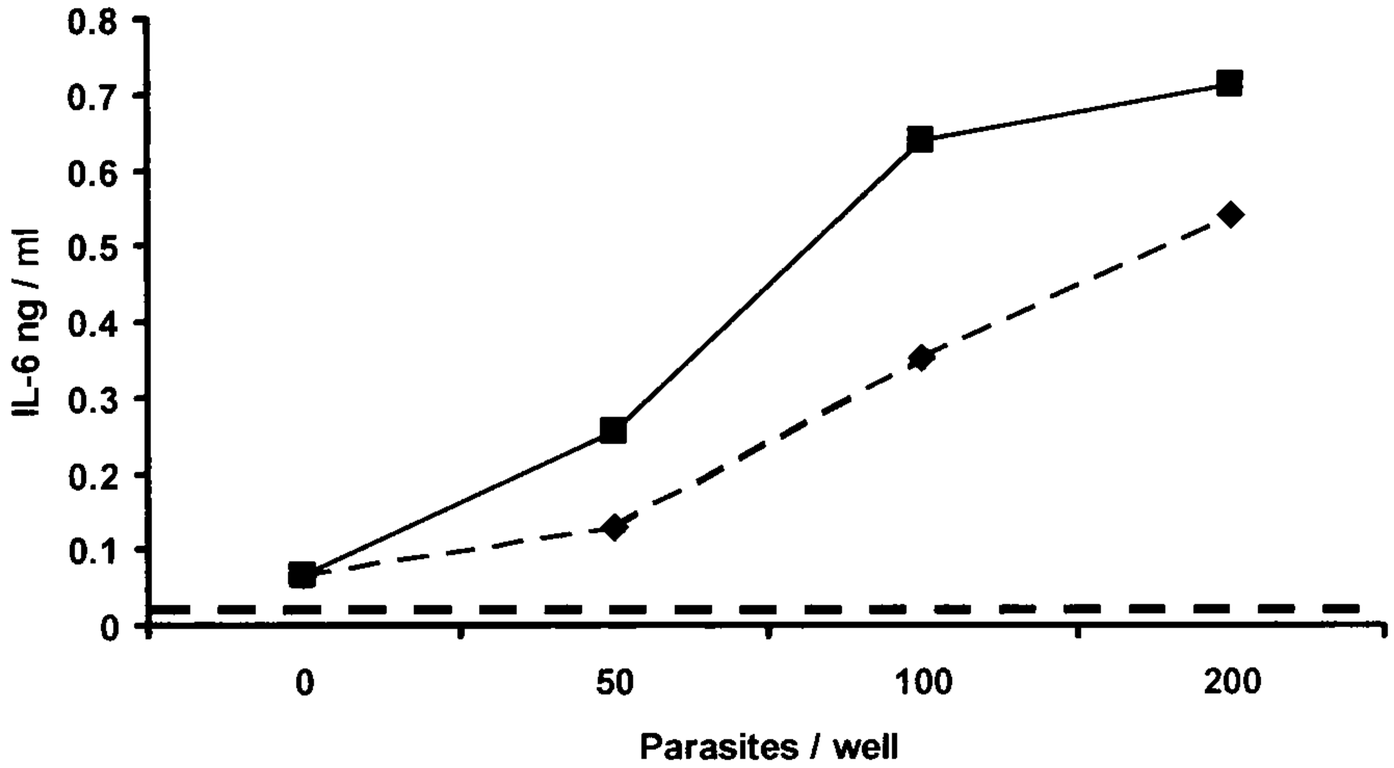


Figure 3.16 **Mechanically-transformed cercariae are more stimulatory than untransformed cercariae.** iMϕ from C57Bl/6 were cultured overnight alone, or with different concentrations (50 - 200 parasites / well) of cercariae (CercN; ◆), or mechanically-transformed cercariae (CercT; ■). All iMϕ were cultured in the presence of PMB (3 μg / ml) throughout. Supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-6. Dashed line denotes the lower limit of detection of ELISA. Data is representative of 2 experiments.

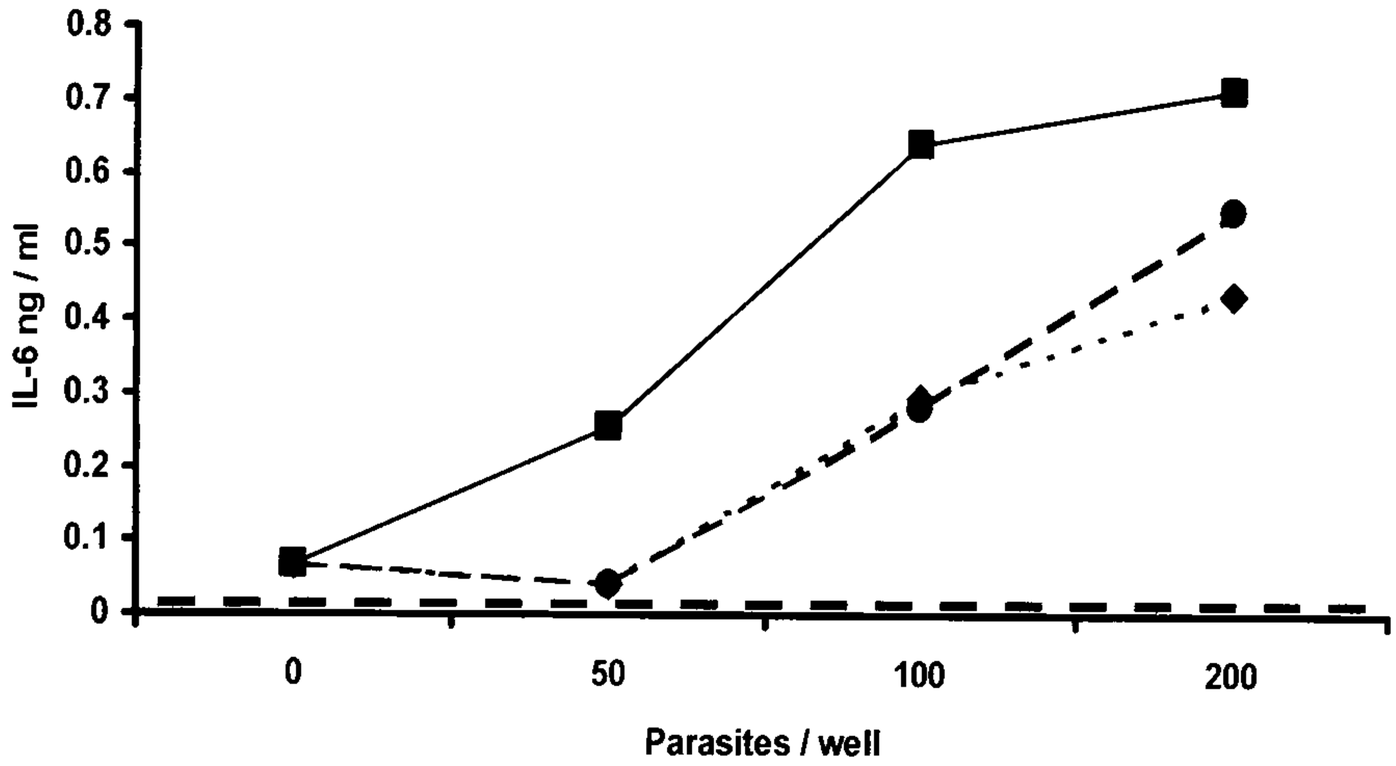


Figure 3.17 **The stimulatory properties of artificially-transformed cercariae are shared between the products released by larvae and the larval bodies.** iMϕ from C57BL/6 mice were cultured overnight alone, or with different concentrations (50 - 200 parasites / well) of mechanically-transformed cercariae (CercT; ■), mechanically-transformed cercariae that had been previously cultured *in vitro* for 3 hr (3hSom; ●), or the SN from the 3hr *in vitro* cultured cercariae (0-3hSN; ◆). All iMϕ were cultured in the presence of PMB (3 μg / ml) throughout. iMϕ supernatants from triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-6. Dashed line denotes lower level of detection of ELISA. Data is representative of 2 experiments.

3.4 DISCUSSION

In this chapter, I have demonstrated that the soluble material released by transforming schistosome cercariae (0-3hRP) is a potent stimulator of IL-6 and IL-10 production by iMϕ from a variety of mouse strains. Moreover, when administered in parallel with IFNγ, 0-3hRP stimulates high levels of the Th1-associated pro-inflammatory cytokine IL-12p40.

3.4.1 The Mϕ-based assay

At the start of this investigation, considerable time was spent to validate and optimise the iMϕ-based assay used to screen the stimulatory capacity of schistosome PAMP preparations. From heterogeneous inflammatory PECs, a highly enriched iMϕ population was generated using adherence-based selection. The vast majority were large, highly granular, and expressed the Mϕ markers F4/80 and CD11b. Since the expression of these markers varies greatly with Mϕ activation and maturation states (McKnight and Gordon 1998), these results suggest that this technique generates a heterogeneous adherent Mϕ population. Furthermore, numerous parameters (including concentration of cells, IFNγ, and schistosome PAMPs) were optimised for *in vitro* stimulation of iMϕ cytokine production. The kinetics of IL-6, IL-10, and IL-12p40 production by iMϕ were shown to be approximately similar, all reaching peak levels by 6 hr post-stimulation and remaining at a plateau until at least 24 hr post-stimulation. Therefore, for ease of experimental design, further analysis of cytokine production was restricted to 18 hr post-stimulation.

Due to the limited numbers of Mϕ obtainable from individual mice, cells from several animals were often pooled. Consequently, it was generally not possible to look at variation in cytokine production between individual mice. In addition, due to the limited availability of schistosome PAMPs, it was impracticable to analyse variance between stimulation regimes, since assays were restricted to triplicate wells for each treatment, and the volume of supernatant required for multiple cytokine ELISA's meant these wells were pooled. However, experiments were repeated several times, giving greater confidence in the data.

3.4.2 Schistosome PAMPs are concentrated within larval released material

The highly stimulatory properties of 0-3hRP suggest this preparation is rich in schistosome PAMPs. In contrast, the soluble preparations of *in vitro*-cultured schistosomulae (SSPs) were non-stimulatory causing little, if any, IL-6, IL-10, or IL-12p40 production by iMϕ or IFNγ-Mϕ. This suggests that these preparations contain few stimulatory components. The physiological context in which innate immune recognition of schistosomes evolved may reflect the difference in the stimulatory properties of these preparations (0-3hRP *versus* SSPs). The schistosome components most visible to innate accessory cells will be those released by the parasite during migration through the skin (such as 0-3hRP), and larval surface molecules. In contrast, since more than 90% of parasites exit the skin during infection (Wilson and Coulson, 1986), the host accessory cells are unlikely to be exposed to their somatic larval molecules (likely to be the abundant components of the SSPs). Alternatively, it could be argued that some somatic molecules within the SSPs should be stimulatory due to possible homology with host 'Danger Signals' and other pathogen PAMPs, for example highly conserved Heat Shock Proteins (HSPs), or DNA (Section 1.8.1 & 1.8.2). Therefore, the SSPs may also contain inhibitory components that antagonise any stimulatory PAMPs, resulting in net stimulatory properties that are undetectable. In contrast to SSPs, the preparation of whole cercariae (SCP) was observed to have some stimulatory properties. However, compared to 0-3hRP, these properties were low, often verging upon the undetectable. It seems likely that SCP will contain some stimulatory 0-3hRP components but are greatly diluted by non-stimulatory / inhibitory somatic constituents (Section 2.4).

In addition to schistosome PAMP preparations, live parasites stimulated dose-dependent IL-6 production. Cercariae that were mechanical transformed (CercT) were twice as stimulatory as their non-transformed counterparts (CercN). The only physical difference between these two groups is the active release of head gland material and shedding of the glycocalyx by CercT, whereas CercN remained intact in culture not shedding their tails (data not shown). Moreover, the stimulatory capacity of CercT was split approximately 1 : 1 between the material released into the SN during the first 3 hours of culture (0-3hSN), and 3 hr schistosomulae from which the 0-3hSN was removed (3hSom). This provides clear evidence that components within the released material can stimulate Mϕ cytokine

production. Although 0-3hSN released by 100 to 200 cercariae stimulated less IL-6 production than concentrated 0-3hRP, it demonstrates that physiological levels of these released PAMPs remain stimulatory for iMϕ cytokine production. It is noteworthy that the stimulatory nature of 3hSom contrasts with the non-stimulatory nature of the soluble preparation 3hSSP. The apparent increase in stimulatory capacity of the live larvae could be due to the presence of membrane bound surface material present on the schistosomulae (Section 2.4). Alternatively, the material that these larvae continue to release into the supernatant following the first 3 hours of culture could account for this.

3.4.3 Schistosome preparations contain PAMPs of larval origin

One concern of the work presented in this chapter is that the stimulatory nature of 0-3hRP may be due to the presence of naturally occuring endotoxin. However, it must be emphasised that the levels of endotoxin in 0-3hRP can be classed as 'low' (Section 2.4). Because low-levels of LPS endotoxin is detected within these preparations, contamination with other microbial endotoxins, such as bacterial lipoproteins and fungal glycans, cannot be discounted. However, since LPS is the most potent of these microbial stimulants, the contribution of other microbial PAMPs to the stimulatory properties of 0-3hRP, is likely to be much less important. Indeed, yeast-derived Zymosan A (Section 3.3.5) and Gram positive bacterial peptidoglycan and lipoteichoic acid (Takeuchi *et al.*, 1999) are only stimulatory at microgram levels. Because LPS endotoxin was detected at low levels (pg to ng), any other microbial PAMPs present are likely to be at equally low levels, and thus of little significance.

The results of this chapter strongly suggest that the stimulatory properties of 0-3hRP are predominantly due to schistosome PAMPs and not endotoxin. Definitive evidence of schistosome PAMPs in the released material is demonstrated by the fact that transforming-cercariae are almost twice as stimulatory as their non-transforming counter-parts. In these experiments, both parasite groups were derived from exactly the same pool of larvae. Therefore, any microbial / endotoxin contaminants would be present in equal quantities in both cercarial suspensions. Thus, the increase in stimulatory capacity of transforming larvae must be due to the active release and shedding of larval PAMPs. Since 0-3hRP represents a considerably concentrated preparation of this released larval material, the

parasite PAMPs would be expected to account for most of its stimulatory properties. Preliminary studies also suggest that the stimulatory components of 0-3hRP are heat labile (data not shown). Since LPS endotoxin is thought to be heat stable (Smiley *et al.*, 2001), this would demonstrate that the stimulatory properties of 0-3hRP are independent of LPS.

In addition, 0-3hRP retained the majority of its potent IL-12p40, IL-6, and IL-10 stimulating properties in the presence of endotoxin-neutralising PMB. In contrast, PMB dramatically inhibited the stimulatory properties of LPS. PMB exerts this neutralising effect by high-affinity binding and subsequent blocking of the stimulatory Lipid A moiety of LPS. The control LPS used in this study was selected for its highly stimulatory properties from a range of sources by screening different preparations (data not shown) and reviewing the literature (Luchi and Morrison, 2000). Consequently, the potency of this control was probably much higher than the endotoxin contained within 0-3hRP. It has been reported that the stimulatory properties of LPS from some bacterial strains are less effectively blocked by PMB than others (Cavaillon and Haeffner-Cavaillon, 1985). However, at concentrations equivalent to the endotoxin contained in 0-3hRP, PMB still retained blocking capabilities against these 'PMB-resistant' LPS strains (Cavaillon and Haeffner-Cavaillon, 1985). From these results, I conclude that culture with endotoxin-neutralising PMB is a good method for further examination of the effects of 0-3hRP on the innate immune system.

In this chapter I also show that LPS-stimulated cytokine production by iMϕ and IFNγ-Mϕ was dependent on the presence of a functional *TLR4* gene, as demonstrated using cells from LPS-unresponsive C3H/HeJ mice. In contrast, in the absence of TLR4 signalling, 0-3hRP still stimulated production of IL-10 and IL-12p40 by iMϕ and IFNγ-Mϕ respectively, albeit at a lower level than produced by cells from LPS-responsive C3H/HeN Mϕ. Moreover, both iMϕ and IFNγ-Mϕ production of IL-6 was similar in the two strains of mice in response to 0-3hRP, in the presence of PMB. Since the adherent iMϕ populations from both C3H/HeJ and C3H/HeN mice were phenotypically and quantitatively similar (data not shown), it is unlikely that the difference in cytokine production was due to variations in cell population. However, it is conceivable that cells from C3H/HeN mice might be at a higher state of activation (due to endogenous priming by endotoxins within the peritoneal cavity), resulting in increased IL-12p40 or IL-10 production. Nevertheless,

because IL-6 production is so similar between strains, this is unlikely. Therefore, my data strongly suggests that there are both TLR4-dependent and TLR4-independent stimulatory components within 0-3hRP. Although it has recently become apparent that LPS from some bacterial species signals through TLR2 (Hirschfeld *et al.*, 2001; Werts *et al.*, 2001), it is likely that both the TLR4-dependent and independent signalling pathways involved in 0-3hRP recognition are due to schistosome PAMPs and not endotoxin, as both were activated in the presence of PMB. This is quite probable as the complexity of 0-3hRP suggests it may contain numerous ligands for different PRRs. Moreover, TLR4 is a promiscuous receptor, sharing its signalling function with many different PAMPs (Section 1.8.3). One candidate PRR potentially involved is TLR2, since glycolipids from soluble schistosome egg antigen (SEA) that may be common to 0-3hRP, are recognised through this receptor (van de Kleij *et al.*, 2002). Thus, the evidence suggests that 0-3hRP contains potent schistosome PAMPs that stimulate through a variety of different PRRs. Moreover, PMB is useful to examine the stimulatory properties of 0-3hRP in the absence of endotoxin signalling.

3.4.4 Control of cytokine production by schistosome PAMPs

In this chapter, I show that Mϕ can produce a variety of cytokines upon exposure to schistosome PAMPs. Indeed, Mϕ are regarded as a principle source of both pro-inflammatory (*e.g.* TNFα, IL-1β, IL-6, & IL-12) and regulatory cytokine production (*e.g.* IL-10 & IL-6) during innate immune responses. As such, the mechanisms that control Mϕ cytokine production are the object of intense study. Furthermore, I show that the profile of iMϕ cytokine production differs both qualitatively and quantitatively in response to diverse pathogen stimuli, with LPS and 0-3hRP stimulating IL-10, IL-12p40, and high levels of IL-6, and Zymosan A stimulating little IL-6, no IL-10, but higher levels of IL-12p40. These observations support the hypothesis that Mϕ individually tailor responses to specific types of pathogen (Nau *et al.*, 2002).

The initial trigger for cytokine production by pathogens is thought to be the direct or indirect ligation of specific TLRs, or other PRRs, resulting in translocation of cytokine promoter-binding NF-κβ transcription factors to the nucleus (Section 1.9.2). It has been proposed that the differences in macrophage cytokine production to diverse pathogen

products are due to signalling events mediated by the specific TLRs (Hirschfeld *et al.*, 2001; Nau *et al.*, 2002). Indeed, studies have shown that TLR4 agonists but not TLR2 agonists preferentially induce expression of IL-6 (Hirschfeld *et al.*, 2001; Schilling *et al.*, 2002). My work is partially consistent with these findings as TLR4-mediated LPS signalling resulted in high IL-6, whereas Zymosan A, thought to signal in part through a TLR2-dependent pathway (Underhill *et al.*, 1999b), resulted in low IL-6 production. However, 0-3hRP poses an anomaly to this model, since it stimulated high levels of IL-6 in the absence of TLR4. This suggests there is a TLR4-independent pathway of high level IL-6 production through which 0-3hRP signals.

The control of IL-6 production has been linked to the adapter proteins through which TLRs mediate signalling for transcription of cytokine genes. Specifically, all TLRs utilise the MyD88 pathway. However, TLR4 can signal via a MyD88-independent pathway. This alternative TLR4 signalling pathway utilises the MyD88 adaptor-like (MAL) adaptor protein (also known as Toll-IL-1R domain containing adapter protein [TIRAP]). Moreover, it is this TIRAP-dependent pathway which is thought to be required for maximal IL-6 expression by Mϕ (Schilling *et al.*, 2002). Although the majority of work on Zymosan A suggests it signals through a TLR2 / MyD88 pathway, a MyD88-independent pathway has also recently been described (Edwards *et al.*, 2002), which could explain the residual levels of IL-6 that were produced in response to this PAMP. However, responses to 0-3hRP appear to provide evidence of a TLR4 / TIRAP-independent alternative pathway for maximal IL-6 production by iMϕ. Moreover, my data appears to show redundancy in the IL-6 signalling pathway, since the majority of the IL-12p40 / IL-10 stimulating capacity of 0-3hRP was dependent on TLR4, yet IL-6 production was not, suggesting TLR4-dependent signalling can be replaced by the alternative pathway. In this case, the alternative pathway of IL-6 production signals weakly for IL-12p40 and IL-10 production. Notably, other TLRs are now thought to utilise a TIRAP pathway (Horng *et al.*, 2002), and it may be those through which 0-3hRP is co-recognised. Indeed, with very recent work it is becoming evident that other MyD88-independent signalling pathways exist in humans (Oshiumi *et al.*, 2003), and thus probably exist in mice, through which PRRs may signal for maximal IL-6 production. With the intense amount of work taking place in this area, these other IL-6 transcription pathways may soon be described.

In addition to TLRs, it is also possible that components of 0-3hRP may signal for cytokine production through other PRRs. Although, there is limited evidence of direct signalling by non-TLR PRRs for cytokine production, the mannose receptor has been implicated in IL-6 and IL-12 production in response to a variety of PAMP ligands (Shibata *et al.*, 1997; Yamamoto *et al.*, 1997). Indeed, the J774A.1 cell-line is reported to lack surface expression of the MR (Diment *et al.*, 1987), which could explain the apparent lack of responsiveness by this and the related J7.DEF.3 cell-line to stimulation with 0-3hRP and the other schistosome PAMP preparations. This proposition will be explored more fully in Chapter 4. Moreover, the J774A.1 cell-line could also lack other PRRs whose functions may be important in schistosome PAMP recognition. Alternatively, this line may have a higher threshold of stimulation that must be reached before detectable cytokine production occurs since these cells were also less sensitive to stimulation with LPS than primary Mϕ.

The activity of the Mϕ was also critically important for the cytokine profile produced. Priming iMϕ with exogenous IFNγ dramatically increased the levels of IL-12p40 whilst decreasing the level of IL-10 produced in response to both 0-3hRP, SCP, and LPS. It is well documented that priming with IFNγ is required for iMϕ to produce high levels of IL-12p40 in response to stimulation with a variety of PAMPs (Chensue *et al.*, 1995; Murphy *et al.*, 1995; Skeen *et al.*, 1996), and live pathogens (Flesch *et al.*, 1995; Camargo *et al.*, 1997). IFNγ acts by enhancing the binding of PAMP-induced, NF-κβ heterodimer, p50 / c-REL transcription factor complex to the IL-12p40 promoter (Murphy *et al.*, 1995). This explains why IFNγ alone does not stimulate IL-12p40 production. The decrease in IL-10 production by IFNγ-Mϕ compared to iMϕ, has previously been documented (Fiorentino *et al.*, 1991b), but less is known about the intracellular mechanism involved. In contrast to IL-12p40 and IL-10, IFNγ had inconsistent effects on the production of IL-6 stimulated by 0-3hRP and SCP. Reviewing the literature, the capacity of IFNγ to effect IL-6 production remains controversial. Many groups have demonstrated that IFNγ enhances IL-6 production in response to a variety of PAMP stimuli but the potency of this effect is limited and not as great as for IL-12p40 (Fiorentio *et al.*, 1991b; Goodridge *et al.*, 2001; Schilling *et al.*, 2002). In contrast, other groups demonstrated that IFNγ-priming had no effect on iMϕ production of IL-6 in response to LPS (Shnyra *et al.*, 1998; Takeuchi *et al.*, 1999). Perhaps differences in the activity of the iMϕ used in my experiments, due to variables such as mouse age, or priming within the peritoneal cavity by endogenous

endotoxin, could account for this effect. This could also explain the considerable variation in overall levels of IL-12p40 produced by iMϕ between experiments (Figure 3.3 and 3.4).

3.4.5 Stimulatory components within 0-3hRP

0-3hRP is a complex mixture of components (Section 2.4) retaining stimulatory properties although it is thought to contain potentially anti-inflammatory factors (*i.e.* prostaglandins and Sm 16.8; Section 2.4). Many of the documented components of 0-3hRP have possible immunological properties, such as glycans containing the Le^x epitope, and the abundant protease component. For example, some proteases including those released by cercariae can stimulate basophils to produce IL-4 and histamine (Machado *et al.*, 1996), although the effect of schistosome proteases on Mϕ cytokine production is not known. Furthermore, the protease component complies with the PAMP-criterion of being essential for the survival / pathogenicity of an organism (Medzhitov and Janeway, 1997), since protease inhibitors are able to block parasite penetration (Lim *et al.*, 1999). Alternatively, the schistosome may have evolved virulence factors, which sequester host pathways that result in cytokine production and inflammation (be that via PRRs or other surface receptors). This could be beneficial to the parasite as the inflammation and oedema produced by injection of cercarial homogenate actually enhances parasite infectivity (Fallon *et al.*, 1996). In this respect, 0-3hRP probably contains several homologues of host proteins that may have stimulatory functions. For example, the homologue of TCTP can stimulate histamine production by mast cells (Rao *et al.*, 2002). Thus, 0-3hRP most likely represents a mixture of potentially stimulatory PAMPs, potentially stimulatory host homologues, and as discussed above, potentially inhibitory factors. These may act on iMϕ directly, via recognition through PRRs, or by utilising other host signalling pathways, resulting in a profile of cytokines similar to LPS.

In summary, the material released by *S. mansoni* cercariae upon transformation is highly enriched for putative parasite PAMPs. A soluble preparation of these PAMPs (0-3hRP) stimulates pro-inflammatory and regulatory cytokine production by iMϕ. This cytokine profile is similar to that stimulated by the TLR4 agonist LPS but not the TLR2 agonist Zymosan A. Furthermore, the stimulatory properties of 0-3hRP are semi-dependent on signalling through TLR4, although 0-3hRP stimulates high levels of IL-6 via a TLR4-

independent pathway. This data provides a basis for further analysis of the effect of these putative parasite PAMPs on cells of the innate immune system, and on the priming of acquired immune responses.

CHAPTER 4

SCREENING FOR MACROPHAGE MANNOSE RECEPTOR LIGANDS WITHIN THE SCHISTOSOME PAMP REPERTOIRE

4.1 INTRODUCTION

In Chapter 3, I demonstrated that skin-stage schistosomes express PAMPs that stimulate cytokine production by murine Mϕ. These PAMPs were concentrated within the material released by cercariae during transformation, and their recognition involved both a TLR4-dependent and a TLR4-independent pathway.

Many host PRR's are involved in the recognition of PAMPs by innate accessory cells, including members of the TLR family (leucine-rich proteins), intergrins, and C-type lectins (Medzhitov and Janeway, 1997). One such C-type lectin is the Mϕ mannose receptor (MR), which has been implicated in the recognition of viruses, bacteria, fungi and protozoa (reviewed by Linehan and Gordon, 2000). In addition to its well-defined role in microbe phagocytosis and endocytosis (*e.g.* Sallusto *et al.*, 1995; Tan *et al.*, 1997), the MR is also thought to be involved in the induction of cytokine production (including IL-6 and IL-12), upon recognition of PAMP ligands (Garner *et al.*, 1994; Yamamoto *et al.*, 1997; Shibata *et al.*, 1997).

The MR is a complex receptor with 8 carbohydrate recognition domains (CRD), and an additional lectin activity mediated by its N-terminal cysteine-rich domain (MR-Cys). CRDs are lectins with affinity for mannose, fucose and N-acetyl-glucosamine. Co-operative binding by multiple CRDs generates the high affinity of the MR for multivalent oligosaccharides, such as certain glycans found upon the surface of microbial pathogens (Taylor and Drickamer, 1993). In this respect, the function of the MR in innate recognition of microbial PAMPs has been attributed to these domains (Taylor and Drickamer, 1993), although these regions also bind endogenous ligands, such as lysosomal enzymes and secretory glycoproteins (Linehan *et al.*, 2001). Since schistosomes are known to synthesise many glycans, some with homology to host structures (Cummings and Nyame, 1999), it is a reasonable hypothesis that there may be MR ligands within this schistosome glycan repertoire. Indeed, molecules secreted by schistosome eggs have recently been shown to contain MR ligands (Linehan *et al.*, manuscript submitted). Moreover, the material released by schistosomes during transformation is thought to be highly glycosylated (Veira *et al.*, 1986), and it is possible that MR ligands may be concentrated within this highly stimulatory schistosome compartment. An additional indication that the MR may be

important in schistosome recognition came from observations that the J774A.1 Mϕ cell-line, thought to be deficient in MR expression (Diment *et al.*, 1987), was refractory to stimulation with schistosome PAMPs (Section 3.3.6).

A naturally-occurring soluble form of the mannose receptor (sMR) has been used to identify MR ligands (Martinez-Pomares *et al.*, 1998). However, sMR is only detectable at low levels in murine serum and Mϕ culture supernatant, making it difficult to obtain (Martinez-Pomares *et al.*, 1998). A recombinant fusion protein containing MR CRDs 4 to 7 fused to the Fc portion of human IgG_1 (CRD4-7Fc) has been produced and successfully used to probe for MR ligands within immobilised preparations of host tissue and host tissue-sections (Linehan *et al.*, 2001), and schistosome egg secretions (Linehan *et al.*, manuscript submitted). This MR probe has been shown to possess the same specificity as soluble MR (sMR) when tested with certain glycoconjugate ligands (Linehan *et al.*, 2001). Moreover, a study of the recognition properties of segments of CRDs suggests that CRD4-7 are sufficient to mediate full affinity for high-mannose-type glycans (Taylor and Drickamer, 1993).

The purpose of this short chapter is to study the expression of MR CRD ligands within the different schistosome preparations, using the CRD4-7Fc probe, in order to gain insight into the involvement of this PRR in schistosome PAMP recognition.

4.2 MATERIALS AND METHODS

4.2.1 Lectin blotting

The soluble MR probe (CRD4-7Fc) was a gift from Prof. Siamon Gordon (University of Oxford).

Soluble schistosome preparations (Section 2.2.1) were applied directly to pre-wetted (PBS; pH 7.2) Hybond^{TM} PVDF membranes (Invitrogen) placed in a Bio-Dot^{TM} dot blot apparatus (Biorad Laboratories Ltd., Hemel Hempstead; UK) under suction. Alternatively, preparations were separated by 1-D SDS-PAGE electrophoresis under denaturing conditions (previously described; Section 2.2.2.2), and then electrophoretically transferred

to PVDF membranes (Invitrogen) for 90 min at 30 V in blot transfer buffer (10% methanol and 1% NuPAGE™ antioxidant in NuPAGE® transfer buffer; Invitrogen). Pre-stained molecular weight markers (Biorad Laboratories Ltd.) were used for size comparison. To ensure separated material had transferred, the gels were stained with BBGC (Sigma-Aldrich), as described previously (Section 2.2.2.2).

In some experiments, membranes were treated with sodium periodate prior to blotting. Specifically, membranes were equilibrated in sodium acetate solution (0.1 M; pH 4.5) solution for 30 min, then cut into strips and incubated with sodium meta-periodate (20 mM; Sigma-Aldrich) in sodium acetate solution, or with sodium acetate solution alone, for 1 hr at room temperature (RT) with agitation. Strips were then washed twice with sodium acetate, and incubated with sodium borohydride (50 mM; Sigma-Aldrich) in PBS for 30 min and then washed extensively in Tris buffered saline (TBS; pH 7.6; 137 mM NaCl, 20 mM Tris) containing 0.1% (v / v) Tween 20 and 10 mM $CaCl_2$ (TBS-T). $CaCl_2$ was included in the buffer since MR binding is calcium dependent.

In all subsequent washing and incubation steps, TBS-T was used except where specified. Membranes were blocked overnight at 4 °C in TBS-T plus 5% (w / v) non-fat milk and then rinsed twice before incubation with CRD4-7Fc (1 μg / ml) for 1 hr at room temperature (RT) on an orbital shaker. In some experiments, membranes were incubated with CRD4-7Fc in the presence of 50 mM D-mannose (Sigma-Aldrich) in order to out-compete specific binding. The membrane was then extensively washed (rinsed twice, then washed once for 15 min followed by 3 washes for 5 min), and incubated with sheep HRP-conjugated anti-human IgG (1:2000 dilution; Amersham Pharmacia Biotech UK Ltd., Little Chalfont, UK) for 1 hr with orbital agitation. Finally, membranes were extensively washed as above before bound HRP was detected using chemiluminescence. Specifically, membranes were incubated with ECL Plus™ reagent for 5 min (Amersham Pharmacia), drained, and exposed to autoradiography film (Hyperfilm™-ECL; Amersham Pharmacia). Exposure times were typically in the range 30 - 120 secs. Film was developed using an X-graph.

4.2.2 Phenotypic analysis of MR expression by iMϕ

To determine if iMϕ express the MR, inflammatory PEC were phenotyped using flow-cytometry. PECs from C57Bl/6 mice were elicited using 3% Brewers thiogylcollate broth and harvested as previously described (Section 3.2.2). Red blood cells were lysed with ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA; Sigma Aldrich), and the remaining cells washed three times with RPMI-10 (Section 3.2.2). Cells were stained with optimal concentrations of specific antibodies, or irrelevant isotype matched antibodies, using the method previously described (Section 3.2.3.2). Antibodies against various surface markers were as follows (clone; supplier): FITC-conjugated F4/80 (CI:A3-1; Caltag) and biotin-conjugated MR (5D3; gift from Prof. Siamon Gordon, University of Oxford). Cells stained with biotinylated antibody were then incubated with streptavidin-conjugated QR™ (Sigma-Aldrich). Two-colour staining was used to analyse co-expression of F4/80 and MR.

For two-colour flow-cytometry, the required amount of compensation was set using samples stained with specific antibody for one marker and the irrelevant isotype control for the second marker. Compensation is the use of electronic signal algorithms to prevent one colour's signal spilling over into another.

4.3 RESULTS

4.3.1 CRD4-7Fc recognises schistosome carbohydrates

The recombinant fusion protein CRD4-7Fc was used to probe for ligands within the soluble schistosomal PAMP preparations, using a lectin blotting technique. CRD4-7Fc bound to dot-blots of 0-3hRP, SCP, and 18hSSP (Figure 4.1a). In contrast, RPMIc did not contain ligands for the MR probe. The binding between the schistosome ligands and the MR probe was highly specific, being out-competed by the monomeric ligand mannose (Figure 4.1b).

To test whether CRD4-7Fc binding was dependent upon the glycan constituent of the schistosome preparations, membrane immobilised samples were first oxidised with sodium

periodate and then reduced with sodium borohydride, in order to structurally alter any carbohydrate components. To control for this procedure, an identical membrane underwent a similar procedure except that it was not exposed to sodium periodate. Treatment of the dot-blots with sodium periodate destroyed the ligands for CRD4-7Fc within 0-3hRP, SCP, and 18hSSP (Figure 4.2b), whereas control treatment did not affect the ability of the MR probe to bind to the sample (Figure 4.2a). Interestingly, CRD4-7Fc binding was consistently more intense for SCP and 18hSSP compared to 0-3hRP, even though equal amounts of these preparations were loaded onto the membrane (Figures 4.1 and 4.2).

In order to determine if CRD4-7Fc was binding to similar ligands within the different preparations, samples were first separated by 1-D SDS-PAGE and then probed. The soluble preparation of 3 hr *in vitro*-cultured schistosomulae was also included for reference. The most distinctive difference in the pattern of lectin binding was between the released schistosome molecules and the whole larval preparations. Specifically, the ligands within 0-3hRP did not resolve into distinct bands rather being associated with a 75 - 250 kDa complex of macromolecules (Figure 4.3). In contrast, distinct ligand bands were visible within SCP, 3hSSP, and 18hSSP (Figure 4.3). Moreover, there were numerous different ligands within each of these preparations. One of the most striking features of these bands was their high molecular weight, ranging from approximately 75 to 250 kDa. Although several bands of equal size were shared between these whole larval preparations, SCP contained a band of approximately 100 kDa, which was detected only weakly in 3hSSP and not in 18hSSP. Similarly, both schistosomulae preparations contained a distinct band of just under 150 kDa, which was not present in SCP. There was little difference between the ligand binding patterns of 3hSSP and 18hSSP.

4.3.2 MR is expressed by a proportion of iMϕ

Flow-cytometric analysis confirmed that iMϕ express the MR. $F4/80^+$ cells comprised 60% of inflammatory PEC (Figure 4.4ia). Furthermore, 18% of PEC were MR^+ although the levels of receptor expression by these cells was relatively low (Figure 4.4ib). Subsequent two-colour staining revealed that the MR^+ PEC were $F4/80^+$ (Figure 4.4iia & b), and that they represented approximately one third of the $F4/80^+$ restricted group (Figure 4.4ia & b).

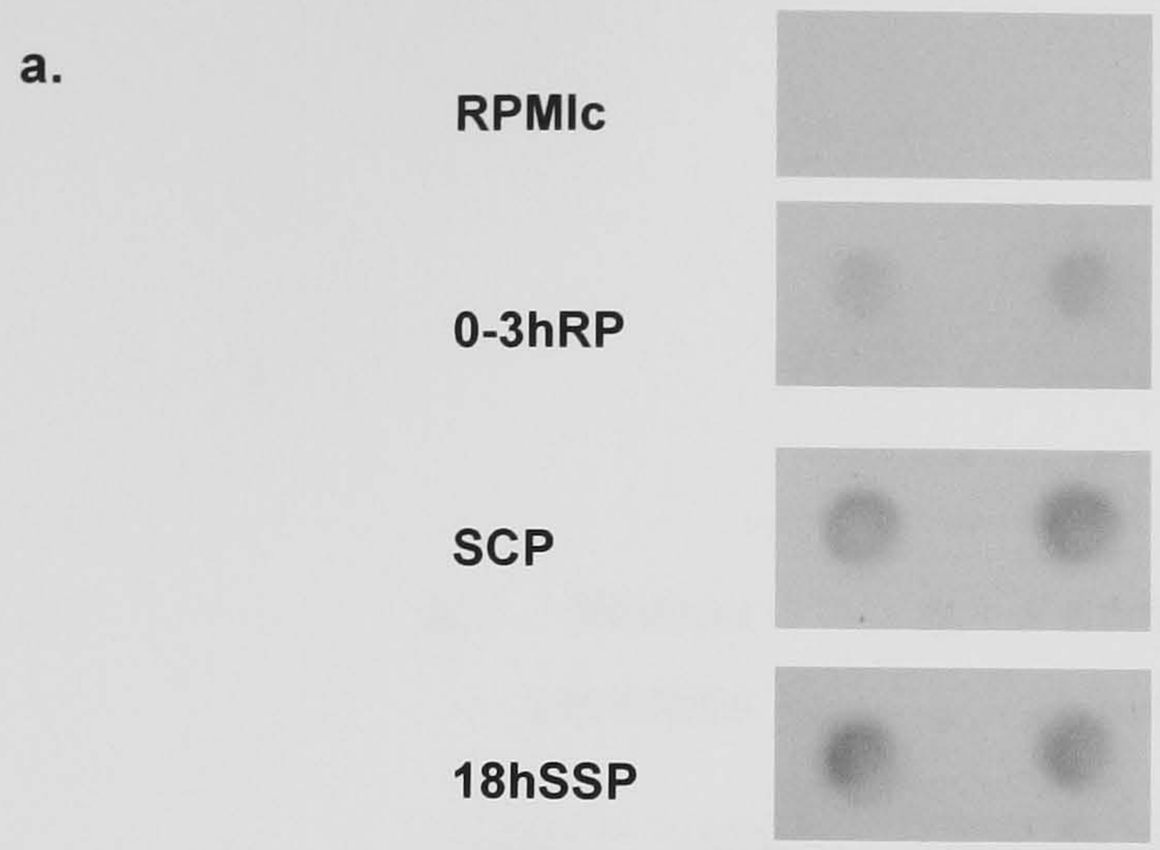


Figure 4.1 **CRD4-7Fc binds to ligands within soluble schistosome preparations.** Soluble schistosome preparations (4 μg) were dotted onto PVDF membranes. In one experiment the membrane was probed with CRD4-7Fc (1 μg / ml) (a). Alternatively the membrane was probed with CRD4-7Fc in the presence, or absence, of D-mannose (50 mM) (b).

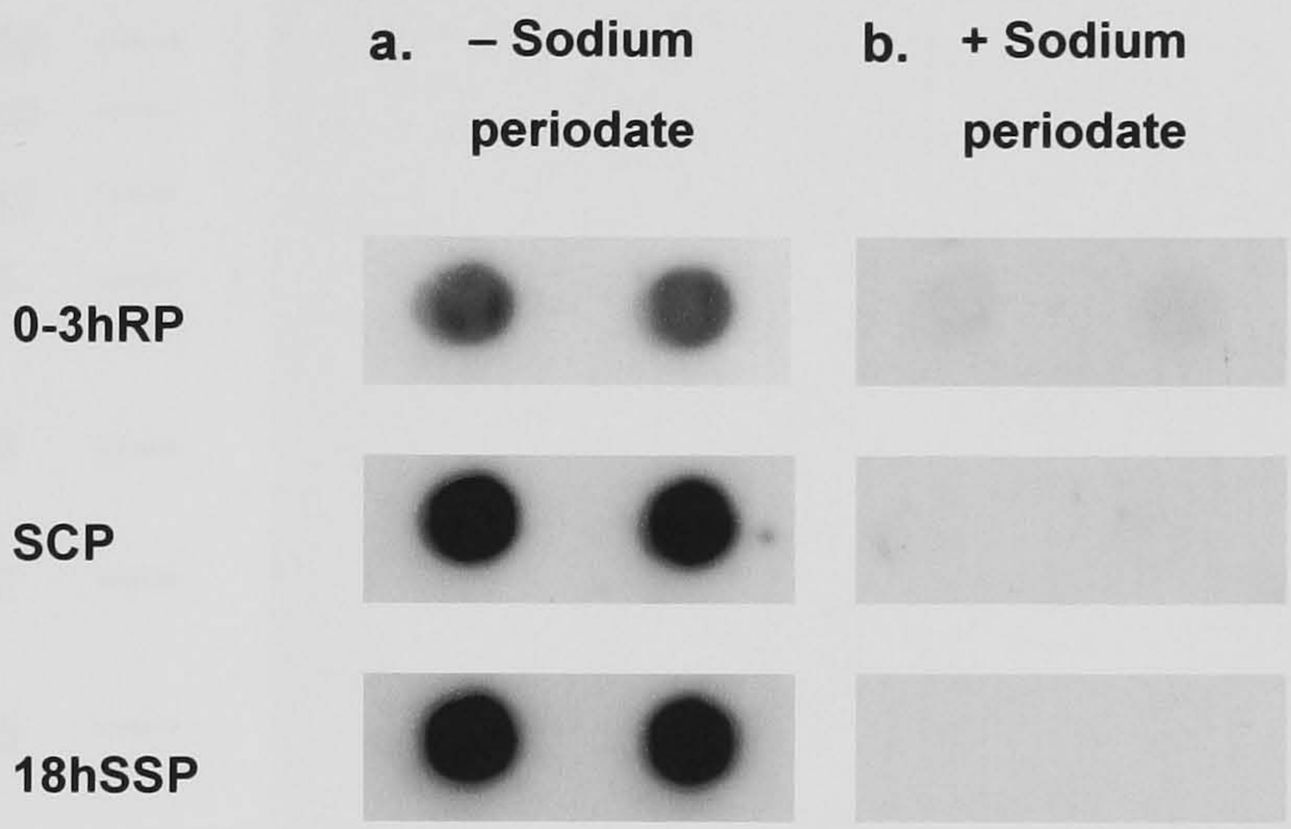


Figure 4.2 **CRD4-7Fc binds to glycans within the soluble schistosome preparations.** Soluble schistosome preparations (4 μg) were dotted onto PVDF membrane. The membrane was cut into strips which were treated with (b), or without (a), sodium meta-periodate. The membranes were then probed with CRD4-7Fc (1 μg / ml).

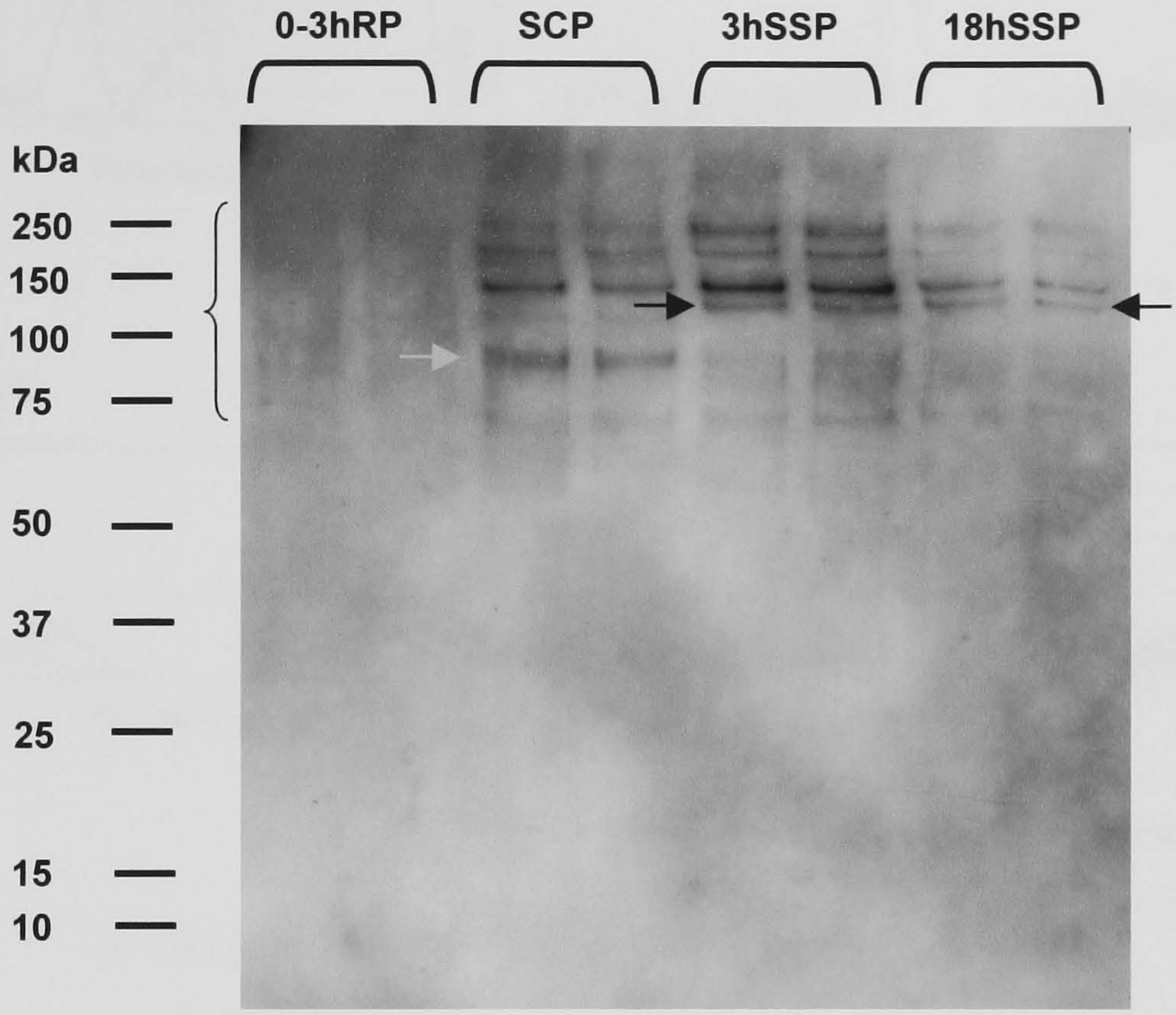


Figure 4.3 **CRD4-7Fc binds to distinct ligand bands within soluble whole larval preparations but not within 0-3hRP.** Soluble schistosome preparations (10 μg / lane) were denatured and separated on a 4% - 12% gradient bis-tris acrylamide gel. The separated material was then transferred to a PVDF membrane and probed with CRD4-7Fc (1 μg / ml). The brace indicates a ligand complex within 0-3hRP. The grey arrow indicates a ligand band visible only with SCP, and the black arrows indicates a ligand band visible only within 3hSSP and 18hSSP.

