In vivo and *in vitro* studies of Immune Responses Induced By Normal Or Attenuated *Schistosoma mansoni* schistosomula.

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ABSTRACT

A schistosome vaccine would be of great value in controlling schistosomiasis. Exposure of experimental animals to live gamma-irradiated cercariae (GI) is highly effective and although not logistically feasible for human vaccination has provided insight into the immune mechanisms which may facilitate development of defined antigen vaccines.

In mice the radiation attenuated vaccines induce IFN-γ dependent immunity following a single exposure and antibody mediated immunity following repeated exposure. In order to induce high levels of immunity the irradiated larvae need to survive for 2-3 weeks and to reach the lung stage in order to "arm" the lungs with sensitized cells, but it has been shown that larval infections with 500 larvae treated with the drug Ro11-3128 at just 2 days post-infection also results in high levels of immunity but with an apparently more focal and truncated exposure to living larvae in the skin. The focus of this thesis is to compare the immune mechanism underlying this Ro11-3128-induced vaccination (RoNI) (which induced >90% protection) with the GI (which induced 60-70% protection) and with infections which are both irradiated and drug treated which induce poor levels of immunity (RoGI) (induced only 30% protection).

RoNI, GI, RoGI and a normal unattenuated infection (NI) all resulted in a Th1 biased response (IFN-γ>IL-4) in the skin-draining lymph nodes (SLN), but RoNI and RoGI induced enhanced and more prolonged responses. RoNI was unique in causing highly elevated antigen specific IFN-γ responses in the spleen suggesting local but protracted stimulation of a strong systemic response. Studies in B6RAG1-/- mice (no B or T cells), IFN-γ -/-, μ-MT mice (no B cells) and by *in vivo* depletion of IFN-γ with neutralizing monoclonal antibody during challenge showed that RoNI is mediated almost entirely by IFN-γ mediated mechanisms and antibody is not involved.

Adding Ro11-3128 to cultures containing RoNI-sensitized SLN cells and living schistosomula enhanced the IFN-γ production in the presence of *in vitro* bone marrow (bm)-derived DCs suggesting that altered antigen presentation is induced by drug treatment. However, the superiority of Ro11-3128 in inducing immunity compared with other drugs could not be attributed simply to its ability to induce membraneous blebs as these were also produced by Ro11-3128-treated irradiated schistosomula. Furthermore, there was no evidence of the drug having a general adjuvant effect.

To try to establish how the larval exposure induces the Th1 biased response, living schistosomula were cultured with bone marrow-derived dendritic cells. This had no effect (up- or down- regulate) on surface activation maker expression (MHCII, CD86, CD40) even if the bm-DCs, were partially activated with LPS or TNF-α. There was also no cytokine production (IL-12p70, TNF-α, IL-10 or IL-6). However, schistosomula induced a differential dose-dependent reduction in cytokine production by LPS-activated bm-DCs (IL-12p70 >IL-6 and TNF-α) but IL-10 was relatively unaffected. Again this was unaffected by addition of Ro11-3128.

This work indicates that it is possible to induce highly effective Th1 mediated systemic immunity in mice by protracted but local stimulation of the skin/SLN suggesting that delivery of defined schistosome antigens by Th1 promoting strategies such as prime-boosting with heterologous recombinant viruses delivered in the skin would be worth investigating. There was no evidence that the Th1 response to larval infection is caused by direct action of larvae or larval products on dendritic cells and in fact there was evidence of an anti-inflammatory effect, which should be investigated further at the mRNA level. It is suggested that schistosomula may interact differently with other cells encountered in the skin e.g. keratinocytes in initiating the Th1 bias.

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DEDICATION

To my dear Mum and Dad

I hope this work is as interesting and inspiring to you as it has been to me.

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LIST OF ABBREVIATIONS

ADCC- Antibody-dependent cell-mediated cytotoxicity.

APC – antigen presenting cell.

BCG- *Mycobacterium bovis* Bacillus Calmette-Guerin.

bm-DC – *in vitro* bone marrow derived Dendritic cells.