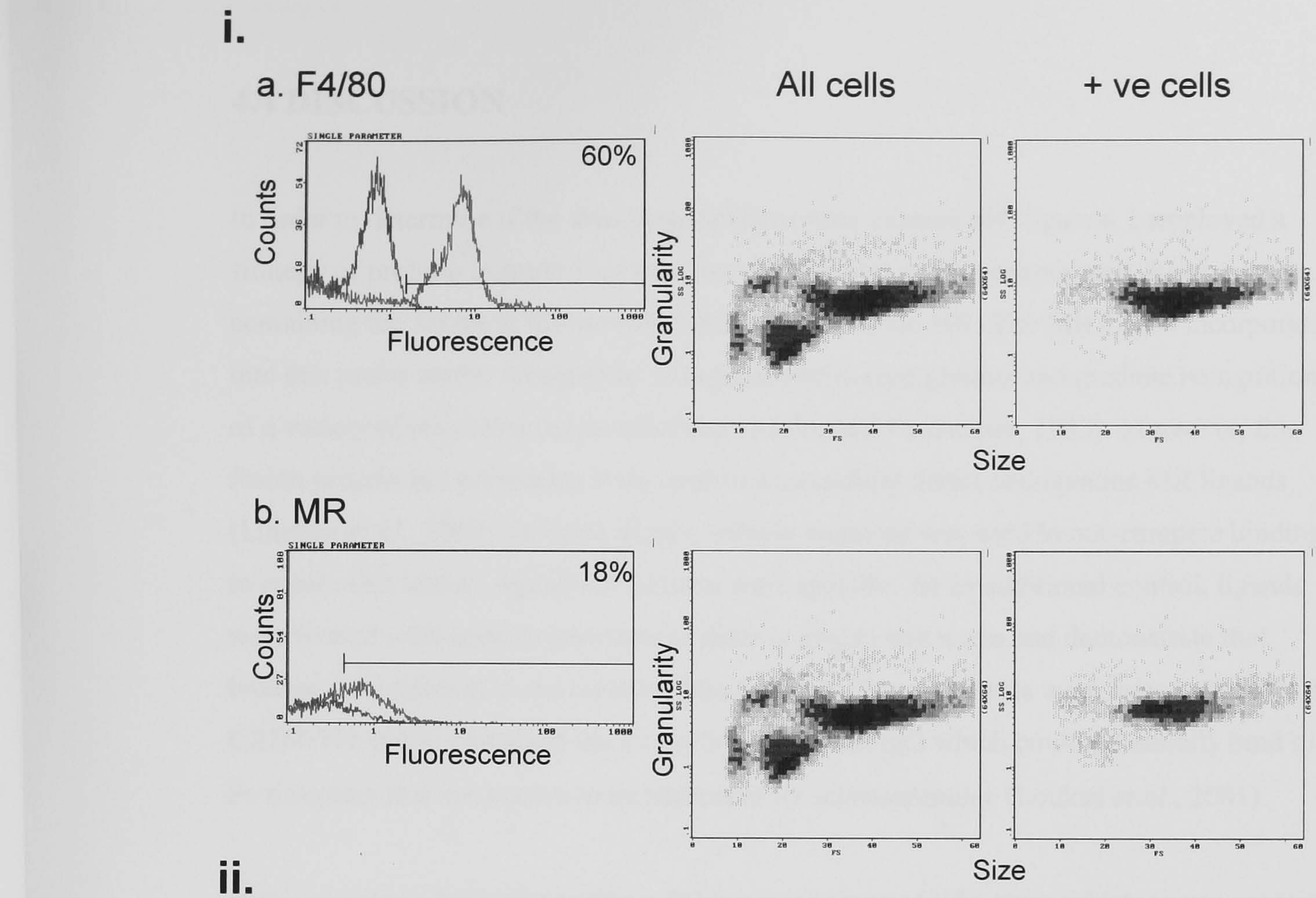


Figure 4.4 **The MR is expressed by a proportion of inflammatory macrophages.**

i) Thioglycollate-elicited PEC were analysed for their surface expression of F4/80 (a) or MR (b). Cells were stained with antibodies specific for the surface marker (red line), or with matched isotype controls (blue line). The values represent the percentage of cells expressing the marker. For each marker, the population of positive cells was characterised upon size and granularity, and compared to the whole cell population. ii) Cells were double stained with antibodies specific for F4/80 and the MR (b) or appropriate isotype controls (a). Control antibody staining is represented by the quadrant boundaries, and values represent the percentage of cells in the particular quadrant.

4.4 DISCUSSION

In order to determine if the skin-stage schistosomes express MR ligands, I employed a strategy of probing immobilised schistosome material with a recombinant fusion protein containing the fourth to the seventh CRDs of the murine MR. The MR CRDs incorporated into this probe confer recognition of high-mannose-type glycans and mediate recognition of a variety of microbial polysaccharides (Taylor and Drickamer, 1993). Moreover, this fusion protein has previously been used to successfully detect endogenous MR ligands (Linehan *et al.*, 2001). In these assays, soluble mannose was used to out-compete binding to ensure that lectin / ligand interactions were specific. As an additional control, ligands were treated with sodium periodate to destroy glycan structures and demonstrate that binding was directed to the carbohydrate moieties. These controls were important since the CRD4-7Fc probe contained the Fc portion of human IgG which could potentially bind to Fc receptors that are known to be expressed by schistosomulae (Loukas *et al.*, 2001).

In this chapter, I have shown that soluble preparations of skin-stage schistosomes contain PAMPs recognised by CRDs of the MR. Therefore, I conclude that MR ligands are expressed in the soluble compartments of cercariae, schistosomulae and the material released by cercariae upon transformation. As might be expected, these ligands were all of high molecular weight, probably due to heavy oligosaccharide chains. The ligand species in the whole larval preparations (SCP, 3hSSP, 18hSSP) formed distinct bands when separated by SDS-PAGE, that were not present in 0-3hRP. This suggests that some ligands are exclusive to the somatic larval material. These ligand bands are most probably internal, or tegument components, since they are unlikely to represent cercarial glycocalyx material, which would also be present in 0-3hRP (Section 2.4). The detection of a schistosomulae specific band suggests that a new ligand could be synthesised upon transformation. However, it could be an enzymatic digest product of a one of the larger ligands contained within the whole larval preparations. Perhaps surprisingly, the ligand material contained within 0-3hRP did not resolve into discrete bands using SDS-PAGE. Since nearly all the proteins within 0-3hRP were below 75 kDa in size (Section 2.3.1), this suggests that the major protein components of 0-3hRP do not contain MR CRD ligands. The high molecular weight complex of ligands within 0-3hRP is probably the same high molecular weight

complex of proteins described in Section 2.3.1, and could well represent schistosome mucins (discussed in Section 2.4).

Binding of carbohydrate ligands through the MR is thought to affect a range of Mϕ and accessory cell immune functions, such as phagocytosis and endocytosis of microbes, and the induction of cytokine production (Garner *et al.*, 1994; Sallusto *et al.*, 1995; Yamamoto *et al.*, 1997; Shibata *et al.*, 1997). Furthermore, MR activity may be important in adaptive immunity, through the uptake of antigens by DC and the delivery of these to MHC II-loading compartments (Sallusto *et al.*, 1995). In this respect MR-mediated up-take of antigen greatly enhances the efficiency of antigen presentation (*e.g.* Tan *et al.*, 1997). Therefore, my data suggests that recognition through the MR may play a role in the innate and adaptive immune responses to schistosome infection. Indeed, approximately one third of $F4/80^+$ PEC, thought to represent iMϕ, were shown to express the MR suggesting that culture of iMϕ with schistosome preparations (described in Chapter 3) would result in MR-mediated recognition of schistosome PAMPs. However, it is difficult to speculate what function MR recognition could have during infection and in the Mϕ responses observed in Chapter 3, since a recent review suggests that down-stream effects of MR ligation are not hard-wired, with many factors affecting MR-induced effector functions, such as the activation-state of the Mϕ, the type of ligand, and co-operation with other lectins (Linehan *et al.*, 2000). This is understandable for a receptor whose functions are though to include clearance of host secretory and circulatory glycoproteins, as well as microbial phagocytosis and immune signalling. This complex effector function could explain why the schistosome preparations containing the greatest quantity of MR ligands (SCP, 18hSSP) did not stimulate Mϕ cytokine production, whereas the preparation with the least quantity of ligands (0-3hRP) was a potent stimulator of cytokine production. One possible interpretation of these results is that schistosome MR ligation does not lead to cytokine production. Indeed, some reports suggest that MR ligands must be particulate in order to stimulate cytokine production (Shibata *et al.*, 1997). This is controversial, since others suggest that soluble ligands are stimulatory (Garner *et al.*, 1994), while a recent report suggests that MR ligation can actually have inhibitory functions on DC cytokine production (Nigou *et al.*, 2001). Thus, a similar inhibitory effect on Mϕ cytokine production could be envisaged. In this respect, 0-3hRP could be most stimulatory for cytokine production because it contains lower levels of 'inhibitory' MR ligands. In

addition, the MR ligands within 0-3hRP and the soluble whole larval preparations were quite different in character as determined by SDS-PAGE separation, and therefore may actually have different effects upon receptor-ligation. It is yet unclear what controls these differences in MR function, and further work is awaited in this area.

It should be noted that the MR could also recognise schistosome PAMPs via its Cys-MR domain, which is not included in the CRD4-7Fc probe. Although only endogenous host ligands have been described for this region of the receptor, one of these, the Le^x oligosaccharide (Martinez-Pomares *et al.*, 1996; Leteux *et al.*, 2000), is thought to be contained within 0-3hRP (Section 2.4), and could affect the outcome of MR-induced effector functions. In order to confirm a role for the MR in innate immune responses to schistosome PAMPs, mice deficient for this receptor could be used in future studies.

In this brief chapter, I have shown that soluble preparations of skin-stage schistosomes contain ligands of CRDs from the murine MR. However, MR ligands were more abundant in whole larval preparations than in the released products of schistosomulae, suggesting that MR recognition of somatic schistosome PAMPs may not be involved in signalling for cytokine production. In this respect, the repertoire of MR CRD ligands within the stimulatory 0-3hRP appear to be quite distinct to that of the whole larval preparations, possibly accounting for the difference in the stimulatory properties of these preparations.

CHAPTER 5

THE ROLE OF Fcγ RECEPTORS IN THE RECOGNTION OF RELEASED SCHISTOSOME PAMPS

5.1 INTRODUCTION

Fcγ receptors (FcγR) are important phagocytic PRRs on accessory cells. These receptors recognise the Fc portion of antibodies of the IgG isotype but also mediate the recognition and phagocytosis of other soluble 'innate' PRRs, such as the pentraxin family members Serum amyloid P and C-reactive protein, when in complex with pathogen PAMPs (Mold *et al.*, 2001; Bharadwaj *et al.*, 2001). FcγR ligation by IgG-opsonised *L. major* amastigotes was recently shown to have a profound effect upon the regulatory function of Mϕ, acting in synergy with PAMPs to induce high-level IL-10 production (Kane and Mosser, 2001). This IL-10 can inhibit pro-inflammatory cytokine production by additional Mϕ populations and enhance susceptibility of these cells to infection by amastigotes. Together with this, the observation that IL-10 $^{-/-}$ mice are able to control *Leishmania* infection has led to the proposal that amastigotes use host IgG as a virulence factor to exploit the inhibitory effects of signalling through FcγRs (Kane and Mosser, 2001). In this respect, FcγR ligation is also known to directly suppress Mϕ production of IL-12 (Sutterwala *et al.*, 1997; 1998; Grazia Cappiello *et al.*, 2001; Gerber and Mosser, 2001). Indeed, FcγR signalling via antibody, or pentraxins, can lead to the abrogation of IL-12p70 production concurrent with the induction of high levels of IL-10 in response to many pro-inflammatory stimuli, including TLR-dependent PAMPs, danger signals, and CD40 ligation (Gerber and Mosser, 2001). Further studies have shown that these effects are mediated through the common FcR γ-chain of the FcγRs (Sutterwala *et al.*, 1997; 1998; Mold *et al.*, 2002), an essential component in the signalling from FcγRI and FcγRIII (Takai *et al.*, 1994).

It is a reasonable hypothesis that production of parasite-specific antibody following infection with schistosomes could also promote the regulation of pro-inflammatory responses stimulated by parasite PAMPs released upon subsequent infections, or during the ensuing larval migration. In addition, opsonisation of parasite PAMPs by pentraxins could have a similar effect, but without requiring a preceding acquired (antibody) response.

The availability in York of a small number of mice that lack the γ-chain of the FcRs (FcR γ $^{-/-}$) allowed us to perform a short series of experiments to examine the potential role of FcγR-mediated regulation of inflammatory responses. A preliminary study by other researchers in the group demonstrated that infection of FcR γ $^{-/-}$ mice resulted in reduced

IL-10 production by skin biopsies on days 4 (prior to schistosome-specific antibody production) and 7 post-infection compared with those from wild-type (WT) mice (pers. com. K. Hogg and A. Mountford). However, there was more than 50% reduction in the production of IL-10 following re-infection of previously vaccinated FcR $\gamma^{-/-}$ mice in which parasite-specific antibodies are abundant.

Therefore, the aim of this study is to further investigate the role that FcγRs have in the recognition of released larval PAMPs, with respect to the resulting profile of IL-10 and IL-12 production by Mϕ. The majority of work investigating FcγR signalling in PAMP-stimulated IL-10 and IL-12 production has been performed using *in vitro* Mϕ-stimulation assays. Since, I have already defined such an assay to study the effects of schistosome PAMPs on cytokine production (Chapter 3), a similar system incorporating inflammatory peritoneal Mϕ will be used in these studies. This chapter will follow three strategies. Firstly, the profile of IL-10 and IL-12p40 production by iMϕ from FcR $\gamma^{-/-}$ mice will be compared to that from WT mice upon culture with larval released material. This will determine whether signalling through the γ-chain is essential to the 'innate' profile of cytokine production stimulated by schistosome PAMPs. Secondly, cells from WT mice will be stimulated with released larval PAMPs in the presence or absence of anti-parasite polyclonal serum (antiserum), in order to determine whether FcγR ligation by anti-parasite antibody / antigen complexes effects the profile IL-10 and IL-12p40 produced by normal Mϕ. Released larval material will be used in these experiments since it should contain PAMPs that stimulate iMϕ directly through PRRs, and it provides the source of parasite antigens to form complexes with the anti-parasite antibody. Finally, responses by FcR $\gamma^{-/-}$ iMϕ will be compared to those of WT upon culture with larval released material, in the presence of antiserum, to determine whether signalling through the γ-chain is essential for the profile of cytokines stimulated by schistosome PAMPs in the presence of parasite-specific antibody.

5.2 MATERIALS AND METHODS

5.2.1 Animals

All mice were maintained in open housing at the University of York animal unit until used. A breeding colony of FcR $\gamma^{-/-}$ mice (Takai *et al.*, 1994) were a gift from Prof. R. Grencis (University of Manchester), and together with wild-type (WT) 129 x C57 mice, were bred in-house.

5.2.2 Preparation of crude released larval material

Infective schistosome cercariae were obtained, concentrated, and transformed, as described in Section 2.2.1. These larvae were then cultured *in vitro* in RPMI 1640, containing 200 U / ml penicillin, 100 μg / ml streptomycin, and 2mM L-glutamine (RPMI-00), at 37 °C. The volume of medium used to culture the larvae depended on the amount of larval material required for the stimulation assays, typically 10 ml. After 3 hr, the culture supernatant was removed and centrifuged at 120 *g*, 4 °C, for 8 min to pellet the heads and tails, which were subsequently discarded. The remaining crude preparation, containing both the soluble and insoluble fractions of released material, was then sterilised by UV-irradiation for 30 min and termed 0-3hRM. Because of the particulate nature of 0-3hRM it was not possible to accurately measure the protein content of the different batches used in these experiments.

5.2.3 Production of anti-parasite polyclonal serum to larval released material

Anti-parasite polyclonal serum (IRS) against larval released material was raised in a rabbit by subcutaneous immunisation with 0-3hRM in 'Complete Freunds adjuvant' and produced 'in house' by Harrop *et al.* (2000). Control rabbit serum was obtained from naïve animals (NRS). The IRS had previously been shown to contain abundant antibodies against 0-3hRM, as judged by ELISA (Harrop *et al.*, 2000). IRS and NRS were heat-inactivated for 30 min at 56 °C, and then sterilised by UV-irradiation for a further 30 min.

5.2.4 iMϕ stimulation assay

Inflammatory peritoneal Mϕ (iMϕ) from WT or FcR γ $^{-/-}$ mice were elicited, harvested, and adherence-purified in 96-well plates as described in Section 3.2.2, except that RPMI-00 was used instead of medium containing FCS, and in one experiment (Experiment 2) cells were cultured at a concentration of 0.5 x 10^6 cells / ml rather than 1 x 10^6 cells / ml (due to limited cell availability). iMϕ were then cultured in a total volume of 200 µl medium for 24 hr with LPS (10 ng / ml), with a 3 : 1 dilution of 0-3hRM, or without any supplements. These stimulation assays were carried out in RPMI-00 containing 5% FCS, 5% NRS, or 5% IRS. Supernatants were then harvested and analysed by ELISA for production of IL-10, or IL-12p40, as described in Section 3.2.6.

5.3 RESULTS

5.3.1 IL-10 production stimulated by 0-3hRM is not effected by the absence of FcγR signalling through the common FcR γ-chain

In order to determine the contribution of FcγRs to the recognition of released schistosome molecules, the response of iMϕ from FcR γ $^{-/-}$ mice to larval PAMPs was compared to that of cells from wild-type (WT) animals. Due to the limited availability of these mice, this study was restricted to 3 experimental repeats.

iMϕ from the different mouse groups were cultured in media containing neutral FCS and either released larval material (0-3hRM), or LPS, in order to determine if they substantially differed in their ability to produce cytokines to these PAMP stimuli in the absence of specific antibody / antigen complexes. iMϕ from FcR γ $^{-/-}$ mice produced marginally greater amounts (1.24-fold) of IL-10 than their WT counterparts in response to 0-3hRM (Experiment 1; Figure 5.1a). Similarly, the absence of this receptor had minimal effect on the level of IL-10 produced in response to LPS. The same pattern was observed in two further experimental repeats (Experiment 2 & 3; Figure 5.1b & c). In contrast, the profile of IL-12p40 produced by these two different Mϕ populations (in response to either 0-3hRM or LPS) varied considerably between experiments, since a dramatic increase seen in the absence of FcγR γ-chain signalling in Experiment 1 (Figure 5.1d) but a decrease was

observed in the repeat (Experiment 3; Figure 5.1e). It is noteworthy that the high background levels of IL-10 and IL-12 production by cells cultured alone observed in Experiment 3 (Figure 5.1c & e), were later confirmed to be due to contaminating endotoxin in the batch of commercially available FCS used for that assay. However, for the purpose of my study, this further demonstrates that IL-10 production did not differ greatly between the two Mϕ populations in response to stimulation with 0-3hRM in the absence of parasite-specific antibody / antigen complexes. In addition, the overall levels of IL-10 and IL-12p40 stimulated by 0-3hRM differed considerably between the experimental repeats, whereas LPS stimulated production of relatively consistent amounts of this cytokine. Since different batches of 0-3hRM (derived from separate cercarial sheds) were used in each experiment, the variation in IL-10 production probably reflects the variation in the actual quantity of schistosome material to which the cells were exposed. In this respect, the batch of 0-3hRM used in Experiment 2, in which the least IL-10 was detected, was thought to contain the lowest amount of parasite material. Furthermore, fewer cells (0.5×10^6 compared to 1×10^6 / ml) were used in this experiment.

5.3.2 0-3hRM-specifc antibody has little effect on the production of IL-10 by iMϕ stimulated with released schistosome PAMPs

To determine if parasite-specific antibody alters the profile of cytokine production by iMϕ resulting from recognition of schistosome PAMPs, iMϕ were cultured with crude larval released material (0-3hRM) in the presence of anti-0-3hRM serum (IRS), or a neutral control serum (NRS). A crude preparation of the larval released material was used (rather than a soluble fraction centrifuged at 100,000 *g*) because it was deemed important to have both the soluble and insoluble components present. In this context, FcγR clustering that leads to the initiation of signalling pathways (following ligation by antigen / antibody complexes) may occur to a greater extent in the presence of particulate antigen. LPS was included as a stimulatory PAMP that should not cause antibody ligation of FcγR in the presence of anti-parasite polyclonal serum. The data from three experiments is presented in Figure 5.2, with the results of Experiment 1 and 2 corresponding to those presented in Figure 5.1. Experiment 4 represents an unrelated assay.

When cultured in neutral NRS, iMϕ produced high levels of IL-10 upon stimulation with 0-3hRM (Experiment 1; Figure 5.2a). Conversely, in the presence of anti-larval IRS, iMϕ produced dramatically lower levels of IL-10 (0.33-fold) when stimulated with 0-3hRM (Experiment 1; Figure 5.2a). In contrast, the levels of IL-10 produced in response to stimulation with LPS did not differ when Mϕ were cultured in IRS compared to NRS. However, in two further experimental repeats (Experiment 2 & 3), IL-10 production stimulated by 0-3hRM did not decrease when the assays were carried out in IRS compared to NRS; in Experiment 3 the levels actually increased 1.2-fold (Figure 5.2b & c). Similarly, there was no clear difference in the levels of IL-12p40 stimulated by 0-3hRM following culture in either NRS, or IRS, when the results from two experiments were compared (Figure 5.2d & e).

5.3.3 Maximal IL-10 production in the presence of anti-schistosome antibodies requires FcγR signalling through the common FcR γ-chain

As an alternative method to analyse the effect of schistosome-specific antibody / antigen complexes on stimulation by larval PAMPs, the cytokine response of iMϕ from FcR $\gamma^{-/-}$ mice to larval PAMPs was compared to that of cells from wild-type (WT) animals following culture in IRS. Again, the data from three experiments is presented (Figure 5.3), and directly correspond to those performed using FCS (Figure 5.1).

Compared to culture with neutral FCS (Figure 5.1), the profile of IL-10 production by FcR $\gamma^{-/-}$ and WT Mϕ stimulated in the presence of anti-0-3hRM serum displayed marked differences. iMϕ from FcR $\gamma^{-/-}$ mice produced considerably lower levels of IL-10 in response to stimulation with either 0-3hRM (0.52-fold) or LPS (0.55-fold) when cultured with IRS, compared to the WT controls (Experiment 1; Figure 5.3a). This was repeatable in two further experiments (Experiment 2 & 3; Figure 5.3b & c), albeit less dramatically in Experiment 2. In contrast, the profile of IL-12p40 produced by the different cell populations was not consistent between experiments (Figure 5.3d & e). However, the relative increase in IL-12p40 production by FcR $\gamma^{-/-}$ iMϕ upon stimulation with 0-3hRM, or LPS, observed in Experiment 1 (Figure 5.3d) mirrored that seen upon stimulation in the presence of FCS, in the same experiment (Figure 5.1d). Similarly, the relative decrease in IL-12p40 production observed in the absence of γ-chain signalling in Experiment 3 (Figure

5.3e) mirrored that in the equivalent experiment in the presence of FCS (Figure 5.1e). It is also noteworthy that in experiments where there was overall high levels of IL-10 there was little IL-12p40 production (Experiment 3; Figure 5.3c & e), whereas low levels of IL-10 corresponded with higher levels of IL-12p40 production (Experiment 1; Figure 5.3a & d).

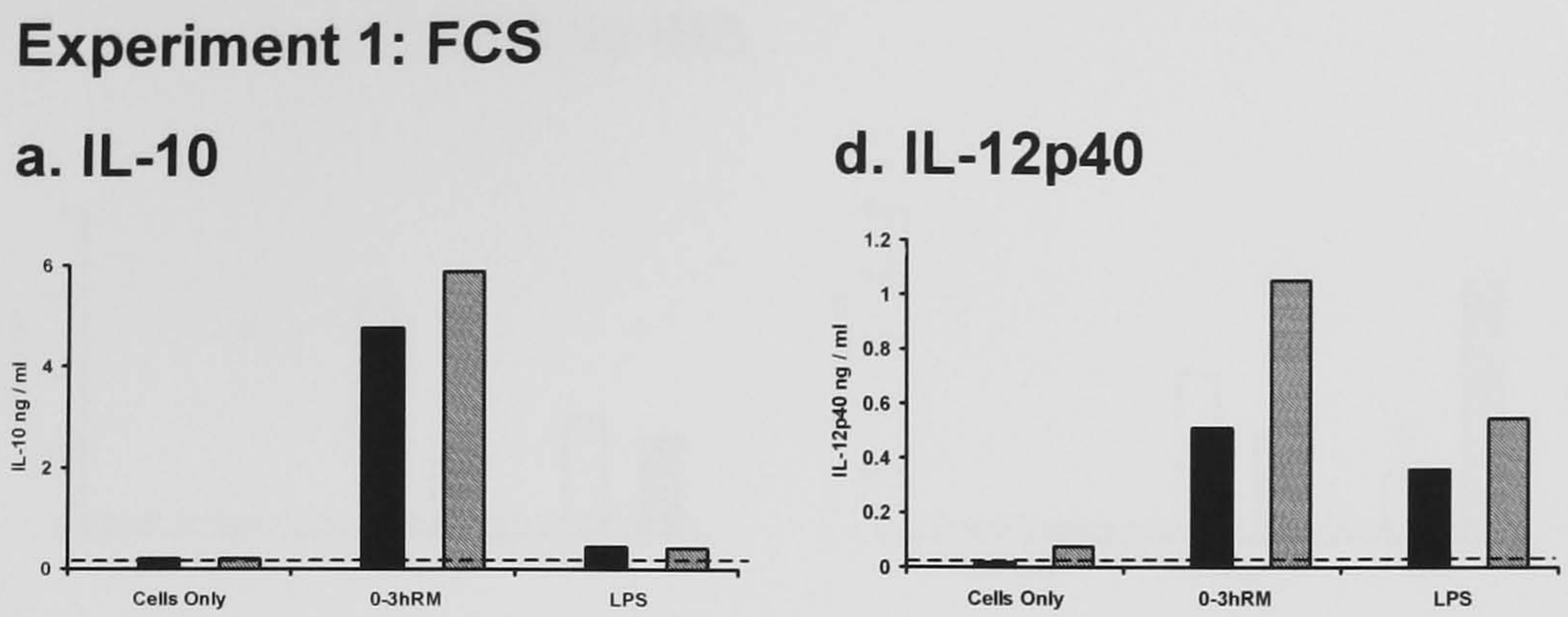


Figure 5.1 **Deficiency of FcR γ-chain-signalling has little effect on IL-10 production in the absence of specific antibody.** iMϕ from WT (solid bars) or FcR $\gamma^{-/-}$ mice (hatched bars) were cultured in medium containing FCS alone, with supernatant from *in vitro*-cultured transforming cercariae (0-3hRM), or with LPS, for 24 hr. Supernatants from duplicate, or triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-10 (a, b,c), or IL-12 (d, e). Dashed lines indicate the lower detection limit of the ELISA. Data from three experiments are presented (Experiment 1 - 3). ND = not done.

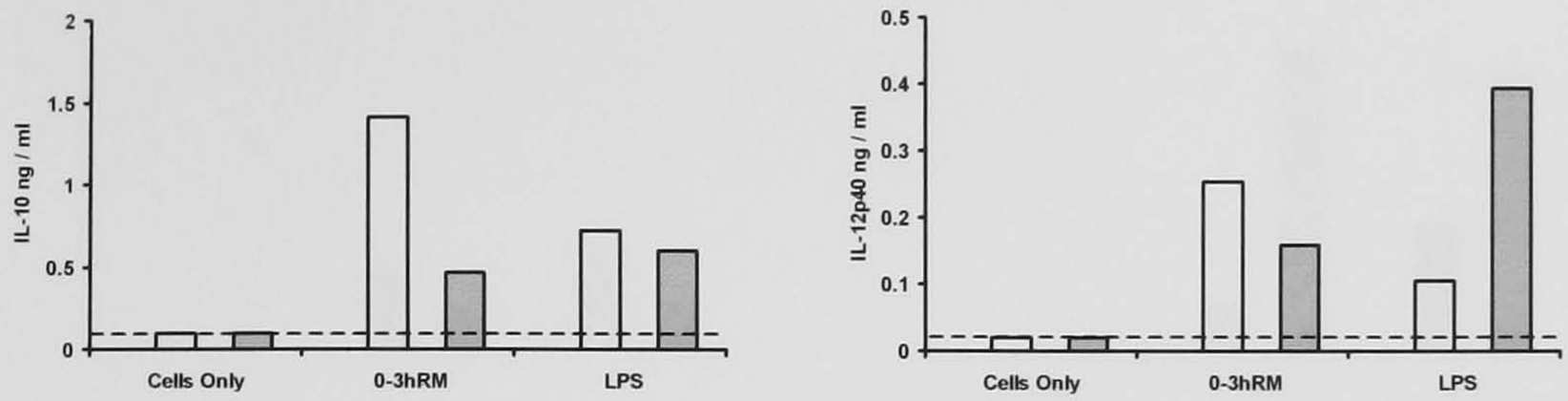
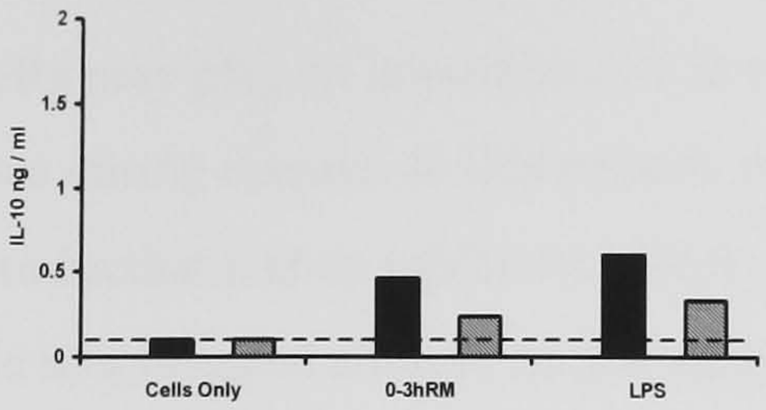


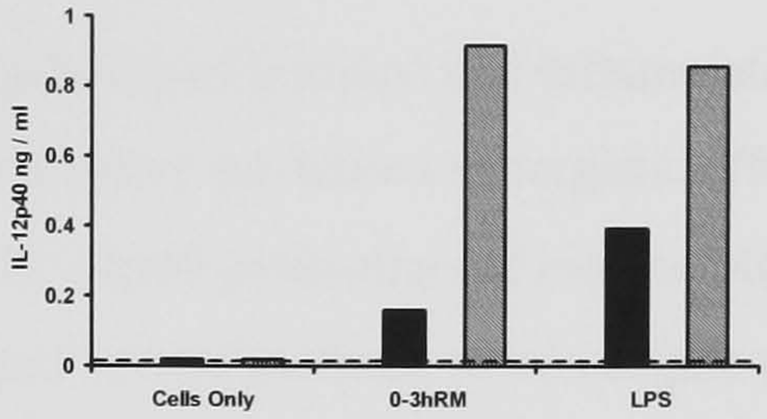
Figure 5.2 **Presence of 0-3hRM-specific antibody does not affect IL-10 production.** iMϕ from WT mice were cultured alone, with supernatant from *in vitro*-cultured transforming cercariae (0-3hRM), or with LPS, for 24 hr in medium containing either normal rabbit serum (open bars), or serum from a rabbit immunised with 0-3hRM (shaded bars). Supernatants from duplicate, or triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-10 (a, b,c), or IL-12 (d, e). Dashed lines indicate the lower detection limit of the ELISA. Data from three experiments are presented (Experiment 1, 2, & 4). Experiments 1 and 2 correspond to those presented in Figure 5.1.

Experiment 1: IRS

a. IL-10

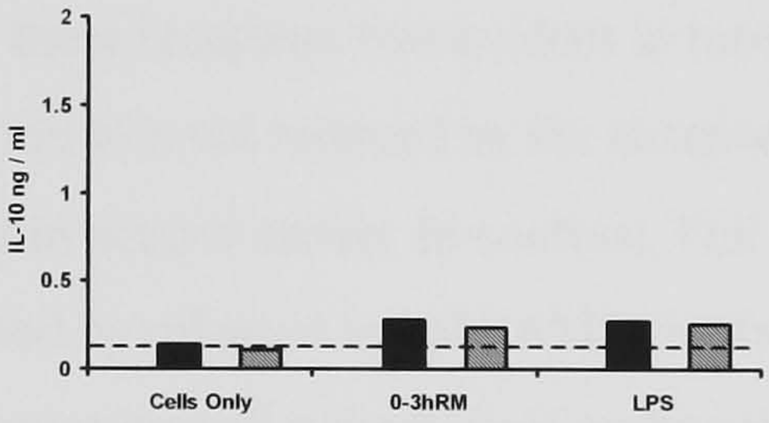


d. IL-12p40



Experiment 2: IRS

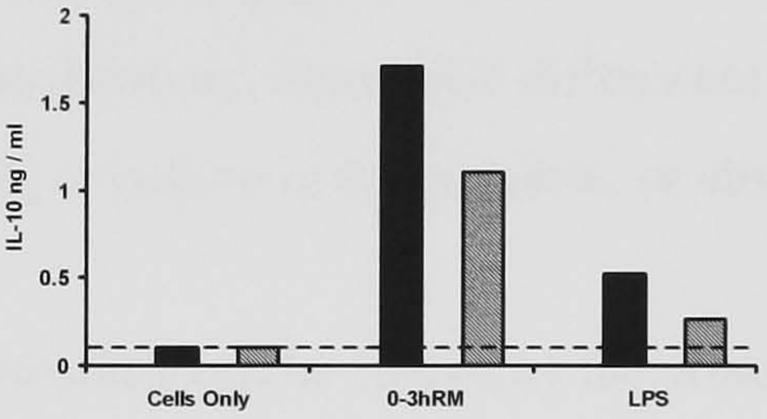
b. IL-10



ND

Experiment 3: IRS

c. IL-10



e. IL-12p40

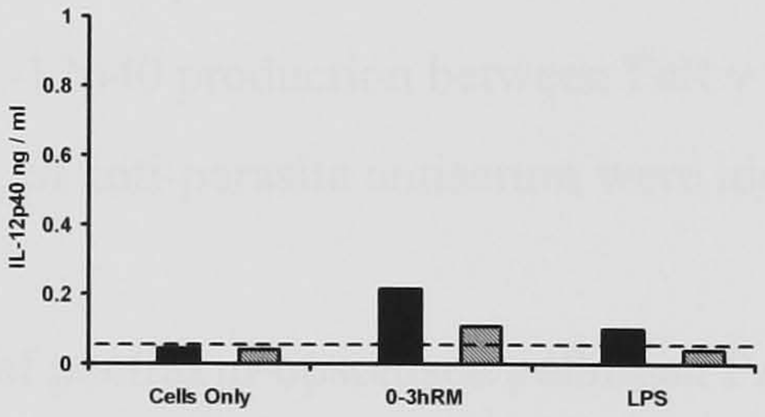


Figure 5.3 **Deficiency of FcR γ-chain-signalling causes reduction in IL-10 production in the presence of specific antibody.** iMϕ from WT (solid bars) or FcR $\gamma^{-/-}$ mice (hatched bars) were cultured alone, with supernatant from *in vitro*-cultured transforming cercariae (0-3hRM), or with LPS, for 24 hr in medium containing serum from a rabbit immunised with 0-3hRM (IRS). Supernatants from duplicate, or triplicate wells of a 96-well plate were pooled and analysed by ELISA for production of IL-10 (a, b,c), or IL-12 (d, e). Dashed lines indicate the lower detection limit of the ELISA. Data from three experiments are presented (Experiment 1 - 3), and correspond to those presented in Figure 5.1. ND = not done.

5.4 DISCUSSION

The FcγRs may play an important role in the regulation of immune and inflammatory responses during disease. In this respect, these receptors can have a synergistic effect upon IL-10 production and an inhibitory effect upon IL-12p40 production by murine Mϕ in response to a range of stimuli. In this study, I used several methods to investigate the effects of FcγR-signalling upon the profile of regulatory and pro-inflammatory cytokine production stimulated by larval PAMPs, to determine if these receptors may function in regulation of the inflammatory responses following exposure to schistosomes. No clear role for these receptors was evident in innate responses to parasite PAMPs, since IL-10 production was not reduced by the absence of signalling through the FcR γ-chain when cultured in neutral serum. In contrast, FcR $\gamma^{-/-}$ iMϕ produced reduced levels of IL-10 stimulated by released larval PAMPs in the presence of anti-parasite polyclonal serum (IRS), suggesting that FcγR-ligation by parasite antigen / antibody complexes could promote IL-10 production during the inflammatory responses initiated by schistosomes. However, no reliable difference was demonstrated between the levels of IL-10 production by WT iMϕ following stimulation with 0-3hRM in the presence of IRS, or a neutral serum, NRS. Furthermore, no reliable differences in IL-12p40 production between FcR $\gamma^{-/-}$ and WT iMϕ, or culture in the presence, or absence, of anti-parasite antiserum were identified.

FcγR signalling can be caused by the presence of pentraxin-opsonised pathogen PAMPs (Mold *et al.*, 2002). The common γ-chain of the FcRs is an essential component of signalling through FcγRI and FcγRIII (Takai *et al.*, 1994), and is required for the up-regulation of IL-10 and inhibition of IL-12p40 production seen in response to pentraxin-opsonised pathogen PAMPs (Mold *et al.*, 2002). In this respect, IL-10 production by whole organ cultures of split pinnae is reduced in FcR $\gamma^{-/-}$ mice compared to WT animals 4 days post-infection with schistosomes, which is prior to the onset of antibody production (pers. com. K. Hogg and A. Mountford).

Therefore, to determine if FcR γ-chain-signalling contributes to the response of iMϕ to released schistosome PAMPs, iMϕ from mice lacking the FcR γ-chain were analysed for their ability to produce IL-10 and IL-12p40 following stimulation with 0-3hRM in the

presence of a neutral serum that lacked parasite-specific antibody. Using this system, 0-3hRM stimulated slightly greater levels of IL-10 production by FcR $\gamma^{-/-}$ compared to WT iMϕ, suggesting that FcR γ-chain signalling does not contribute to IL-10 production stimulated by larval released PAMPs. It is possible that the minimal increase in IL-10 was due to a slightly higher activity of the FcR $\gamma^{-/-}$ iMϕ compared to the WT. Similarly, LPS stimulated slightly greater levels of IL-10 in the absence of FcR γ-chain signalling. Since pentraxin-opsonised LPS has been shown to augment IL-10 production by Mϕ (Mold *et al.*, 2002), it would be expected that LPS-stimulated IL-10 production by FcR $\gamma^{-/-}$ Mϕ would be reduced compared to WT cells. Since this was not the case suggests that the FCS used in these experiments did not contain these opsonins in sufficient quantities to effect LPS-stimulated IL-10 production, or that bovine pentraxins may not have a similar function to their murine counterparts. Therefore, it remains conceivable that schistosome PAMPs could be opsonised *in vivo* by murine pentraxins, and contribute to IL-10 production during the inflammatory responses in the skin via ligation of the FcγRs.

FcγR-signalling is also caused by the presence of IgG-opsonised antigen, and binding of pathogen material opsonised with IgG, or polyclonal antisera, can result in the dramatic up-regulation of IL-10 and inhibition of IL-12p40 produced in response to pathogen PAMPs (Sutterwala *et al.*, 1997; 1998; Gerber and Mosser, 2001). Therefore, to determine whether opsonisation of parasite material by specific polyclonal antibody affects the stimulatory properties of schistosome PAMPs, iMϕ from WT mice were analysed for their ability to produce IL-10 upon stimulation with 0-3hRM in the presence of anti-parasite polyclonal serum (IRS), or neutral serum (NRS).

There was considerable variation in the production of IL-10 between three experimental repeats, with stimulation by 0-3hRM in the presence of IRS resulting in increased, decreased, or the same levels compared to stimulation in the presence of NRS. However, the profile of IL-10 produced in response to LPS in the presence of NRS, or IRS, did not differ between two repeats, suggesting that the variation observed in response to 0-3hRM could reflect differences in the schistosome material (such as concentration) used within each study. Therefore, it is impossible to say what overall effect anti-parasite antibody had upon IL-10 production stimulated by 0-3hRM. However, it is clear that opsonisation by

anti-parasite antibody did not dramatically up-regulate the production of IL-10 stimulated by 0-3hRM.

This obviously contrasts the many recent studies that conclusively demonstrate a dramatic synergistic effect of IgG-, or polyclonal antibody-, opsonisation upon IL-10 production in response to a variety of different stimuli, including LPS, lipoteichoic acid, and hyaluronic acid (Sutterwala *et al.*, 1997; 1998; Kane and Mosser, 2001; Gerber and Mosser, 2001). It is possible that antibodies within the polyclonal rabbit antiserum used in my studies did not efficiently bind FcRs upon the murine Mϕ. However, this is doubtful since LPS opsonised by polyclonal anti-LPS rabbit serum exhibited IL-10 promoting activity (Gerber and Mosser, 2001). Moreover, the IRS used in this study conferred partial protection against schistosome infection to naïve mice, confirming a biological activity within this species (Harrop *et al.*, 2000).

Since the FcR γ-chain is also required for the synergistic effect of antibody-opsonised material upon PAMP-stimulated IL-10 production, iMϕ from FcR $\gamma^{-/-}$ mice were used to further investigate whether opsonised parasite material affects 0-3hRM-induced cytokine production. Using this system, 0-3hRM in the presence of IRS stimulated reduced levels of IL-10 (between 0.5 - 0.8-fold) by FcR $\gamma^{-/-}$ compared to WT iMϕ. This reduction was not due to a general lower activity of the FcR $\gamma^{-/-}$ iMϕ, since cytokine production in the presence of neutral serum (FCS) was greater by these cells (discussed above). Therefore, this data indicates that opsonised parasite material could signal through the FcR γ-chain to increase IL-10 production by Mϕ stimulated with released schistosome PAMPs. Although the common γ-chain is involved in signalling through a variety of other receptors, such as the high affinity IgE receptor, it seems likely the contribution to IL-10 production was made through the FcγRI, which has previously been shown to mediate such effects by IgG complexes (Sutterwala *et al.*, 1998).

It was expected that production of IL-10 stimulated by LPS in the presence of anti-schistosome polyclonal serum, would not be affected by the absence of the FcR γ-chain. Therefore, it is surprising that LPS also stimulated lower levels of IL-10 production by FcR $\gamma^{-/-}$ iMϕ than WT cells in the presence of IRS. The most feasible explanation for this observation is that IRS also contained antibodies against LPS. In hindsight this seems

logical, since the 0-3hRM used to generate this serum probably contained endotoxin to which the rabbit would have produced antibody.

The observation that FcR $\gamma^{-/-}$ iMϕ produce less IL-10 than WT cells in response to 0-3hRM in the presence of IRS, suggests that parasite antibody / antigen complexes contribute to IL-10 production. However, addition of IRS does not cause an increase in IL-10 production by WT iMϕ compared to culture in neutral NRS. It is possible that the IRS and NRS varied in content of a number of biological factors (in addition to antibody) that could have affected Mϕ cytokine production, such as regulatory cytokines (*e.g.* TGFβ). Subsequent titration of both NRS and IRS demonstrated both to have inhibitory effects upon IL-10 and IL-12 production by iMϕ when used at high concentrations, such as used in these studies, which could have masked the effect of antibody complexes (data not shown). Similarly, differences between IRS and FCS could account for the considerable variation in IL-10 production by iMϕ cultured in these serums.

Although this data appears to support a role for FcγR signalling in IL-10 production stimulated by opsonised 0-3hRM, the overall contribution of this to schistosome PAMP-stimulated cytokine production does not appear to be great. It is possible that the 'regulatory' profile of cytokine production by iMϕ (when compared to IFNγ-Mϕ; Section 3.4) is not greatly affected by FcγR signalling due to high levels of IL-10 and low levels of IL-12p40 produced by these cells. Previous studies have focused upon responses by bone marrow-derived Mϕ, which produce a more 'pro-inflammatory' cytokine profile with high IL-12 and low IL-10, and which may be more sensitive to the consequences of FcγR signals. However, it is possible that the effects of opsonised 0-3hRM could be minimal due to cleavage of specific antibodies, or FcRs, by proteases contained within the released larval material.

There was no reliable pattern in IL-12p40 production between two repeat experiments, when responses of WT and FcR $\gamma^{-/-}$ iMϕ to 0-3hRM, or LPS, in the presence of FCS or IRS were compared. Similarly, there was no reliable pattern in IL-12p40 production by WT cells when comparing the effects of culture in NRS to culture in IRS. This variability may reflect a lack of sensitivity in these assays due to the relatively low overall levels of IL-12 produced by iMϕ upon stimulation with 0-3hRM. Since FcR γ-chain signalling

appeared to effect IL-10 production in the presence of IRS, it is surprising that there was not a reciprocal effect upon IL-12p40 production. However, FcR γ-chain signalling could enhance IL-10 production without inhibiting IL-12 production. In this respect, the inhibitory effect of IgG-opsonised LPS upon Mϕ IL-12 production is independent of the IL-10 production by these cells (Gerber and Mosser, 2001).

It is noteworthy that in experiments where there was overall high levels of IL-10 there was little IL-12p40 production (Experiment 3; Figure 5.3c & e), whereas low levels of IL-10 corresponded with higher levels of IL-12p40 production (Experiment 1; Figure 5.3a & d), suggesting that regulation of these two cytokines was tightly balanced. However, the factor that was primarily responsible for the predomination of either IL-12, or IL-10, was independent of PAMP stimulus (*i.e.* a similar profile was observed in response to both LPS and 0-3hRM), and appeared to be independent of the individual cell populations (*i.e.* the profiles were the same for both WT and FcR $\gamma^{-/-}$ cells).

In conclusion, it is unclear whether FcγRs, or other receptors utilising the common γ-chain, contribute to the profile of iMϕ cytokine production stimulated by released schistosome PAMPs in the absence of schistosome-specific antibody. Furthermore, it is not clear from this study whether FcγR-ligation by antibody-opsonised PAMPs affect the iMϕ cytokine production stimulated by 0-3hRM, although this may potentially contribute to the production of IL-10.

CHAPTER 6

INDUCTION OF DC MATURATION BY RELEASED SCHISTOSOME PAMPS

6.1 INTRODUCTION

DC are involved in the initiation of both innate and acquired immune responses during infection with pathogens. Immature DC reside in peripheral sites, such as the dermis, or epidermis, where they are optimally located to act as sentinels against potential pathogens (Steinman, 1991).

Early studies of DC showed that they have high levels of pathogen specific endocytosis, macropinocytosis and phagocytosis (Reis e Sousa *et al.*, 1993; Sallusto *et al.*, 1995). Further characterisation demonstrated that upon exposure / contact with pathogen stimuli, *in vitro*-cultured DC switch from a state of highly active environment sampling and antigen processing to a state of low environmental sampling (Cella *et al.*, 1997). This change corresponded with dramatic up-regulation of MHC II expression and a switch from low to high T-cell stimulatory capacity, therefore allowing antigens captured at the site of infection / stimulation to be presented to T-cells (Cella *et al.*, 1997). These two cellular states have been classified as 'immature', characterised as expressing low levels of MHC II, and 'mature', characterised as expressing high levels of MHC II (Pierre *et al.*, 1997; Banchereau and Steinman, 1998; Sparwasser *et al.*, 1998).

DC have a unique ability to prime $CD4^+$ T-cells (Steinman, 1991). This ability is due partly to their efficient capture and presentation of antigens, and partly to their capacity to migrate to, or their location in, T-cell areas of the local lymphoid tissue (Jenkins *et al.*, 2001).

Studies of both human and murine DC demonstrate that they mature upon contact with a variety of different pathogens, or pathogen products, such as LPS (Cella *et al.*, 1997; Lutz *et al.*, 1999), CpG DNA (Sparwasser *et al.*, 1998), dsRNA (Cella *et al.*, 1999), Zymosan A (Reis e Sousa *et al.*, 1993), and bacterial lipopeptide (Horng *et al.*, 2002). However, it is becoming more fully understood that host molecules may play an important role in the maturation status of DC. Tissue factors, such as prostaglandins (PGE_2) can drive human DC maturation, as can inflammatory cytokines such as TNFα (Rieser *et al.*, 1997; Cella *et al.*, 1997). Indeed, inflammatory cytokines may be critical to the maturation of certain DC subsets (Cumberbatch and Kimber, 1995; Wang *et al.*, 1997; Cumberbatch *et al.*, 1997a).

Moreover, contact with necrotic cells, danger signals, or physical trauma, can result in DC maturation (Gallucci *et al.*, 1999; Johnson *et al.*, 2002, Termeer *et al.*, 2002).

In addition to pathogen and host tissue factors, signals from effector T-cells, such as CD40L, can directly stimulate human and murine DC *in vitro* (Cella *et al.*, 1996; Kelsall *et al.*, 1996) and enhance DC maturation *in vivo* (Schulz *et al.*, 2000). This provides evidence that feedback received by DC from T-cells during antigen presentation, could ultimately affect the outcome of priming. Indeed, it appears that ligation of CD40 can act as an amplification signal, enhancing the maturation phenotype of DC following stimulation with PAMPs and allowing the full display of maturation-associated factors (Schulz *et al.*, 2000; Edwards *et al.*, 2002).

Upon maturation, both human and murine DC up-regulate a variety of different cytokines, such as IL-12, IL-6, and IL-10, and membrane bound co-stimulatory factors, such as CD40, CD80, CD86, and OX40L. Further studies will undoubtedly continue to identify additional factors that can define the mature DC phenotype. However, it is only recently that the plasticity of the DC response to different stimuli has been fully appreciated. Several elegant studies have demonstrated that depending upon the source of stimuli, DC mature to different phenotypic states (d'Ostiani *et al.*, 2000; Whelan *et al.*, 2000; Huang *et al.*, 2001; MacDonald *et al.*, 2001; de Jong *et al.*, 2002). These host factor / pathogen programmed responses are so specific that the DC can discriminate between different cellular states of the same pathogen (d'Ostiani *et al.*, 2000). Moreover, the difference in maturation state leads directly to selective polarisation of the Th cell population during priming (d'Ostiani *et al.*, 2000; Whelan *et al.*, 2000; MacDonald *et al.*, 2001; de Jong *et al.*, 2002; McGuirk *et al.*, 2002). Thus, depending upon the DC maturation signal, DC are instructed to mature to different states capable of priming for Th1, Th2, or 'Treg' polarisation.

In the context of schistosome infection, there is evidence of epidermal APC maturation, corresponding with up-regulated MHC II and CD86 expression (Angeli *et al.*, 2001a). Moreover, MHC II^+ cells accumulate in the dermis directly below schistosome larvae following infection (Riengrojpitak *et al.*, 1998), and data emerging from our laboratory suggests that LC are stimulated to migrate from the epidermis, and IL-12-producing 'myeloid' DC spontaneously migrate from the skin following vaccination with irradiated

cercariae (Kumkate *et al.*, 2003; Hogg *et al.*, 2003a). However, it is still not known if schistosome PAMPs directly stimulate these cells, or if tissue-derived inflammatory / 'danger' signals produced during infection result in LC or DC maturation. Moreover, the maturation phenotype of DC exposed to schistosome PAMPs is not known. Since PAMPs released by transforming cercariae are potent stimulators of iMϕ cytokine production, it is a reasonable hypothesis that these PAMPs will also drive DC maturation. However, in order to test this hypothesis, and to assess the maturation state that is induced, an *in vitro* stimulation assay is required.

DC are rare within both lymphoid and non-lymphoid tissues, making it difficult to isolate sufficient numbers for the *in vitro* analysis of cell function. Several methods have been developed to increase the number of DC *in vivo*, enabling their subsequent recovery *ex vivo*. Injection of mice with Flt3 ligand increases the numbers of DC found within a variety of organs, including LN and bone marrow (Maraskovsky *et al.*, 1996). In addition, mice over-expressing GM-CSF had 3-fold more DC in their LN, than wild-types (Vremec *et al.*, 1997), although injection of GM-CSF did not have the same result (Maraskovosky *et al.*, 1996). However, these methods still do not result in sufficient cell numbers to study the effect of schistosome PAMPs on DC maturation and function.

As an alternative to studying *ex vivo* DC, a method was developed for the generation of DC from murine blood borne precursors cultured *in vitro* with GM-CSF (Inaba *et al.*, 1992a). However, the yield from this technique was low, and it was quickly superseded by one using murine bone-marrow (BM) precursor cells cultured *in vitro* with GM-CSF, which generated a much higher yield (Inaba *et al.*, 1992b). Since then, this technique has undergone numerous adaptations by different groups to produce DC of greater purity and higher yield (Lutz *et al.*, 1999; Son *et al.*, 2002). In turn, this has enabled the interaction between DC, pathogens, and host factors, to be studied in more detail. Therefore, this method was chosen to generate DC with which to study the effect of schistosome PAMPs.

The literature is inconclusive over a single preferred method for the *in vitro* culture of BM-derived DC, due to the many adaptations made by individual groups. Important culture factors frequently vary between different reports, including the type of culture vessel, length and regime of culture, deletion of erythrocytes before culture, deletion of non-

adherent cells during culture, and the cellular, or GM-CSF, concentration. Therefore, a number of factors relevant to the *in vitro* culture of BM-derived DC will be optimised at the start of this study. Four criteria were judged to be important in the optimisation process: 1) large scale generation of immature DC (iDC), 2) purity of iDC after six days of BM culture with GM-CSF, 3) sensitivity of iDC to stimulation with PAMPs, as judged by the fold increase in activation / maturation markers compared to non-stimulated cells, and 4) purity of mature DC (mDC) resulting from overnight stimulation of iDC with PAMPs.

The aim of this chapter is to determine whether schistosome PAMPs stimulate DC to mature. In this respect, the phenotype of the resulting mDC will be studied in detail. In order to gain perspective on the outcome of DC stimulation with schistosome PAMPs, the maturation phenotype of the resulting cells will be compared to that of DC matured using classical pathogen PAMPs (LPS & Zymosan A). These PAMPs will be chosen based upon their ability to 'instruct' DC to prime for Th1 or Th2 polarised responses. In addition, the outcome of CD40 ligation during PAMP-induced DC maturation will be studied.

6.2 MATERIALS AND METHODS

6.2.1 Animals

All mice were maintained in open housing at the University of York animal unit. C57Bl/6 mice were bred in house, while BALB/c mice were obtained from Harlan UK. C57Bl/6 mice were used in all experiments unless specified.

6.2.2 Generation of DC from bone marrow

Two methods for the generation of iDC from bone marrow precursors were compared to determine which produced the most suitable cells for screening stimulatory properties of PAMPs. For these experiments, the purity of iDC and mDC, and the analysis of mDC maturation states, was determined by analysis of myeloid differentiation marker expression using flow cytometry.

Method 1: Culture 'Flask' generation. This method, previously described by Whelan *et al.* (2000) and adapted from Inaba *et al.* (1992b), was learnt whilst visiting Kevin Rigley's laboratory at the Edward Jenner Institute for Vaccine Research. The culture medium (DCF) consisted of Iscoves MEM Glutamax I, containing 200 U / ml penicillin, 100 μg / ml streptomycin, 50 μM β-mercaptoethanol (Invitrogen), and 10% heat-inactivated low-endotoxin FCS (Harlan Seralab). Bone marrow cells were removed from the femurs and tibias of sacrificed C57Bl/6 mice as previously described (Lutz *et al.*, 1999). Briefly, bones were cleaned of muscle and sterilised in 70% alcohol for 1 min. Bone marrow was then flushed-out with DCF using a 23-gauge needle, and cell clusters dissociated by gentle pipetting. Cells were cultured in 75 cm^2 tissue-culture flasks (1 x 10^6 cells / ml) in 25 ml DCF supplemented with 20 ng / ml GM-CSF (Peprotech, London, UK), at 37 ^oC, in a humidified incubator. On day 4, the spent media (containing the non-adherent cell fraction) was carefully removed and discarded, and the flask containing the adherent and semi-adherent cells replenished with 25 ml fresh DCF, containing 20 ng / ml GM-CSF. Semi-adherent cells were defined as those that would detach from the adherent cell monolayer by gentle agitation of the culture medium. Two days later, the non-adherent and semi-adherent cell populations were recovered by gentle agitation and used as iDC.

Method 2: Culture 'plate' generation. This method, previously described by Son *et al.* (2002) and adapted from Inaba *et al.* (1992b), was provided by Melanie Leech (University of Manchester). The culture medium (DCP) consisted of RPMI 1640 containing 2mM L-glutamine (Invitrogen) and all supplements as added to DCF media. Bone marrow was removed as described above. Prior to culture, red blood cells were lysed with ACK buffer (Section 4.2.2) and the remaining cells washed three times with DCP. The cells were then cultured in 6-well tissue-culture plates (Nalge Nunc) at a concentration of 1.8 x 10^6 cells / well in 3 ml of DCP supplemented with 20 ng / ml GM-CSF. On day 4, a further 3 ml of DCP, containing 40 ng / ml GM-CSF, was added. Two days later, non-adherent and semi-adherent cells were recovered by gentle agitation and used as iDC.

6.2.3 DC stimulation and maturation

For the comparison of DC generation techniques, iDC were seeded into 24-well, or 96-well tissue-culture plates (Nalge Nunc), at 1 x 10^6 cell / ml, in DCF or DCP containing 20 ng /

ml GM-CSF. The cells were then cultured for 24 hr alone, or in the presence of LPS (10 ng / ml; see below).

Subsequently, iDC generated using the selected method were used to determine the outcome of activation with different pathogen, or parasite, PAMPs. Specifically, iDC derived from C57Bl/6, or BALB/c, bone marrow were cultured for 24 hr in DCP containing 20 ng / ml GM-CSF as follows:

- In media alone.

Or with:

- 0-3hRP, SCP, 3hSSP, 18hSSP (all 1.1 - 40 μg / ml; prepared as described in Section 2.2.1).
- Concentrated medium control RPMIc (for 0-3hRP).
- Zymosan A (0.2 - 5 μg / ml; Sigma-Aldrich).
- LPS (1 - 100 ng / ml; *E. coli* serotype 0111:B4; Sigma-Aldrich).

Assays were carried out in the presence, or absence, of PMB (0.1 - 27 μg / ml; Sigma-Aldrich) where indicated. Additionally, in some experiments low-endotoxin anti-CD40 antibody (5 μg / ml; clone HM40-3; BD PharMingen) was added to the culture at the time of exposure to PAMPs.

After culture overnight with the different PAMPs, the semi-adherent mDC were harvested and analysed for expression of surface markers. Culture supernatants were frozen and stored at -20 °C to await detection of cytokines by ELISA.

6.2.4 Phenotypic characterisation of DC populations

To determine the purity of DC cultures, iDC were analysed for the surface expression of myeloid (CD11c, F4/80, Gr-1) and lymphoid (B220, CD4, CD8α) differentiation markers. In addition, mDC resulting from culture with different PAMPs were analysed for surface expression of CD11c and maturation / activation markers (MHC II, CD40, CD80, CD86 and OX40L). Cells were stained with specific antibodies, or irrelevant isotype matched antibodies using the method previously described (Section 3.2.3.2). Antibodies against

various surface markers were as follows (clone; supplier): FITC- or PE-conjugated CD11c (HL3; BD PharMingen), CD40 (3/23; BD PharMingen), CD80 (RMMP-1; Caltag), CD86 (RMMP-2; Caltag), Gr-1 (RB6-8C5; Caltag), F4/80 (CI:A3-1; Caltag), B220 (RA3-6B2; BD Pharmingen), CD4 (CT-CD4; Caltag), CD8α (53-6.7; BD PharMingen), or biotin-conjugated I-$A^{b,d}$ (28-16-8S; Caltag) and biotin-conjugated OX40L (RM134L; PharMingen), followed by streptavidin-conjugated Quantum Red™ (QR; Sigma). Where specified, two-colour staining was used to analyse co-expression of activation markers and CD11c on mDC. In this context, the respective antibodies were conjugated to different fluorochromes.

To determine the number of dead cells, 10 µl of PI (200 mg / ml PI in PBS) was added to relevant cell aliquots and incubated for 20 sec before flow-cytometric analysis. Those staining positive for PI were considered dead.

In some experiments, DC were also analysed for expression of intracellular IL-12p40, or IL-4, as previously described (Hogg *et al.*, 2003a). Briefly, Golgi Plug™ (1µg / ml; BD PharMingen) was added to the mDC culture 6 hr before harvesting. DC were first labelled with CD11c-FITC as described above. Cells were then fixed and permeabilized with Cytofix / Cytoperm™ (BD PharMingen) according to manufacturer's instructions, and stained with PE-labelled IL-12p40 mAb (clone #C15.6; BD PharMingen), or IL-4 mAb (clone BVD6-24G2; Caltag).

Cells were analysed by one-colour, or two-colour, flow-cytometry as previously described (Section 3.2.3.4 - 3.2.3.5, or Section 4.2.2, respectively). Low expression of I-A and CD86 is constitutive on some DC but levels of these markers can be up-regulated following stimulation. In order to determine the percentage of cells expressing high levels of I-A or CD86, the cursor was set to exclude all negative and low-level expressing events.

6.2.5 Cytokine ELISAs

In addition to the cytokines studied in Chapter 3, DC culture supernatants were tested for production of IL-12p70, IL-4, and IFNγ, using ELISA (Section 3.2.6). Supernatants were tested neat, or at 1:2, 1:5, 1:10, or 1:20 dilutions. Lower limits of detection are shown on

figures as dashed lines, or arrows. The IL-12p70 ELISA incorporated the same recombinant standard and detecting antibody as the IL-12p40 ELISA (Section 3.2.6), but uses an IL-12p70-specific capture antibody (Clone 9A5; BD PharMingen). The antibodies for the other additional cytokines were also obtained from BD PharMingen as follows: (coating clone; detecting clone): IL-4 (BVD1-1D11; BVD6-24G2) and IFNγ (R4-6A2; XMG1.2). Recombinant standards were as follows: IL-4 (BD PharMingen) and IFNγ (2A11 CHO cell supernatant).

6.2.6 Statistics

Data comparisons were tested for significance using the one-tailed Students *t*-test, assuming equal variance if $p > 0.05$ when tested with the Levene's test. Values where $p \leq 0.05$ were considered to be significant, whereas values where $p > 0.05$ were considered not significant. The following nomenclature was used to denote the value of the significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

6.3 RESULTS

6.3.1 Generation of maturation-inducible iDC from BM precursor cells.

'Plate' and 'Flask' methods of generating iDC from BM precursors were compared. The major differences in these techniques are summarised in Table 6.1. Between the two techniques, no difference was observed in the purity of iDC generated from BM after six days of culture with GM-CSF, as determined by expression of the DC marker CD11c (40% *versus* 39% for 'Plate' and 'Flask' methods respectively; Table 6.1). In contrast, the yield of putative iDC compared to the starting cell number was twice as high using the 'Plate' technique compared to the 'Flask' technique (2.2-fold *versus* no increase; Table 6.1).

After transfer to 24-well plates and further overnight culture in the presence of GM-CSF, the purity of $CD11c^{+}$ cells increased in both 'Plate' and 'Flask' generated populations, although the former were considerably purer (53% *versus* 44%, respectively; Table 6.2a). Matured DC (mDC) generated using both techniques showed greatly up-regulated expression of high levels of MHC II ($MHC\ II^{high}$) after culture with LPS (48% 'Plate'; 32%

'Flask') compared to DC cultured alone (27% 'Plate'; 20% 'Flask'; Table 6.2a). Moreover, the percentage of mDC expressing CD40, also increased upon stimulation with LPS (58% 'Plate'; 45% 'Flask') compared to DC cultured alone (34% 'Plate'; 31% 'Flask'; Table 6.2a). However, the increase in levels of expression of MHC II^{high} and CD40 on LPS-stimulated DC compared to DC cultured alone, was slightly greater in the 'Plate'-generated population (1.8-fold and 1.7-fold respectively) compared to the 'Flask'- generated population (1.6-fold and 1.5-fold respectively; Table 6.2b). Thus, the 'Plate' generation technique was used for all subsequent experiments, due to the higher yield of iDC, the higher purity of $CD11c^+$ cells after further overnight culture of iDC, and the greater sensitivity of iDC to stimulation with LPS.

Further adaptations to the 'Plate' generation method (*e.g.* using only BM from femurs) increased the purity of $CD11c^+$ cells in the 6 day old iDC population to 59% (Figure 6.1a), although in many experiments the purity of $CD11c^+$ cells reached 70% (data not shown). To ensure reasonable consistency between experiments, iDC were routinely tested for CD11c expression. Analysis of size and granularity of the cells shows that granularity increases with size in a continuum, such that there are not any distinct individual cell populations (Figure 6.1b). However, further analysis shows that the $CD11c^+$ population comprises the largest and most granular cells, whereas the $CD11c^-$ population comprises of the smallest and least granular cells (Figure 6.1c & d). Moreover, the vast majority of the large granular population are $CD11c^+$. Indeed, a quadrate placed on the size *versus* granularity scattergram effectively distinguishes the $CD11c^+$ and $CD11c^-$ populations, with 85% of $CD11c^+$ cells and only 11% of $CD11c^-$ cells in box (ii).

More in-depth phenotypic characterisation of the iDC population demonstrated that nearly half (48%) of the cells were MHC II^+ (Figure 6.2i.a). Moreover, two distinct groups existed within this population, one expressing high levels, and one expressing low levels of MHC II (Figure 6.2ia). Importantly, the MHC II low expressing cells were greater in number (27% compared to 21%), suggesting that the majority were of an immature state, and justifying their description as iDC. Most of the MHC II^+ cells were located in the large granular population (Figure 6.2ib). As most of the large granular cells were $CD11c^+$ (Figure 6.1), this suggests that the MHC II^+ cells are also $CD11c^+$. Indeed, further characterisation using two-colour flow-cytometry, showed that 95% of the MHC II^+ cells

were $CD11c^+$ (Figure 6.2iia & b). Moreover, 95% of the $CD11c^-$ cells were MHC II^- (data derived from Figure 6.2ii).

Analysis of other myeloid differentiation markers, demonstrated that 46% of iDC were $F4/80^+$ and 24% were $Gr\text{-}1^+$ (Figure 6.2ia). The $F4/80^+$ cells were of the large granular population, suggesting that they were also $CD11c^+$ (Figure 6.2ib). In contrast, the majority of the $Gr\text{-}1^+$ cells were of the small / low granular population, and so were distinct from the $CD11c^+$ cells (Figure 6.2ib). Analysis with other leukocyte markers showed that there were no $CD4^+$, or $CD8^+$, cells (data not shown) and only 2% of cells were $B220^+$ (Figure 6.2i.a).

6.3.2 Optimisation of PMB concentration for use in iDC stimulation assays.

In order to determine the effects of schistosome PAMPs upon iDC, PMB was used to inhibit the potential stimulatory effects of low levels of contaminating endotoxin. PMB concentration had previously been optimised to inhibit endotoxin stimulation of iMϕ following culture with parasite PAMPs (Section 3.3.3). However, due to potential differences between these accessory cells, the concentration of PMB was optimised for culture with iDC using *E. coli* LPS as a model endotoxin. Both concentrations of LPS (10 and 100 ng / ml) stimulated high levels of IL-12p40 and IL-6 production compared to iDC cultured alone (Figure 6.3). However, these stimulatory properties were blocked by PMB in a dose-dependent manner, with 1 μg / ml PMB completely blocking 10 ng / ml LPS, and 3 μg / ml PMB completely blocking 100 ng / ml LPS. Therefore, 3 μg / ml PMB was used in further assays to ensure sufficient blocking of endotoxin. In addition, this concentration of PMB had little effect on the IL-12p40 and IL-6 production produced in response to Zymosan A (Figure 6.4), suggesting it would not interfere with the stimulatory properties of schistosome PAMPs. Furthermore, PMB (0.1 - 9 μg / ml; tripling dilution) had no effect on the viability of iDC cultured alone, as determined by PI staining (data not shown).

6.3.3 0-3hRP but not other schistosome preparations, stimulates cytokine production by iDC.

The stimulatory properties of the different schistosome PAMP preparations were screened using iDC cultured in the presence of PMB. 0-3hRP stimulated a dose-dependent increase

in both IL-12p40 and IL-6 production by iDC (Figure 6.5a & b). In contrast, the soluble whole larval preparations, SCP, 3hSSP and 18hSSP, and the RPMIc control, did not stimulate increased production of either cytokine (Figure 6.5a & b). Similarly, in separate experiments the soluble whole larval preparations 3dSSP, 5dSSP, and 8dSSP, did not stimulate increased cytokine production (data not shown). No IL-10 was detected in any of the supernatants. It is noteworthy that in the absence of PMB, 0-3hRP stimulated production of 3- to 7-fold more IL-12p40, and 9- to 12-fold more IL-6, than in the presence of PMB. This demonstrates the sensitivity of iDC to the low levels of endotoxin within 0-3hRP and supports the continued use of PMB to analyse the effect of schistosome PAMPs on iDC in the absence of endotoxin signalling.

Since 0-3hRP exhibited greater stimulatory properties compared to other schistosome preparations, the study focused upon the response of iDC to these released molecules. Since 40 μg / ml of 0-3hRP stimulated the greatest levels of IL-6 and IL-12p40 production, this concentration was used in all further studies, unless indicated.

6.3.4 Cytokine response of DC to 0-3hRP compared to other PAMPs.

There were no significant differences in IL-12p40 or IL-6 production by DC cultured with PMB, RPMIc + PMB, or LPS + PMB, compared to DC cultured alone (Figure 6.6a & b). Conversely, in the presence of PMB, 0-3hRP stimulated a significant increase in the levels of IL-12p40 (2-fold) and IL-6 (10-fold) produced compared to all negative controls (Figure 6.6a & b). However, the increase in IL-12p40 was small compared to that stimulated by LPS (198-fold) and Zymosan A (308-fold) (Figure 6.7a). Similarly, 0-3hRP + PMB stimulated only a small increase in IL-6 production (10-fold) when compared to LPS (282-fold) and Zymosan A (165-fold) (Figure 6.7b). Interestingly, LPS and Zymosan A stimulated different profiles of cytokine production, the former stimulating the highest level of IL-6, and the latter stimulating the highest levels of IL-12p40.

6.3.5 $CD11c^{+}$ cells are the source of IL-12p40 stimulated by PAMPs.

Two-colour flow-cytometry was used to identify the cellular source of the IL-12p40 detected in the culture supernatants of the enriched DC population. It is clear that after

culture with RPMIc, 0-3hRP, LPS, or Zymosan A, the vast majority of the IL-12p40$^+$ cells were CD11c$^+$ (94%, 96%, 87%, and 84%, respectively; Figure 6.8). Moreover, the percentage of CD11c$^+$ IL-12p40$^+$ cells increased after culture with 0-3hRP (13%), LPS (58%) and Zymosan (56%), compared to the RPMIc control (6.6%). In addition, the CD11c$^+$ IL-12p40$^+$ cells had higher intensity of cytokine staining (as judged by the median fluorescence value) after culture with 0-3hRP (1.5), LPS (6.8) and Zymosan A (5.2) compared to the RPMIc control (1.1). This control was chosen as it had the highest background percentage of IL-12p40$^+$ cells relative to DC cultured alone, DC cultured with PMB, or DC cultured with LPS + PMB (data not shown). However, stimulation with 0-3hRP resulted in a lower percentage of CD11c$^+$ IL-12p40$^+$ cells and a lower intensity of IL-12p40 staining than LPS and Zymosan A, as would be expected from the profiles of cytokine released into the culture supernatant (Figure 6.7a).

6.3.6 Phenotype of DC activated with 0-3hRP compared to other PAMPs.

The up-regulation of MHC II complexes and co-stimulatory molecules during maturation greatly contributes to the APC function of DC. Therefore, expression of MHC II, and the co-stimulatory molecules CD40, CD80, CD86, and OX40L, on DC stimulated with 0-3hRP was compared to DC stimulated with LPS, or Zymosan A. Two-colour flow-cytometry was used to compare the expression of CD11c *versus* MHC II by the DC. However, due to limitations on availability of cells for flow-cytometric analysis, it was not possible to perform similar two-colour staining for co-stimulatory molecules. Therefore, analysis of CD40, CD80, CD86, and OX40L expression, was performed on the whole population of enriched DC. In the following experiments, expression of MHC II and co-stimulatory molecules was also determined on DC cultured alone, or with PMB, LPS + PMB, or RPMIc + PMB. However, there was no significant difference between these controls. Therefore, only the data for DC cultured alone, or with RPMIc + PMB, which were the controls with the greatest background staining for MHC II and all the co-stimulatory molecules, are shown in the following figures.

The CD11c expression on DC changed after stimulation with different PAMPs (Figure 6.9a & b). Indeed, LPS and Zymosan A led to an increase in the percentage of CD11c$^+$ cells within box A. Most importantly, 0-3hRP (and RPMIc) had little effect on CD11c expression compared to DC cultured alone. However, any variation in the proportion of

$CD11c^+$ cells between stimulation regimes would lead to difficulty in analysing the relative expression of co-stimulatory molecules upon the iDC using only one-colour staining. Therefore, only the larger most granular cells, previously shown to contain the vast majority of $CD11c^+$ cells (Figure 6.1), were analysed by gating on box H (Figure 6.9c). Consequently, the difference in CD11c expression between DC cultured alone and DC stimulated with Zymosan A, or LPS, was dramatically reduced (Figure 6.9d). However, even gated on box H, there was still a slight increase in CD11c expression on cells cultured with LPS, or Zymosan A, but because the overall levels of $CD11c^+$ expression were very high (88% - 92%), this difference is much less relevant. Thus, for the surface expression of CD40, CD80, CD86, and OX40L, only the large granular cells within H were analysed. This also increased the sensitivity of the system, as any change in the surface expression of co-stimulatory molecules on the $CD11c^+$ cells would become more apparent.

0-3hRP appeared to stimulate an increase in the percentage of $CD11c^+$ cells expressing MHC II (Figure 6.10a). Moreover, the proportion of $CD11c^+$ cells expressing MHC II^{high} appeared to be greater for 0-3hRP-matured DC than for DC cultured alone, or with RPMIc (Figure 6.10b). However, in both cases this was not significant and the levels of expression were lower than that stimulated by LPS and Zymosan A (Figure 6.10a & b).

0-3hRP stimulated slight up-regulation of both CD40 and high levels of CD86 ($CD86^{high}$) expression compared to DC cultured alone or with RPMIc, but these levels were not significantly different (Figure 6.11a & b). In contrast, DC stimulated with LPS or Zymosan A dramatically up-regulated CD40 ($p < 0.05$, and $p < 0.01$, respectively) and $CD86^{high}$ ($p < 0.05$, and $p < 0.05$, respectively) expression. 0-3hRP did not stimulate an increase in the proportion of cells expressing CD80 or OX40L, compared to DC cultured alone or with RPMIc (Figure 6.11c & d). LPS and Zymosan A had differential effects on the expression of these co-stimulatory molecules. LPS appeared to up-regulate expression of CD80, but did not up-regulate expression of OX40L, whereas Zymosan A up-regulated expression of OX40L ($p < 0.01$) but not CD80.

6.3.7 The response of BALB/c iDC to 0-3hRP compared to other PAMPs.

In order to determine if maturation with 0-3hRP effected the capacity of DC to prime and polarise T-cells, it was necessary to use DC on a BALB/c background (Chapter 7).

Therefore, the maturation phenotypes of BALB/c DC were characterised after stimulation with 0-3hRP, or the control pathogen PAMPs.

6.3.7.1 Cytokine production.

0-3hRP stimulated a highly significant increase in both IL-12p40 ($p < 0.001$) and IL-6 ($p < 0.001$) production by BALB/c DC compared to controls (Figure 6.12a & b). This was dose-dependent, with a 7-fold and 18-fold increase in IL-6, and a 4-fold and 9-fold increase in IL-12p40, after stimulation with 20 μg / ml and 40 μg / ml of 0-3hRP respectively. However, 0-3hRP stimulated dramatically lower levels of IL-6 and IL-12p40 than LPS, or Zymosan A (Figure 6.13a & b). Notably, this was the same when a lower dose of LPS (1 ng / ml), or a higher dose of Zymosan A (5 μg / ml) was used (data not shown). 0-3hRP did not stimulate detectable production of IL-12p70, IL-10, or TNFα, in contrast to LPS and Zymosan A (Figure 6.13c, d, e). Moreover, 0-3hRP did not stimulate production of IL-1β, in contrast to LPS (Figure 6.13f). It is worth noting that LPS and Zymosan A stimulated different profiles of cytokine production compared to each other. LPS stimulated higher levels of IL-6, IL-1β, and IL-10, whereas Zymosan A stimulated higher levels of IL-12p70. Notably, IL-4 and IFNγ were not detected in any of the supernatants.

Similar to C57Bl/6 DC, the percentage of IL-12p40$^+$ CD11c$^+$ cells increased after culture with 0-3hRP (11%), LPS (72%) and Zymosan (30%), compared to the negative control (6%) (Table 6.3). Again, similar to C57Bl/6 DC, the vast majority of the IL-12p40$^+$ cells were CD11c$^+$ (up to 94%) (Table 6.3).

In contrast to the absence of detectable levels of IL-4 in DC culture supernatants, an increase in the percentage of IL-4$^+$ cells was observed after stimulation with 0-3hRP (14%), or LPS (30%), compared to control (10%) (Table 6.3). In contrast, stimulation with Zymosan A appeared to decrease the number of IL-4$^+$ cells (7%). Similar to IL-12p40, the vast majority of the IL-4$^+$ cells were CD11c$^+$ (up to 94%).

6.3.7.2 MHC II and co-stimulatory molecule expression.

As in Section 6.3.6, MHC II and co-stimulatory molecule expression upon BALB/c DC cultured alone, or with PMB, LPS + PMB, or RPMIc + PMB, was determined. However, since there was no significant difference between these controls, only the data for DC cultured alone and RPMIc + PMB are shown.

All PAMPs, including 0-3hRP, stimulated an increase in the percentage of $CD11c^+$ cells expressing MHC II, although this was only significant ($p < 0.05$) for LPS and Zymosan A treatments (Figure 6.14a). However, 0-3hRP stimulated a significant ($p < 0.05$) increase in the percentage of $CD11c^+$ cells expressing MHC II^{high} (70%) compared to negative controls (50%), but this was not as high as stimulated by LPS (76%) and Zymosan A (82%) (Figure 6.14b). A lower dose of 0-3hRP (20 μg / ml) also up-regulated expression of MHC II and MHC II^{high}, although to a lesser extent than 40 μg / ml (data not shown). It is noteworthy that similar to iDC, analysis of all MHC II^+ cells within the mature DC populations revealed that the vast majority (approximately 95%) were $CD11c^+$ (data not shown).

Similar to the study of C57Bl/6 DC, analysis of co-stimulatory factor expression upon BALB/c DC was focused on the large granular cells (within box H) of the mDC population. Again, the vast majority of these cells were $CD11c^+$ (Figure 6.15), with very few $CD11c^+$ excluded by this method (< 10%; data not shown). Moreover, the proportion of $CD11c^+$ cells within this population did not significantly vary between the differently stimulated DC groups (Figure 6.15).

0-3hRP stimulated significant ($p < 0.05$) up-regulation of CD40 and $CD86^{high}$ expression but not to the same extent as LPS or Zymosan A. (Figure 6.16a, & b). Again, a lower dose of 0-3hRP (20 μg / ml) also up-regulated expression of CD40 and $CD86^{high}$, although to a lesser extent than 40 μg / ml (data not shown). As observed using C57Bl/6 DC, 0-3hRP did not stimulate up-regulation of CD80 and OX40L, whereas the differential effect of LPS and Zymosan A on these co-stimulatory molecules was clear. In this respect, LPS up-regulated CD80 ($p < 0.05$) but not OX40L expression, whereas Zymosan A up-regulated OX40L ($p < 0.05$) but not CD80 expression (Figure 6.16c & d). In addition, the phenotype

of DC stimulated with a lower dose of LPS (1 ng / ml) was similar to those stimulated with 100 ng / ml (data not shown).

6.3.8 Ligation of CD40 effects the profile cytokines produced in response to 0-3hRP.

Ligation of CD40 can amplify the maturation phenotype of differentially stimulated DC (Edwards *et al.*, 2002). In order to determine if the cytokine profile produced by stimulation with 0-3hRP is modified by CD40-signalling, DC were co-cultured with anti-CD40 antibody. The presence of the antibody substantially increased IL-12p40 production by both non-stimulated DC (10-fold) and by 0-3hRP-stimulated DC (4-fold) (Figure 6.17a). In contrast, ligation of CD40 had much less of an effect on IL-6 production with 0-3hRP-stimulated DC producing just 1.6-fold more cytokine in the presence of the antibody (Figure 6.17b). Moreover, anti-CD40 stimulated only a marginal increase (1.3-fold) in spontaneous IL-6 production by DC cultured alone. In some experimental repeats IL-6 production was up to 3-fold greater in the presence of anti-CD40, however, the effect on IL-12p40 production was always greater (data not shown). No IL-10 (Figure 6.17c), IL-12p70, IFNγ, or IL-4 (data not shown), was detected in any of the supernatants.

In addition, culture with the anti-CD40 antibody greatly increased the proportion of $CD11c^+$ cells staining positive for IL-12p40 after culture alone (3.5-fold), or with 0-3hRP (2-fold), or Zymosan A (2-fold) (Table 6.4). In contrast, anti-CD40 treatment did not increase the percentage of $IL\text{-}4^+$ cells after stimulation with 0-3hRP, or Zymosan A (Table 6.4). Indeed, fewer DC cultured in the absence of PAMPs were $IL\text{-}4^+$ in the presence of anti-CD40 antibody. Only the DC population stimulated with LPS appeared to contain more $IL\text{-}4^+$ cells in the presence of the antibody, although this increase was slight (1.2-fold).

	6-well 'Plate' Generation	*75 cm^2 'Flask' Generation*
$CD11c^+$ Purity of day 6 iDC [a]	**40%**	**39%**
Day 0 Cell Number [b]	1.8 x 10^6 / well	25 x 10^6 / Flask
Day 6 Cell Number [c]	4 x 10^6 / well	26 x 10^6 / Flask
Cell Yield **Day 0:Day 6 Cell Number**	**2.2**	**1**
Depletion of erythrocytes within fresh BM	Yes	No
Depletion of non-adherent cell population during culture	No	Yes
GM-CSF	20 ng / ml	20 ng / ml
Medium	RPMI 1640 + 10% FCS	Iscoves MEM + 10% FCS
Culture Regime	Day 3: Add 100% more media, and then GM-CSF to 20 ng / ml concentration throughout.	Day 4: Discard 100% of spent culture medium, replace with new, and add 20 ng / ml GM-CSF.

Table 6.1 **Comparison of BM-derived iDC culture techniques.**

[a]Values represent the percentage of cells staining positive with specific Ab compared to matched isotype controls, as determined by flow-cytometry.

[b]Values represent the number of BM cells.

[c]Values represent the number of non- and semi-adherent cells.

a.

	'Plate' Generation		*'Flask' Generation*	
LPS Treatment ng / ml	*0*	*10*	*0*	*10*
CD11c	53%	56%	44%	46%
MHC II $^{high\ a}$	27%	48%	20%	32%
CD40	34%	58%	31%	45%

b.

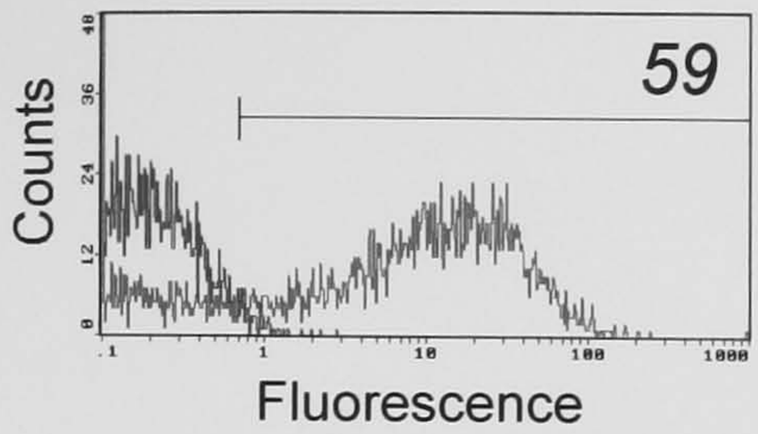
	'Plate' Generation	*'Flask' Generation*
MHC II $^{high\ a}$	**1.8**	1.6
CD40	**1.7**	1.5

Table 6.2 **Effect of LPS stimulation on the expression of surface markers on iDC generated by different methods.** BM precursor cells were grown in the presence of GM-CSF for 6 days in culture flasks, or 6-well plates. Non- and semi-adherent cells were then recovered and cultured overnight alone, or with LPS (10 ng / ml). Values represent the percentage of semi-adherent cells staining positive with antibody to CD11c, MHC II, or CD40 (a). Alternatively, values represent the fold increase in the number of positive cells upon stimulation with LPS compared to cells cultured alone (b).

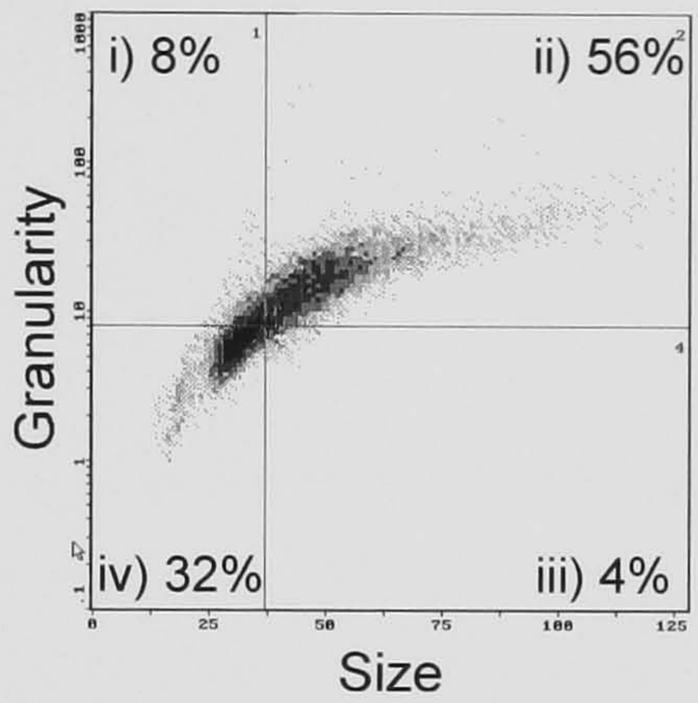
[a] represents the cells expressing high levels of the surface marker.

Figure 6.1 **Phenotype of the iDC population generated from BM precursors cultured for six days in the presence of GM-CSF.** A distinct population of dead cells, shown to be positive for PI, was identified and gated out from all subsequent analysis. Cells were stained with antibody specific for CD11c (red line), or with a matched isotype control (blue line) (a). The value in italics represents the percentage of cells expressing CD11c (a). All live cells (b), and those positive (c), or negative (d), for CD11c were characterised on their size and granularity. The quadrate over-laid on the size / granularity scattergrams arbitrarily divides the cells into 4 populations (i-iv). Data is representative at least 3 experiments.

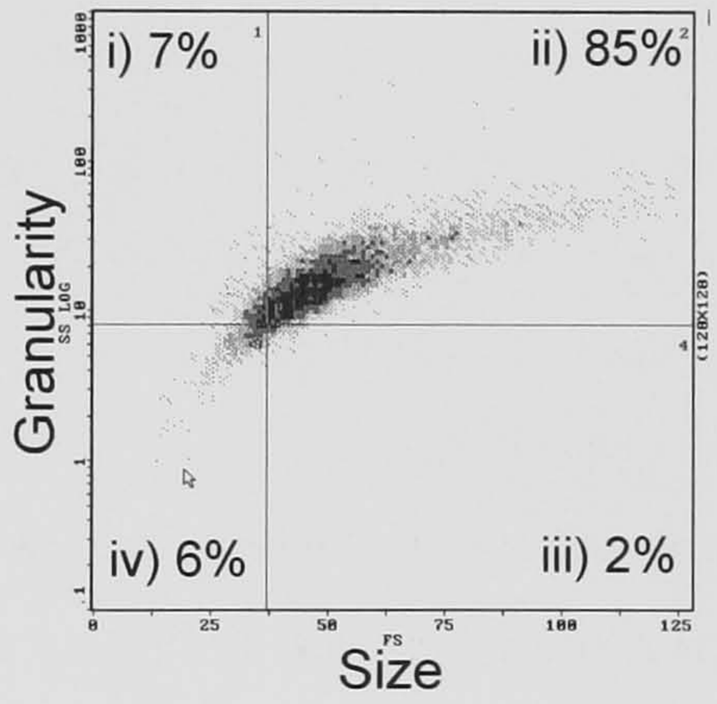
a. CD11c



b. All Live Cells



c. CD11c^{+} cells



d. CD11c^{-} cells

Figure 6.2 **Phenotype of the iDC population generated from BM precursors cultured for six days in the presence of GM-CSF.**

i) A distinct population of dead cells, shown to be positive for PI, was identified and gated out from all subsequent analysis. Cells were stained with antibody specific for the markers indicated (red line), or with matched isotype controls (blue line). The values in italics represent the percentage of cells expressing the marker and the values in red italics represent the percentage of cells expressing high / low levels of the marker (a). For each marker, the population of positive (b), or negative (c), cells was characterised on size and granularity. The quadrate over-laid on the size / granularity scattergrams arbitrarily divides the cells into 4 populations (i-iv), and is in the same position as on Figure 4.1. Data is representative at least 3 experiments.

ii) In a separate experiment, cells were double-stained with antibodies specific for CD11c and MHC II (b) or isotype matched controls (a). Control antibody staining is represented by the quadrant boundaries and values represent the percentage of cells staining positive in the particular quadrant. The values in italics represent the $CD11c^+$ MHC^+ cells, or the $CD11c^-$ MHC^+ cells, as a percentage of the total MHC^+ cells.

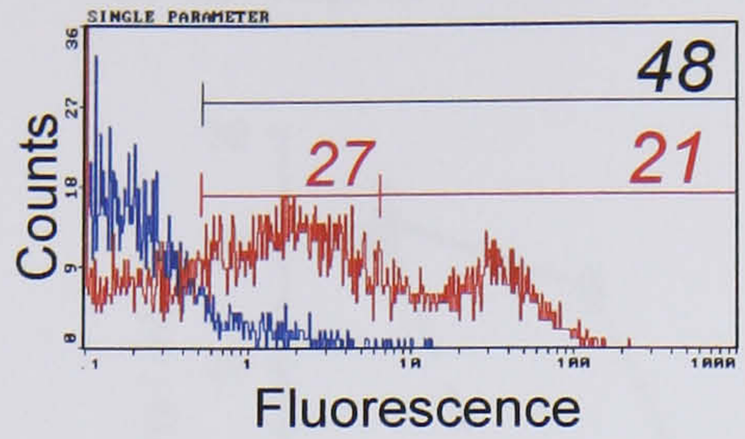
i.