CC – (Challenge Controls). Naïve mice.

cDMEM – Complete (+10%foetal calf serum) Dulbecco's modified Eagle's medium.

CHO- carbohydrate moiety.

CMI – cell-mediated immunity.

Con A – Concanavilin A.

CTF - cercarial transformation fluid.

DC- dendritic cell.

DC-SIGN - DC-specific ICAM (intercellular adhesion molecule)-3-grabbing nonintegrin.

DMSO – dimethyl sulphoxide.

DOC-SCAP – sodium deoxycholate extract of the aqueous insoluble fraction of cercariae

ELISA - enzyme linked immunosorbent assay.

EU – Endotoxin unit

FACS – Fluorescence-activated cell sorter.

F/T – Freeze-Thawed.

GI – (Gamma irradiated infection) i.e. percutaneous exposure to 500 cercariae irradiated with 20krad.

GM-CSF – Granulocyte-macrophage colony stimulating factor.

i.d. – intradermal.

IL- interleukin.

i.p. – intraperitoneal.

KO – knockout (applied to gene knockout mice, otherwise designated -/-).

krad – kilorad.

LC - Langerhans cells.

Le^X -Lewis x trissacharide.

LN – lymph node.

LPS – lipopolysaccharide.

mAb - monoclonal antibody.

MHCII – major histocompatibility complex class II.

MS – Mechanically transformed schistosomula.

Mu – murine.

NI – (Normal Infection) i.e. percutaneous exposure to 500 unirradiated cercariae.

NK – natural killer (cell).

NS – Not significant.

OD – optical density.

PAMPs - pathogen-associated molecular patterns.

PG – prostaglandin.

p.i. – post-infection.

PRR - pattern recognition receptors.

rMU – recombinant murine.

RT-PCR – reverse transcription – polymerase chain reaction.

Ro11-3128 – the Hoffmann la Roche designation for the benzodiazepine commonly known as Methylclonazepam.

RoCC – (Ro11-3128 treated Challenge Controls) i.e. naïve mice used as controls for mice given drug-terminated infections and so they are treated with 200mg/kg Ro11-3128 at the same time as the immunized groups.

RoGI - (Ro11-3128 treated Gamma irradiated infection) i.e. percutaneous exposure to 500 cercariae irradiated with 20krad followed by treatment with 200mg/kg Ro11-3128.

RoNI - (Ro11-3128 treated Normal Infection) i.e. percutaneous exposure to 500 unirradiated cercariae followed by treatment with 200mg/kg Ro11-3128.

SCAP/SCA – aqueous extract preparation of *S. mansoni* cercariae.

SD – Standard deviation.

SDD - serum derived drug.

SEM – standard error of the sample mean.

SLN – Skin draining lymph nodes.

WHO – World Health Organization.

WT – wild type.

CHAPTER 1

INTRODUCTION

1.1 Schistosomiasis - History and Health:

Among human parasitic diseases, schistosomiasis is one of the great endemic diseases of the tropics and subtropics. It is ranked only second behind malaria in terms of socio-economic and public health importance. It has been a disease since ancient times, as reviewed by Grove (1990) and Ouma and Fenwick (1991), who reported that from hieroglyphic records and from parasitological examination of mummified remains that human schistosomiasis was endemic in Dynastic Egypt. It was not until 1852, however, that the adult worms were first discovered from a human body in Cairos's Kasr ElAini hospital in Egypt by Theodor Bilharz, a young German pathologist, from whom the disease took its original name, Bilharziasis, a name which is still occasionally used. A further 65 years elapsed before the three main human schistosome species and their complete life cycles were clearly described (reviewed by Taylor, 1994).