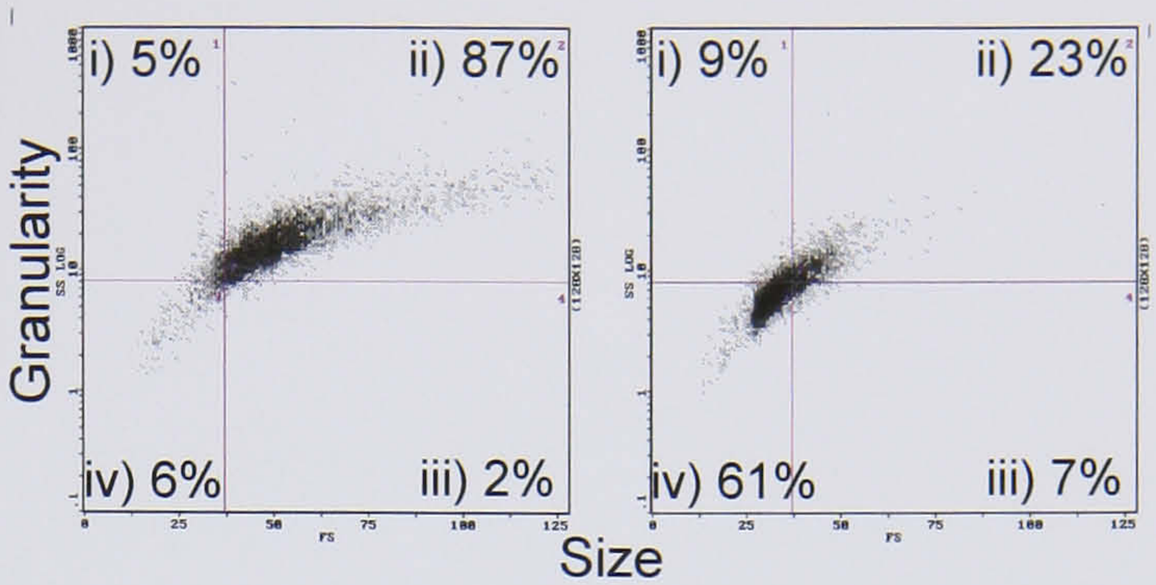
a. Fluorescence Intensity

b.+ve cells

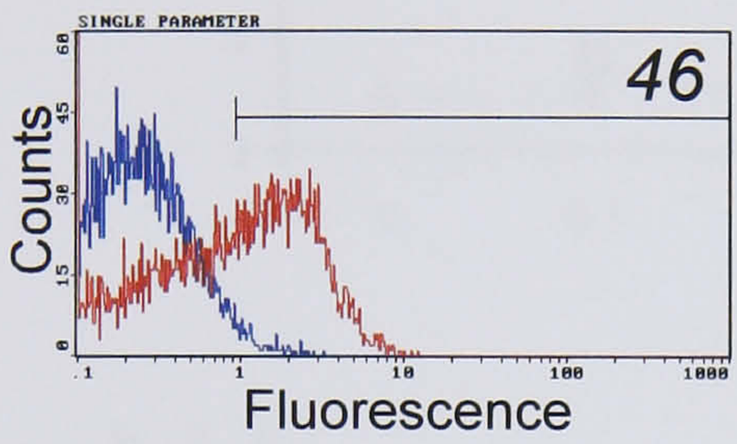
c. -ve cells

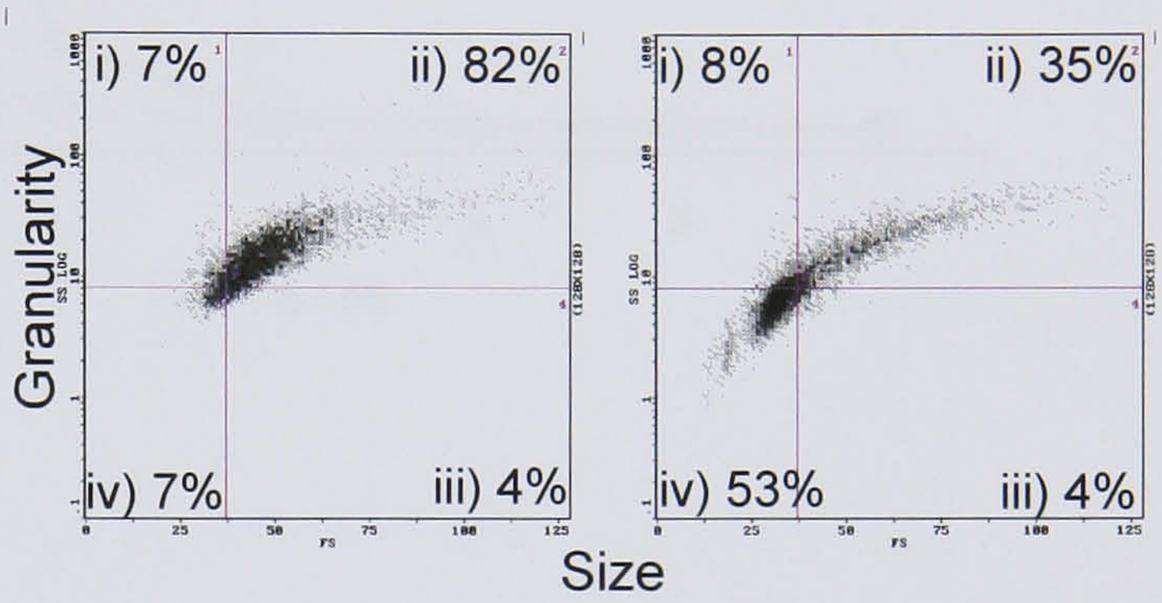
MHC II



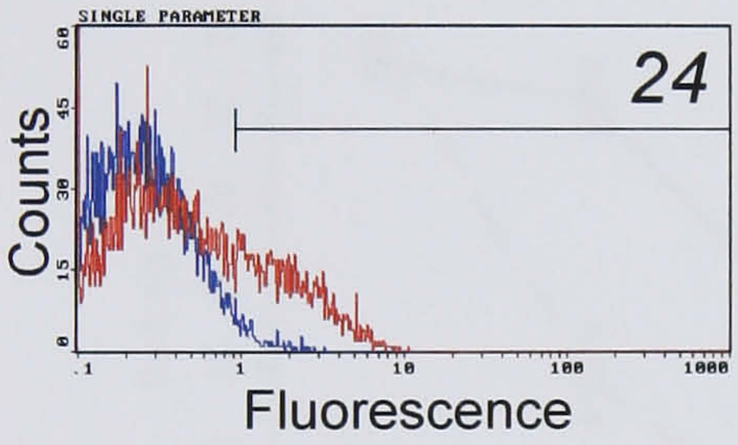


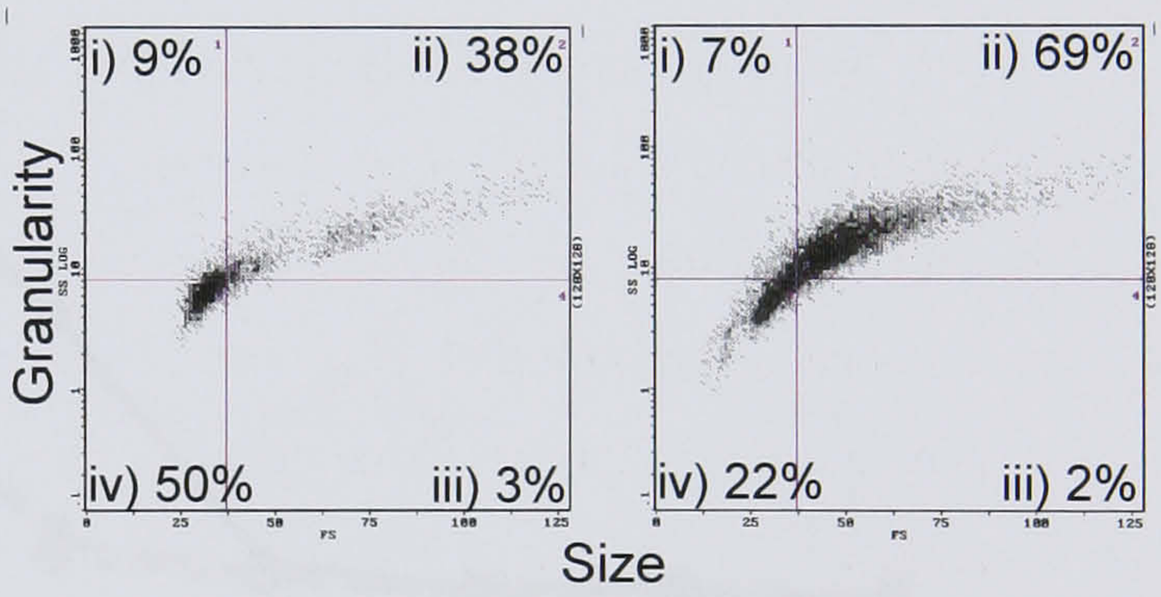
F4/80



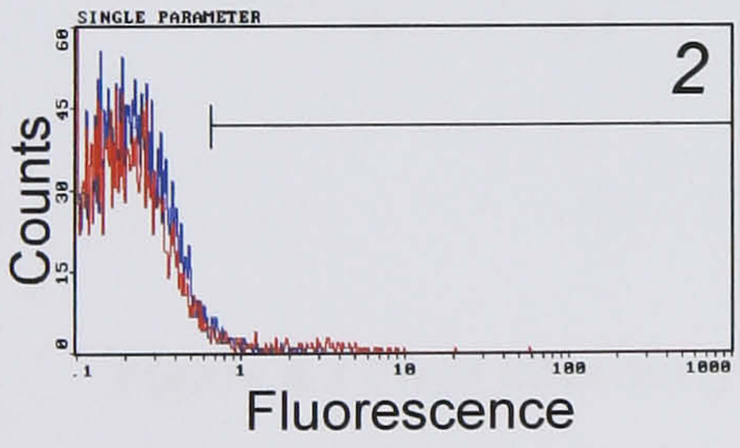


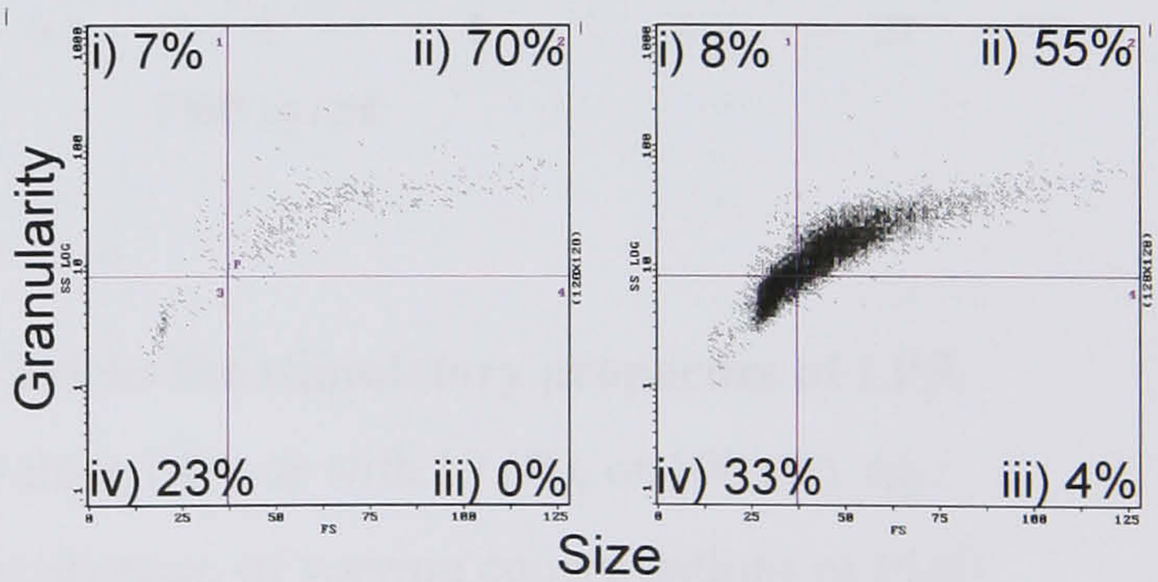
Gr-1





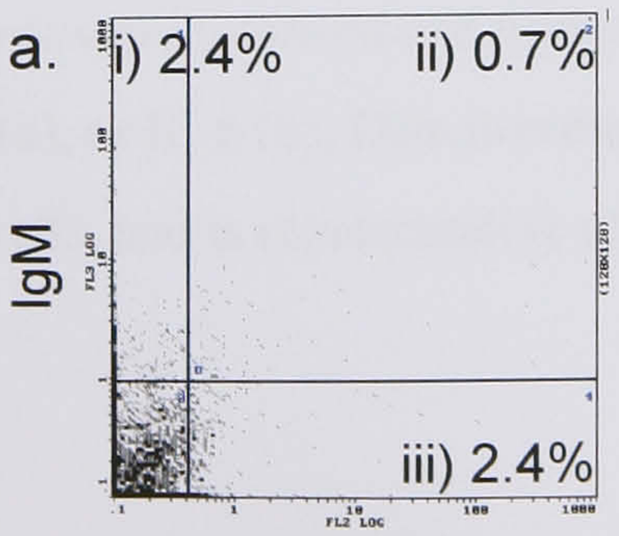
B220



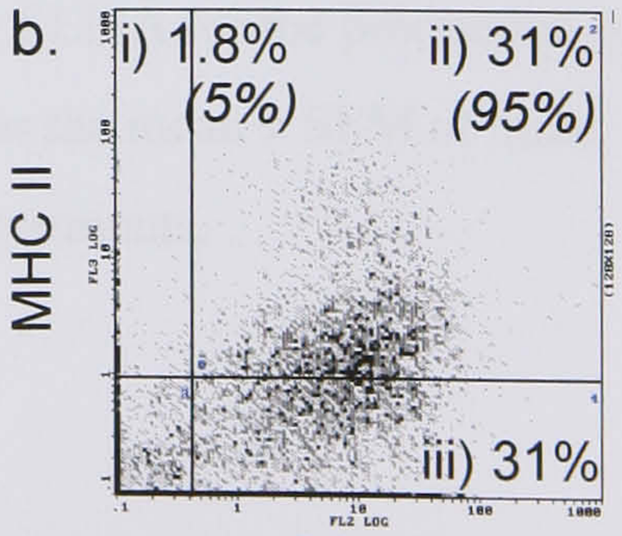


ii. CD11c *vs* MHC II

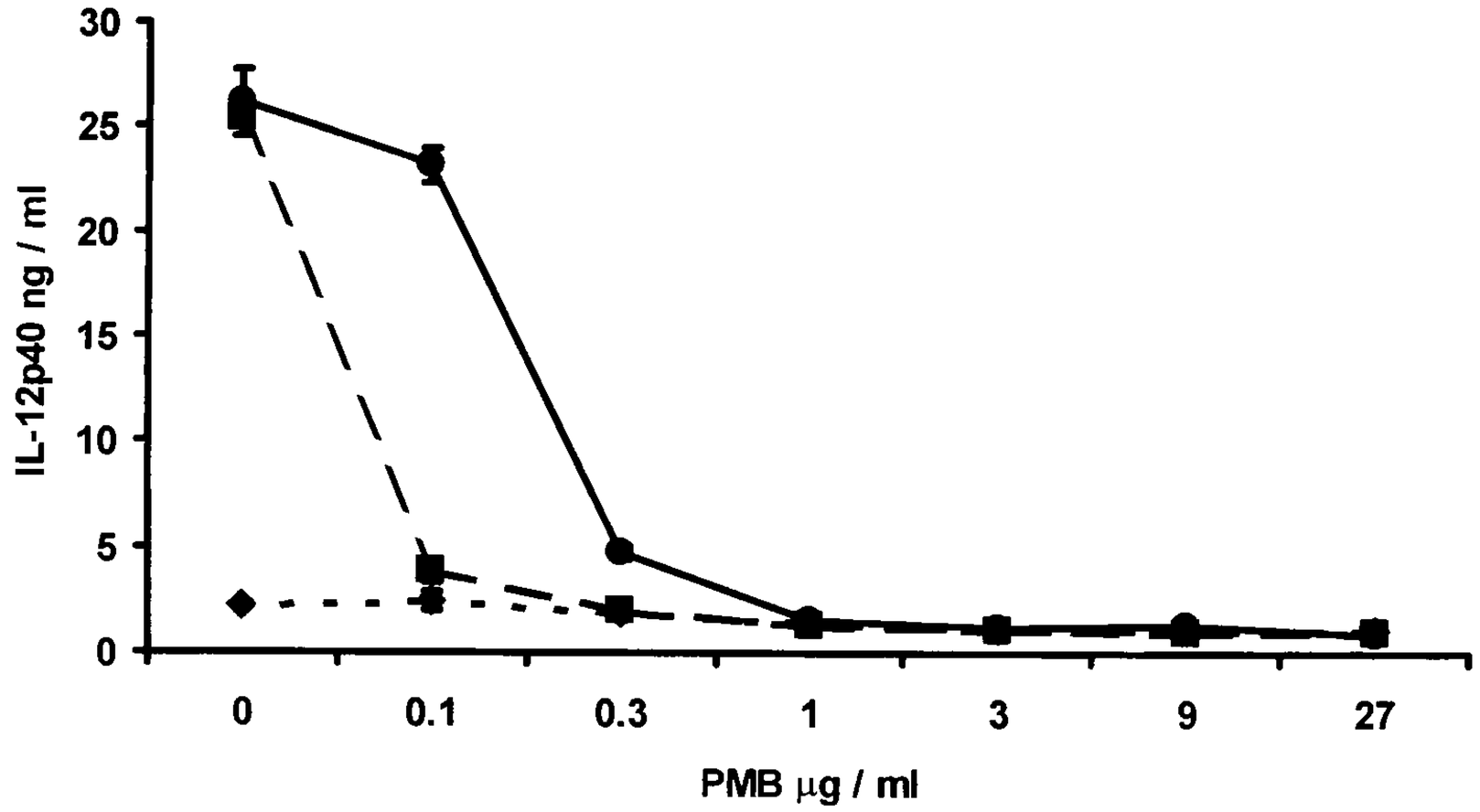
a.



b.



a. IL-12p40



b. IL-6

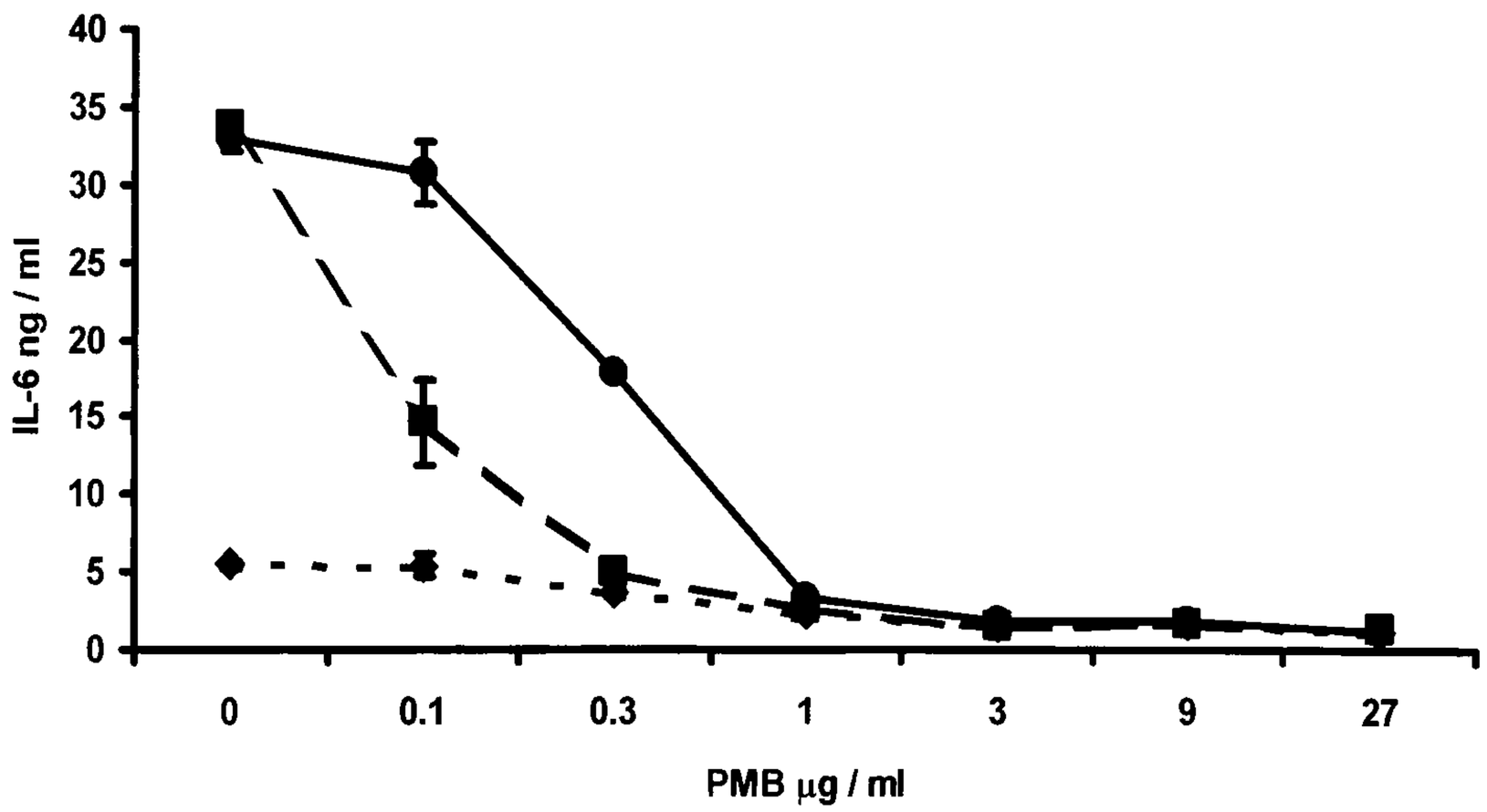
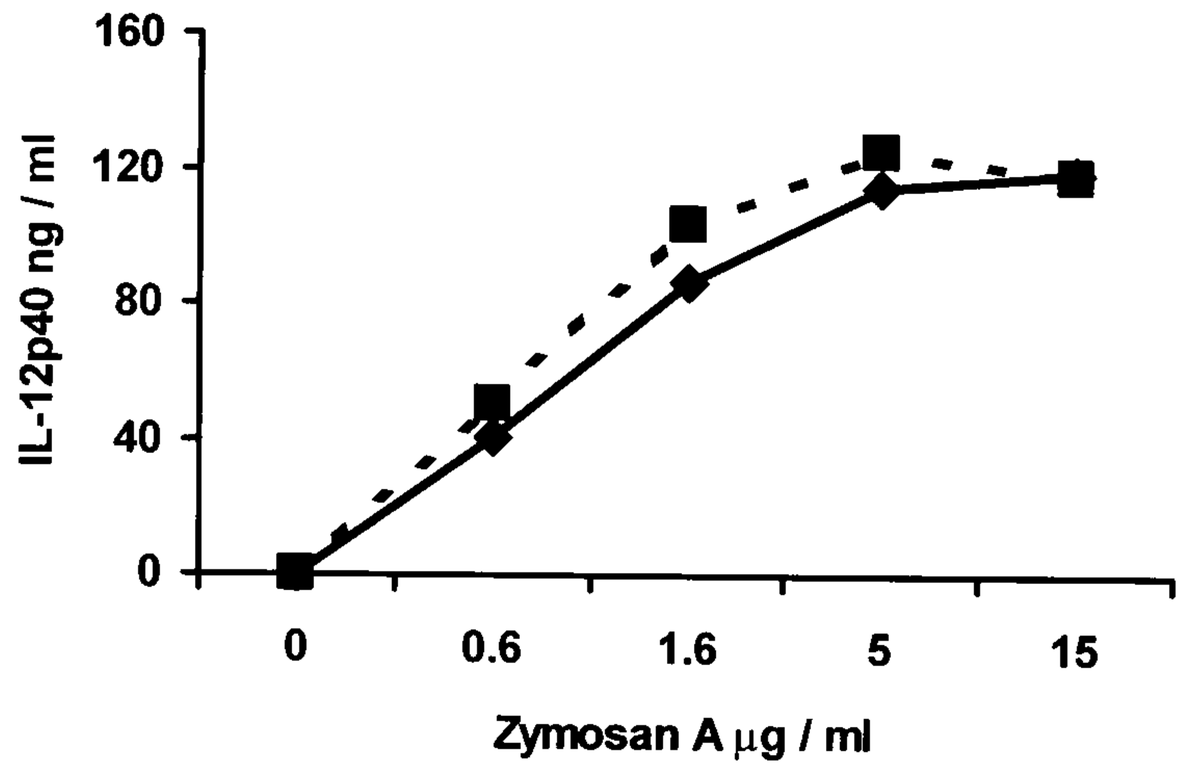


Figure 6.3 **PMB effectively blocks the stimulatory properties of LPS.** iDC were cultured overnight alone (◆), or with 10 (■), or 100 (●), ng / ml of LPS, in the presence, or absence, of varying concentrations of PMB. Supernatants were removed and analysed by ELISA for the production of IL-12p40 (a), or IL-6 (b). Data is presented as the mean ± SEM of three separate wells, and is representative of 2 experiments.

a. IL-12p40



b. IL-6

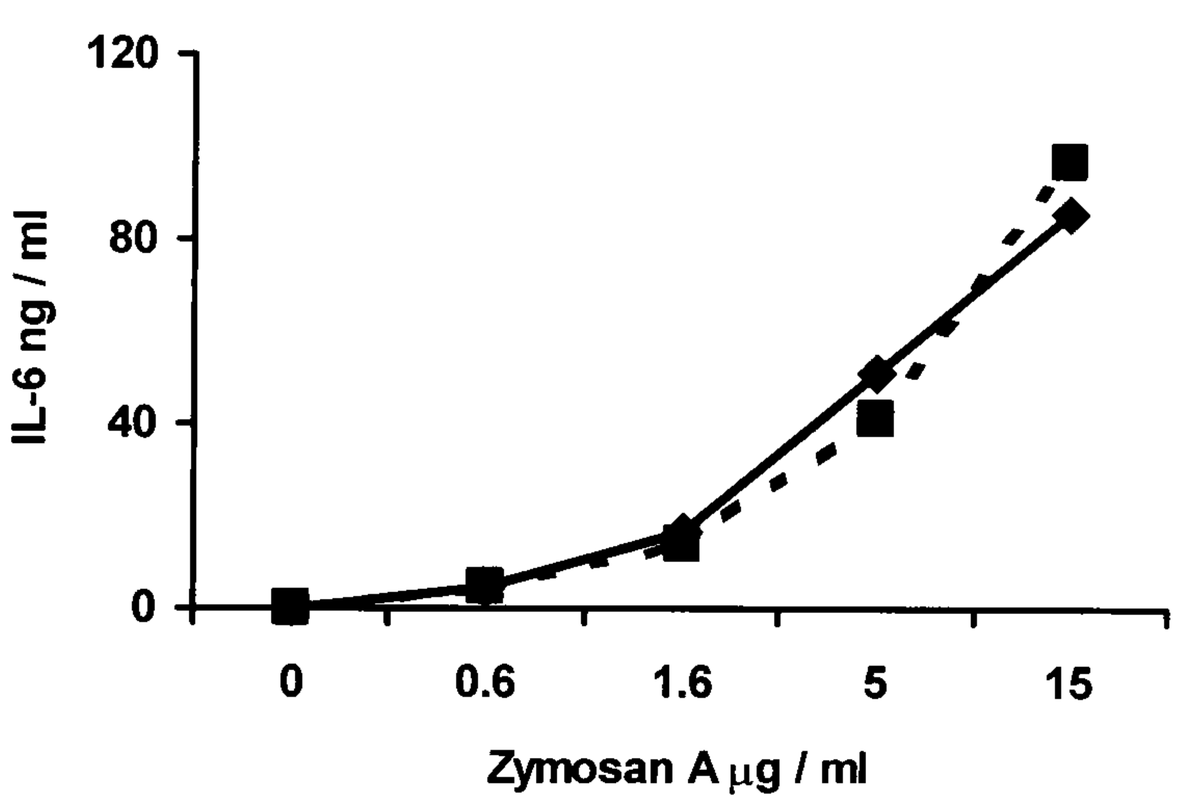
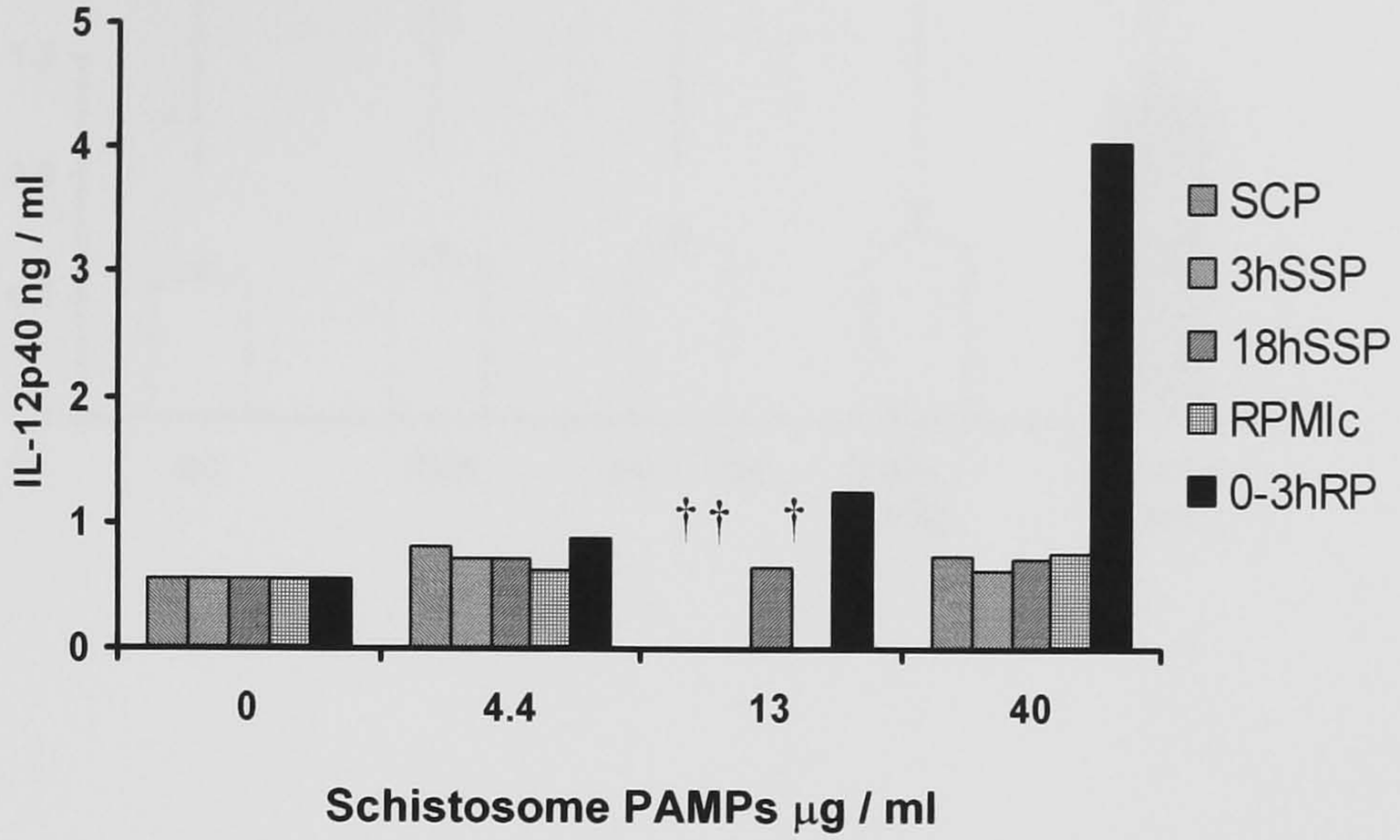


Figure 6.4 **PMB does not effect on the stimulatory properties of Zymosan A.** iDC were co-cultured with 0 (◆), or 3 (■), μg / ml PMB and the indicated concentrations of Zymosan A. Supernatants from triplicate wells were pooled and analysed by ELISA for production of IL-12p40 (a) or IL-6 (b). Data is representative of two experiments.

a. IL-12p40



b. IL-6

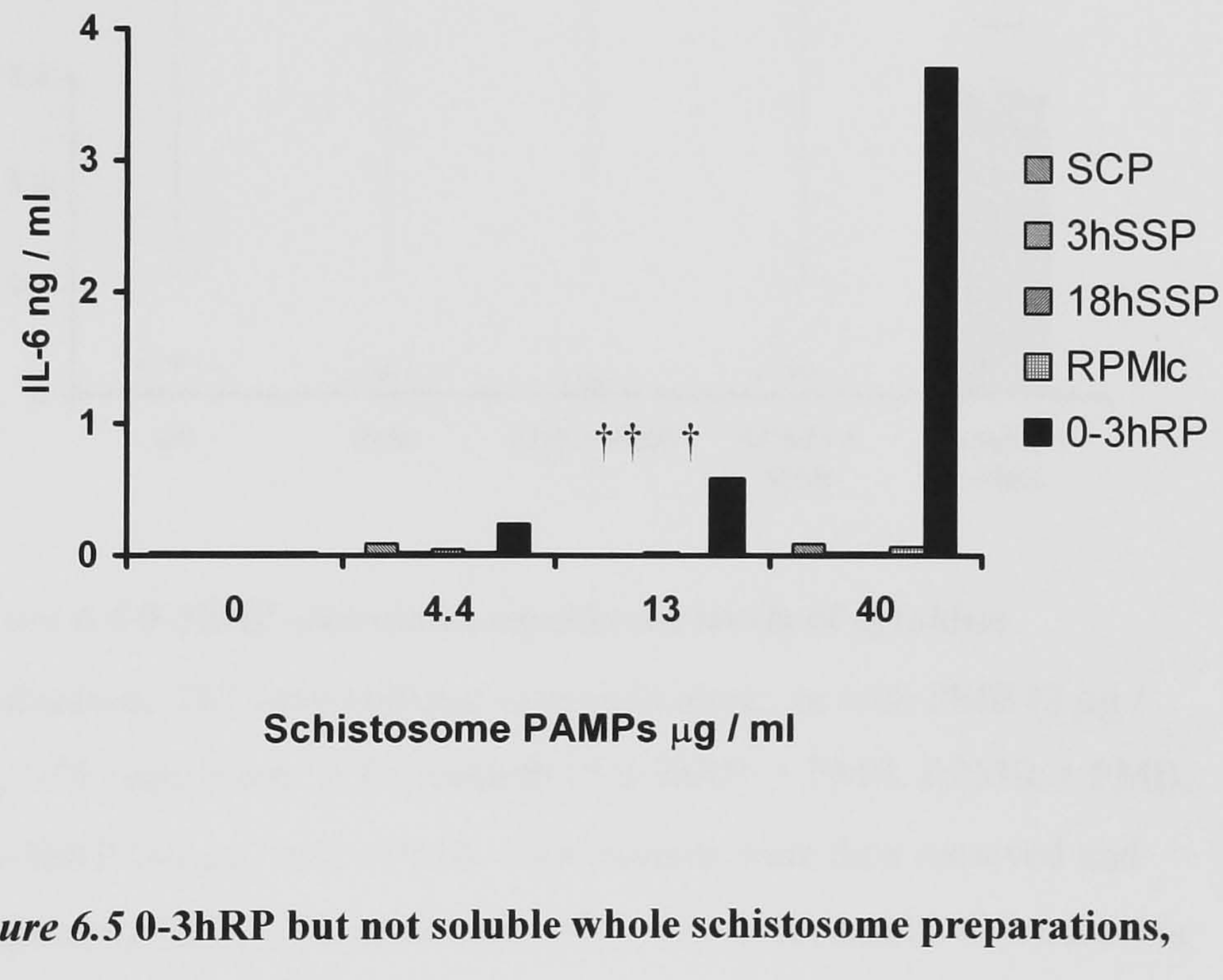


Figure 6.5 **0-3hRP but not soluble whole schistosome preparations, stimulates up-regulation of cytokine production by DC.** iDC were co-cultured overnight with PMB (3 μg / ml) and the indicated concentrations of different parasite preparations. Supernatants from triplicate wells were pooled and analysed by ELISA for the production of IL-12p40 (a) and IL-6 (b). † = not done.

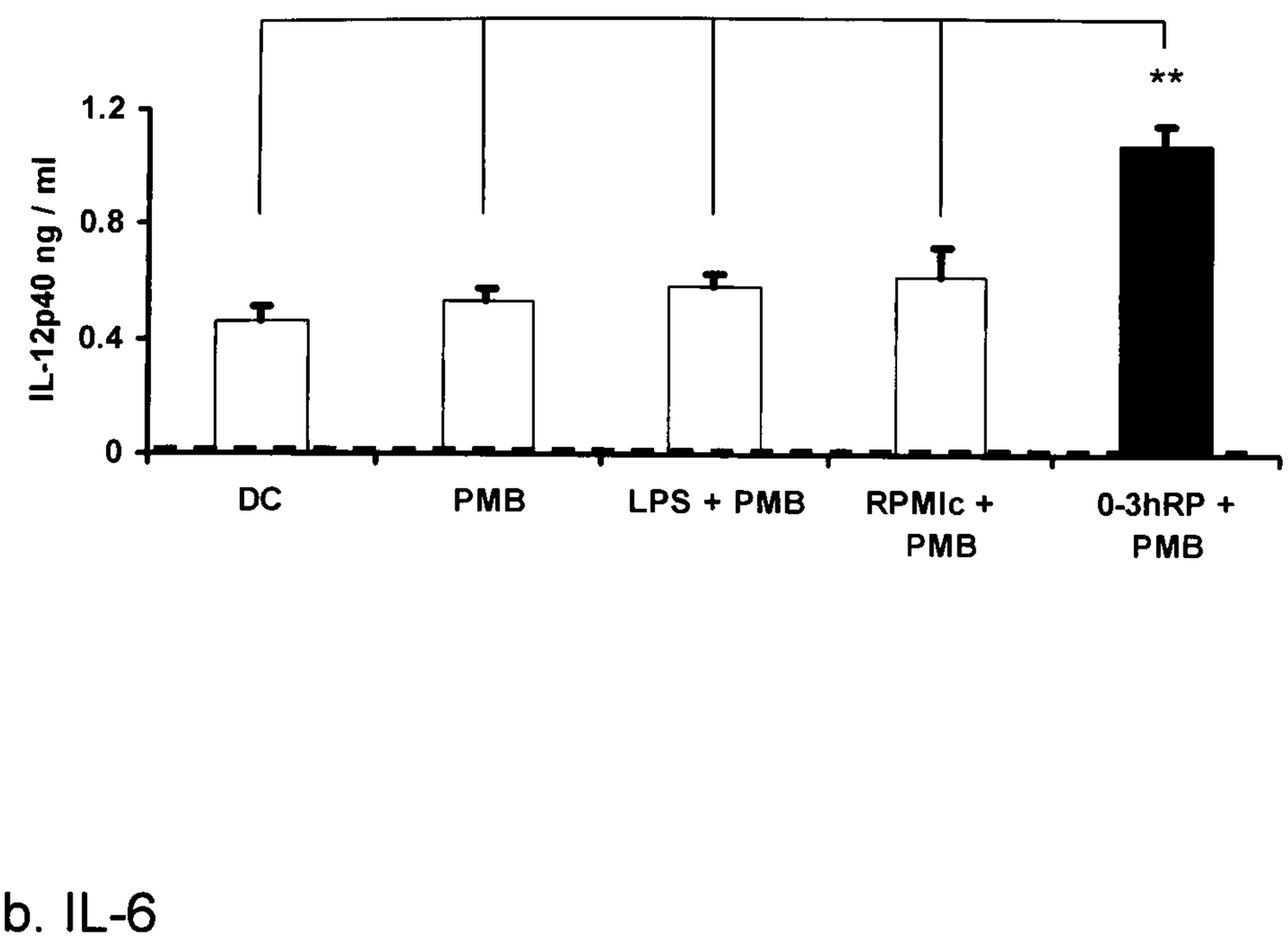
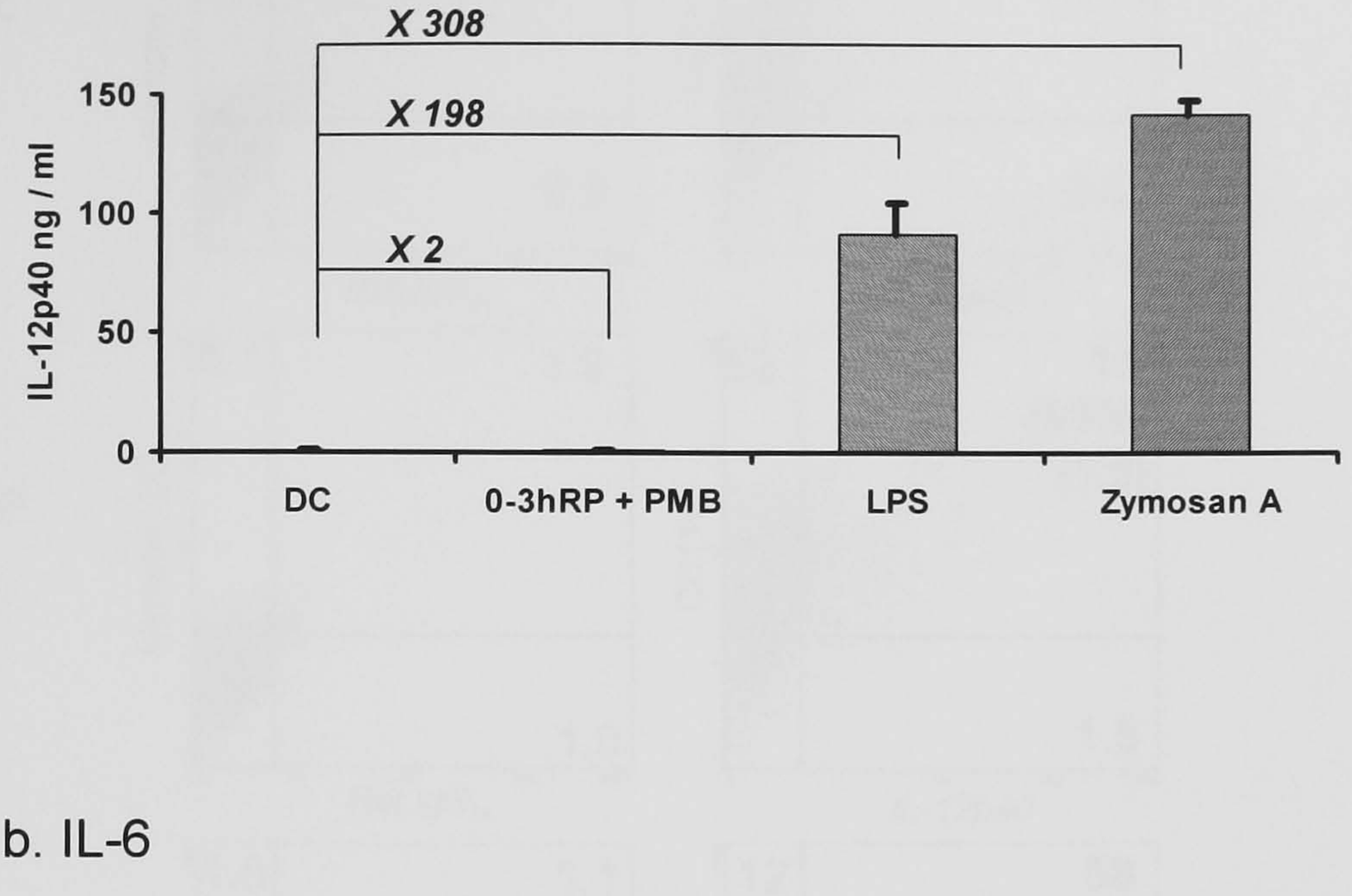


***Figure 6.6* 0-3hRP stimulates significant levels of cytokine production.** iDC were cultured overnight alone, or with PMB (3 μg / ml), LPS (equivalent to EU content of 0-3hRP) + PMB, RPMIc + PMB, or 0-3hRP (40 μg / ml) + PMB. Supernatants were then removed and analysed by ELISA for production of IL-12p40 (a) and IL-6 (b). Data is presented as the mean ± SEM of four individual wells and is representative of 3 experiments. Dashed line denotes the lower detection limit of ELISA. Levels of significance, as determined by Students *t* test, are indicated as: ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

a. IL-12p40



b. IL-6

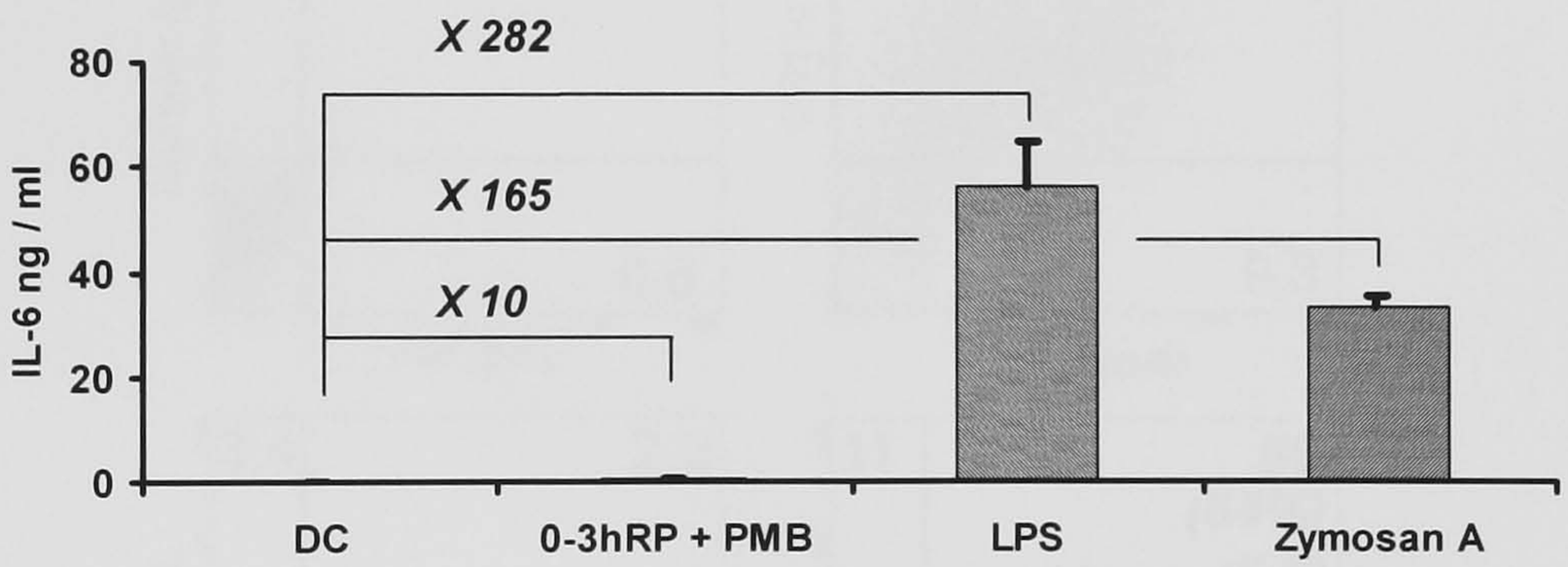


Figure 6.7 **Stimulation by 0-3hRP is dwarfed by the response to other PAMPs.** iDC were cultured overnight alone, or with LPS (1 ng / ml), Zymosan A (5 μg / ml), or 0-3hRP (40 μg / ml) + PMB (3 μg / ml). Supernatants were then removed and analysed by ELISA for production of IL-12p40 (a) and IL-6 (b). Data is presented as the mean ± SEM of four individual wells and is representative of 3 experiments. Numbers in italics represent the fold-increase in cytokine production compared to DC alone.

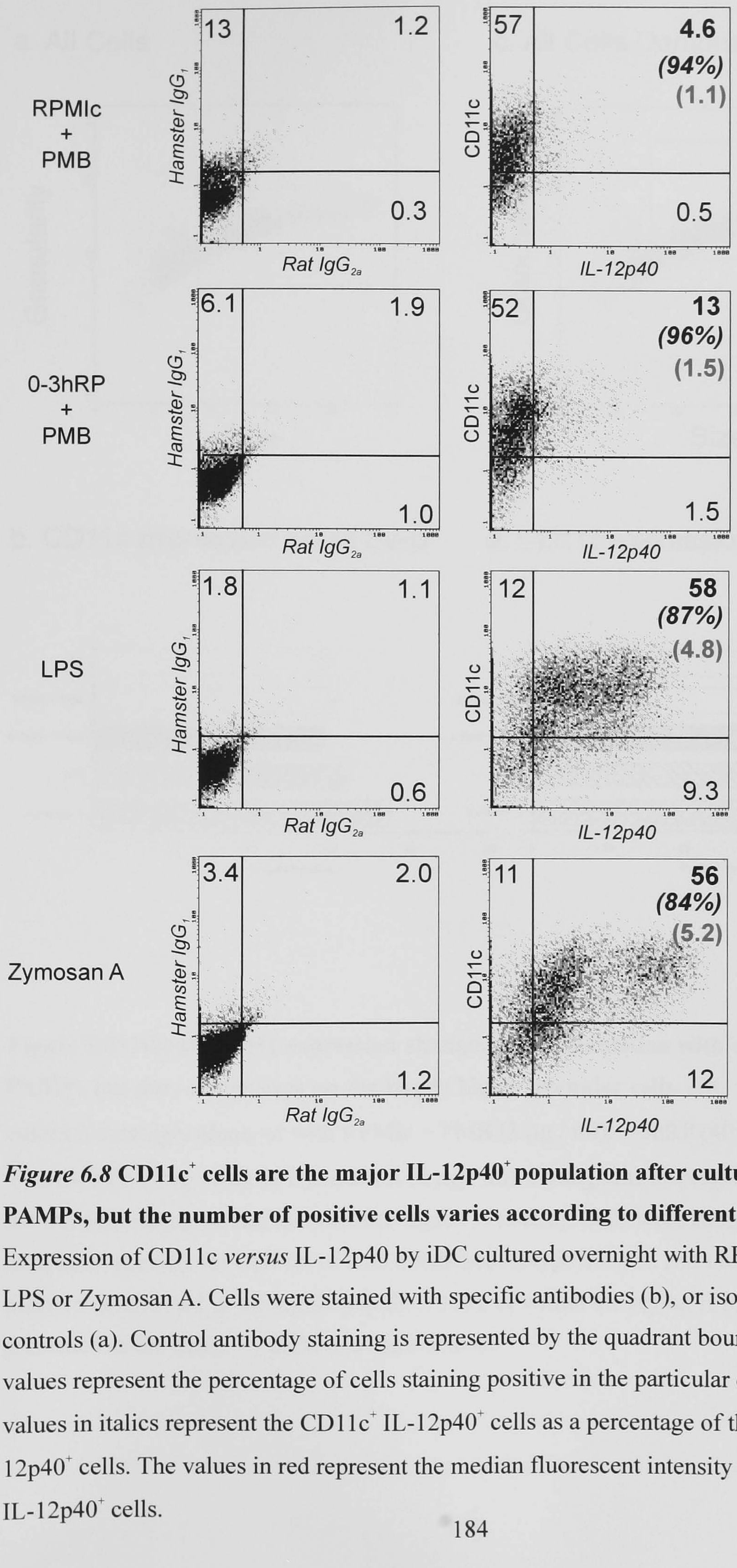


Figure 6.8 **CD11c$^+$ cells are the major IL-12p40$^+$ population after culture with PAMPs, but the number of positive cells varies according to different stimuli.** Expression of CD11c *versus* IL-12p40 by iDC cultured overnight with RPMI, 0-3hRP, LPS or Zymosan A. Cells were stained with specific antibodies (b), or isotype matched controls (a). Control antibody staining is represented by the quadrant boundaries and values represent the percentage of cells staining positive in the particular quadrant. The values in italics represent the CD11c$^+$ IL-12p40$^+$ cells as a percentage of the total IL-12p40$^+$ cells. The values in red represent the median fluorescent intensity of staining for IL-12p40$^+$ cells.

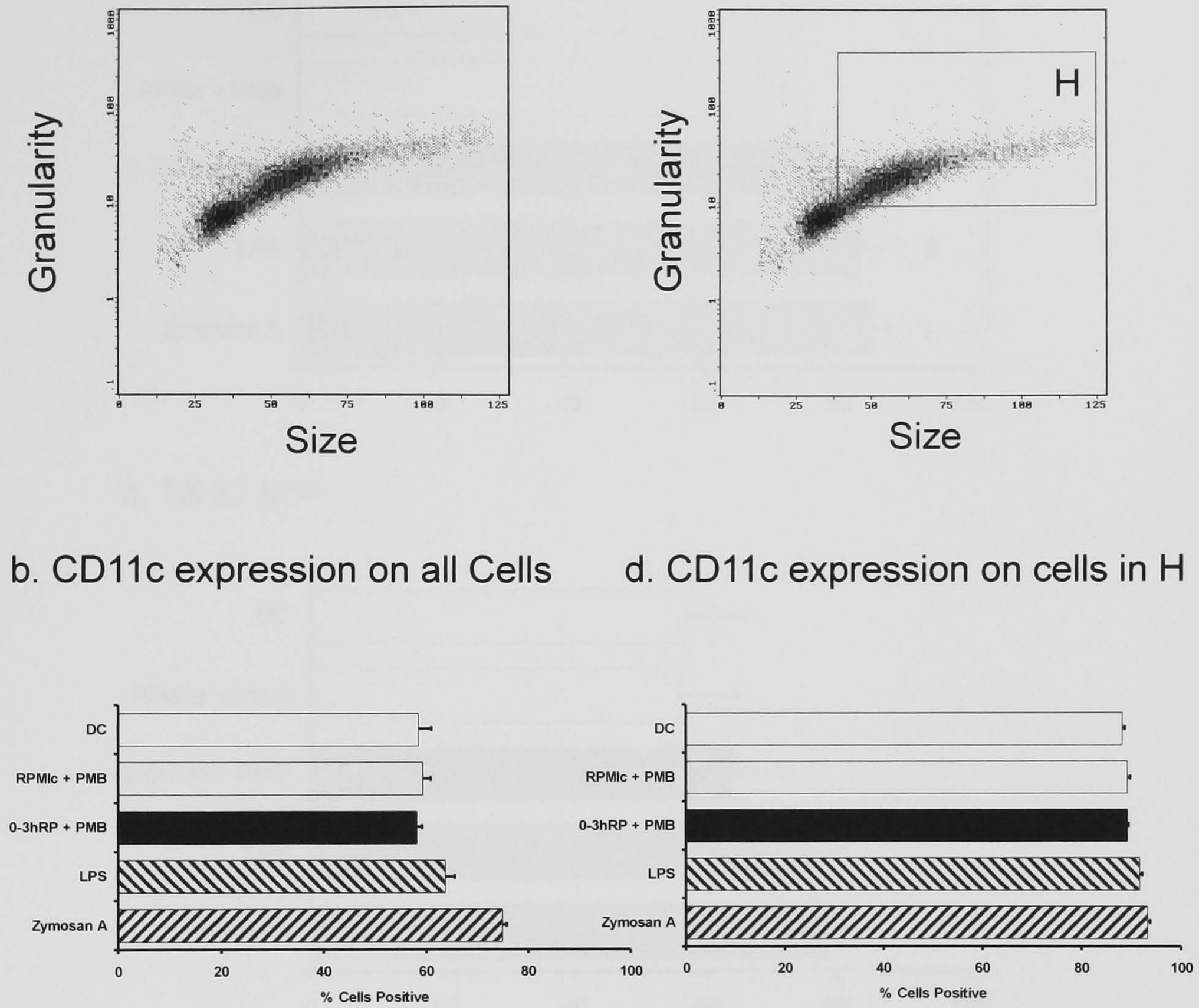


Figure 6.9 **Overall CD11c expression changes after stimulation with different PAMPs but does not change on the large / highly granular cells.** iDC were cultured overnight alone, or with RPMIc + PMB (3 μg / ml), 0-3hRP (40 μg / ml) + PMB, LPS (1 ng / ml), or Zymosan A (5 μg / ml). Histograms display all events for DC cultured alone and demonstrate region H (a & c). Cells were stained with antibodies specific for CD11c, or with a matched isotype control, and the percentage positive determined in the whole population (b), or within the region H (d). Data is presented as the mean +/- SEM of 3 experiments.

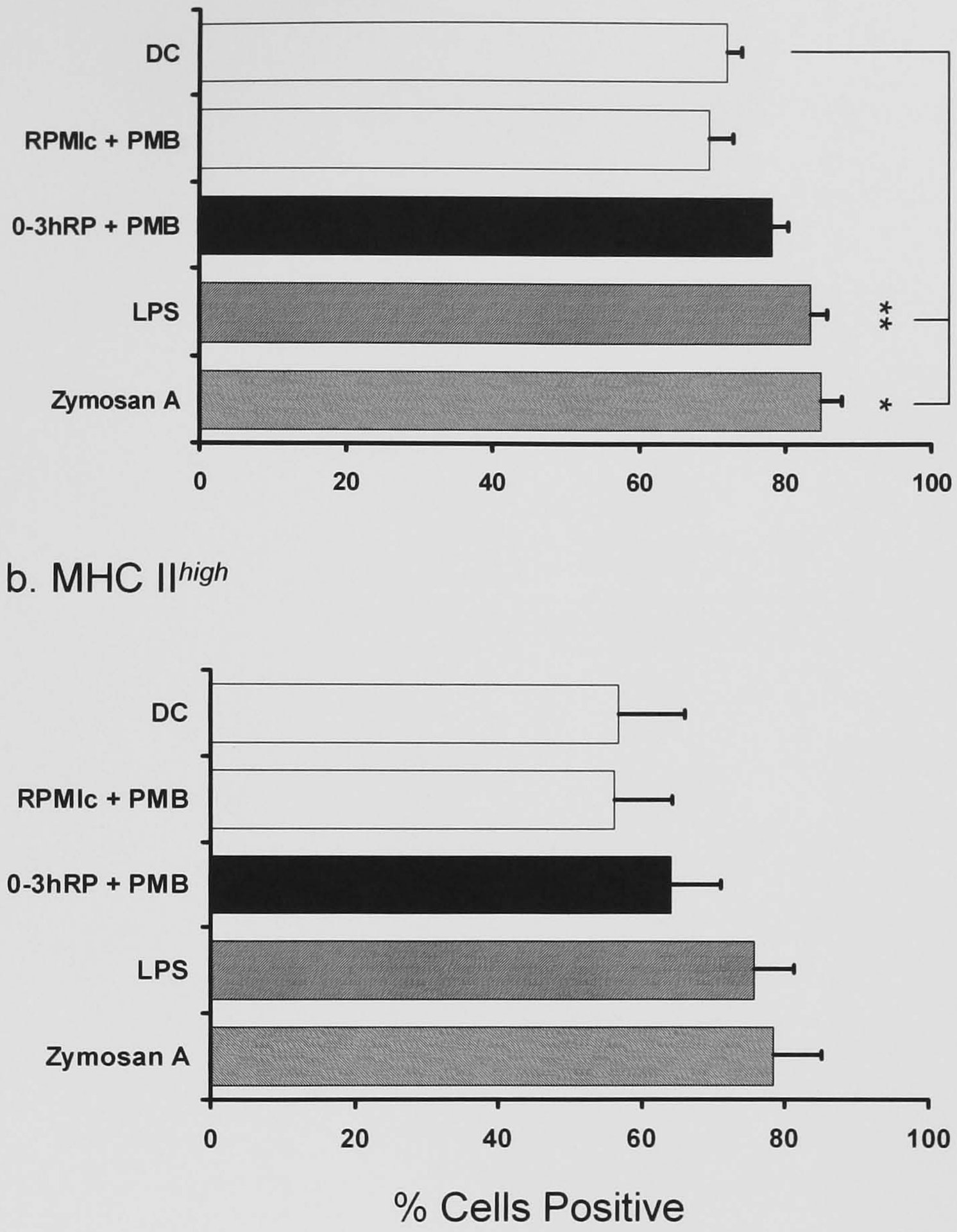
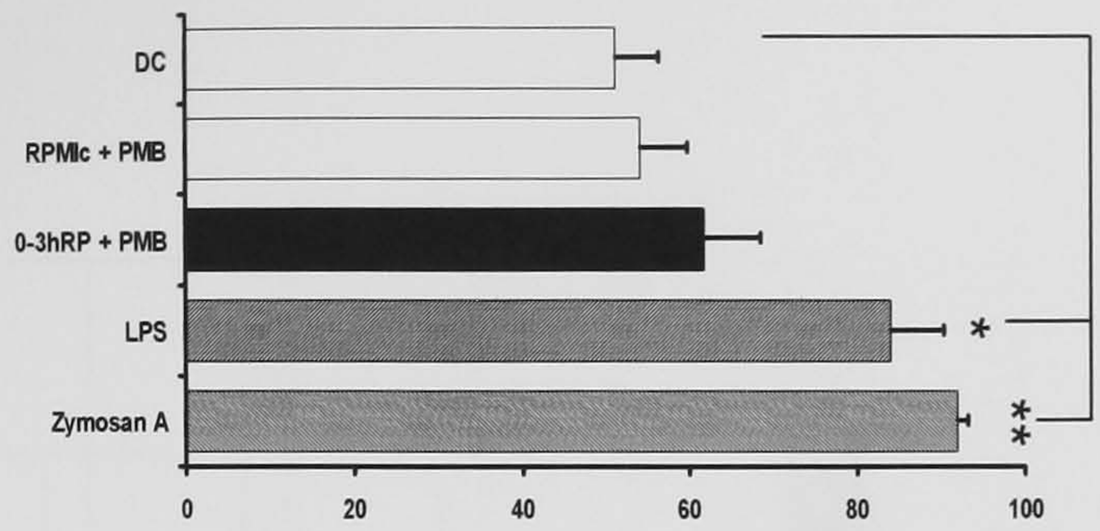


Figure 6.10 **MHC II expression on mDC cultured with different PAMPs.** Percentage of $CD11c^+$ cells expressing MHC II (a), or high levels of MHC II (b) was determined after iDC were cultured alone, or with RPMIc + PMB (3 μg / ml), 0-3hRP (40 μg / ml) + PMB, LPS (1 ng / ml), or Zymosan A (5 μg / ml). Cells were stained with antibodies specific to CD11c and MHC II, or with matched isotype controls. Gating on all $CD11c^+$ cells, the percentage positive for MHC II and high levels of MHC II was determined. Data is presented as the mean ± SEM of 3 experiments. Levels of significance, as determined by Students *t* test, are indicated as: * = $p \leq 0.05$, ** = $p \leq 0.01$.

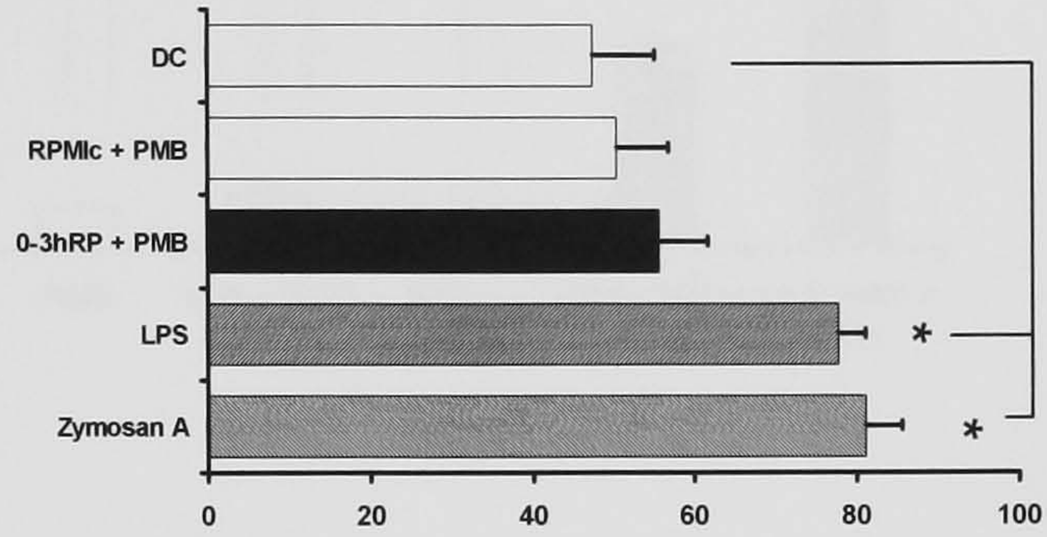
Figure 6.11 **Phenotype of mDC cultured with different PAMPs.**

Percentage of cells expressing CD40 (a), $CD86^{high}$ (b), CD80 (c), or OX40L (d), was determined after iDC were cultured alone, or with RPMIc + PMB (3 μg / ml), 0-3hRP (40 μg / ml) + PMB, LPS (1 ng / ml), or Zymosan (5 μg / ml). Cells were stained with antibodies specific to CD40, CD80, CD86, or OX40L, or with matched isotype controls. Analysis was restricted to large granular cells within box H. Data is presented as the mean ± SEM of 3 experiments, except CD80 which is derived from 2 experiments. Levels of significance, as determined by Students *t* test, are indicated as: * = $p \leq 0.05$ and ** = $p \leq 0.01$.

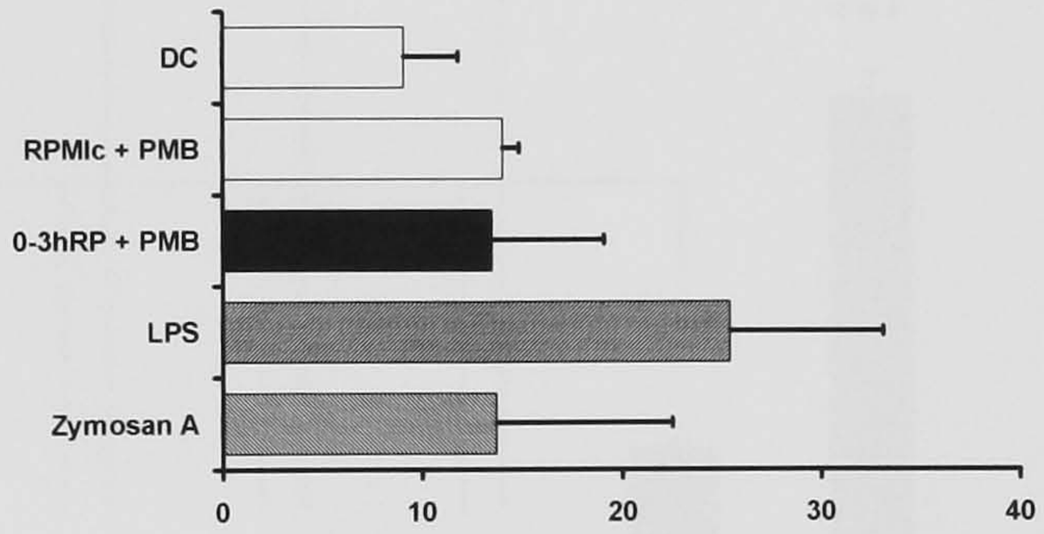
a. CD40



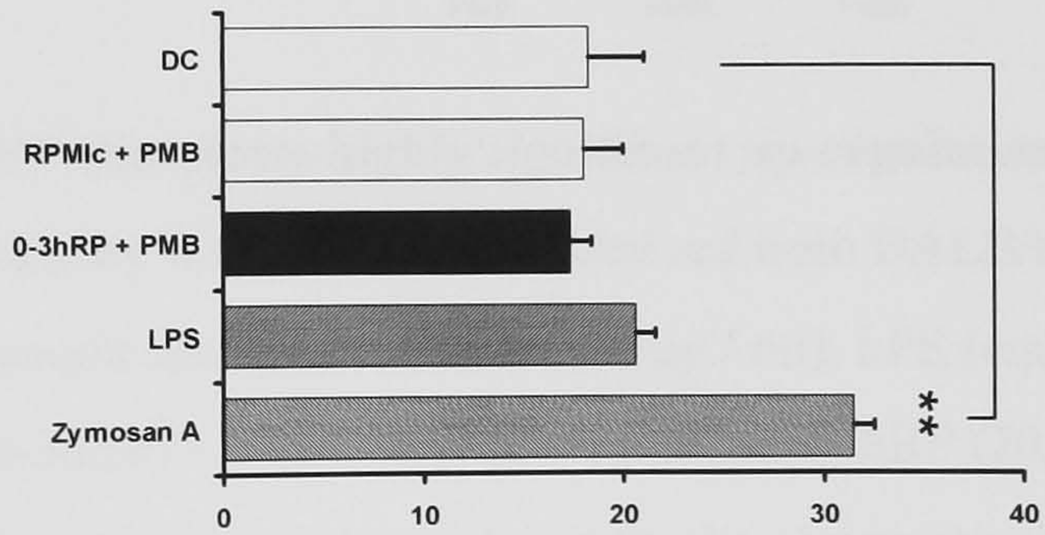
b. CD86*high*



c. CD80

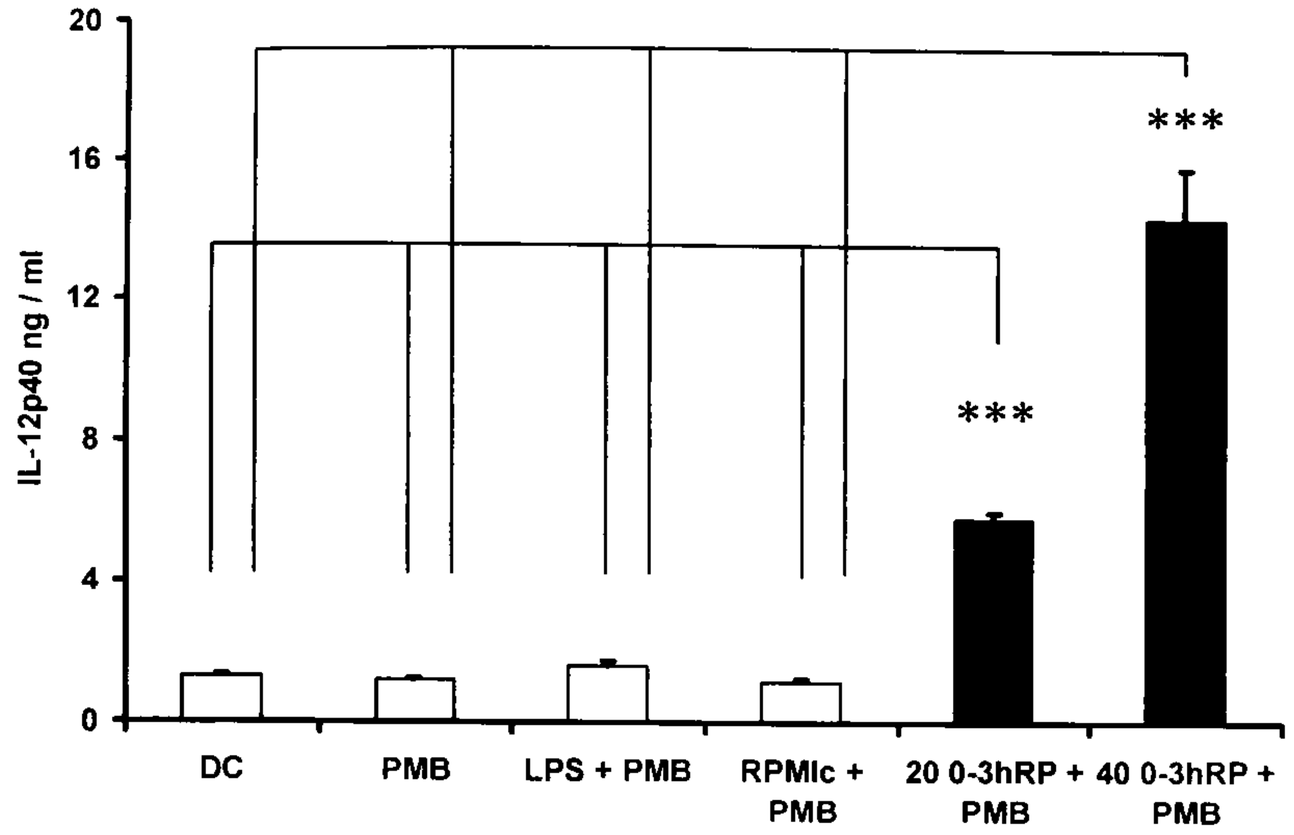


d. OX40L



% Cells Positive

a. IL-12p40



b. IL-6

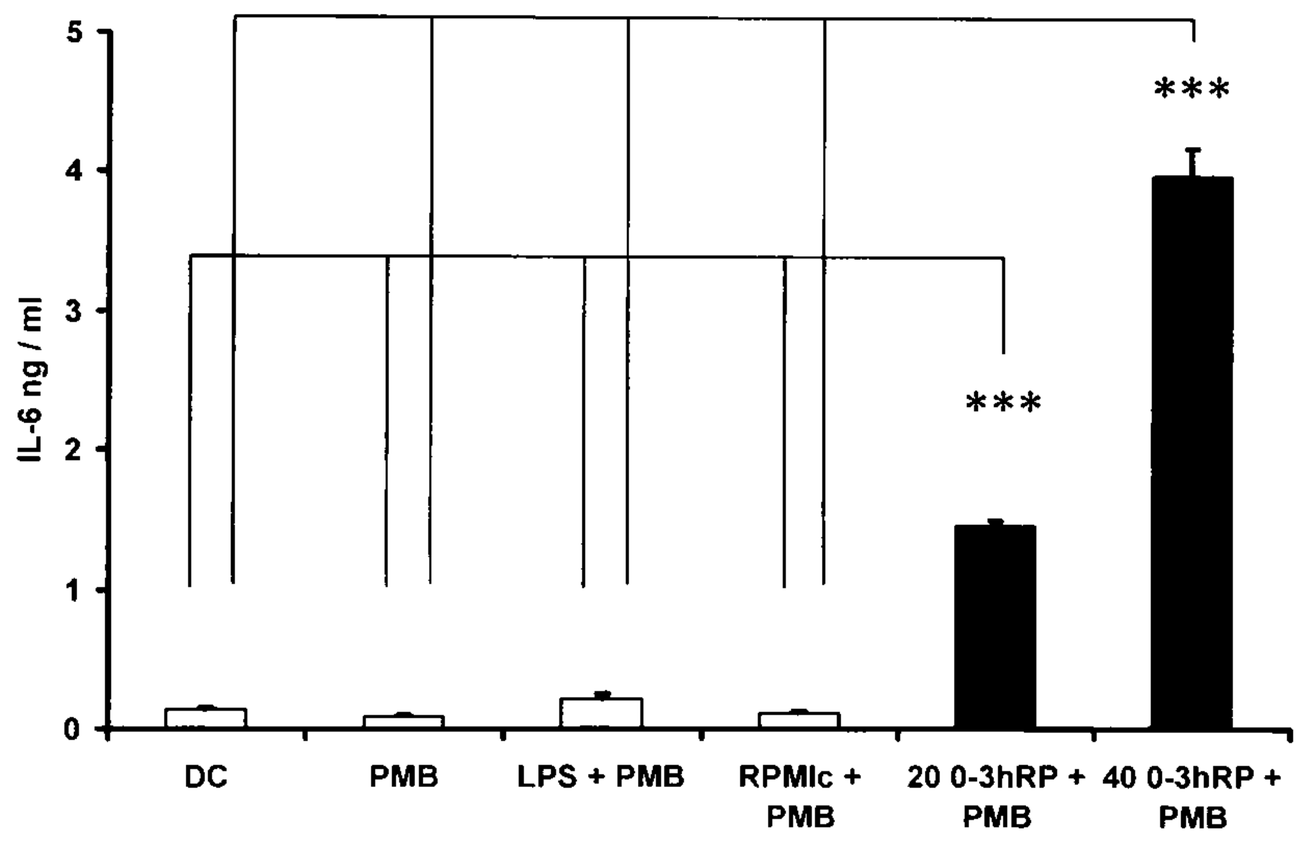


***Figure 6.12* 0-3hRP stimulates highly significant up-regulation of cytokine production by BALB/c DC.** iDC derived from BALB/c BM were cultured overnight alone, or with PMB (3 μg / ml), LPS (equivalent to EU content of 0-3hRP) + PMB, RPMIc + PMB, or 0-3hRP (20, or 40, μg / ml) + PMB. Supernatants were analysed for the expression of IL-12p40 (a), or IL-6 (b), by ELISA. Data is presented as the mean ± SEM of 4 wells, and is representative of at least 4 experiments. Levels of significance, as determined by Students *t* test, are indicated as: *** = $p \leq 0.001$.

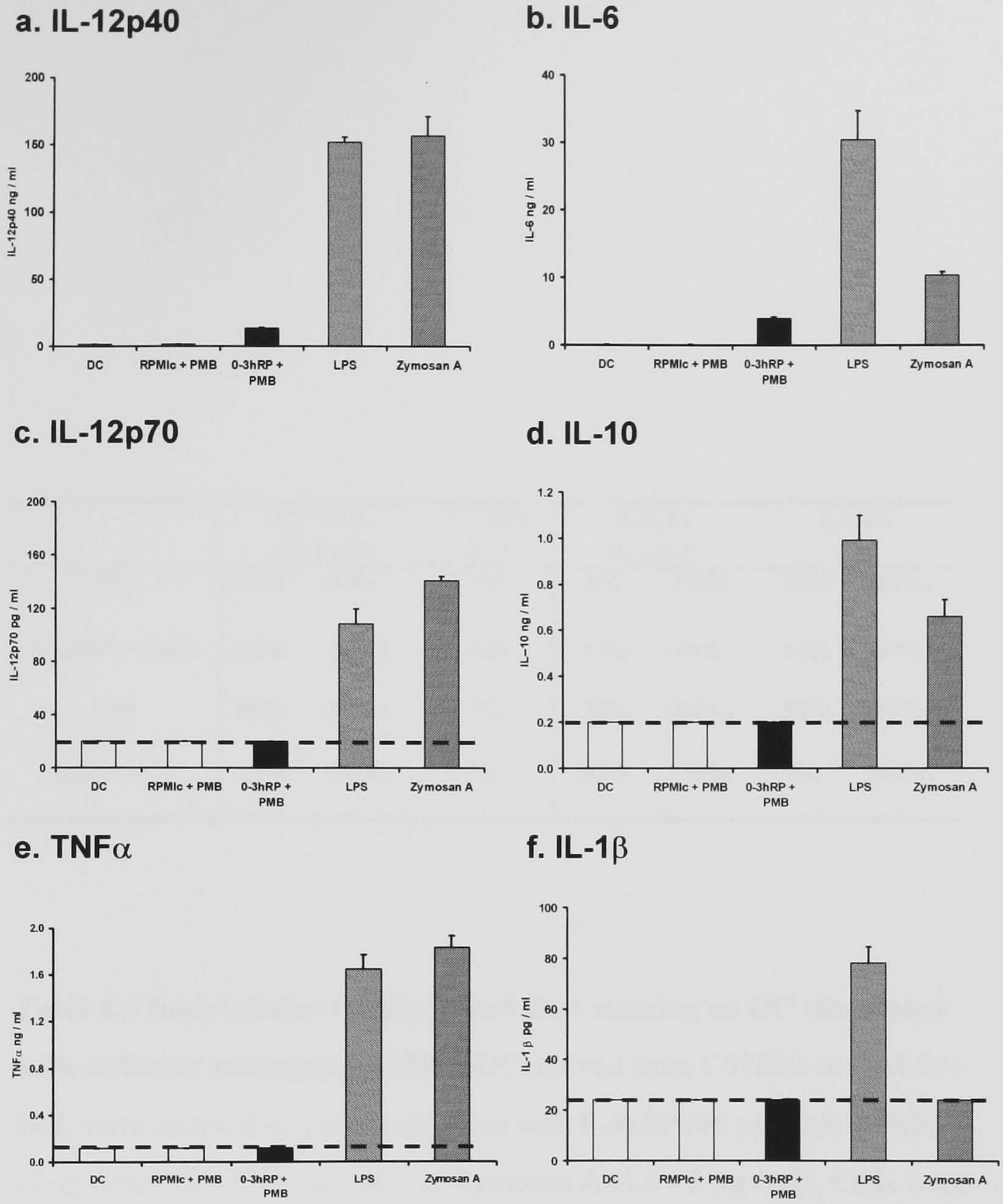


Figure 6.13 **Different pathogen PAMPs stimulate contrasting profiles of DC cytokine production.** iDC derived from BALB/c BM, were cultured overnight alone, or with RPMIc + PMB (3 μg / ml), 0-3hRP (40 μg / ml) + PMB, LPS (100 ng / ml), or Zymosan A (1.6 μg / ml). Supernatants were removed and analysed for expression of IL-12p40 (a), IL-6 (b), IL-12p70 (c), IL-10 (d), TNFα (e), or IL-1β (f), by ELISA. Data is presented as the mean ± SEM of 4 wells, and is representative of at least 2 experiments. Dashed lines represent the lower limit of detection of ELISA.

	C57Bl/6 *IL-12p40*	*C57Bl/6* *IL-4*	*BALB/c* *IL-12p40*	*BALB/c* *IL-4*
DC	**10%** (94%)	ND	**6%** (78%)	**10%** (87%)
0-3hRP + PMB	**22%** (96%)	ND	**11%** (94%)	**14%** (94%)
LPS	**85%** (87%)	ND	**72%** (89%)	**30%** (89%)
Zymosan A	**87%** [a] (84%)	ND	**30%** [b] (90%)	**7%** [b] (82%)

Table 6.3 **Intracellular IL-12p40 and IL-4 staining on DC stimulated with different pathogen PAMPs.** iDC derived from C57Bl/6 or BALB/c BM, were cultured overnight alone, or with 0-3hRP (40 μg / ml) + PMB (3 μg / ml), LPS (100 ng / ml), or Zymosan A (1.6 - 5 μg / ml). Cells were stained with antibodies specific for CD11c, and IL-12p40, or IL-4, or with matched isotype controls, and analysed by 2-colour flow-cytometry. The values shown in bold represent the percentage of $CD11c^+$ cells that were positive for IL-12p40, or IL-4. The values in brackets represent the $CD11c^+$ $cytokine^+$ DC as a percentage of the total $cytokine^+$ cells. Data presented for C57Bl/6 DC is derived from the experiment shown in Figure 6.8.

[a] 5 μg / ml Zymosan A.

[b] 1.6 μg / ml Zymosan A.

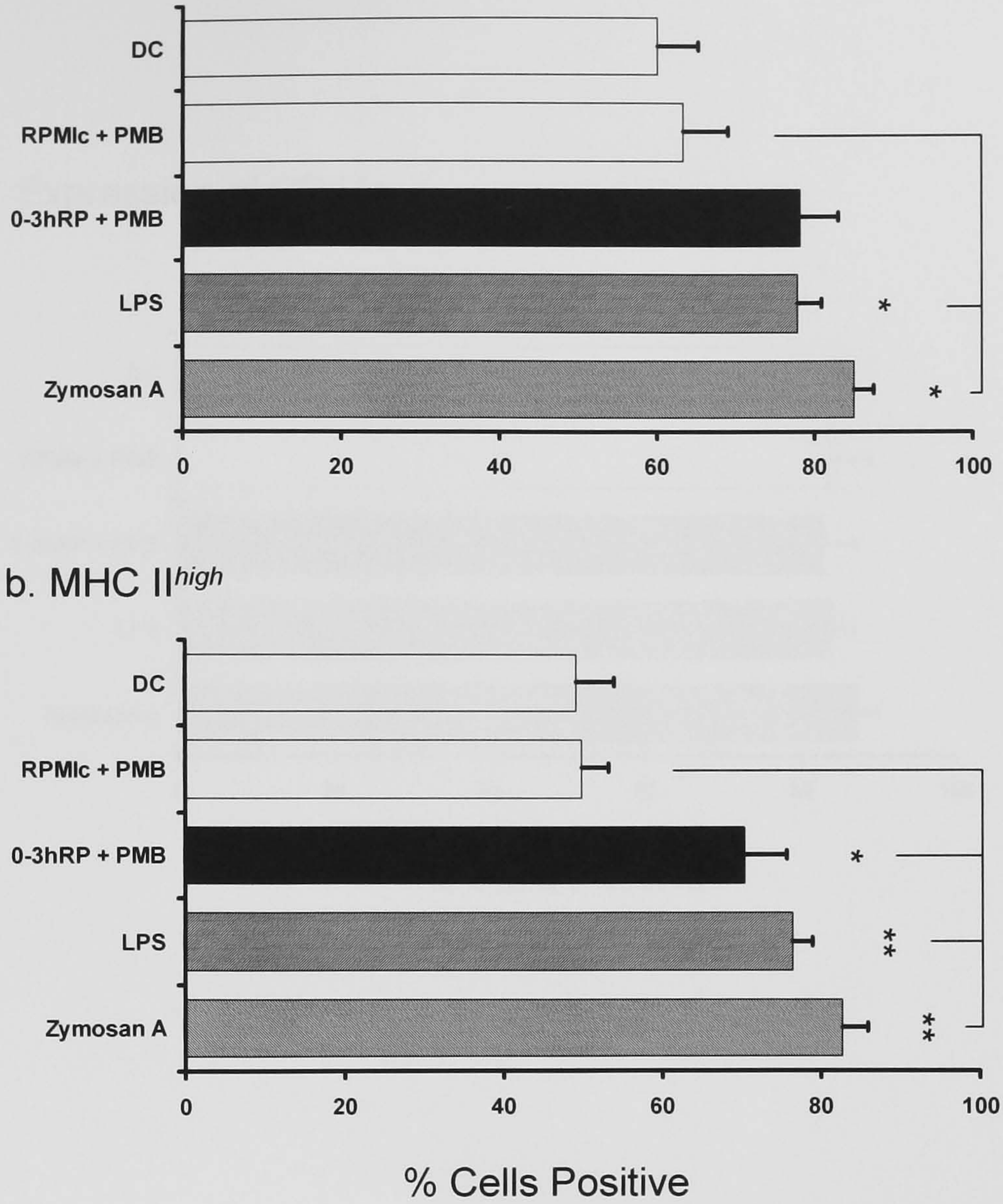


Figure 6.14 **0-3hRP stimulates up-regulation of MHC II expression on DC derived from BALB/c BM.** The percentage of $CD11c^+$ cells expressing MHC II (a), or high levels of MHC II (b), was determined after iDC were cultured alone, or with RPMIc + PMB (3 μg / ml), 0-3hRP (40 μg / ml) + PMB, LPS (100 ng / ml) or Zymosan A (1.6 μg / ml). Cells were stained with antibodies specific to CD11c and MHC II, or with matched isotype controls. Gating on $CD11c^+$ cells, the percentage positive for MHC II (a) and high levels of MHC II (b) was determined. Data is presented as the mean ± SEM of 3 experiments. Levels of significance, as determined by Students *t* test, are indicated as: * = $p \leq 0.05$ and ** = $p \leq 0.01$.

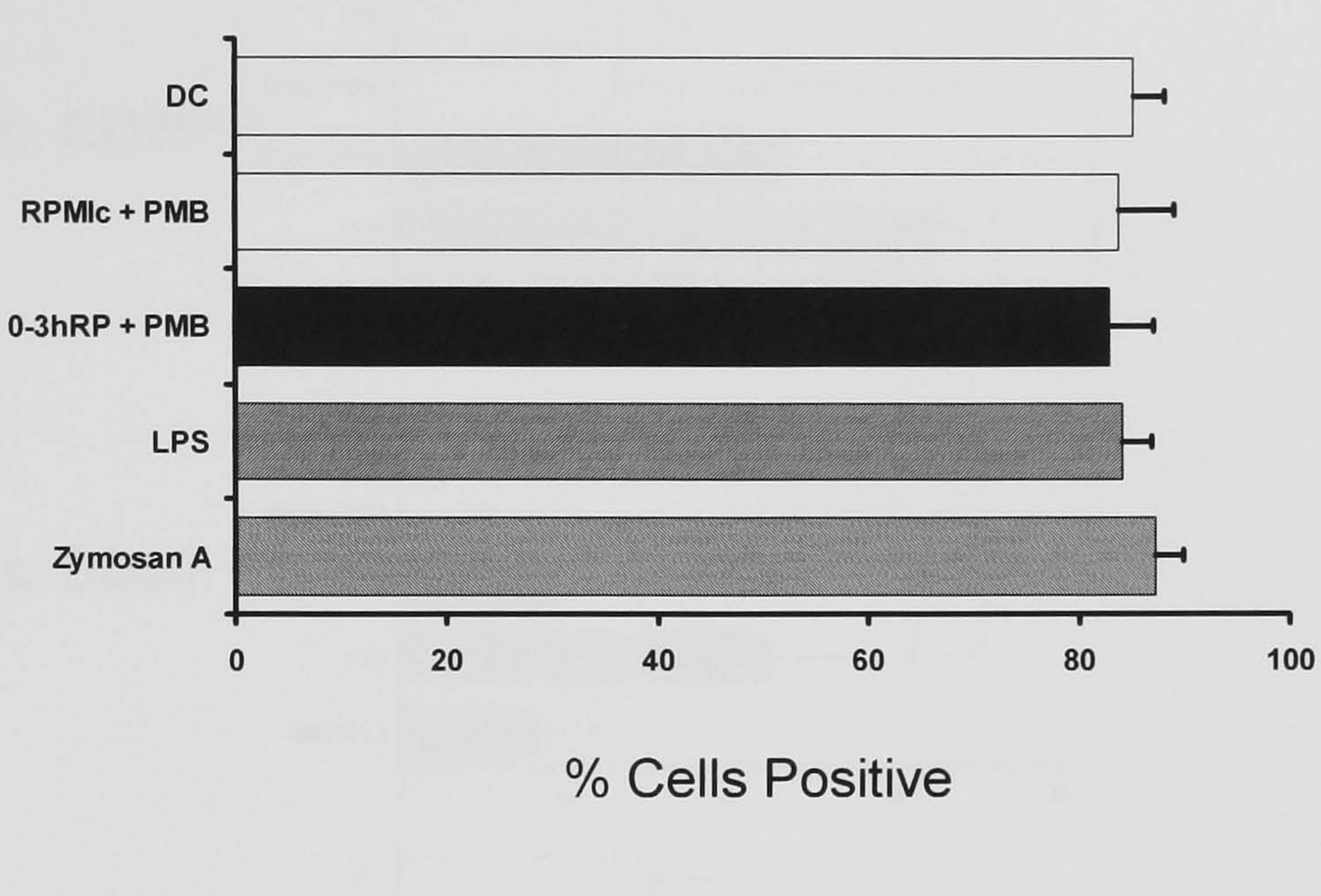


Figure 6.15 **Percentage of cells expressing CD11c$^+$ within the large granular population does not differ after culture with different PAMPs.** iDC derived from BALB/c BM were cultured overnight alone, or with RPMIc + PMB (3 μg / ml), 0-3hRP (40 μg / ml) + PMB, LPS (100 ng / ml), or Zymosan A (1.6 μg / ml). Cells were stained with antibodies specific for CD11c, or with a matched isotype control. Data is presented as the mean ± SEM of three experiments. Analysis was performed on the large granular cell population within box H (see Figure 6.9).

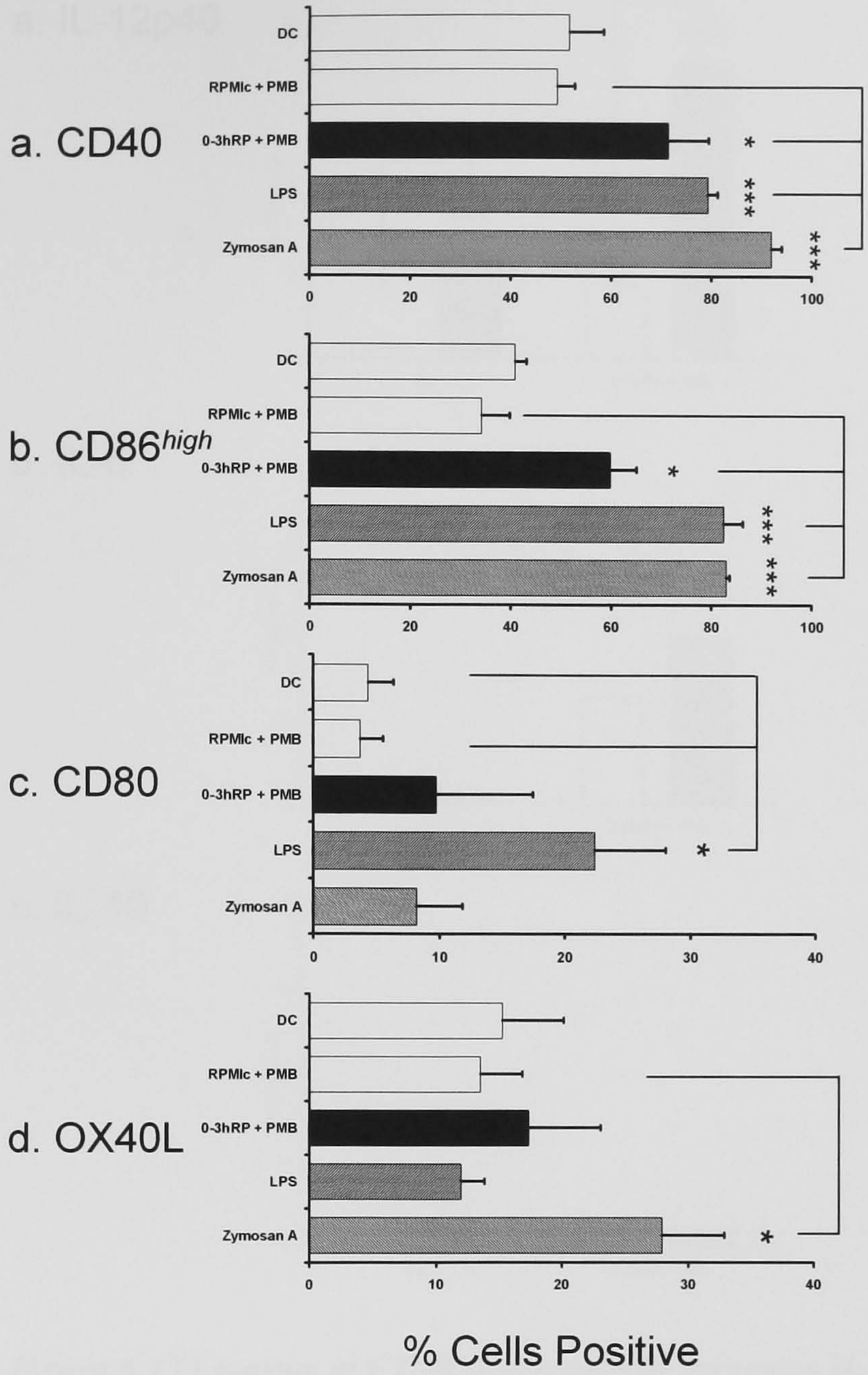
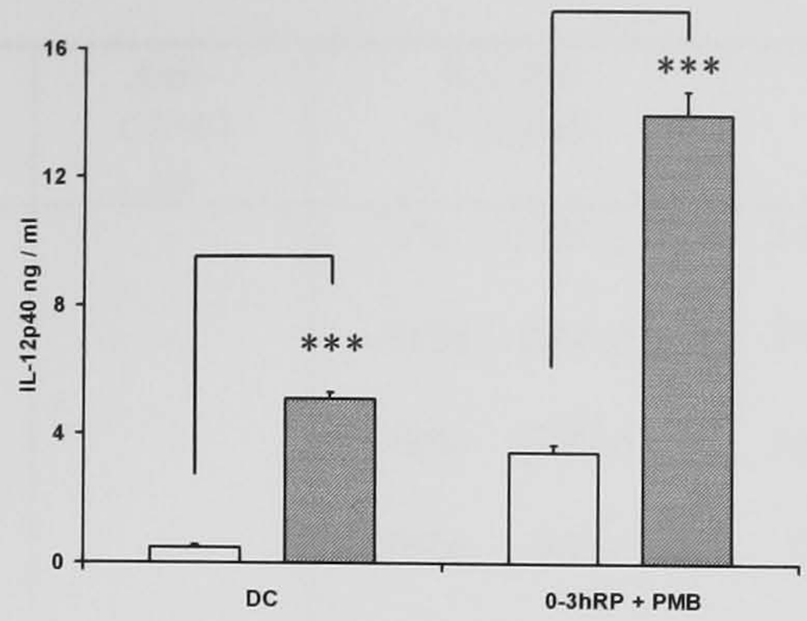
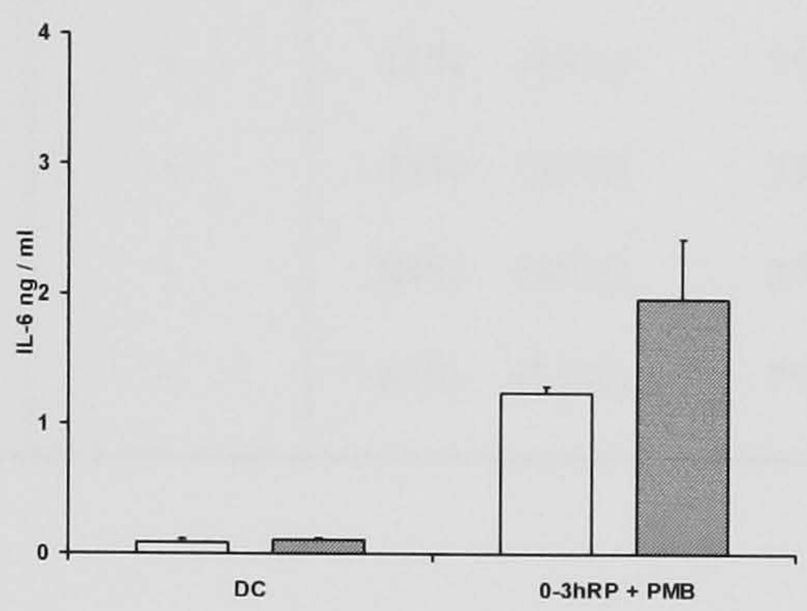


***Figure 6.16* Expression of co-stimulatory molecules on DC cultured with different PAMPs.** Percentage of cells expressing CD40 (a), $CD86^{high}$ (b), CD80 (c), or OX40L (d), was determined after BALB/c iDC were cultured alone, or with PMB (3 μg / ml), RPMIc + PMB, 0-3hRP (40 μg / ml) + PMB, LPS (100 ng / ml), or Zymosan A (1.6 μg / ml). Cells were stained with antibodies specific to CD40, CD80, CD86, OX40L, or with matched isotype controls. Analysis was restricted to large granular cells within box H. Data is presented as the mean ± SEM of 3 experiments. Levels of significance, as determined by Students *t* test, are indicated as: * = $p \leq 0.05$ and *** = $p \leq 0.001$.

a. IL-12p40



b. IL-6



c. IL-10

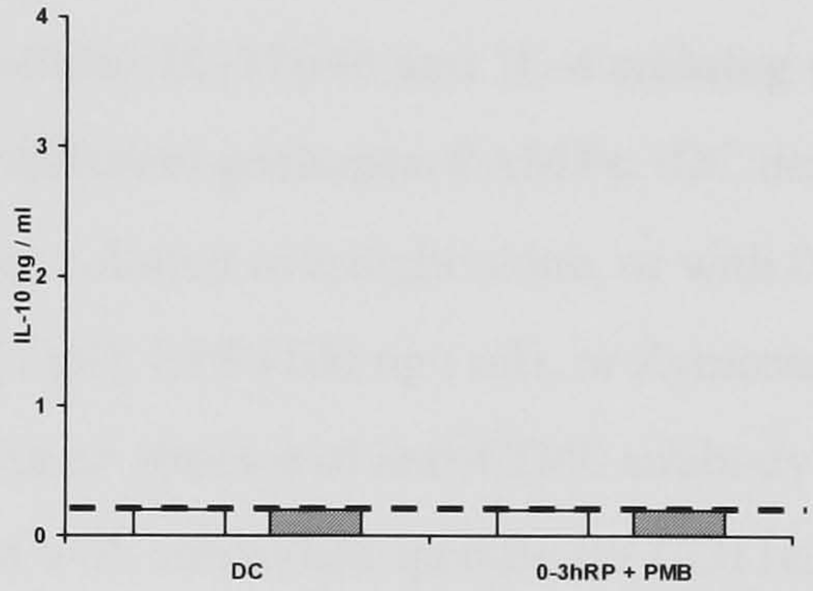


Figure 6.17 **Ligation of CD40 preferentially increases IL-12p40 production by unstimulated and 0-3hRP-stimulated DC, compared to other cytokines.** iDC derived from BALB/c BM, were cultured overnight alone, or with 0-3hRP (40 μg / ml) + PMB (3 μg / ml), in the presence (hatched bars), or absence (open bars), of anti-CD40 antibody (5 μg / ml). Supernatants were removed and analysed for expression of IL-12p40 (a), IL-6 (b), or IL-10 (c), by ELISA. Data is presented as the mean ± SEM of 3 wells, and is representative of 3 experiments. Dashed lines represent the lower limit of detection of ELISA. Levels of significance, as determined by Students *t* test, are indicated as: *** = $p \leq 0.001$.