According to recent current WHO assessment (WHO website, 2003), the disease is endemic in 74 developing countries, with an estimated 600 million people worldwide exposed to the risk of infection, while more than 200 million people are currently infected. Of these, 20 million individuals suffer severe consequences of the disease, and an estimated 120 million are symptomatic. The disease causes a great deal of morbidity (1932 thousand disability-adjusted life years, DALYs) and an estimated 20,000 deaths each year. This mortality is mostly due to bladder cancer or renal failure associated with urinary schistosomiasis and to liver fibrosis and portal hypertension associated with intestinal schistosomiasis.

1.2 Causative Agents:

The major forms of schistosomiasis in humans are caused by several different species of blood flukes which are water borne flatworms (Trematodes) of the genus *Schistosoma.* Three species are the principle causes of disease in man. *Schistosoma haematobium* is responsible for urinary symptoms and disease, S*chistosoma mansoni* and *Schistosoma japonicum* cause intestinal symptoms and hepatosplenic disease.

1.3 Geographical Distribution:

Schistosomiasis is a waterborne, snail-transmitted infection. The geographical distribution of the schistosomes roughly corresponds to the distribution of susceptible fresh water intermediate snail hosts, which are present in many tropical and subtropical regions. Snails are found in many different habitats including permanent or semi-permanent small ponds, marshes, swamps, rivers and streams and large permanent water bodies such as lakes, dams, irrigation channels and rice fields. Those snail hosts are specific for each species of schistosome. *S. mansoni* and *S. haematobium* are transmitted by aquatic snail hosts of the genus *Biomphalaria* and *Bulinus*, respectively, whilst amphibious snails of the genus *Oncomelania* transmit *S. japonicum* (Jordan, Webbe and Sturrock, 1993).

S. mansoni is the most widespread species, being prevalent in 53 countries in Africa, the Middle East, the Caribbean and South America. *S. haematobium* has a similar distribution to *S. mansoni* in the Old World, where it is endemic in 54 countries in Africa and the Eastern Mediterranean (WHO, 1993). In Africa, *S.mansoni* and *S.haematobium* often coexist, and mixed infections are common. Apart from a possible small focus of *S. haematobium* in India, neither *S.mansoni* nor *S.haematobium* occurs in central or east Asia. Oriental or Asian intestinal schistosomiasis mostly caused by *S. japonicum* is endemic in seven countries in South-East Asia and in the Western Pacific Region (WHO, 1993).

1.4 The Life Cycle of Schistosomes:

The complex life cycle of schistosomes (see Figure 1) involves the mammalian definitive host and the snail intermediate host. The cycle is initiated when the free living larvae (cercariae), shed from the intermediate snail host, locate a mammalian host in the water, and burrow into the skin epidermis. On entering the mammalian host, the cercariae shed their tails and form the schistosomula stage of the parasite. The schistosomula then enter the peripheral lymphatic vessels or the blood venules and are carried via the lungs to the liver. Based largely on studies in mice, the larvae spend on average 2 to 5 days in the skin (Miller & Wilson, 1978), after which they penetrate the nearest blood vessel and gain entry to the blood. Once within the capillary they are rapidly transported by the blood flow to the

lungs. Those few (about 10-20%) that penetrated the lymphatics (Wheater & Wilson, 1979) enter the circulation from the thoracic duct. On reaching the lungs, schistosomula