	Anti-CD40 Ab	*BALB/c IL-12p40*	*BALB/c IL-4*
DC Only	-	**6%** (78%)	**10%** (87%)
0-3hRP + PMB	-	**11%** (94%)	**14%** (94%)
LPS	-	**72%** (89%)	**30%** (89%)
Zymosan A	-	**30%** (90%)	**7%** (82%)
DC Only	+	**21%** (88%)	**7%** (86%)
0-3hRP + PMB	+	**23%** (92%)	**13%** (89%)
LPS	+	**85%** (86%)	**37%** (88%)
Zymosan A	+	**64%** (91%)	**7%** (84%)

Table 6.4 **Intracellular IL-12p40 and IL-4 staining on DC stimulated with different pathogen PAMPs.** iDC derived from BALB/c BM, were cultured overnight alone, or with 0-3hRP (40 μg / ml) + PMB (3 μg / ml), LPS (100 ng / ml), or Zymosan A (1.6 μg / ml), in the presence or absence of anti-CD40 antibody (5 μg / ml). Cells were stained with antibodies specific for CD11c, and IL-12p40, or IL-4, or with matched isotype controls, and analysed by 2-colour flow-cytometry. The values shown in bold represent the percentage of $CD11c^+$ cells that were positive for IL-12p40, or IL-4. The values in brackets represent the $CD11c^+$ $cytokine^+$ DC as a percentage of the total $cytokine^+$ cells. The data presented for cells cultured in the absence of anti-CD40 antibody is derived from the same experiment but was presented in Table 6.3, and therefore is shown in grey type.

6.4 DISCUSSION

In this chapter, I demonstrate that the PAMP material released by transforming schistosomes (0-3hRP) stimulates maturation of iDC with a phenotype commensurate with them being potent APC. Indeed, 0-3hRP-induced maturation of iDC corresponds with up-regulation of IL-12p40 and IL-6 production, and elevated surface expression of MHC II and the co-stimulatory factors CD40 and CD86. However, 0-3hRP stimulated lower levels of cytokine production and co-stimulatory factor expression when compared to the classic pathogen PAMPs LPS and Zymosan A.

6.4.1 Generation of immature BM-derived DC

Initially, I selected and developed a robust technique for bulk *in vitro* generation of enriched iDC based on the culture of BM precursors with GM-CSF. Of two techniques, 'Plate' generation more fully satisfied the criteria set out in Section 6.2.1, and was used in subsequent experiments to generate iDC with which to study the stimulatory potential of schistosome PAMPs. Due to the notorious difficulty in establishing iDC culture of high purity, this technique was further optimised, resulting in cell populations highly enriched (up to 70%) for $CD11c^+$ DC. Indeed, following continuous optimisation, the iDC populations used in the latter experiments of this chapter, and in Chapter 7, were routinely 70% $CD11c^+$ (data not shown).

CD11c is one of the most commonly used surface markers to identify DC, and is expressed on all subsets including myeloid, lymphoid and plasmacytoid DC (Kelsall *et al.*, 2002). Further phenotypic characterisation demonstrated that the iDC used in my study were negative for the lymphoid DC marker, CD8α, and only 2% were positive for the plasmacytoid DC marker, B220. As such, I conclude the $CD11c^+$ DC used in this study were myeloid in origin. In addition, most of the $CD11c^+$ cells expressed low levels of F4/80. Although F4/80 is predominantly a Mϕ marker, it is expressed on LC (Hume *et al.*, 1983), some *ex vivo* myeloid DC (Henri *et al.*, 2001), and at low levels on BM-derived DC (Lutz *et al.*, 1999; Reid *et al.*, 2000), further supporting the conclusion that the cells generated in this study were myeloid DC.

A large proportion of the iDC population were also MHC II^+ (48%). Two-colour flow-cytometry revealed that 95% of the MHC II^+ cells were $CD11c^+$. The expression of MHC II is indicative of the maturation state of the DC. In this context, less than 50% of the cells expressed high levels of MHC II, demonstrating that the majority of DC were immature. Therefore, the DC culture technique used in this study is more appropriate than the recently described 'higher yield' method, which utilises BM cultured with both IL-4 and GM-CSF, and results in DC with a MHC II^{high} mature phenotype (Son *et al.*, 2002). In this context, the iDC used in this study more closely resemble DC which schistosome larvae would likely encounter in the skin. The spontaneous maturation of a proportion of the iDC in my cultures may have been caused by physical trauma during the culture period (*i.e.* through pipetting / changing of media), to which DC are sensitive (Gallucci *et al.*, 1999).

The minor population of $CD11c^-$ cells were almost completely MHC II^- (95%), and partially $Gr\text{-}1^+$, corresponding to previous reports that iDC generated after 6 days also contain low levels of myeloid precursor cells and granulocytes (Lutz *et al.*, 1999). In this respect, the $CD11c^+$ and $CD11c^-$ cells within the iDC, and mDC, populations could be divided upon the basis of their size and granularity. This allowed for analysis of surface marker expression to focus upon the large highly granular population, confident that this contained nearly all the $CD11c^+$ cells, and was almost exclusively $CD11c^+$.

6.4.2 Schistosome PAMPs stimulate DC maturation: cytokine production

Having defined the enriched iDC population, these cells were used to analyse the effect of schistosome PAMPs on DC maturation. In an initial screen of the schistosome PAMP preparations only 0-3hRP had the capacity to induce pro-inflammatory and regulatory cytokine production. This work supports the results obtained in Chapter 3 using iMϕ, which show that the material released by schistosome larvae upon transformation is highly enriched for parasite PAMPs compared to soluble preparations of whole larvae. Therefore, the effect of 0-3hRP upon DC was further characterised; the DC response was also compared to that induced by LPS or Zymosan A. In addition to acting as positive controls, these well-documented pathogen PAMPs were chosen as examples of molecules that instruct DC to mature to a Th1-inducing phenotype (LPS; Whelan *et al.*, 2000; MacDonald *et al.*, 2001), or a Th2-inducing phenotype (Zymosan A; Manickasingham *et al.*, 2003).

I show for the first time that molecules released by transforming schistosome larvae (0-3hRP) stimulate up-regulation of IL-12p40 production by iDC. This corresponds with an increase in the number and intensity of staining of IL-12p40$^+$ CD11c$^+$ mDC. Importantly, staining for intracellular IL-12p40 was restricted to the CD11c$^+$ population (94% - 96%), demonstrating that the myeloid DC were the source of this cytokine. Although splenic myeloid DC appear to produce little IL-12p40 upon stimulation *in vitro* (Edwards *et al.*, 2002), BM-derived iDC are well known to produce large amounts in response to maturation with numerous different pathogens or pathogen PAMPs, such as LPS, CpG DNA motifs (Sparwasser *et al.*, 1998), whole live *Mycobacterium tuberculosis* (Hickman *et al.*, 2002), or *Propionebacterium acnes* (MacDonald *et al.*, 2001). In addition, myeloid DC are the source of abundant IL-12p40 produced by *in vitro* cultured skin biopsies from mice vaccinated with γ-irradiated *S. mansoni* cercariae (Hogg *et al.*, 2003a).

IL-12 is a pro-inflammatory cytokine (Ma and Trinchieri, 2001). Moreover, IL-12 increases blastogenesis of human T-cells (Kubin *et al.*, 1994) and has potent Th1 polarising activity (Manetti *et al.*, 1994; Macatonia *et al.*, 1995; Trinchieri, 2003). Although the biologically active heterodimer IL-12p70, which possesses the pro-inflammatory / Th1 polarising function, was not detected in the culture supernatant of DC stimulated with 0-3hRP (DC/0-3hRP), it is probably released but at lower levels than detected by the ELISA assay. In this respect, analysis of the supernatants from DC stimulated with LPS (DC/LPS) or Zymosan A (DC/Zymosan A) demonstrated that IL-12p70 is released at concentrations approximately 1000-fold less than IL-12p40. Since IL-12p70 is a heterodimer of the p40 and p35 subunits, most of the IL-12p40 detected probably represents monomeric p40, or the IL-12p70 antagonist p40 homo-dimer (Gillessen *et al.*, 1995). However, the p40 sub-unit also complexes with a p19 sub-unit cytokine to form IL-23, which is known to be produced by DC (Oppmann *et al.*, 2000). IL-23 is thought to share many of the properties of IL-12p70, including driving IFNγ production by T-cells (Oppmann *et al.*, 2000), although a recent report suggests that it may possess greater pro-inflammatory properties (Cua *et al.*, 2003). Therefore, the presence of IL-23 in the DC supernatant cannot be excluded.

In contrast to IL-12p40, 0-3hRP did not stimulate production of IL-10, a potent anti-inflammatory cytokine, thought to promote Th2 polarisation through the inhibition of Th1 responses. In addition, IL-10 is also involved in the development of 'Treg' cells (McGuirk *et al.*, 2002). IL-10 is known to regulate IL-12p40 production in the skin and sdLN following vaccination with irradiated cercariae (Hogg *et al.*, 2003b). Moreover, IL-12 (and / or IL-23?) exerts reciprocal regulation of IL-10 expression in the skin (Hogg *et al.*, 2003b). Therefore, the control of IL-12p40 and IL-10 production may considerably affect the outcome of the innate and acquired responses during normal infection. In this respect, the profile of cytokines produced by DC stimulated with 0-3hRP greatly contrasts that of iMϕ, which produced high levels of IL-10 and low levels / no IL-12p40 (section 3.3.3). Thus, the balance of IL-12 *versus* IL-10 production in the skin during infection with normal schistosomes may reflect the relative dominance of DC *versus* Mϕ responses.

In this chapter I also show that 0-3hRP stimulates up-regulation of IL-6 production by iDC. Similar to IL-12, iDC are known to produce IL-6 in response to live pathogens, or PAMPs (Sparwasser *et al.*, 1998; MacDonald *et al.*, 2001). Therefore, although intracellular staining for IL-6 was not performed, it is most likely that $CD11c^+$ DC were the cellular source of this cytokine. Similar to IL-12, IL-6 also acts directly upon T-cells to increase proliferation during priming (Vink *et al.*, 1990). Thus, the up-regulated production of IL-6 and IL-12p40 by 0-3hRP-matured DC suggests these cells will be more effective at priming T-cells.

The direct effect of IL-6 early in the process of T-cell polarisation remains unclear, although it has been linked to Th2 induction (Rincon *et al.*, 1997; LaFlamme *et al.*, 2000; Moser, 2001; Diehl *et al.*, 2000; 2002). On the other hand, IL-4 is known to be a potent driver of Th2 responses, and therefore production of this cytokine was determined. Using intracellular flow-cytometry, an increase in $IL\text{-}4^+$ DC was observed after stimulation with 0-3hRP, although no IL-4 was detected in the culture supernatants. It has been reported that DC can be positive for intracellular IL-4 (Kelleher *et al.*, 1999), yet it has only rarely been detected in culture supernatants (d'Ostiani *et al.*, 2000). Indeed, my data contrasts with that of MacDonald *et al.* (2001) who found no up-regulation of IL-4 mRNA by BM-derived DC stimulated with a variety of PAMPs (including LPS), although it is possible that differential regulation of this cytokine occurs at translation rather than transcription.

Since IL-4 has potent autocrine effects on DC, including up-regulation of MHC II (Son *et al.*, 2002), it has been suggested that IL-4 may be released by stimulated DC but immediately bound by the surface receptors (Kelleher *et al.*, 1999). Although IL-4 was not detected in the DC/0-3hRP supernatants, the intimate localisation of T-cells with DC during priming could result with paracrine functions of this cytokine.

The profile of DC cytokine production stimulated by 0-3hRP differs greatly to that stimulated by the classical pathogen PAMPs LPS and Zymosan A. DC/0-3hRP produced considerably less IL-12p40 and IL-6 than either DC/Zymosan, or DC/LPS. Moreover, both DC/Zymosan and DC/LPS produced detectable quantities of IL-12p70, IL-10, and TNFα. This suggests that 0-3hRP instruct DC in a different manner to these PAMPs, leading to an 'intermediate' state of maturation. There was also a significant difference in the profile of cytokine production stimulated by Th1-associated LPS compared to Th2-associated Zymosan A. Therefore, my data supports the hypothesis that DC possess substantial plasticity in their cytokine response to different PAMPs (Whelan *et al.*, 2000; MacDonald *et al.*, 2001; de Jong *et al.*, 2002; Edwards *et al.*, 2002). Interestingly, Zymosan A stimulated more IL-12p70 but less IL-10 and IL-6, compared to LPS, which is more suggestive of a Th1 profile, contrasting recent findings that all splenic DC subsets produce IL-10 rather than IL-12 in response to Zymosan A (Edwards *et al.*, 2002). In addition, LPS stimulated a large increase in the number IL-4$^+$ DC, which is indicative of Th2 promotion. Therefore, from the cytokine profiles it is very difficult to predict whether DC matured with 0-3hRP would preferentially prime for a Th1 or Th2 response. Indeed, a recent study using adoptive transfer of IL-12p40-, or IL-4-, deficient DC demonstrated that production of IL-4 was not necessary to prime a Th2 response, whereas production of IL-12p40, although required for maximal T-cell IFNγ production, was not necessary to prime a Th1 response (MacDonald and Pearce, 2002).

6.4.3 Schistosome PAMPs stimulate DC maturation: MHC II expression

Up-regulation of MHC II expression is explicitly important for T-cell priming, and high-level expression of MHC II is commonly used to define mature DC. In this chapter I demonstrate that 0-3hRP stimulates maturation of iDC, corresponding with an increase in the number of MHC II^{high+} cells, suggesting that DC/0-3hRP will have a greater capacity

to present antigen to T-cells. However, compared to DC/LPS, or DC/Zymosan A, fewer DC matured with 0-3hRP expressed high levels of MHC II, concurrent with the hypothesis that 0-3hRP matures DC in a different manner to LPS, or Zymosan A, and leads to an intermediate state of activation.

6.4.4 Schistosome PAMPs stimulate DC maturation: co-stimulatory factor expression

Up-regulation of co-stimulatory factor expression is another way in which DC enhance T-cell priming. Indeed, ligation of T-cell CD28 by co-stimulatory CD80, or CD86, augments TCR-mediated proliferation, IL-2 production, and T-cell survival (Boise *et al.*, 1995), and is essential for normal T-cell cytokine responses (Whelan *et al.*, 2000), while the absence of this co-stimulatory pathway promotes T-cell anergy (Van Gool *et al.*, 1999). Expression of CD40 on DC also contributes to the survival signal, helping to prevent T-cell anergy by CD154 (CD40L) signalling (Van Gool *et al.*, 1999). In addition, T-cell expansion is maintained by anti-apoptotic signals received through OX40 via DC-expressed OX40L (Rogers *et al.*, 2001).

In my studies, 0-3hRP was shown to induce the up-regulation of surface CD40 and CD86 but not CD80 upon DC. Since the expression of either CD80, or CD86, by DC is sufficient for normal T-cell priming through CD28 (Whelan *et al.*, 2000), 0-3hRP-stimulated DC should more effectively prime T-cell responses, through greater ligation of CD28 by CD86 and CD154 by CD40. Although DC/0-3hRP did not up-regulate surface OX40L compared to DC cultured alone (DC/media), the constitutive low level expression of this factor may be sufficient to prevent apoptosis of expanding T-cells. However, both LPS and Zymosan A stimulated greater up-regulation of CD40 and $CD86^{high}$ expression than 0-3hRP, again suggesting that schistosome PAMPs drive an 'intermediate' state of maturation. There was also a considerable difference in expression of CD80 and OX40L between DC/LPS and DC/Zymosan A. Therefore, my data agrees with the observations that, in addition to cytokine production, DC possess substantial plasticity in their regulation of co-stimulatory factor expression in response to stimulation with different PAMPs (Whelan *et al.*, 2000; MacDonald *et al.*, 2001; de Jong *et al.*, 2002).

The role of co-stimulatory factors in T-cell polarisation is the subject of much debate, fuelled by observations that they are differentially regulated upon exposure to different

PAMPs. Since only LPS stimulated up-regulation of CD80, this could suggest this co-stimulatory molecule may have a role in Th1 induction. Indeed, polar roles for CD80 and CD86 have been reported, with CD80 involved in Th1 and CD86 involved in Th2 commitment (Kuchroo *et al.*, 1995). However, CD86 has been implicated in Th1 priming by splenic DC (Moser, 2001). Conversely, another group reported that co-stimulation through CD28 *per. se.* promoted Th2 responses (Rulifson *et al.*, 1997), supporting observations that CD28-deficient mice generate diminished Th2 but not Th1 cytokine production, in response to deposition of schistosome eggs (King *et al.*, 1996b). More recent data suggests that the role of CD80 / CD86 may chiefly be to promote T-cell responses rather than influencing T-cell differentiation (Schweitzer *et al.*, 1997; Whelan *et al.*, 2000).

Co-stimulation via OX40L expression has also been linked to T cell polarisation (Flynn *et al.*, 1998; Ohshima *et al.*, 1998; de Jong *et al.*, 2002) but again its role is controversial (MacDonald *et al.*, 2001). My results conform with a possible role of OX40L in Th2 responses, since Th2-associated Zymosan A but not Th1-associated LPS, stimulated an increase in the number of DC expressing OX40L. Since 0-3hRP did not stimulate increased OX40L expression this suggests that it may not drive Th2 polarisation.

The effect of CD40 / CD154 interactions on T-cell priming and polarisation is also the focus of intense study. There is a strong case for the involvement of CD40 / CD154 in Th2 induction. *In vitro*, human T-cell production of IL-4 induced by anti-CD3 and anti-CD28 requires co-stimulation through CD154 (Blotta *et al.*, 1996). Furthermore, adoptive transfer of CD40 $^{-/-}$ DC into wild type recipients demonstrated that CD40 expression is essential for the Th2 response induced by SEA-matured DC but not for the Th1 response driven by DC matured with *Propionibacterium acnes* (MacDonald *et al.*, 2002a). In addition, the Th2 responses that develop during *S. mansoni* infection, or after injection with schistosome eggs, are impaired in CD154 $^{-/-}$ mice (MacDonald *et al.*, 2002a; 2002b). However, DC expression of CD40 has also been associated with IL-12-dependent induction of Th1 responses by murine and human DC *in vitro* (Cella *et al.*, 1996; Reudl *et al.*, 2000).

In addition to its role as a co-stimulatory molecule for T-cells, CD40 also allows DC to receive feedback from T-cells during priming. Since T-cells express surface CD154 immediately upon activation by APC (Schonbeck and Libby, 2001), the feedback DC

receive is restricted to antigen-specific interactions. There is an increasing body of evidence to suggest that CD154 is expressed on the surface of some accessory cells, including certain DC subsets, indicating that accessory cells could provide feedback to each other via CD40 to amplify innate responses (Schonbeck and Libby, 2001). A recent study suggests that CD40 / CD154 interactions are essential for maintaining DC activation during infections that result in either Th1 (*T. gondii*) or Th2 (*S. mansoni*) responses (Straw *et al.*, 2003). It is well established that ligation of CD40 can cause increased / high level production of IL-12 by both non-stimulated murine and human DC *in vitro* (Koch *et al.*, 1996; Cella *et al.*, 1996; Reudl *et al.*, 2000), and PAMP-stimulated murine DC *in vivo* (Schulz *et al.*, 2000). Indeed, in this chapter I showed that signalling via CD40 following addition of anti-CD40 antibody increased IL-12p40 production by DC/0-3hRP 10-fold, corresponding with an increased frequency of IL-12p40$^+$ cells. Increased IL-12 production following CD40 ligation could help amplify developing Th1 responses (Cella *et al.*, 1996; Schulz *et al.*, 2000), suggesting that 0-3hRP may have Th1-driving capacity. However, it has recently been hypothesised that CD40 signalling acts more as an amplification signal for DC to increase the cytokine profile already programmed by PAMP recognition. In this respect, DC can produce increased IL-12, or IL-10, depending on the initial PAMP stimulus received (Edwards *et al.*, 2002). Since stimulation of DC/0-3hRP with anti-CD40 antibody resulted in increased IL-12p40 production but no increase in the frequency of IL-4$^+$ cells, no detectable IL-10 production, and only a marginal increase in IL-6 production, suggests that DC matured with 0-3hRP will not display increased Th2-associated cytokines during T-cell priming, and will therefore have limited Th2-driving capacity.

6.4.5 Schistosome PAMPs stimulate DC maturation: an intermediate state?

A recent report suggests that because of the extensive plasticity of DC responses, maturation cannot be defined by a simple set of markers (Huang *et al.*, 2001). In this respect, the 'intermediate' DC maturation phenotype induced by 0-3hRP is reminiscent of that revealed in studies of the nematode products ES-62 (Whelan *et al.*, 2000) and SEA (MacDonald *et al.*, 2001). Specifically, SEA matures BM-derived DC to express intermediate levels of MHC II compared to LPS, yet without any increase in production of IL-12p40, IL-12p70, IL-6, IL-4, or TNFα, or any increase in expression of CD40, CD80, CD86, or OX40L (MacDonald *et al.*, 2001). Similarly, ES-62 matures BM-derived DC

without any increase in co-stimulatory factor expression and with limited up-regulation of IL-12p70, compared to LPS (Whelan *et al.*, 2000). Despite these very limited activation states, DC matured with ES-62, or SEA, were both potent drivers of Th2 responses (Whelan *et al.*, 2000; MacDonald *et al.*, 2001). However, although the maturation state induced by 0-3hRP bears similarity to that reported for SEA and ES-62, DC/0-3hRP do appear to have a more highly activated phenotype, evident by their increased IL-12p40 and IL-6 production, and up-regulated CD40 and CD86 expression. Moreover, in contrast to 0-3hRP, both ES-62 and SEA stimulated some up-regulation of IL-10 production. Therefore, whilst the limited activation state of DC/0-3hRP might suggest a potential to drive Th2 responses, the up-regulated pro-inflammatory cytokine production and co-stimulatory factor expression suggests otherwise.

As a point of note, when compared to DC generated from C57Bl/6 mice, BALB/c iDC appeared to be more sensitive the stimulatory actions of 0-3hRP, demonstrated by a greater fold-increase in cytokine production and expression of MHC II and co-stimulatory factors. One potential explanation for this observation could be the differential expression of PRRs upon these cells (Liu *et al.*, 2002). Alternatively, there could be differences in the responses to receptor ligation between mouse strains, however, this is an area in which further research is awaited.

6.4.6 Summary

In summary, soluble schistosome PAMPs released during transformation (0-3hRP) stimulate maturation of iDC, corresponding with up-regulation of MHC II and co-stimulatory factor expression, and increased cytokine production. This suggests that DC stimulated with 0-3hRP may play a role in developing the innate response, and have an increased capacity to prime T-cell responses. However, the maturation state of DC/0-3hRP appears limited compared to that induced by Th1-driving LPS and Th2-driving Zymosan A, which both stimulated greater overall expression of cytokine, MHC II, and co-stimulatory factors. The specific maturation profiles of these control PAMPs were significantly different to each other, but because maturation with 0-3hRP differed from both LPS, and Zymosan A, it is difficult to speculate what effect DC/0-3hRP would have on T-cell polarisation. In this respect, 0-3hRP stimulated a DC maturation state not unlike that reported for the potent helminth-derived Th2 drivers, ES-62 and SEA. Therefore,

further investigation is required into the outcome of T-cell priming by DC matured with 0-3hRP in order to more fully understand how interactions between schistosome PAMPS and the innate immune system may effect the polarisation of the acquired immune response.

CHAPTER 7

PRIMING AND POLARISATION OF THE ACQUIRED IMMUNE RESPONSE BY DC MATURED WITH RELEASED SCHISTOSOME PAMPS

7.1 INTRODUCTION

The generation of cell-mediated or humoral acquired immunity can be critical to the outcome of infections, and is characterised by selective clonal expansion of Th1 or Th2 effector cells respectively. Generation of the correct type of response can confer protection against pathogens or disease, whereas the incorrect response may lead to immune-related, or disease-related, pathology. Many factors have been implicated in influencing differentiation of naïve T-cells towards committed Th1 or Th2 effector lineages, but of critical importance is the outcome of the innate immune response during infection (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997).

In this respect, DC form the link between the innate immune system and the acquired immune response, initiating naïve Th cell expansion and differentiation by delivering antigenic (signal 1) and co-stimulatory (signal 2) messages. Furthermore, recent work has demonstrated that DC are highly plastic, with the ability to express a range of differential maturation phenotypes upon interpretation of the nature of the pathogen (via PAMPs), or the disease state (via tissue factors and cytokines). This flexibility allows the DC to prime for either biased Th1-type or Th2-type responses, or mixed Th1 / Th2 responses (possibly representing Th0 cells) (Whelan *et al.*, 2000; MacDonald *et al.*, 2001; de Jong *et al.*, 2002; Manickasingham *et al.*, 2003), and has led to the concept that DC deliver a third 'polarising' signal (signal 3) to the expanding T-cell population (Kalinski *et al.*, 1999).

In Chapter 6, I have described how 0-3hRP drives DC maturation which is characterised by up-regulated MHC II expression, co-stimulatory factor expression, and cytokine production. However, whether stimulation with 0-3hRP increases the ability of DC to prime naïve T-cells remains to be established. Furthermore, despite analysis of their maturation state, it remains difficult to predict whether DC matured with 0-3hRP would drive a biased Th1 or Th2 T-cell response, or a non-polarised response.

To determine the outcome of T-cell priming by 0-3hRP-matured DC requires a neutral antigen-presentation assay from which the T-cell response can be quantified and differentiation towards the Th1 or Th2 poles established.

Both *in vitro* and *in vivo* assays of T-cell priming have been described. Since many factors can affect the outcome of priming, including the antigen dose (*e.g.* Reudl *et al.*, 2000; Bonnstra *et al.*, 2003), and APC : T-cell ratio (Tanaka *et al.*, 2000; Manickasingham *et al.*, 2003), *in vitro* T-cell priming assays, in which DC are cultured directly with T-cells, have the benefit that conditions can be much better defined, and are useful for determining how effective DC are in driving primary responses. In comparison, *in vivo* T-cell priming assays, in which antigen-loaded DC are injected into recipient mice and effector cells subsequently re-stimulated *in vitro*, offer the distinct advantage that the function of the DC can be determined in physiological context.

Transgenic (*Tg*) mice are available that contain re-arranged TCRα and TCRβ genes, such that their germline DNA encodes a TCR specific for a peptide fragment. Several types of TCR *Tg* mice have been created, such as the DO11.10 strain which express TCRs specific for a chicken ovalbumin peptide ($OVA_{323-339}$) bound to I-A^d MHC II (Murphy *et al.*, 1990), or a strain which recognise a pigeon cytochrome C peptide bound to I-E^k. Using antigen-restricted *Tg* T-cells in both *in vitro* and *in vivo* priming assays has the benefit that the frequency of antigen-specific responder cells is greatly increased, allowing for greater sensitivity in detecting differences between T-cell responses. In addition, this system controls for any variation in the antigenicity of different PAMP preparations, such that only the effect on DC polarising ability is studied. This antigen-restricted T-cell priming system has become widely used, although similar studies have employed various different assay regimes (Whelan *et al.*, 2000; Manickasingham *et al.*, 2003; Boonstra *et al.*, 2003). Most importantly, in some studies DC are matured with PAMPs prior to *in vitro* culture with T-cells (Whelan *et al.*, 2000), whereas others report co-culture of T-cells and DC in the presence of PAMPs (Manickasingham *et al.*, 2003). Since some potential components of 0-3hRP may cause T-cell apoptosis and inhibition of proliferation (Vieira *et al.*, 1986; Chen *et al.*, 2002), the former system has the distinct advantage that these PAMPs can be removed prior to T-cell assay, and will thus be used in this study.

Th1 and Th2 cell populations are predominantly characterised by their differential production of IFNγ and IL-4, respectively. Therefore many studies determine levels of T-cell polarisation by measurement of these 'signature' cytokines. In addition, other cytokines can also be used to indicate Th2 polarisation, such as IL-5. Since it is generally accepted that IL-4 is more potent than IFNγ, direct comparisons of cytokine quantities are

obsolete, rather it is more relevant to compare cytokine ratios. Therefore, the T-cell polarising capacity of DC matured with 0-3hRP will be compared to those matured alone, with LPS, or with Zymosan A, as controls for cells priming mixed Th1 / Th2, or polarised Th1, or Th2 responses respectively (Whelan *et al.*, 2000; Manickasingham *et al.*, 2003).

Therefore, the effect of 0-3hRP on the ability of DC to prime both T-cell proliferation and polarisation will be assessed using an *in vitro* assay. In addition, the effect of 0-3hRP on the T-cell polarising capacity of DC will be determined *in vivo*. Polarisation will be determined by production of 'signature' T-cell cytokines, and will be compared to mixed Th1 / Th2, and polarised Th1 and Th2 control profiles.

7.2 MATERIALS AND METHODS

7.2.1 Mice

A breeding colony of DO11.10 αβ TCR *Tg* mice (on a BALB/c background) were a gift from Paul Garside (University of Glasgow), and were maintained in-house at the University of York. BALB/c mice were obtained from Harlan UK Ltd, Bicester, UK. Mice were age and / or sex matched in individual experiments.

7.2.2 Generation of differentially-matured DC

Bone marrow from BALB/c mice (Harlan UK Ltd) was used to generate iDC by the 'Plate' method, as previously described (Section 6.2.1). The iDC were stimulated overnight in 24-well plates with the various pathogen PAMPs, as previously described (Section 6.2.3). Briefly, cells were cultured overnight alone, or with 0-3hRP (20 - 40 μg / ml) and PMB (3 μg / ml), LPS (100 ng / ml), or Zymosan A (1.6 μg / ml). In some experiments, additional controls of iDC cultured with PMB, or PMB and a concentration of LPS equivalent to the low levels of endotoxin contained within 0-3hRP, were included. For assays in which the resulting PAMP-matured DC (mDC) were to be used for *in vivo* T-cell priming (Section 7.2.4), $OVA_{323-339}$ (323-ISQAVHAAHAEINEAGR-339) peptide (100 nM; Albachem, University of Edinburgh, UK) was also added the DC cultures. Culture of iDC with $OVA_{323-339}$ alone did not result in increased cytokine production, providing confirmation that the peptide was free of endotoxin. Additionally, in some

experiments low-endotoxin anti-CD40 antibody (5 μg / ml; clone HM40-3; BD PharMingen) was added to the culture at the time of DC exposure to PAMPs. After overnight maturation, mDC were collected and washed 3 times in 5 ml RPMI 1640 prior to use in either *in vitro* (Section 7.2.3), or *in vivo* (Section 7.2.4), T-cell priming assays.

7.2.3 *In vitro* T-cell priming assay

7.2.3.1 Purification of splenic $CD4^+$ cells

$CD4^+$ cells were purified from spleens of DO11.10 *Tg* mice, using Magnetic-Activated Cell Sorting (MACS), following the manufacturer's instructions. Throughout this process the cells were kept sterile. Briefly, spleens were removed and placed in DCP media, consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin (200 U / ml), streptomycin (100 μg / ml), L-glutamine (2 mM), and β-mercaptoethanol (50 μM). Single cell suspensions were created using 40 μm cell strainers (Falcon, Becton-Dickenson, Oxford, UK). Cells were centrifuged at 350 *g*, for 5 min, and the supernatant discarded. The cells were then incubated with ACK buffer (10 ml; Section 4.2.2) for 5 min at RT to lyse red blood cells. ACK buffer was then quenched with DCP (10 ml) and the cells washed once in DCP (10 ml). Splenocytes were counted using a haemocytometer and re-suspended in chilled de-gassed MACS buffer (MACSB; pH 7.2; PBS containing 2mM EDTA and 0.5% BSA; 90 μl / 10^7 cells). Anti-CD4 microbeads (Miltenyi Biotec, Bisley, UK ; 10 μl / 10^7 cells) were incubated with the cell suspension for 15 min at 4 °C. The splenocytes were then washed and resuspended in MACSB, and $CD4^+$ cells positively selected by passing the suspension through a MACS MS^+ column (Miltenyi Biotec). Flow-cytometric analysis determined the purified cells to be > 90% $CD4^+$ (data not shown).

7.2.3.2 Priming of DO11.10 $CD4^+$ splenocytes by differentially matured DC

T-cell proliferation assays were performed in 96-well plates (200 μl), whereas cytokine assays were performed in 96-well (200 μl), or 24-well (1 ml), plates. Purified $CD4^+$ splenocytes (2.5 x 10^5 cells / ml) were cultured with γ-irradiated (1500 krads) mDC (2.5 x 10^4 cell / ml; Section 7.2.2) for 72 hr in DCP media with, or without, various concentrations of $OVA_{323-339}$ (1 - 100 nM).

For the proliferation assay, cells were then pulsed with [^{3}H]-Thymidine (0.5 μCi / well; Amersham Pharmacia), and harvested 18 hr later using a Packard Cell Harvester (Packard, Pangbourne, UK). Cell proliferation was determined according to the incorporation of ^{3}H-thymidine into DNA using a TopCount^{TM} scintillation counter (Packard).

Alternatively, to determine T-cell cytokine production, phorbol myristate acetate (50 ng / ml) and ionomycin (500 ng / ml; Sigma-Aldrich) were added to the cells after the first 72 hr of culture. The culture supernatants were then removed 24 hr later and frozen at -20 °C.

7.2.4 *In vivo* T-cell priming assay

For *in vivo* T-cell priming, mDC (3 - 4.5 x 10^5 cells in 100 μl) were administered to recipient naïve DO11.10 *Tg* mice by subcutaneous injection over the sternum. After 7 days, the spleens and axillary lymph nodes (sdLN) were removed aseptically into RPMI 1640 and single cell suspensions created using 40 μm cell strainers. Cells were then washed, transferred to 96-well plates (sdLN cells, 2 x 10^5 cells / well; splenocytes, 4 x 10^5 cell / well), and cultured with $OVA_{323-339}$ peptide (100 - 1000 nM), or plate-bound anti-CD3ε mAb (0.25 μg / well; Clone 145-2C11; BD PharMingen). To prepare plate-bound anti-CD3ε mAb, wells were coated with antibody in PBS (30 μl), incubated for 2 hr at 37 °C, and then washed 3 times with PBS (150 μl). Cells were cultured in RPMI 1640 supplemented with 3% heat-inactivated normal mouse serum (instead of FCS; produced in-house), penicillin (200 U / ml), streptomycin (100 μg / ml), L-glutamine (2 mM), and β-mercaptoethanol (50 μm). After 3 days, culture supernatants were removed and frozen at -20 °C to await detection of cytokines by ELISA.

7.2.5 Cytokine ELISAs

Culture supernatants were tested neat, or at a 1:2 dilution, for the presence of IL-4, IL-5 and IFNγ, using ELISA (Section 3.2.6 & 6.2.5). Lower limits of detection are shown on figures as dashed lines or arrows. The coating and detecting antibody clones for IL-5 (TRFK5 and TRFK4, respectively) were obtained from BD PharMingen, whereas the recombinant standard was from R&D Systems.

7.2.6 Statistics

Comparisons of data within an individual experiment were tested for significance using the two-tailed Students *t*-test, assuming equal variance if $p > 0.05$ when tested with the Levene's test. Alternatively, data was expressed as the log value of the fold-increase in cytokine production when compared to the mean value produced by cells primed with DC/media. These values were then pooled from multiple individual experiments. The mean log data from pooled experiments was then tested for significance difference from a theoretic value of 0 (equivalent to a 1-fold increase, which represents cells primed with DC/media) using the two-tailed one-sample Students *t*-test. Values where $p \leq 0.05$ were considered to be significant, whereas values where $p \geq 0.05$ were considered not significant. The following nomenclature was used to denote the value of significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

7.3 RESULTS

7.3.1 DC matured with 0-3hRP have increased capacity to prime T-cells

An *in vitro* 'antigen-restricted' assay was used to determine whether DC matured with 0-3hRP, or other control PAMPs, acquired a phenotype exhibiting increased capacity to prime T-cells. In this assay, differentially-matured DC were cultured with purified $CD4^+$ T-cells from DO11.10 *Tg* mice, in the presence of varying concentrations of the model antigen (OVA). Subsequent proliferation was assessed by measurement of DNA synthesis, via the incorporation of ^{3}H-Thymidine.

Compared to DC matured with media alone (DC/media), or with LPS and PMB (DC/LPS + PMB), DC matured with 0-3hRP and PMB (DC/0-3hRP) primed for a significant increase in antigen-specific T-cell proliferation in the presence of 1 and 10 nM OVA (Figure 7.1a). Moreover, this heightened capacity to prime T-cell proliferation was enhanced by maturing DC with a greater concentration of 0-3hRP (Figure 7.1a). Background proliferation of purified $CD4^+$ splenocytes was minimal (up to 16-fold less than DC-media; Figure 7.1a). As expected, γ-irradiated DC cultured without T-cells did not proliferate at all, demonstrating that they did not contribute to the proliferation observed in

the mixed T-cell cultures (data not shown). DC matured with LPS (DC/LPS) or Zymosan A (DC/Zymosan A) also exhibited an increased capacity to drive T-cell proliferation compared to DC-media (Figure 7.1b).

7.3.2 0-3hRP instructs DC to prime for Th2 polarisation *in vitro*

To determine if DC/0-3hRP polarised the expanding T-cell population, the profile of Th1-signature (IFNγ) and Th2-signature (IL-4) cytokine production was compared to that of T-cells cultured with the neutral DC/media control. A DC to T-cell ratio of 1:10 was used as previous studies demonstrated this to result in a mixed Th response, characterised by both IFNγ and IL-4 production (Whelan *et al.*, 2000; Manickasingham *et al.*, 2003). DC matured with either Th1-associated LPS (Whelan *et al.*, 2000), or Th2-associated, Zymosan A (Manickasingham *et al.*, 2003), were assayed as positive controls. The data from two individual experiments are presented (Figure 7.2).

DC matured with PMB (DC/PMB) and DC/LPS + PMB primed T-cells for slightly increased IFNγ production compared to DC/media (Figure 7.2a). DC/0-3hRP also primed for increase IFNγ production compared to DC/media, but this was not more than that primed by the DC/PMB and DC/LPS + PMB negative controls (Figure 7.2a). Conversely, priming with DC/LPS led to a much greater increase in IFNγ production than priming with any other mDC. In contrast to IFNγ, DC/0-3hRP drove increased production of the reciprocal cytokine IL-4 compared to controls, whereas DC/LPS did not (Figure 7.2b). A similar pattern was observed in a repeat experiment, in which DC/0-3hRP primed T-cells for increased IL-4 but with little increase in IFNγ production compared to DC/media, whereas DC/LPS primed for increase IFNγ but not IL-4 production (Figure 7.2c & d). Additionally, DC/Zymosan A were also analysed in the second experiment, and primed for a similar cytokine profile to DC/0-3hRP, with increased IL-4 production but little increase in IFNγ (Figure 2.2c & d). DC or $CD4^+$ cells cultured alone did not produce detectable cytokine (data not shown). Taken together, the data from these experiments suggests that DC/0-3hRP promotes Th2 polarisation. Indeed, increasing the dose of 0-3hRP appeared to enhance the DC capacity to drive Th2 polarisation (Figure 7.3a & b). It is noteworthy that in these experiments increased production of one polar cytokine (*i.e.* IFNγ or IL-4) did not

necessarily result in inhibition of the reciprocal polar cytokine when compared to DC/media.

7.3.3 0-3hRP instructs DC to prime for Th2 responses *in vivo*

During percutaneous infection with schistosomes, initial T-cell priming occurs in the sdLN (Constant *et al.*, 1990; Pemberton *et al.*, 1991). Therefore, I wanted to examine whether iDC matured *in vitro* with 0-3hRP could prime for Th2 responses *in vivo*, after delivery via the skin. Consequently, DO11.10 mice were injected subcutaneously with differentially matured DC previously pulsed with OVA antigen; the outcome of T-cell priming was determined after 7 days by *in vitro* re-stimulation of sdLN cells with OVA. As with the *in vitro* assays, Th1- and Th2-associated, or neutral DC, were included as markers of polarisation. In addition to IL-4, production of another Th2 signature cytokine, IL-5, was also analysed.

Re-stimulation with limiting dilutions of OVA (100 - 1000 nM) revealed that sdLN cells from mice injected with LPS-matured DC produced approximately 5-fold greater IFNγ than sdLN cells from mice injected with DC/media (Figure 7.4a). In contrast, sdLN cells from mice injected with DC/0-3hRP, or DC/Zymosan A, produced no detectable IFNγ upon re-stimulation with 100 nM OVA and only two-fold more than DC/media control, when re-stimulated with 1000 nM OVA. Analysis of Th2 signature cytokines revealed that sdLN cells from DC/0-3hRP recipients produced dramatically increased levels of IL-4 and IL-5 upon re-stimulation compared to cells from DC/media recipients (Figure 7.4b & c). Moreover, DC/LPS stimulated no significant increase in IL-4 production and only a little increase in IL-5 production. Similarly to DC/LPS, DC/Zymosan A did not prime for increased IL-4 production, and only a limited increase in IL-5 production. However, in experimental repeats, DC/0-3hRP and DC/LPS appeared to drive little increase in IFNγ production, whereas DC/Zymosan A stimulated increased IFNγ, IL-4, and IL-5 production. Therefore, the data from three experiments (representing 3 individual mice) was collated and analysed. Due to variations in overall cytokine production between the experiments, the data was expressed as the log fold-increase in cytokine production compared to the DC/media control. Therefore, in this analysis the DC/media control assumes the value of zero. In one experiment, sdLN cells from mice

injected with DC/LPS appeared to produce little cytokine, and so this data was disregarded.

Analysis of the pooled data demonstrated that sdLN cells from DC/0-3hRP recipients appear to produce an overall decreased level of IFNγ production upon re-stimulation with either concentration of OVA, compared DC/media (Figure 7.5a). In contrast, DC/0-3hRP primed for increased IL-4 and IL-5 production at both concentrations of OVA, compared to DC/media (Figure 7.5b & c). It is noteworthy that DC/0-3hRP-primed sdLN cells produced significantly more IL-4 and IL-5 than DC/media-primed cells in two of the three individual experimental repeats when re-stimulated with 100 nM OVA, and in all repeats when re-stimulated with 1000 nM OVA (data not shown). Overall, DC/LPS appeared to drive increased IFNγ production, although the variation between experiments was considerable (Figure 7.5a). In addition, DC/LPS primed for little, or no, increase in IL-4 and IL-5 production compared to that primed by DC/0-3hRP (Figure 7.5b & c). Surprisingly, DC/Zymosan A primed sdLN cells for increased IFNγ and IL-5 production but with little increase in IL-4, apart from a small elevation upon re-stimulation with 1000 nM OVA, and therefore appear to be less potent drivers of Th2 polarisation than DC/0-3hRP.

Interestingly, the log fold-increases in IFNγ production primed by DC/LPS and DC/Zymosan A were quite low when compared to the log-fold increases in IL-4 and IL-5 production primed by DC/0-3hRP. This is probably due to the relatively high levels of IFNγ but low levels / undetectable levels of IL-4 or IL-5 produced by DC/media (Figure 7.4).

To determine if subcutaneous injection of the differentially matured DC also affected the systemic polarisation of the T-cell population, splenocytes from recipient mice were re-stimulated *in vitro*, in the same manner as for sdLN cells. Splenocytes from mice injected with DC/0-3hRP produced higher levels of IFNγ when re-stimulated with 100, or 1000, nM OVA compared to those from DC/media recipients but lower levels than splenocytes from mice injected with DC/LPS (Figure 7.6a). Splenocytes from mice injected with DC/Zymosan A also produced high levels of IFNγ similar to, or less than, that produced by DC/LPS recipients upon re-stimulation with 1000, or 100, nM OVA respectively. Analysis

of 'Th2 signature' cytokines demonstrated that both splenocytes from mice injected with DC/0-3hRP and those injected with DC/Zymosan A, produced dramatically increased levels of IL-4 compared to DC/media, whereas splenocytes primed with DC/LPS produced little (1000 nM OVA), or no (100 nM OVA), increase (Figure 7.6b). In addition, splenocytes from DC/0-3hRP recipients appeared to produce relatively high levels of IL-5 compared to those from DC/media recipients (Figure 7.6c). In contrast, DC/LPS and DC/Zymosan A primed for less, or no, increase in IL-5 production.

The data from three individual experiments were pooled, as for sdLN, allowing greater confidence in the trends observed in cytokine production. All PAMP-stimulated DC appeared to drive increased IFNγ production compared to DC/media (Figure 7.7a). Moreover, this increase in IFNγ production was similar between DC/0-3hRP, DC/LPS, and DC/Zymosan A. In contrast, splenocytes from mice injected with DC/0-3hRP and DC/Zymosan A but not DC/LPS produced increased IL-4 and IL-5 compared to cells from DC/media recipients (Figure 7.7b & c). Indeed, when splenocytes were re-stimulated with 100 nM OVA, DC/0-3hRP appeared to be a more potent promoter of IL-4 and IL-5 production than DC/Zymosan A. Moreover, cells from DC/LPS recipients appeared to produce an overall decreased level of IL-5 production compared to splenocytes primed with DC/media, when re-stimulated with 1000 nM OVA.

It is noteworthy that splenocytes cultured with anti-CD3 antibody (which non-specifically stimulates all T-cells) produced similar cytokine profiles to that of their OVA-re-stimulated counter-parts. In this respect, splenocytes from mice injected with DC/0-3hRP, DC/LPS and DC/Zymosan A, all produced similarly increased levels of IFNγ compared to DC/media primed splenocytes, but only splenocytes from DC/0-3hRP and DC/Zymosan A recipients produced increased IL-4 and IL-5 (Figure 7.8a, b, c). Moreover, when the CD3-stimulated cytokine production was averaged for 3 separate experiments, the profiles were also very similar (Figure 7.9a, b, c). Considerable variation in IFNγ production was observed between experiments due to high-level production by cells from DC/media treated mice in one experiment (data not shown). Although a large variation in the log fold-increase of IL-5 production compared to DC/media was apparent (Figure 7.9c), it is noteworthy that in each individual experiment, both DC/0-3hRP and DC/Zymosan A primed for significant increases compared to DC/media. In contrast, in only one

experiment did DC/LPS prime for a significant increase in IL-5 production, with significantly reduced levels apparent in one of the repeats.

7.3.4 Culture of DC with anti-CD40 antibody leads to a heighten ability to drive antigen-specific responses but impairs the generation of Th2 polarised responses

One of the factors thought to play a pivotal role in the activation and function of DC is the interaction between CD40 and CD154. In this respect, co-culture of DC with 0-3hRP and anti-CD40 antibody increased their production of IL-12, a cytokine known to contribute to Th1 development, whilst having limited effects on Th2-associated cytokines (Section 6.3.8). However, the role of CD40-signalling in T-cell polarisation is controversial (discussed in Section 6.4). Therefore, the *in vivo* priming assay was used to determine whether stimulation through CD40 affects the ability of DC/0-3hRP to drive Th2 polarised responses.

Upon re-stimulation, sdLN cells from mice injected with anti-CD40-treated DC/media produced 3-fold more IFNγ, compared to cells from mice injected with untreated DC/media (Figure 7.10a). Similarly, DC/0-3hRP treated with anti-CD40 antibody primed for 2-fold greater IFNγ production compared to the levels produced by sdLN cells from DC/0-3hRP recipients. In addition, analysis of Th2 cytokines revealed that sdLN cells from anti-CD40-treated DC/media recipients also produced increased levels of IL-4 and IL-5 (3- to 4-fold, respectively) compared to the low levels produced by DC/media-primed cells (Figure 7.10b & c). In contrast, compared to the high levels of IL-4 and IL-5 primed for by DC/0-3hRP, sdLN cells from anti-CD40-treated DC/0-3hRP recipients produced significantly less Th2 cytokines (2- to 3-fold, respectively).

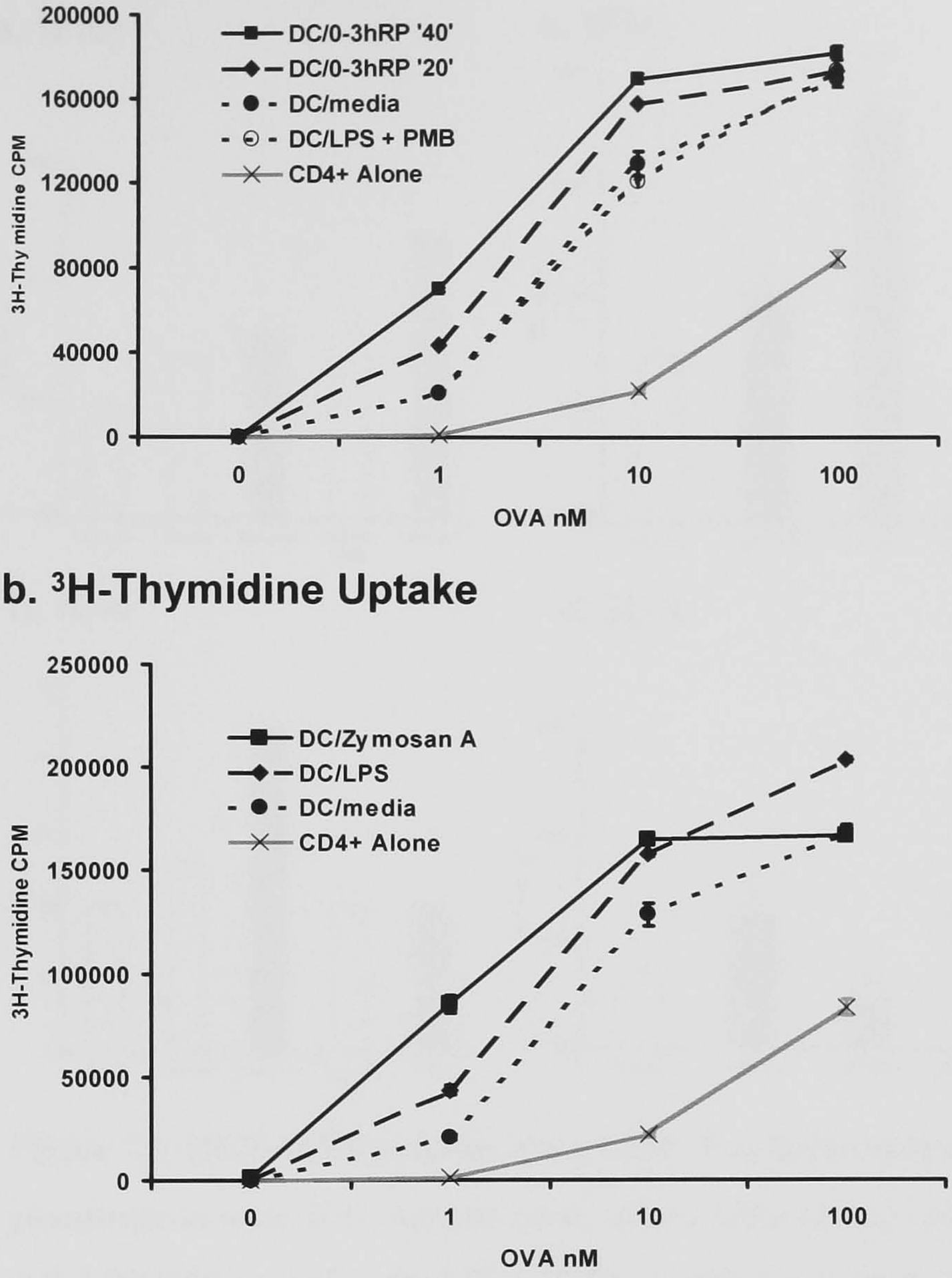


Figure 7.1 **DC/0-3hRP promote T-cell proliferation**. Splenic $CD4^+$ cells from DO11.10 mice were cultured alone (**X**), or with mDC previously matured alone (●), with '20' μg / ml (◆) or '40' μg / ml (■) 0-3hRP in the presence of PMB, or with LPS and PMB (O) (a). In the same experiment, $CD4^+$ cells were also cultured with mDC previously matured with LPS (100 ng / ml; ◆), or Zymosan A (1.6 μg / ml; ■) (b). Cells were then cultured in the presence of OVA peptide (0 - 100 nM) for 72 hr, before measuring DNA synthesis by uptake of ^{3}H-Thymidine. Data is presented as the mean ± SEM of 6 wells. Data is representative of 2 experiments.

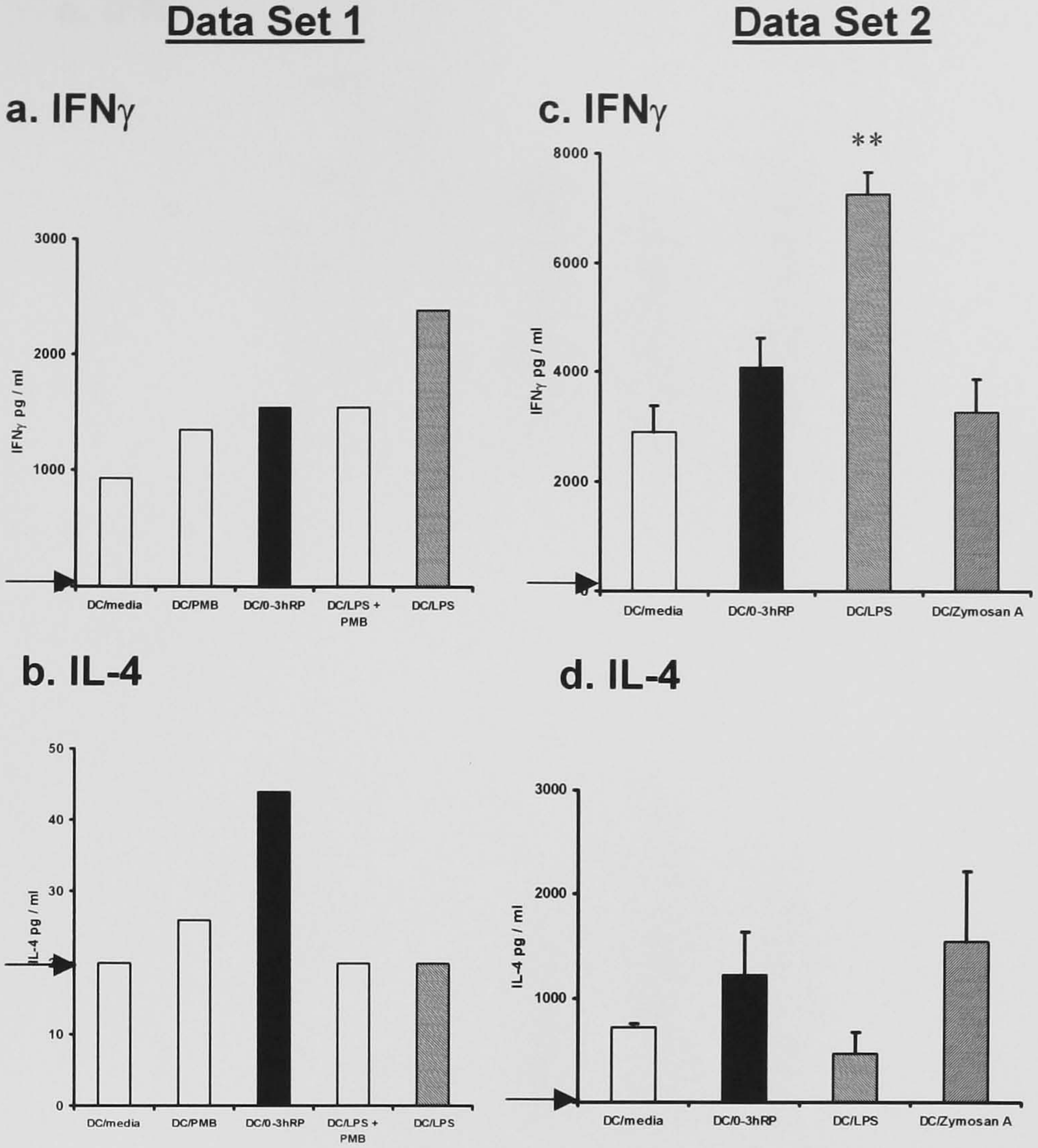
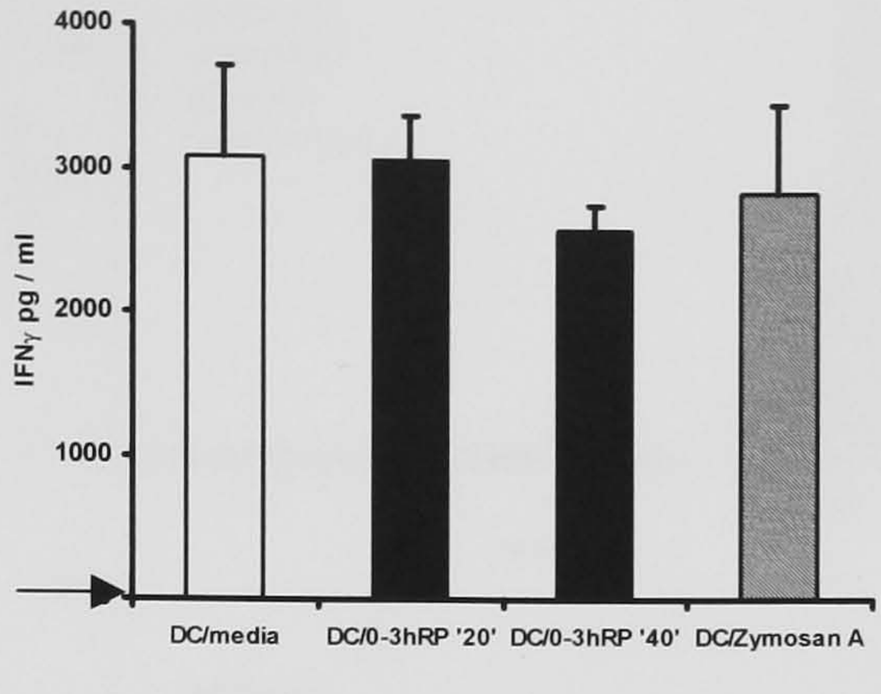


Figure 7.2 **DC/0-3hRP polarise naïve CD4$^+$ T-cells towards a Th2 phenotype *in vitro*.** iDC matured alone, with 0-3hRP (20 μg / ml) + PMB, with LPS (100 ng / ml), with LPS + PMB, or with Zymosan A (1.6 μg / ml), were co-cultured with purified splenic CD4$^+$ cells from DO11.10 mice in the presence of OVA peptide (10 nM). After 3 days, cells were pulsed overnight with phorbol myristate acetate and ionomycin. The supernatants were analysed by ELISA for production of IFNγ (a & c) and IL-4 (b & d). Data from two different experiments is presented (note that low levels of IL-4 in Data Set 1 were probably due to omission of β-mercaptoethanol from the culture medium). Data Set 1 represents values from pooled wells (a & b). Data Set 2 is presented as the mean ± SEM of 3 wells (c & d). Arrows denote the lower detection limit of ELISA. Levels of significance are between DC/media control and test groups.

a. IFNγ



b. IL-4

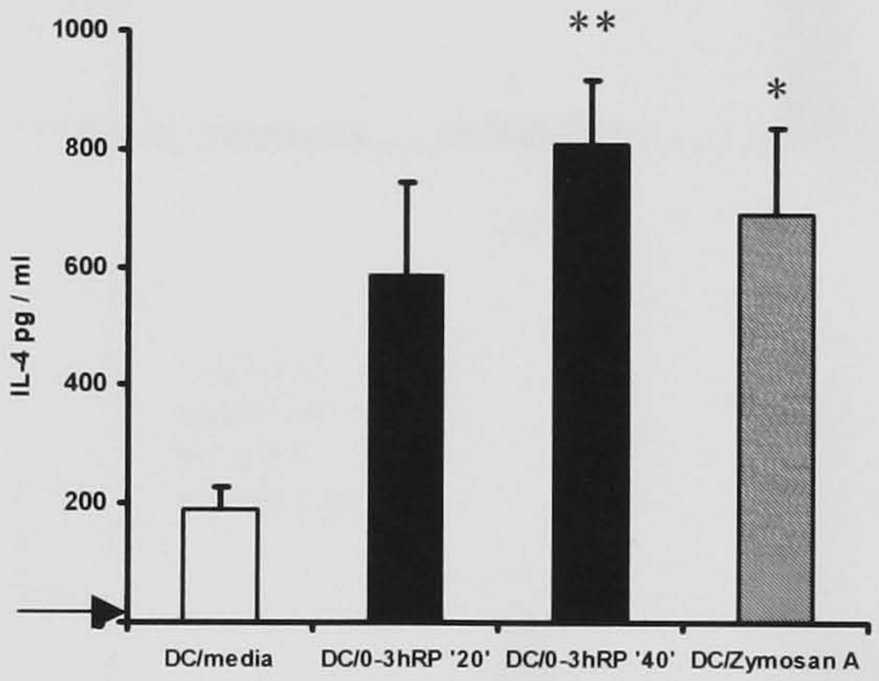


Figure 7.3 **Th2-polarising function of DC is dependent on 0-3hRP dose.** iDC matured alone, with 0-3hRP (20 - 40 μg / ml) + PMB, or with Zymosan A (1.6 μg / ml), were co-cultured with purified splenic $CD4^+$ cells from DO11.10 mice, in the presence of OVA peptide (10 nM). After 3 days, cells were pulsed overnight with PMA and ionomycin. The supernatants were analysed by ELISA for production of IFNγ (a) and IL-4 (b). Data is presented as the mean ± SEM of 5 wells. Arrows denote the lower detection limit of ELISA. Levels of significance are between DC/media control and test groups.

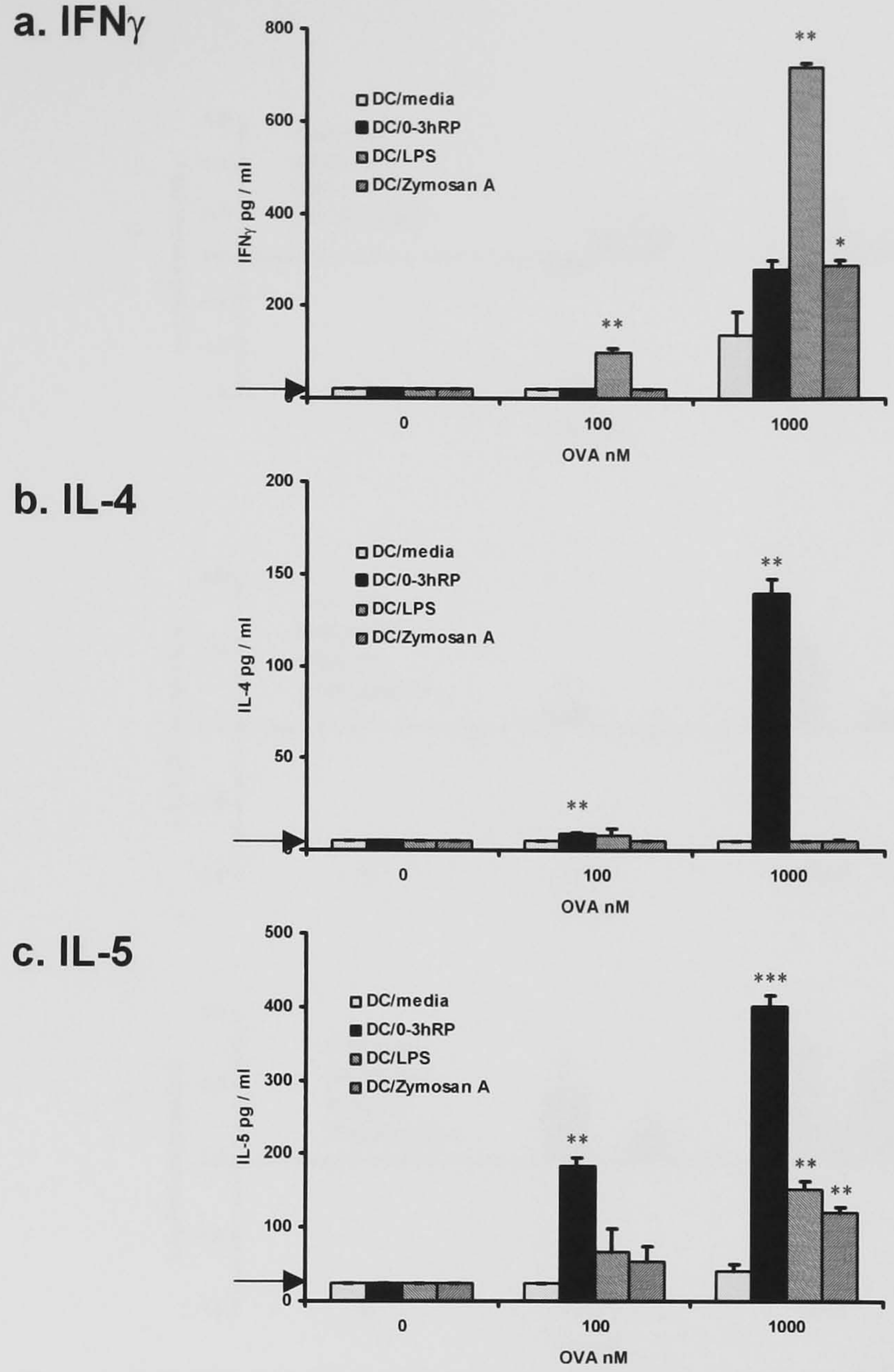
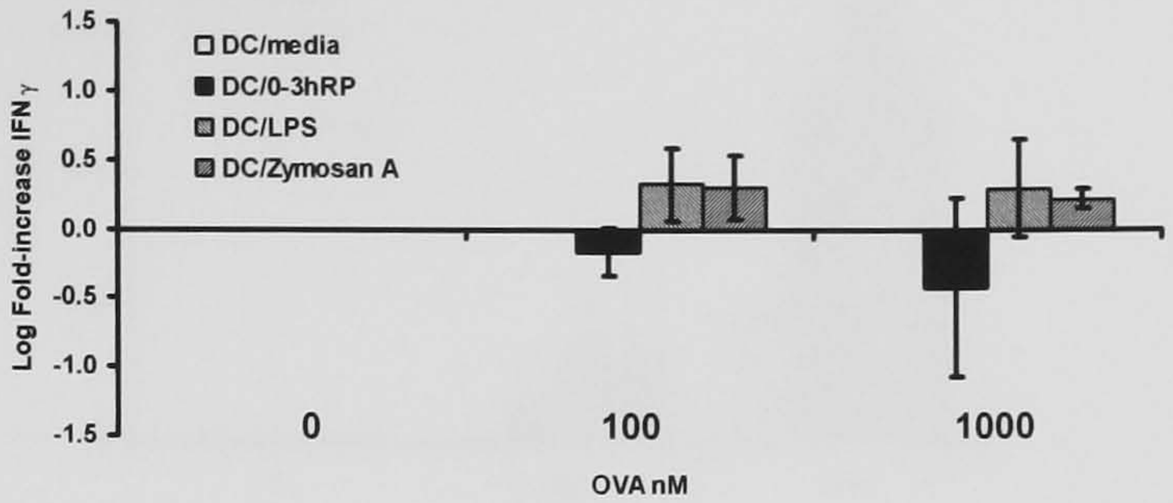
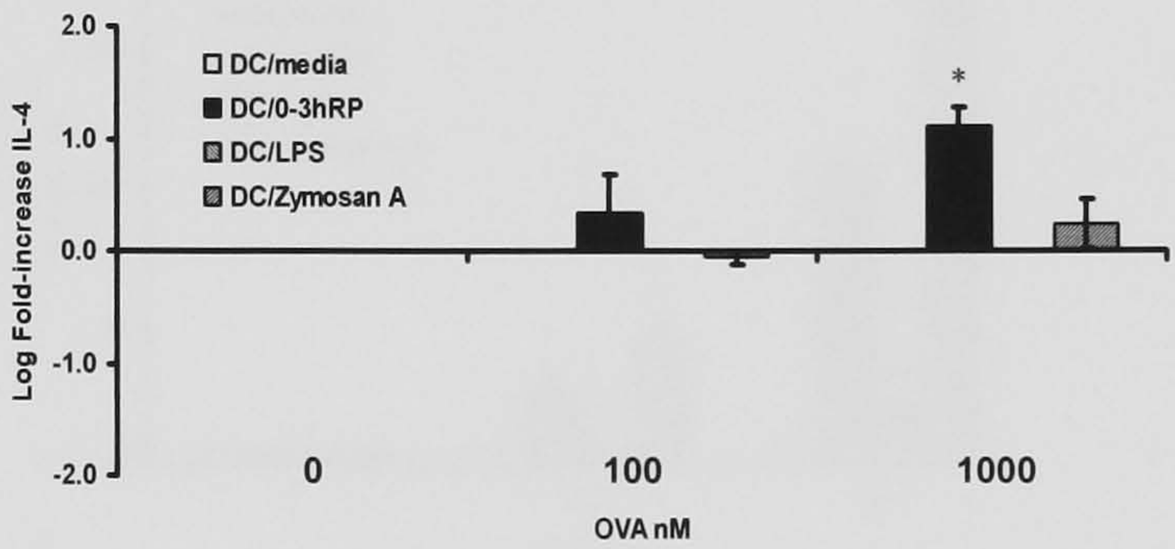


Figure 7.4 **DC/0-3hRP drives polarisation towards a Th2 phenotype *in vivo*.** DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, with 0-3hRP (40 μg / ml) + PMB, with LPS (100 ng / ml), or with Zymosan A (1.6 μg / ml). After 7 days, sdLN cells were re-stimulated *in vitro* with OVA (0 - 1000 nM) for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is presented as the mean ± SEM of 3 wells. Arrows denote the lower detection limit of ELISA. Levels of significance are between DC/media control and test groups.

a. IFNγ



b. IL-4



c. IL-5

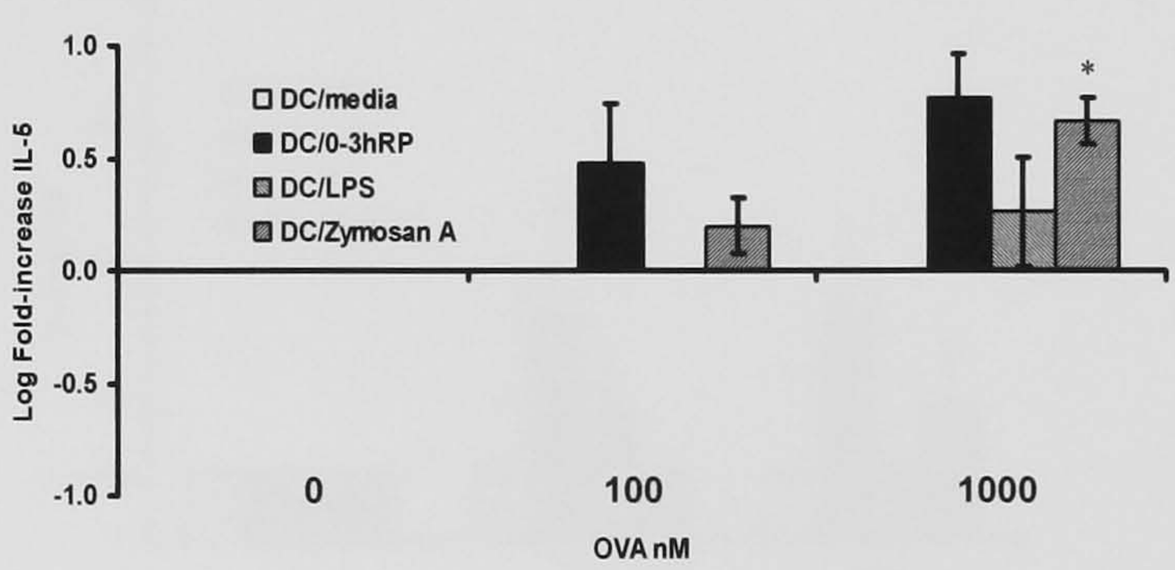


Figure 7.5 **DC/0-3hRP drives polarisation towards a Th2 phenotype *in vivo*.** In three separate experiments DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, with 0-3hRP (40 μg / ml) + PMB, with LPS (100 ng / ml), or with Zymosan A (1.6 μg / ml). After 7 days, sdLN cells were re-stimulated *in vitro* with OVA (0 - 1000 nM) for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is expressed as log fold-increase in cytokine production compared to DC matured with media. Data is presented as the mean ± SEM of 3 individual experiments, except LPS for which n = 2. Levels of significance are between DC/media control and test groups.

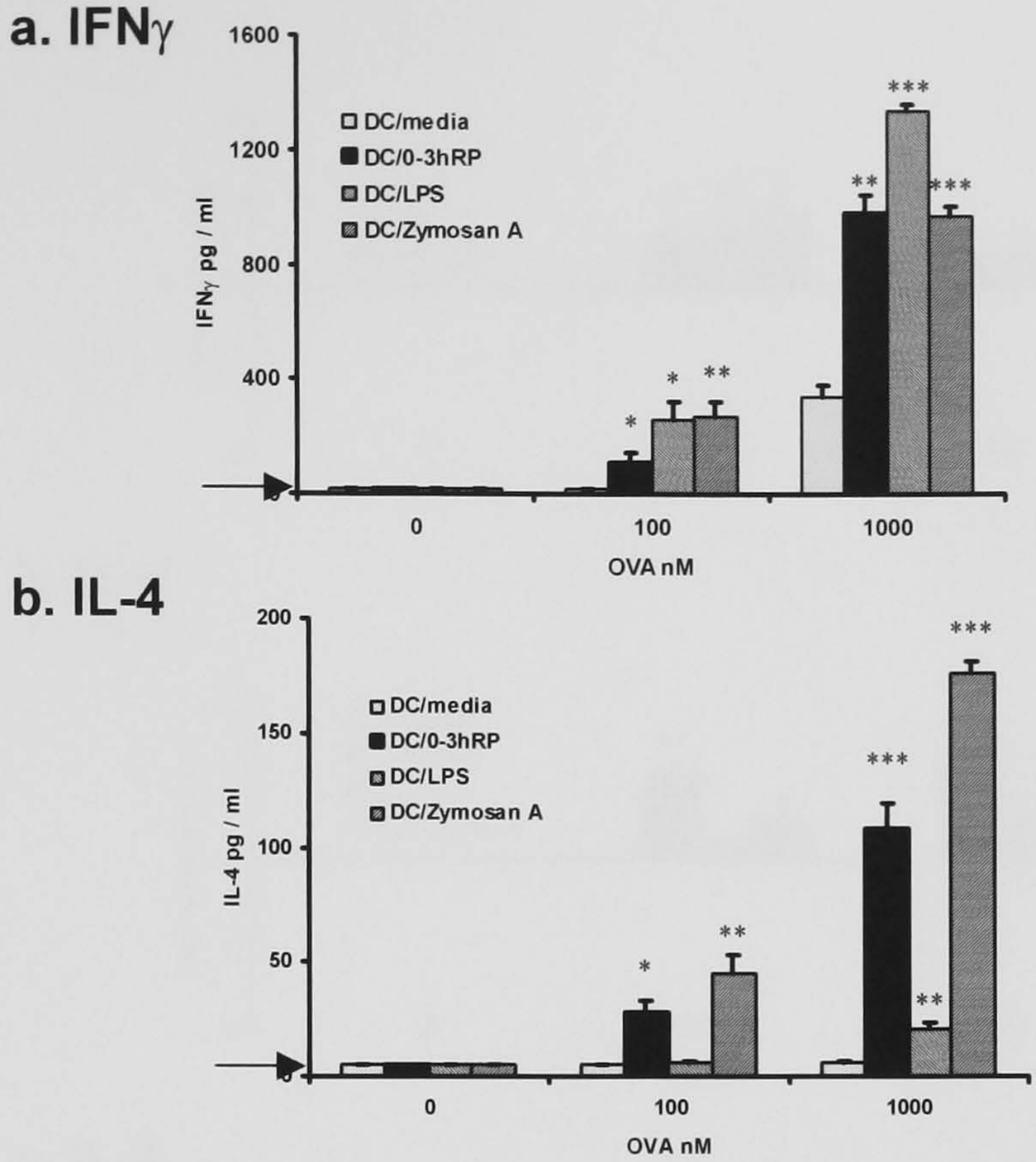
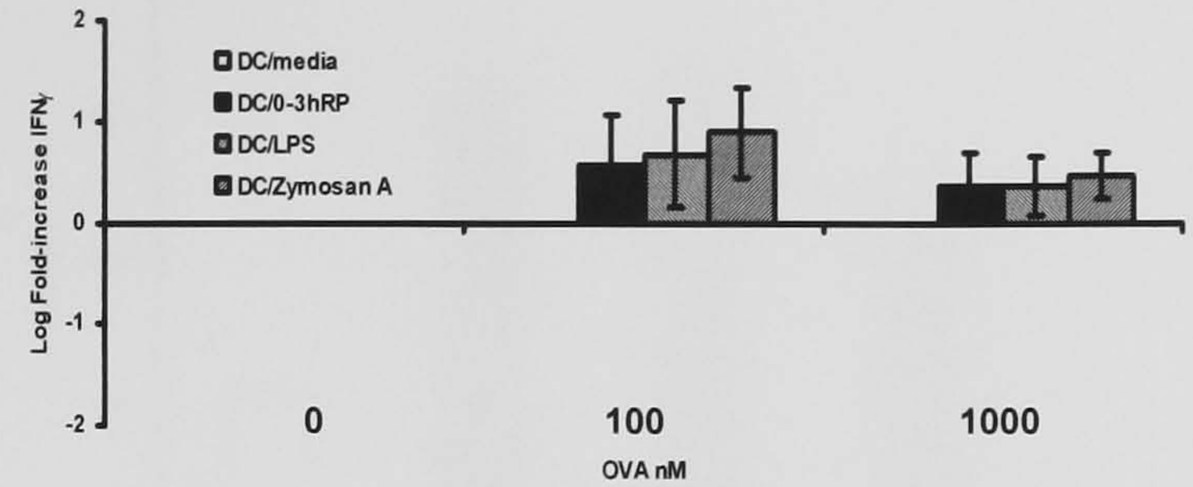
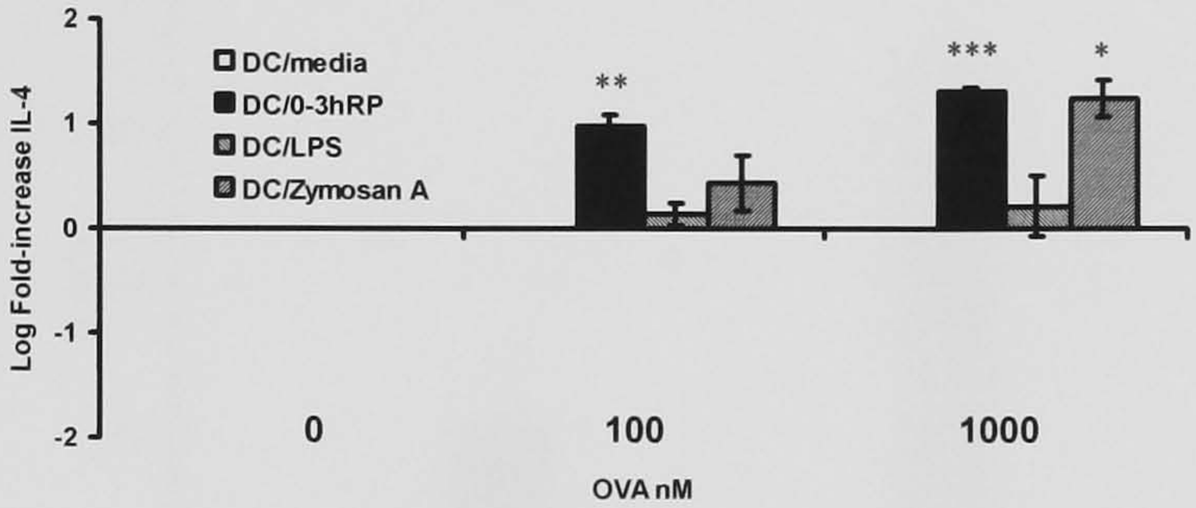


Figure 7.6 **DC/0-3hRP drives systemic Th2 polarisation.** DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, with 0-3hRP (40 μg / ml) + PMB, with LPS (100 ng / ml), or with Zymosan A (1.6 μg / ml). After 7 days, splenocytes were re-stimulated *in vitro* with OVA (0 - 1000 nM) for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is presented as the mean ± SEM of 3 wells. Arrows denote the lower detection limit of ELISA. Levels of significance are between DC/media control and test groups.

a. IFNγ



b. IL-4



c. IL-5

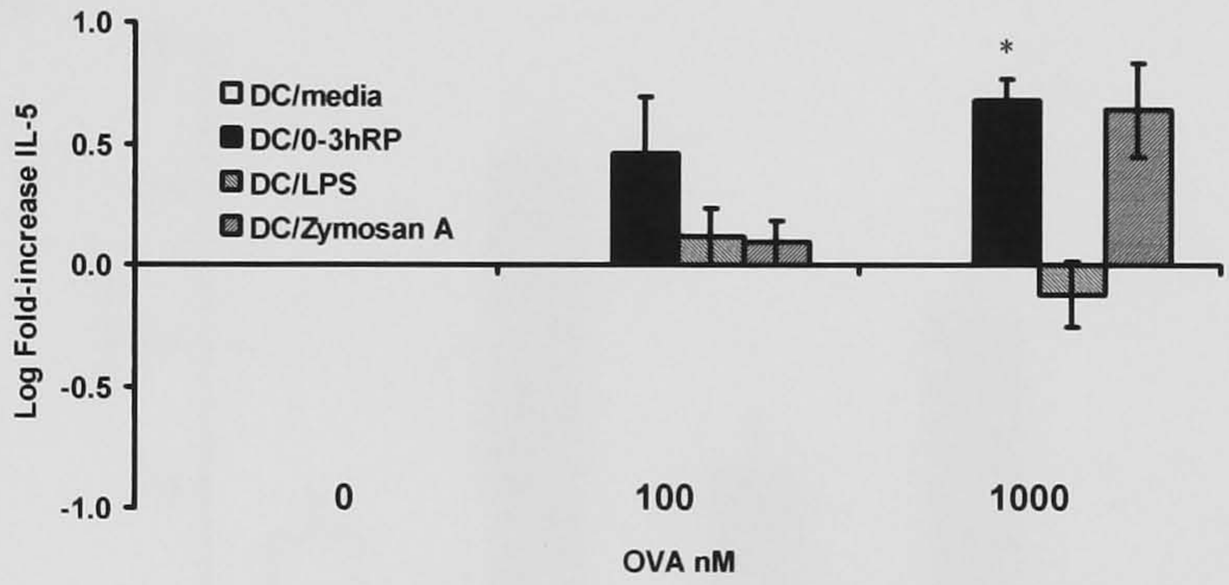


Figure 7.7 **DC/0-3hRP drives systemic Th2 polarisation.** In three separate experiments DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, with 0-3hRP (40 μg / ml) + PMB, with LPS (100 ng / ml), or with Zymosan A (1.6 μg / ml). After 7 days, splenocytes cells were re-stimulated *in vitro* with OVA (0 - 1000 nM) for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is expressed as log fold-increase in cytokine production compared to DC matured with media. Data is presented as the mean ± SEM of 3 individual experiments. Levels of significance are between DC/media control and test groups.

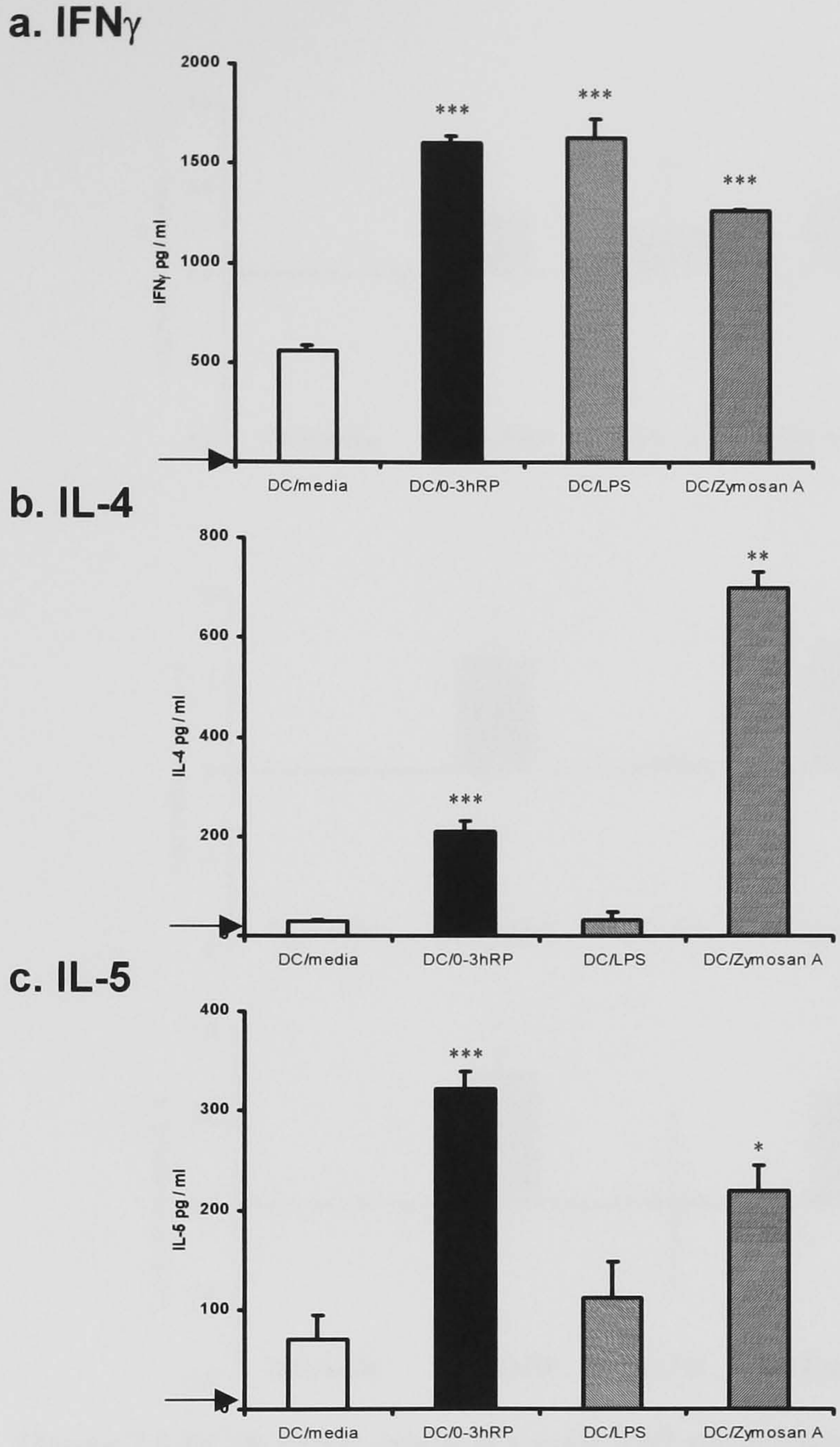
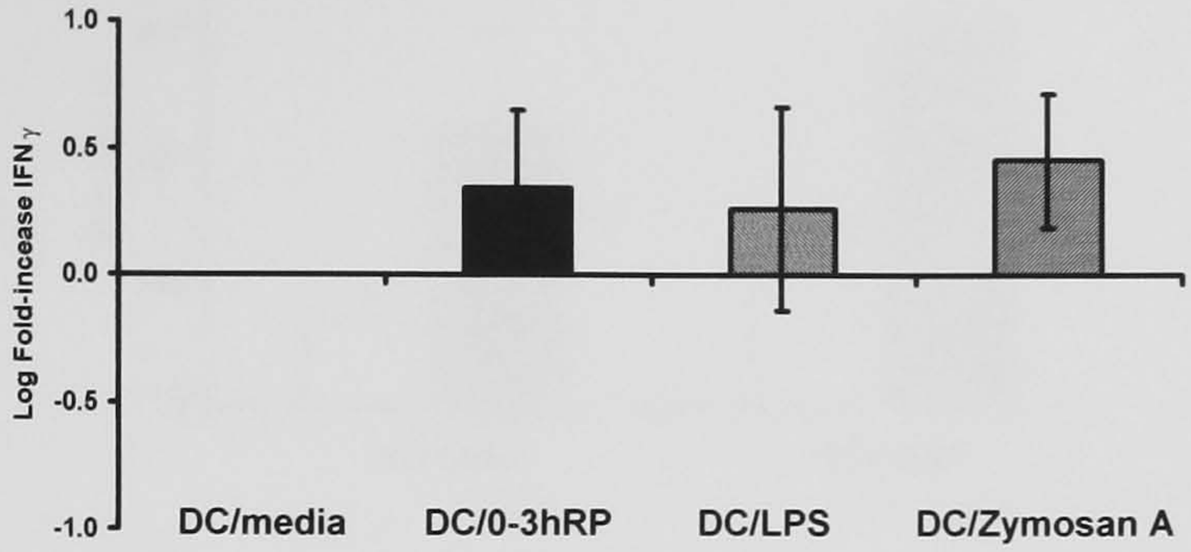
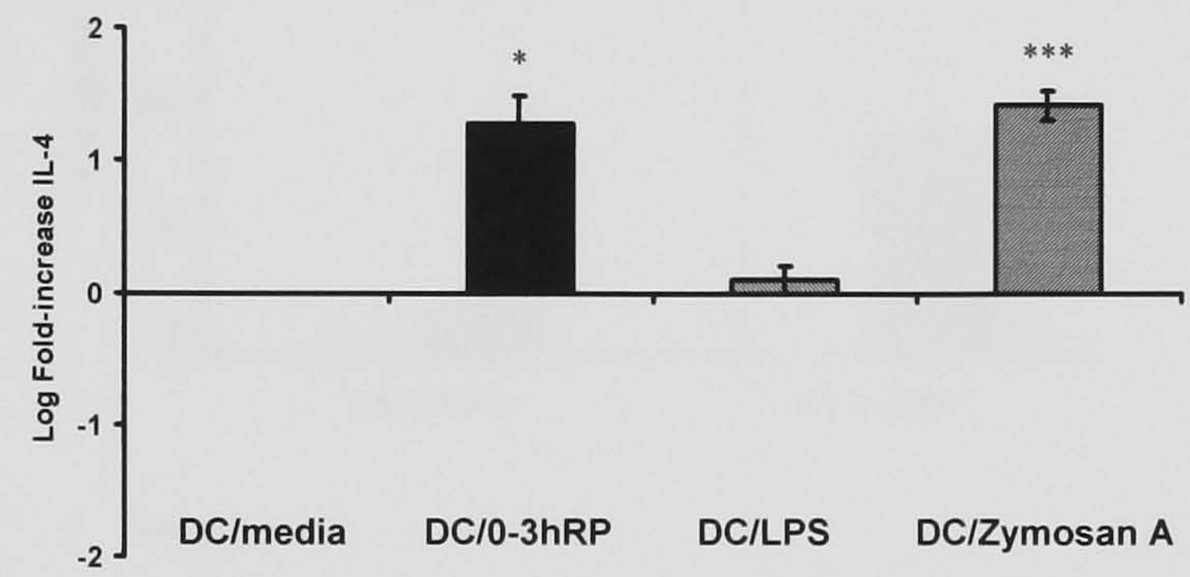


Figure 7.8 **DC/0-3hRP drives systemic Th2 polarisation.** DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, with 0-3hRP (40 μg / ml) + PMB, with LPS (100 ng / ml), or with Zymosan A (1.6 μg / ml). After 7 days, splenocytes were re-stimulated *in vitro* with anti-CD3 antibody for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is presented as the mean ± SEM of 3 wells. Arrows denote the lower detection limit of ELISA. Levels of significance are between DC/media control and test groups.

a. IFNγ



b. IL-4



c. IL-5

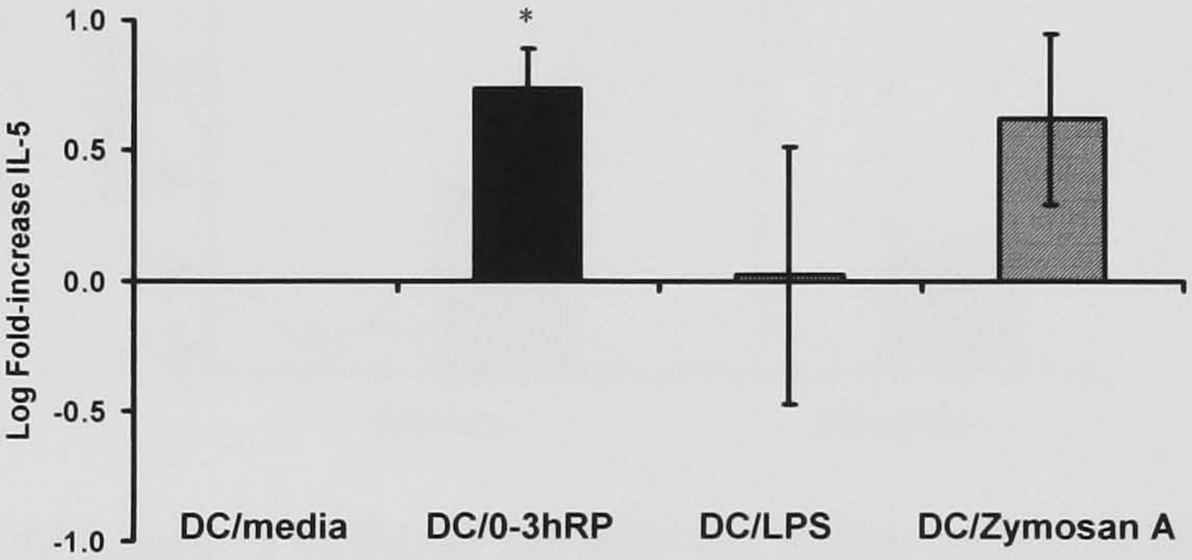


Figure 7.9 **DC/0-3hRP drives systemic Th2 polarisation.** In three separate experiments DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, with 0-3hRP (40 μg / ml) + PMB, with LPS (100 ng / ml), or with Zymosan A (1.6 μg / ml). After 7 days, splenocytes were re-stimulated *in vitro* with anti-CD3 antibody for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is expressed as log fold-increase in cytokine production compared to DC matured with media. Data is presented as mean ± SEM of 3 individual experiments. Levels of significance are between DC/media control and test groups.