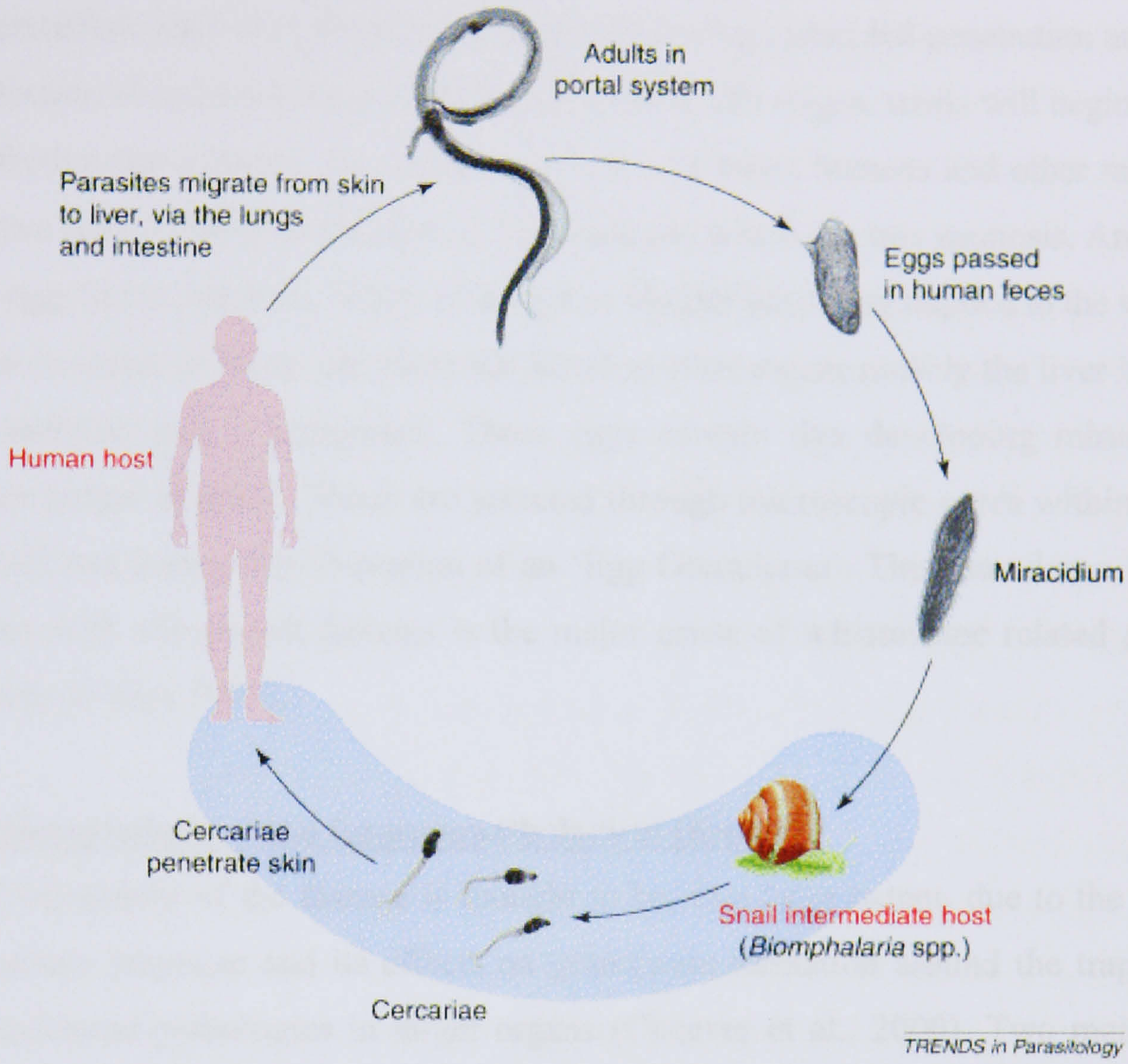


Figure 1. Life cycle of *S. mansoni* (taken from Mckerrow and Salter, 2002)

spend 3-4 days (Wilson et al., 1978) crossing the capillary bed of the lungs and are then carried around the body by the systemic circulation, a proportion reaching the liver as they circulate with the majority of the parasites reaching the liver by 10-15 days after infection (Wilson et al., 1986). Here the worms mature and by 6 weeks post infection nearly all worms have paired before migrating, via the hepatic portal vessels, to either the mesenteric veins in the case of *S .mansoni* and *S. japonicum* or to the vesical plexus around the bladder and ureters in the case of *S. haematobium*, where the female begins to produce eggs. The worms which may live for an estimated 3-7 years in man (Fulford et al., 1995), remain *in copulo* with the female laying hundreds (*S. mansoni* and *S. haematobium*) to thousands (*S. japonicum*) of eggs every day. Many of these eggs are released by the female parasites within the vasculature; they cross the endothelium and basement membrane of the vein, and traverse the intervening tissue, basement membrane and epithelium of the

intestine or bladder tissues from where they exit the body via the faeces (*S. mansoni* and *S. japonicum*) or urine (*S. haematobium*). The eggs contain a developing embryo, the miracidium, which when mature hatches in a suitable fresh water environment and locates its intermediate snail host. Four to five weeks following miracidial penetration and asexual reproduction of intramolluscan schistosome parasite life stages, snails will begin shedding the infective larval stages, the cercariae, which will infect humans and other mammalian definitive hosts, notably in the case of *S. japonicum*, which is a true zoonosis. Around 50% of the eggs fail to reach the lumen of the gut or bladder becoming trapped in the wall of the bladder or ureter or being carried by the blood to other organs notably the liver in the case of *S. mansoni* and *S. japonicum*. These eggs contain live developing miracidia that produce potent antigens. These are secreted through microscopic pores within the rigid egg shell and induce the formation of an 'Egg Granuloma'. This granuloma formation together with subsequent fibrosis is the major cause of schistosome related pathology (Cheever & Yap, 1997).

1.5 Schistosomiasis – "An Immunopathological Disease":

The development of the disease is thought to be, to a large extent, due to the nature of the immune response and its effects on granuloma formation around the trapped eggs and associated pathologies in target organs (Cheever et al., 2000). Two main clinical conditions are recognized in *S. mansoni*-infected individuals- Acute schistosomiasis and Chronic schistosomiasis.

1.5.1 Acute/Chronic disease:

- **Acute Schistosomiais:**

In humans, Katayama fever, which is the acute form of schistosomiasis is a debilitating febrile illness that can occur before the appearance of eggs in the stool. It is thought generally to peak between 6 and 8 weeks after the host is infected by cercariae (Rabello, 1995). This febrile illness seems to be uncommon in individuals who live in areas that are endemic for schistosomiasis, and only occurs in individuals who have no previous history of exposure who become infected after travelling into an endemic area. During this acute illness, there is a measurable level of tumour-necrosis factor (TNF-α) in the plasma, and peripheral blood mononuclear cells (PBMCs) produced large quantities of

TNF-α, interleukin-1 (IL-1) and IL-6 (de Jesus et al., 2002). The PBMCs from patients with acute schistosomiasis were stimulated by parasite antigen to produce elevated levels of IFN-γ and low levels of IL-5 as compared to PBMCs from patients with chronic disease, thus reflecting a dominant Th1, rather than Th2 response in the acute form of the disease (de Jesus et al., 2002).

In mice, the acute prepatent phase of infection is also associated with the antigen-specific production of Th1 cytokines (IFN-γ, IL-2 and TNF-α) (Grzych et al., 1991) but as soon as eggs are produced this switches to a Th2 dominated response, but one in which there is an underlying Th1 response (Rutitzky et al., 2001). The inability to develop a Th2 response to regulate the initial pro-inflammatory response that is associated with acute schistosomiasis is lethal. This was first observed when C57BL/6 IL-4 deficient mice were infected with *S. mansoni*. These mice suffered from cachexia and significant mortality on the onset of parasite egg production (Brunet et al., 1997) eventhough they developed relatively normal hepatic granulomas (although they lacked an eosinophil component). Their intestines, on the other hand, showed pathological changes which included non-haemorrhagic lesions on the mucosal surface (Brunet et al., 1997) that were associated with the inefficient passage of eggs into the lumen (Fallon et al., 2000b). Analysis of the immune responses of infected IL-4 deficient mice showed that there was a correlation between elevated levels of nitric oxide (NO) and disease severity (Brunet et al., 1997).