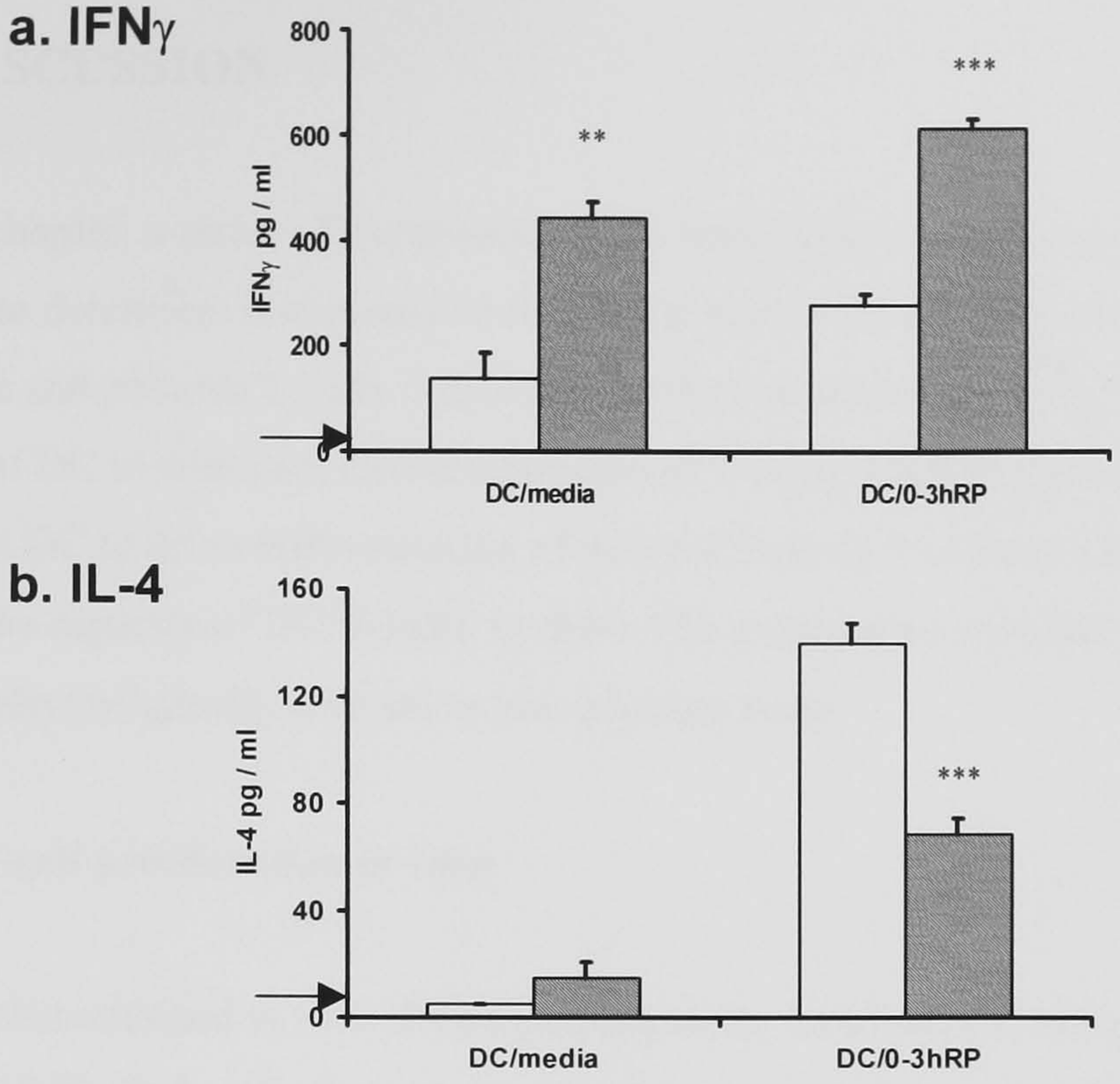


Figure 7.10 **Ligation of CD40 inhibits the ability of DC/0-3hRP to drive Th2 polarisation.** DO11.10 mice were injected subcutaneously with OVA-pulsed DC previously matured alone, or with 0-3hRP (40 μg / ml) + PMB, in the presence (hatched bars), or absence (open bars), of anti-CD40 antibody (5 μg / ml). After 7 days, sdLN cells were re-stimulated *in vitro* with 1000 nM OVA for 72 hr. Supernatants were analysed by ELISA for production of IFNγ (a), IL-4 (b), and IL-5 (c). Data is presented as mean ± SEM of 3 wells, and was obtained from the same experiment presented in Figure 7.4. Data is representative of 2 experiments. Arrows denote the lower detection limit of ELISA. Levels of significance are between cells cultured with, or without, anti-CD40 antibody.

7.4 DISCUSSION

In this chapter, a series of experiments were performed using an antigen-restricted TCR *Tg* system to determine if exposure to released schistosome PAMPs affects the capacity of DC to prime and polarise T-cells. I demonstrate that maturation with 0-3hRP increases the ability of DC to stimulate clonal expansion of T-cells. Furthermore, exposure to 0-3hRP instructs DC to drive differentiation of the proliferating T-cells towards the Th2 pole *in vitro*. The capacity of DC/0-3hRP to drive Th2 polarisation was further demonstrated using a more physiologically relevant *in vivo* priming assay.

7.4.1 T-cell proliferation *in vitro*

A well-characterised *in vitro* T-cell priming assay, in which DC were co-cultured with purified TCR *Tg* T-cells in the presence of model antigen, was used to show that DC matured with 0-3hRP activate T-cells to undergo greater clonal expansion than DC matured alone. Clonal expansion of naïve T-cells requires signalling through both the TCR (signal 1), and co-stimulatory receptors (signal 2). This occurs following ligation of these receptors by specific antigen-MHC II complexes and co-stimulatory factors expressed upon APC. Importantly, the strength of signalling, determined by the amount of receptor ligation, is proportional to subsequent T-cell proliferation (Murtaza *et al.*, 1999). Moreover, cytokines such as IL-12 and IL-6 are also co-stimulatory factors, increasing T-cell expansion during priming (Vink *et al.*, 1990; Kubin *et al.*, 1994). Therefore, the increased T-cell proliferation stimulated by DC/0-3hRP probably directly relates to their up-regulated surface expression of MHC II and the co-stimulatory factors CD86 and CD40, and their up-regulated production of IL-6 and IL-12p40 (Section 6.3.7). This confirms that released schistosome PAMPs stimulate maturation of DC and highlights the relevance of this to the functional phenotype of these cells. Moreover, this study indicates that APC exposed to released schistosome PAMPs at the onset of infection may have an increased capacity to prime schistosome-specific T-cell responses in the host.

Both LPS and Zymosan A stimulated greater expression of MHC II and co-stimulatory factor expression upon DC compared to that stimulated with 0-3hRP, yet this did not confer greater levels of T-cell proliferation. This suggests that other factors also affect the

rate of clonal expansion. Indeed, IL-10 produced by both DC/Zymosan A and DC/LPS (Section 6.3.7) could act in an autocrine manner to regulate the APC function of these cells. In addition, Th cells could limit their own proliferation, through mechanisms such as IL-10 production. Alternatively, incorporation of ^{3}H-thymidine into DNA only measures proliferation between two time points, thus representing a 'snapshot' of cell proliferation. Therefore, it is possible that peak proliferation could have occurred earlier in cultures containing DC/Zymosan A, or DC/LPS. Assessment of actual T-cell numbers may more accurately address this issue.

7.4.2 T-cell polarisation *in vitro*

The same 'antigen-restricted' *in vitro* T-cell priming assay was also used to determine the T-cell polarising capacity of DC/0-3hRP compared to the control DC. As a point of note, the cytokine production measured in these experiments also represents a 'snapshot' of polarisation at one point post-priming. Although this is sufficient for the purpose of my study, further analysis of the kinetics of cytokine production may produce a more detailed account of T-cell differentiation following priming with the differentially-matured DC. DC/media primed for production of the Th1 cytokine IFNγ and the Th2 cytokine IL-4. In contrast to this mixed or Th0 profile, DC/LPS primed for increased production of IFNγ but not IL-4, whereas DC/Zymosan A primed for increased production of IL-4 but not IFNγ. Therefore, as expected DC/LPS promoted a more Th1-biased response, whereas DC/Zymosan A promoted a more Th2-biased response, agreeing with previous reports (Whelan *et al.*, 2000; MacDonald *et al.*, 2001; Manickasingham *et al.*, 2003). In comparison to DC/media, and other negative control DC, DC matured with 0-3hRP primed for increased production of IL-4, but little or no increase in IFNγ production. Therefore, I conclude that DC/0-3hRP promote Th2 polarisation *in vitro*. It is noteworthy that DC matured with 0-3hRP appeared equally as potent as those matured with Zymosan A in driving towards Th2 polarisation.

The responder T-cell population used in these assays may have contained low levels of memory T-cells, thought to exist by expressing an additional endogenously rearranged TCR, which allows them to respond to cross-reactive environmental antigens (Lee *et al.*, 1996). Although originally linked to IL-4 production (Macatonia *et al.*, 1995), the presence

of these memory cells is now thought to preferentially skew the T-cell population towards IFNγ rather than IL-4 production when stimulated with similar levels of OVA to those used in my study (Hosken *et al.*, 1995; Lee *et al.*, 1996; Manickasingham *et al.*, 2003). Thus, the possible presence of Th1-promoting memory cells in the responder T-cell population emphasises the potency of DC/0-3hRP and DC/Zymosan A to drive Th2 responses, and DC/LPS to enhance Th1 responses. Differences in the number of these cells in responder T-cell populations could explain the variation in overall cytokine production between individual experiments. Many studies have used naïve T-cells selected for expression of $CD62L^{high}$ as responder cells. However, this requires access to a flow-assisted cell sorter, which unfortunately was not possible during the course of my studies. Furthermore, recent studies demonstrate that memory T-cells can be $CD62L^{high}$ (London *et al.*, 1999; Ben-Sasson *et al.*, 2000), thus questioning the validity of using this marker to purify naïve cells.

7.4.3 T-cell polarisation *in vivo*

The same 'antigen-restricted' system was used to determine the *in vivo* T-cell priming capability of DC/0-3hRP. This assay required adoptive transfer of 'antigen-loaded' mature DC into naïve recipient *Tg* mice, and subsequent re-stimulation of sdLN, or splenic, effector cells *in vitro*. Due to restrictions on available mice, one mouse per DC-type were compared in individual experiments but the results from three experiments were pooled to give greater confidence in the data. However, due to variations in the overall levels of cytokine production between experiments, the data was first expressed as the log of the fold-increase in cytokine production over the DC/media control. The cytokine profiles generated in repeat experiments did vary, as is evident by the standard error of the pooled data, although this was more notable in the profile of IFNγ production, than IL-4 or IL-5, possibly reflecting differences in antigen-responsive memory populations within the individual mice. Further experimental repeats should reduce the error within this data. Moreover, repeating this study with a greater number of mice per experimental group would be an advantage, removing inherent errors incurred by the direct comparison of data from different experiments. However, in all experiments DC/0-3hRP primed for a dramatic increase in IL-4 and IL-5 production by sdLN cells compared to DC/media and DC/LPS. This is reflected by the significance of the pooled experimental data. Although in one

experiment, priming with DC/0-3hRP also resulted in a limited increase in IFNγ production, analysis of the pooled data demonstrated that there was an overall decrease in production of this cytokine compared to priming with the DC/media control. This leads to the conclusion that DC matured with 0-3hRP promote Th2 polarisation in the sdLN, reinforcing the observations from the *in vitro* assays. Furthermore, DC/0-3hRP were more potent than DC/Zymosan A at driving Th2 polarisation. Indeed, DC/Zymosan A appeared to prime for a more mixed Th1/Th2 response, with overall greater levels of IFNγ and IL-5 produced compared to DC/media primed sdLN cells. Similarly, DC/LPS appeared to prime for both increased IFNγ and IL-5. The overall increase rather than qualitative difference in the cytokine responses induced by DC/Zymosan A and DC/LPS compared to DC/media could reflect a greater overall level of *in vivo* T-cell priming by these DC, as might be expected from their increased capability to drive T-cell expansion *in vitro*.

Injection of differentially primed DC also led to the polarisation of responder T-cells in the spleen. This may have occurred through the immigration of effector T-cells arising in the sdLN. Alternatively, the transferred DC may have immigrated from the sdLN to spleen. However, it is possible that following injection some DC bypassed the sdLN, and primed naïve T-cells upon their arrival in the spleen. Indeed, since the APC to T-cell ratio is known to affect polarisation (Tanaka *et al.*, 2000; Manickasingham *et al.*, 2003), differences in the number of DC reaching the LN or spleen between experiments could account for the variation observed in the resulting cytokine profiles.

Similar to cytokine profiles produced by sdLN cells, splenocytes primed *in vivo* with DC/0-3hRP produced dramatically increased levels of IL-4 and IL-5. However, DC/0-3hRP also primed splenocytes for increased IFNγ production. Indeed, both DC/Zymosan A and DC/LPS also primed for similar increases in IFNγ production compared to DC/media (significant in 2 of the 3 individual experiments). However, only DC/Zymosan and not DC/LPS also primed for increased production of Th2 cytokines. Again, the increase in both Th1 and Th2 cytokine production primed for by PAMP-matured DC suggests a greater level of *in vivo* T-cell priming in the spleen, compared to that by DC/media. One interpretation of this data is that DC/0-3hRP and DC/Zymosan A prime more for a mixed Th1 / Th2, or Th0 response in the spleen, and that DC/LPS drive only weak Th1 polarisation. Alternatively, polarisation of T-cell responses represents a continuum

between highly differentiated Th1 and Th2 poles, and thus, it could be interpreted that DC/0-3hRP and DC/Zymosan A prime more towards a Th2 response simply because they drive co-production of IL-4 and IL-5, in addition to IFNγ. In this respect, DC/LPS may be described as Th1-polarising simply by not driving Th2 cytokine production. However, it is noteworthy that the log mean fold-increase in IFNγ production driven by DC/LPS and DC/Zymosan A in both the spleen and sdLN was low compared to the high log mean fold-increases in IL-4 or IL-5 production primed by DC/0-3hRP and DC/Zymosan A (especially at the highest concentration of OVA). This suggests that the assay system could be biased towards Th1 cytokine production, making it difficult to detect further Th1 polarisation.

It is interesting that *in vivo* DC/0-3hRP appeared to prime for a greater increase in IFNγ production by spleen cells than by sdLN cells, possibly reflecting a difference in function, or in a biased potential for T-cell polarisation, of these organs. However, since priming in the sdLN might be expected to precede that in the spleen, these observations may reflect differences in the kinetics of priming in these organs. In this respect, developing Th2 responses may first go through transient Th0 phases *in vivo* (Vella and Pearce, 1992), and IFNγ production is thought to precede IL-4 by expanding T-cells (Bird *et al.*, 1998).

7.4.4 DC function in T-cell priming

Although the DC populations used in these experiments contain a number of myeloid precursor cells / granulocytes, the vast majority of the MHC II^+, co-stimulatory $factor^+$ and $cytokine^+$ cells were $CD11c^+$ (Section 6.4). Thus, it is reasonable to conclude that enriched $CD11c^+$ DC are responsible for the *in vitro* polarisation of the T-cell population. This conclusion is supported by a similar study in which the outcome of priming was the same whether purified $CD11c^+$, or non-fractionated whole BM-derived DC populations were used (Whelan *et al.*, 2000). Furthermore, although DC can exchange antigen / MHC II complexes with each other (Bedford *et al.*, 1999), it seems most that likely that *in vivo* polarisation was primed directly by the PAMP-matured DC.

The data presented in this chapter confirms the growing evidence that DC can distinguish between the nature of different pathogens, or their products, subsequently allowing them to drive either Th1, or Th2, responses (Whelan *et al.* 2000; MacDonald *et al*, 2001; de Jong *et*

al., 2002). This directly contrasts the view that DC subsets are pre-programmed to prime either Th1, or Th2 responses (Rissoan *et al.*, 1999; Maldonado-Lopez *et al.*, 1999; Moser 2001). Moreover, recent work shows the plasticity of DC to drive either Th1 or Th2 polarisation is universal between DC subsets, and not just an aberrant trait of myeloid BM-derived DC (Manickasingham *et al.*, 2003; Boonstra *et al.*, 2003), suggesting that 0-3hRP could stimulate many DC subsets to prime Th2 responses. Interestingly, a bias in the potential of human and murine DC subsets to polarise T-cells could be explained by their selective expression of PRRs, which effectively restricts the class of PAMPs to which they can respond (Kadowaki *et al.*, 2001; Kelsall *et al.*, 2002; Boonstra *et al.*, 2003). Since there have been no studies on the repertoire of PRR expressed on LC or dermal DC, it is difficult to know if they would be limited in the range of PAMPs to which they could respond. However, *in vivo* evidence of APC activation and accumulation at the site of schistosome infection / vaccination (Reingrojpitak *et al.*, 1998; Angeli *et al.*, 2001a; Hogg *et al.*, 2003a; Kumkate *et al.*, 2003) would indicate that these cells do respond to molecules released by penetrating schistosomes.

The data presented in this chapter and Chapter 6 suggests that DC/0-3hRP prime for Th2 responses in the absence of high levels of polarising cytokines and co-stimulatory factors. Similarly, the Th2 responses driven by DC matured with nematode ES-62, or schistosome SEA, occur in the absence of increased co-stimulatory factor expression or cytokine production (Whelan *et al.*, 2000; MacDonald *et al.*, 2001). Together with these studies, my work supports the view that Th2 differentiation represents a default pathway in the absence of strong polarising signals. This is consistent with observations that T-cells can be rescued from ES-62-driven Th2 polarisation by addition of IL-12 to cultures (Whelan *et al.*, 2000), whereas Th1 responses do not develop, or are impaired, using DC from IL-12 $^{-/-}$ mice, or can be ablated by addition of anti-IL-12 antibody to cultures (Whelan *et al.*, 2000; Moser, 2001; MacDonald and Pearce, 2002; Jankovic *et al.*, 2002). Moreover, simultaneous blocking of a number of pro-inflammatory cytokines (IL-12, IL-18 and IFNα) in human DC / T-cell co-cultures switched Th1-dominated responses to Th2-dominated responses (de Jong *et al.*, 2002). In addition, mice deficient for MyD88 develop Th2 instead of Th1 responses to mycobacterial PAMPs, or *T. gondii* infection, corresponding with a decrease in the activation state of their DC (Schnare *et al.*, 2001; Jankovic *et al.*, 2002).

Another way in which priming with DC/0-3hRP could result in increased IL-4 and IL-5 production is through the expression of low levels of MHC II-peptide complexes, which has been linked to Th2 induction (Ruedl *et al.*, 2000). Although 0-3hRP did stimulate increased expression of MHC II compared to DC/media, this was lower than on DC/LPS. This intermediate up-regulation of MHC II could allow increased T-cell priming, but result in Th2 rather than Th1 polarisation.

My data also suggests that Th2 polarisation need not require IL-10 production, since DC/0-3hRP did not produce this cytokine (Section 6.3.7). Similarly, Th2 polarisation driven by ES-62 is independent of IL-10 (Whelan *et al.*, 2000). It is possible that DC/0-3hRP-derived IL-4 and IL-6 production may be involved in Th2 polarisation, although DC can drive Th2 responses in the absence of IL-4 (MacDonald and Pearce, 2002). Furthermore, I demonstrate that Th1 polarisation can occur in the presence of considerable amounts of IL-10, IL-6, and IL-4, since DC/LPS produced high levels of these cytokine in addition to high levels of pro-inflammatory cytokines, emphasising that the balance between these different signals is probably important to the outcome of T-cell polarisation.

In contrast to DC/0-3hRP, DC/Zymosan A also appear to 'instruct' Th2 differentiation yet have a 'highly' activated maturation state, characterised by production of considerable quantities of polarising cytokines, such as IL-12p70, and high level expression of co-stimulatory factors and MHC II. It is noteworthy that at the doses used in this study DC/Zymosan A did not appear to be as potent as DC/0-3hRP in driving Th2 polarisation *in vivo*, since they also primed for increased IFNγ by sdLN cells. It is quite possible that this is a result of the high levels the pro-inflammatory cytokines produced by DC/Zymosan A. In this respect, although Manickasingham *et al.* (2003) reported that DC stimulated with Zymosan A drive Th2 polarisation, their study focused upon splenic DC which have previously been shown to preferentially produce IL-10 rather than IL-12 upon exposure to this PAMP (Edwards *et al.*, 2002).

This data further highlights the complexity of the DC / T-cell interactions that result in the polarisation of the effector population, and demonstrate that there might be several independent mechanisms that lead to Th2 induction. Indeed, DC/Zymosan A but not DC/0-3hRP up-regulated expression of OX40L, which has been implicated in Th2 polarisation

(discussed in Section 6.4). One similarity between DC/0-3hRP and DC/Zymosan A is that, in contrast to DC/LPS, neither up-regulated expression of CD80, suggesting this factor could be involved in promoting Th1 polarisation, although this is highly debatable (discussed in Section 6.4). However, it possible that there is an essential factor for PAMP-driven Th2 polarisation, common to both DC/0-3hRP and DC/Zymosan A, which has not yet been identified, or was not analysed in Chapter 6.

One factor thought to play a critical role in DC activation and function is the interaction of CD40 and CD154. In this chapter, I demonstrate that simultaneous culture of DC with anti-CD40 antibody dramatically increases their ability to drive antigen-specific acquired responses. This concurs with similar findings that CD40-activation of human DC results in increased T-cell proliferation and cytokine production *in vitro* (Cella *et al.*, 1997). Furthermore, I demonstrate that anti-CD40 treatment of DC/0-3hRP reverses their capacity to prime Th2 responses, instead causing the induction of Th1 responses. Indeed, *in vivo* administration of anti-CD40 antibody can reverse the Th2 response resulting from injection of schistosome eggs, in favour of a Th1 response (Martin *et al.*, 2000). One way anti-CD40 treatment may exert this pro-Th1 effect is by stimulating increased IL-12p40 but not Th2-associated cytokine production (Section 6.3.8). Indeed, CD40 ligation on DC can lead directly to Th1 polarisation through the increased production of IL-12 (Cella *et al.*, 1996; Ruedl *et al.*, 2000). Concurrent with this, the inhibition of Th2 cytokine production observed in response to co-injection of schistosome eggs with anti-CD40 antibody was dependent upon IL-12 (and IL-23?) production (Martin *et al.*, 2000). Alternatively, the anti-CD40 antibody used in my experiments, and that of Martin *et al.* (2000) may act to mask CD40, thus blocking signals received, or given, through CD154, since these are important for Th2 development (Poudrier *et al.*, 1998). Indeed, DC generated from CD40 $^{-/-}$ mice have an impaired ability to drive Th2 but not Th1 responses, and CD154 $^{-/-}$ mice do not mount Th2 responses to schistosome eggs or schistosome infection (MacDonald *et al.*, 2002a; 2002b). This work suggests that exposure of DC to released schistosome material during infection will lead to an increased ability to prime Th2 responses, but that feedback from CD154^{+} cells would potentially aid the development of Th1 responses.

It has been shown that T-cells from mice on BALB/c backgrounds default to a Th0 / Th2 phenotype upon priming in neutral conditions (Hsieh *et al.*, 1995). These mice also

preferentially mount Th2 responses in experimental leishmanial infection, compared to the protective Th1 response seen in C57Bl/6 mice (Heinzel *et al.*, 1989; Scott, 1991). Moreover, they develop Th2-dependent mechanisms of protection following schistosome vaccination (Mountford *et al.*, 2001), compared to more protective highly Th1-polarised response mounted by seen in C57Bl/6 mice. Together, these observations have lead to the suggestion that BALB/c are naturally 'Th2' mice. This bias may lie intrinsically with the T-cell (Hsieh *et al.*, 1995), however, recent work shows that differences in DC responses also play a role (Kuroda *et al.*, 2000; Liu *et al.*, 2002). In this respect, BALB/c DC were more responsive to 'Th2 inducing' 0-3hRP, than their C57Bl/6 counterparts (Chapter 6), suggesting that part of the 'Th2' phenotype of BALB/c may be due to their ability to respond to Th2 driving PAMPs. However, SEA can stimulate DC to drive Th2 responses in C57Bl/6 mice (MacDonald *et al.*, 2001). Repeats of these experiments using TCR *Tg* mice on a C57Bl/6 background would determine if 0-3hRP retains its 'Th2' properties in this Th1-associated strain.

7.4.5 Summary

In summary, PAMPs released during the transformation of schistosomes instruct immature DC to mature to a phenotype promoting T-cell proliferation. Moreover, these DC drive Th2 polarisation of antigen-specific responses. The differentiation towards the Th2 pole may occur due to a default mechanism of T-cells in the absence of polarising cytokines. In this respect, early ligation of CD40 on DC leads to increased IL-12p40 production and a switch from Th2 to Th1 priming. However, it is possible that other factors may be involved in active polarisation of Th2 responses by 0-3hRP-stimulated DC.

CHAPTER 8

GENERAL DISCUSSION

The overall goal of this thesis was to determine if host accessory cells recognise molecules derived from schistosome larvae. The secondary objective was to establish what effect innate schistosome recognition may have upon the development of the acquired response. I have shown that molecules released from schistosome larvae in the first 3 hours following transformation are potent stimulators of innate accessory cells. Furthermore, APC exposed to these molecules acquire the capacity to drive Th2 responses.

8.1 Innate recognition of schistosome larval molecules

My studies appear to show that schistosomes directly activate the innate immune system through the expression of molecules with PAMP-like activity. In this respect, larval components are capable of stimulating IL-6 and IL-10 production by inflammatory Mϕ (Chapter 3), and IL-6 and IL-12p40 production by IFNγ-activated Mϕ (Chapter 3) and BM-derived DC (Chapter 6). Furthermore, I show that these stimulatory larval components are highly concentrated within the secretory material released by the parasite (0-3hRP) (chapters 3 & 6). This would appear logical since released larval components have the greatest potential to be encountered by the hosts' immune system. In addition, it is possible that larval surface components may also have stimulatory properties since iMϕ become activated following culture with live larvae. In the *in vivo* context, skin accessory cells are likely to be exposed to both secreted material and surface molecules.

In contrast to 0-3hRP, soluble somatic preparations from whole larvae had little stimulatory capacity, suggesting either the absence or dilution of stimulatory components, and / or the presence of potentially inhibitory molecules. Although this thesis has focused entirely upon the identification of stimulatory schistosome material, analysis of the inhibitory properties of the larval preparations may further reveal important biological interactions that occur between parasite and host at the onset of infection. In this respect, eicosanoids produced by larvae upon contact with host precursor material (*e.g.* linoleic acid) may exert such an inhibitory effect *in vivo*, but are unlikely to be a factor in my preparations.

One major concern throughout my studies has been the possible effects of low-level endotoxin contained within the larval preparations. In Chapter 3, several lines of

experimentation, including co-culture with polymixin B, were used to conclude that schistosome molecules (representing putative schistosome PAMPs) stimulate accessory cells independently of contaminating endotoxin. Further strengthening this conclusion, data presented in chapters 6 and 7 demonstrate that the schistosome PAMPs have a quite different effect on iDC compared to other classical microbial PAMPs, including the major endotoxin constituent LPS. This implies that iDC matured with 0-3hRP have not been stimulated in a manner suggestive of endotoxin contamination. Therefore, under conditions where endotoxin contamination is eliminated, my studies show that 0-3hRP contains stimulatory molecules that represent novel innate ligands of larval origin.

I have termed the stimulatory larval components as schistosome PAMPs, supported by evidence of their interaction with PRRs, such as the TLR4 and the MR (chapters 3 & 4). However, they could instead represent virulence factors, which Medzhitof (2001) states are not PAMPs due to their restriction to a limited number of pathogens. In this respect, the skin inflammatory response to cercarial secretions is known to aid parasite penetration (Fallon *et al.*, 1996). Although coverall terms, such as 'Modulins' have been used more recently to describe pathogen molecules that exert effects upon the innate and acquired immune systems (Whelan *et al.*, 2000), the general title of putative PAMPs will suffice for this thesis.

Activation of innate accessory cells occurs following ligation of their PRRs. The stimulatory effects of 0-3hRP upon IL-10 and IL-12p40 production was partly dependent upon the TLR4, since iMϕ and IFNγ-Mϕ from C3H/HeJ mice, which lack a functional form of this receptor, produced considerably reduced levels of these cytokines compared to cells from C3H/HeN mice. The TLR4 is known to be a promiscuous PRR and my data suggests the existence of TLR4-ligands of schistosome origin, although another interpretation is that there was an intrinsic difference in the activation state of Mϕ from these mice. The availability of cell-lines expressing individual TLRs, additional TLR-gene deficient mice, and antibodies that block TLR signalling should enable us to more fully assess the roles of these receptors in schistosome PAMP recognition. An interesting issue raised by my results was that high-level IL-6 production stimulated by 0-3hRP appeared to be largely independent of TLR4 recognition. Further work upon the signal transduction events downstream of PRRs that lead to IL-6 production may help to explain these

observations. With a complex mixture of PAMPs, as represented by 0-3hRP, it is quite possible that simultaneous recognition occurs through several receptors. Indeed, although FcR γ-chain signalling appeared to have little role in IL-10 production stimulated by 0-3hRP (Chapter 5), the released material did contain molecules recognised by carbohydrate-recognition domains of the MR (Chapter 4). However, MR ligands were much more abundant in the relatively non-stimulatory preparations of whole larvae, and represented molecular species distinct from those within 0-3hRP. Studies using MR $^{-/-}$ mice will allow further dissection of the role of this receptor in the innate responses to schistosomes. Fractionation of 0-3hRP would also allow identification of the individual schistosome PAMPs responsible for these stimulatory (or possibly inhibitory) properties, and the identification of the receptors through which they signal.

8.2 Innate recognition of schistosomes: APC and the development of acquired immune responses

The successful priming of an acquired immune response first requires activation of the local APC population, and there is clear evidence that this occurs in the skin following exposure to schistosome larvae (Hogg *et al.*, 2003a). Consequently, immature BM-derived DC were used to study the effects of schistosome PAMPs on professional APCs (Chapter 6). 0-3hRP stimulated the maturation of BM-derived DC corresponding with an increased number expressing MHC II^{high}, CD40, CD86^{high}, and increased production of IL-12p40 and IL-6. However, when compared with the 'high-level' of maturation induced by the classic pathogen PAMPs LPS and Zymosan A, DC activated with 0-3hRP were shown to be of an 'intermediate' maturation state, reminiscent (yet potentially distinct) of that stimulated by other helminth products (Whelan *et al.*, 2000; MacDonald *et al.*, 2001).

An *in vitro* antigen-restricted priming assay was used to show that DC matured with 0-3hRP had an increased capacity to prime for proliferation of CD4^{+} cells from DO11.10 αβ TCR mice in the presence of the model antigen (OVA peptide) (Chapter 7). This demonstrates that schistosome PAMPs have adjuvant-like qualities and that DC exposed to these molecules during the onset of infection would gain the capacity to prime acquired responses.

Comparison of the results from chapters 3 and 6 demonstrates clear differences between the responses of iMϕ and DC to 0-3hRP. In terms of the amount of cytokine production, 0-3hRP had broadly similar effects on iMϕ compared to LPS and Zymosan A. However, 0-3hRP caused only minimal increases in cytokine production by iDC compared to the high levels stimulated by LPS and Zymosan A. One interpretation of this is that there are differences in the responses of DC and iMϕ to ligation of the PRRs responsible for recognition of 0-3hRP. In this context, signalling via the MR has been implicated in the inhibition of IL-12 production by human dendritic cells (Nigou *et al.*, 2001) but has cytokine promoting effects upon Mϕ (Yamamoto *et al.*, 1997). Alternatively, there may be differential expression of PRRs upon these cells, such that each is restricted in the repertoire of PAMPs to which they can respond. Analysis of the differences in the expression of PRRs between accessory cells remains to be fully investigated, and could lead to a greater understanding of the roles of individual accessory cells in responses to different pathogens. In this respect, differences in responsiveness of iDC and iMϕ to larval PAMPs could reflect the relative contribution of these cells to the inflammatory response that occurs in the skin following infection. Furthermore, iMϕ produced a regulatory repertoire of cytokines (IL-10, IL-6, low IL-12p40) upon stimulation with 0-3hRP, whereas the cytokine profile produced by DC was much more pro-inflammatory (IL-12p40 and IL-6), mimicking that produced by IFNγ-primed Mϕ. In this respect, DC could indirectly enhance IL-12 production by Mϕ during infection, through IL-12-mediated IFNγ production from NK cells. Identifying exactly how the schistosome interacts with the range of other accessory cells in the skin, and how these cells co-operate to orchestrate the inflammatory response *in vivo* are major questions for further research.

8.3 Innate recognition of schistosomes: polarisation of the acquired immune response

Innate responses appear to exert control over the selection of acquired effector responses, via the polarisation of the Th cell population. As the bridge between these two immune systems, DC are in a unique position to directly effect the outcome of Th cell priming, and further work in Chapter 7 focused upon the ability of DC exposed to released schistosome PAMPs to polarise acquired responses. Controls that were expected to prime for 'neutral', 'Th1' and 'Th2' polar responses were used to provide reference points, allowing the outcome of priming to be gauged. Using the *in vitro* antigen-restricted priming assay, DC

activated with 0-3hRP primed $CD4^+$ cells from DO11.10 αβ TCR *Tg* mice to secrete increased IL-4 but little IFNγ similar to DC activated with Zymosan A. In contrast, $CD4^+$ cells primed by LPS-matured DC only produced abundant IFNγ. Furthermore, inoculation of DO11.10 mice with DC matured by 0-3hRP in the presence of OVA, also led to *in vivo* development of polarised Th2-type responses in both the sdLN and spleen.

A major area of research on T-cell regulation is identification of factors controlling the development of Th1 and Th2 responses, since these represent potential targets for immuno-therapies that either promote protective, or interfere with deleterious immune responses. In this respect, the PRRs that are involved in the induction of Th2 responses are not well defined compared to the relative wealth of knowledge on TLR- and MyD88-dependent Th1 responses. Much work has focused on the hunt for factors expressed by DC, or other APC which contribute to polarisation. A clear role for certain cytokines, most obviously IL-12, has been defined in the promotion of Th1 responses but factors involved in Th2 polarisation are less well understood. One current theory suggests that Th2 responses develop as a default of T-cell-priming by activated DC that do not produce known polarising cytokines (Kelsall *et al.*, 2002). My data tends to support this view, since DC/0-3hRP are defined by a state of 'intermediate' maturation, and low-level cytokine production.

Several studies have highlighted a critical involvement of individual co-stimulatory signals in the development of Th2 but not Th1 responses. However, since many cytokines also act as co-stimulatory factors, it may be that high-level cytokine production by Th1-inducing DC compensates for the loss of any one co-stimulatory survival signal. Indeed, the absence of certain co-stimulatory signals results with the loss of Th2 responses without the development of compensatory Th1 responses (MacDonald *et al.*, 2002a; 2002b). However, the Th2-driving capacity of highly-activated DC/Zymosan A (Chapter 7) which produce high levels of cytokines and express high levels of co-stimulatory molecules supports the alternative theory that certain factors (possibly cytokines or co-stimulatory molecules) may actively drive Th2 polarisation, rather than by a passive default mechanism. One co-stimulatory partnership that clearly affects the differentiation of acquired responses is CD40 / CD154. I show that ligation of CD40 following anti-CD40 antibody treatment of DC matured with 0-3hRP increased their production of IL-12 and reversed their capacity to

prime Th2 responses, instead causing the induction of a more Th1-type response. CD40 ligation could exert its effects by further activating DC to produce increased IL-12p40, although the antibody used in this study could also mask CD40 and potentially block Th2-dependent signals between CD40 and CD154. The use of CD154-expressing fibroblasts to ligate CD40 rather than anti-CD40 antibody would allow the dissection of the individual roles of CD40 and CD154 signalling in the switch from Th2 to Th1 induction by DC. Furthermore, gene-array, or proteomic, analysis of differentially-matured DC could identify new markers to better define DC maturation and highlight novel factors differentially expressed by Th1- or Th2-driving DC that could be involved in polarisation.

8.4 Relevance to infection

This is the first study to demonstrate that DC activated by released products from schistosome larvae acquire the capacity to drive Th2 acquired immune responses. A fundamental question is whether Th2 responses are important to host protection. In the context of schistosome infection, the widely accepted view is that exposure to normal cercariae results in a mixed Th1 / Th2 response compared to the more Th1 polarised response induced by vaccination. My data suggests that DC activated during natural infection should skew the acquired response towards the Th2 pole. However, the response of local APCs in the skin and sdLN to released schistosome PAMPs may be only one of many factors involved in polarisation during infection. In this respect, it is possible that the presence of live larvae *in situ* in the skin and sdLN might contribute to the induction of Th1 rather than Th2 responses through the release of Th1-inducing PAMPs by later-stage parasites, or the surface expression of such molecules. Alternatively, endogenous danger signals released in the skin / sdLN due to tissue damage following parasite penetration and migration, could be involved in Th1 induction.

What about the development of immune responses following repeated exposure of the host to cercariae? In this respect, mice that are multiply-infected with either *S. mansoni*, or *T. regenti*, exhibit Th2-associated immediate hypersensitivity reactions and cercarial dermatitis upon re-infection (Section 1.3.4). Indeed, single infection with *T. regenti* appears to result in a mixed Th1 / Th2 response in the sdLN, but in multiply-infected mice re-infection results in extreme Type-2 polarisation (Kourilova *et al.*, 2003). Therefore, exposure of DC to released schistosome PAMPs may contribute to the development of

immediate hypersensitivity within the host, and repeat infection could enhance this by further polarising the Th2 response. Production of anti-schistosome antibody may also contribute to Th2 polarisation upon exposure to 0-3hRP, via the augmentation of IL-10 production via FcR γ-chain signalling (Chapter 5).

How come many individuals in endemic areas are repeatedly exposed to high levels of infectious cercariae yet do not develop correspondingly massive worm burdens? Very little is known about the dermal responses of humans whom are repeatedly exposed to cercariae. However, it is possible that Th2 responses induced by exposure to released larval material contribute to anti-schistosome immunity, potentially operating at the level of immediate hypersensitivity responses in the skin and lungs. Indeed, the natural parasite attrition that occurs following single infection is significantly decreased in IgE $^{-/-}$ mice (King *et al.*, 1996b). In addition, parasite death is more rapid during immediate-hypersensitivity reactions following the re-infection of mice multiply-infected with *T. regenti*, compared to death following primary infection.

The early induction of Th2 responses by parasite larvae may also predispose the host to develop Th2 responses upon egg deposition. Whether the schistosome molecules that drive Th2 responses to eggs are the same as the immunologically active components in 0-3hRP, or whether they act through the same PRRs, remains to be determined but these biological compartments share similar glycosylated molecules.

Manipulation of the innate immune system could provide an important avenue to potentiate the development of protective immunity to infection. However, the results presented in this thesis raise important questions for the development of an effective schistosome vaccine. Although PAMPs within 0-3hRP have adjuvant properties, they may be of only limited use within a Th1-inducing vaccine due to their promotion of Th2 responses. Whilst these responses may provide protection, the development of vaccines that induce Type-2 response have the potential hazardous side-effects of inducing allergies to bystander antigens. Moreover, although strong Th1 responses can provide protection to infection (Wynn *et al.*, 1995; 1996), repeated exposure to the Th2-inducing PAMPs released by cercariae (which would presumably occur in areas of endemic infection) could potentially revert vaccine-induced Th1 responses towards mixed Th1 / Th2, or Th2 responses.

8.5 Summary

In conclusion, it is fundamentally important that we understand the early innate events that follow exposure to schistosome cercariae and lead to the development of inflammatory responses in the skin and acquired responses in the sdLN. The work contained in this thesis furthers our knowledge of how accessory cells are stimulated during infection and the effect this has on promotion of Th2-type acquired immune responses. These events could be key to the development of immediate hypersensitivity to schistosomes, and may present a considerable obstacle to further infection. Indeed, the role of Th2 responses in host protection represents an exciting area for further study. A more precise understanding of the events that lead to the inflammatory response and polarisation of the acquired immune response will better enable intervention, or induction of protection against disease.

ABBREVIATIONS

-/-	Gene deficient
0-3hRM	0-3 hr released material
0-3hRP	Soluble 0-3 hr released preparation
0-3hSN	0-3 hr released material
3hSom	3 hr schistosomulae
3dSSP	3 day SSP
5dSSP	5 day SSP
8dSSP	8 day SSP
3hSSP	3 hr SSP
18hSSP	18 hr SSP
1-D	1-dimension
2-D	2-dimension
ACK	Ammonium chloride buffer
APC	Antigen presenting cells
BAL	Broncho-alveolar lavage
BBGC	Brilliant Blue G-Colloidal Concentrate
BLAST	Basic local alignment search tool
BM	Bone marrow
BSA	Bovine serum albumin
CCR	Chemokine receptor
CercN	Normal cercariae
CercT	Transformed cercariae
CPM	Counts per minute
CR	Complement receptor
CRD	Carbohydrate recognition domains
COX	Cyclo-oygenase
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EDTA	Ethylene diamine tetra-acetic acid

ELISA	Enzyme-linked immunosorbant assay
EU	Endotoxin units
Fc	Antibody constant region
FcγR	Fcγ receptor
FcR γ	Fc receptor γ chain
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulation factor
GPI	Glycophosphoinsitol
GST	Glutathione-s-transferase
HRP	Horseradish peroxidase
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-XR	IL-X receptor
IL-1Ra	IL-1 receptor antagonist
iDC	Immature dendritic cells
iMϕ	Inflammatory peritoneal macrophages
IFNγ-Mϕ	IFNγ-activated peritoneal macrophages
IRS	0-3hRM immunised rabbit serum
IRAK	IL-1 receptor associated kinase
LAL	Limulus Amoebocye Lysate
LBP	LPS-binding protein
LC	Langerhans cells
Le^x	Lewis X
LNFP	Lacto-N-fucopentose
LPS	Lipopolysaccharide
Mϕ	Macrophages
MACS	Magnetic activated cell sorting
MACSB	Magnetic activated cell sorting buffer
MAL	MyD88 adaptor-like
mDC	Mature dendritic cells

MHC	Major histocompatibiltiy complex
MIP	Macrophage inflammatory protein
mLN	Mediastinal lymph node
MR	Mannose receptor
MR-Cys	Mannose receptor N-terminal cysteine-rich domain
mRNA	Messenger RNA
NF	Nuclear factor
NK	Natural killer
NKT	Natural killer T-cell
NRS	Normal rabbit serum
OVA	Chicken ovalbumin peptide
PAGE	Poly-acrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PB	Phenobuffer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS-T	Phosphate buffered saline tween
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEC	Peritoneal exudate cells
PG	Prostaglandin
PI	Propidium iodide
PMB	Polymyxin B
PRR	Pattern recognition receptor
PVDF	Polyvinylidene difluoride
QR	Quantum red
rIL	Recombinant Interleukin
RANTES	Regulated on activation, normally T-cell expressed and secreted
RNA	Ribonucleic acid
RPMIc	Concentrated RPMI preparation
RT	Room temperature
RT-PCR	Reverse-transcription polymerase chain reaction
SCP	Soluble cercarial preparation
sdLN	Skin-draining lymph node

SEA	Soluble egg antigen
SEM	Standard error of the mean
SDS	Sodium dodecyl sulphate
Sm	*S. mansoni* protein
SMAF	*S. mansoni*-derived apoptosis inducing factor
SN	Supernatant
SOCS	Suppressor of cytokine signalling
SSP	Soluble schistosomulae preparation
STAT	Signal transducer and activation of transcription
SWAP	Soluble adult worm preparation
TBS-T	Tris buffered saline tween
TCR	T-cell receptor
TCTP	Translationally controlled tumour protein
Tg	Transgenic
TGF	Transforming growth factor
Th	T-helper
TIR	Toll / IL-1R domain
TIRAP	Toll-IL-1R domain containing adapter protein
TLR	Toll-like receptor
TMB	Tetra-methylbenzidine
TNF	Tumour necrosis factor
TNFR	TNF receptor
Tr	Regulatory T-cell
T_{reg}	Regulatory T-cell
T½	Time taken for half the event to occur
U	Units
WHO	World health organisation
WT	Wild-type

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