- **Chronic Schistosomiasis:**

Chronic disease is graded according to severity. The liver granulomas themselves do not generally affect the liver function, but pathology occurs by blockage of portal blood flow (the granuloma is around 100 times the size of the egg itself). In some respects, the pathology in mice is comparable with that observed in humans (Fallon, 2000). Impairment of the hepatic blood flow leads to the development of portal hypertension and hepatosplenomegaly. This may be further complicated by fibrosis, which may progress to irreversible Symmers' clay pipe-stem fibrosis caused by calcification around branches of the portal veins (Lambertucci, 1993). In severe cases portal hypertension leads to the formation of the collateral circulation, which enables blood to by-pass the liver and carry eggs to organs such as the lungs, kidneys or central nervous system (CNS) where

granulomas may also form. The collateral circulation can carry blood to thin walled oesophageal varices which can bleed or rupture causing haematemesis. In the case of *S.haematobium* infections calcification of the bladder and ureters can take place leading to loss of bladder, ureter and kidney function. The immunological basis of this pathology is considered below.

1.5.2 The Egg Granuloma:

The cell-mediated delayed-type host granulomatous inflammatory reactions (Boros and Warren, 1970) that develops around the schistosome egg is composed of eosinophils, T cells, mast cells, B cells, epitheloid cells, fibroblasts, macrophages, basophils, giant cells and some neutrophils. Research in mice has shown that the granuloma structure is influenced by antibodies (Jankovic et al., 1998), chemokines (Park et al., 2001), cytokines (Cheever et al., 1998), adhesion molecules (Ritter and McKerrow, 1996) and apoptosis (Lundy et al., 2001; Rumbley et al., 2001). Because of the constant turnover of cell populations, granulomas change in size during their development and resolution. Their circumoval localisation and dense cellularity of the granulomas isolates the egg from the healthy liver parenchyma thus rendering protection of the host. This concept was shown from infection studies in T cell-depleted mice (Mathew and Boros, 1986), thymectomised mice (Domingo and Warren, 1967; Dunne & Doenhoff, 1983; Cheever et al., 1985), SCID mice (Cheever et al., 1999) and nude mice (Cheever at al., 1989), where the host is unable to form a well-organised, circumoval granuloma, there is circumoval parenchymal necrosis and the mice die. It has been demonstrated by Doenhoff et al., (1981) and Dunne and Doenhoff, (1983) that certain *S. mansoni* egg molecules are highly toxic to liver parenchyma cells, thus confirming that if a mature circumoval granuloma is not formed during *S. mansoni* infections then these 'hepatotoxic' egg antigens can cause extensive liver damage.

1.5.3 What type of immune response do schistosome eggs induce?

The formation of granulomas during murine infection has been shown to be dependent upon T cell, and specifically CD4+ T-cell, responses (Cheever et al., 1985, Mathew and Boros, 1986). Infection of mice deficient for CD4+ T cells (Fallon et al., 2000a) led to a similar pathological outcome as infected thymectomised, SCID, nude or T cell-depleted mice, where the granuloma inflammation was abrogated, liver damage took place and

mortality rates increased. In humans, a study of CD4+ T cell-deficient humans (HIV co-infected) also suggested that CD4+ve T cells are involved in granuloma formation (Karanja et al., 1997). It had previously been shown that T-cell deprived mice showed reduced faecal egg output and it was reasoned that the granuloma formation was necessary for this excretion (Doenhoff et al., 1981; Cheever et al., 1999). Similarly the HIV and schistosome co-infected individuals had a significant defect in egg excretion in comparison to HIV negative-schistosome positive individuals suggesting that they also had impaired granuloma formation (Karanja et al., 1997).

In mice the involvement of CD4+ T cells in pathology has been further defined as a requirement for the production of Th2 cytokines. A number of studies in mice have demonstrated that down-regulation in the production of Th1 type cytokines e.g. IFNγ and IL-2 and an up-regulation in Th2 cytokines such as IL-4, IL-5 and IL-10 (although not exclusively a Th2 cytokine), occurs at the onset of egg production (Pearce et al., 1991; Sher et al., 1991; Grzych et al., 1991). This also appears to be the same in human infections (Parra et al., 1992; Williams et al., 1994; El Ridi et al., 1997), although Montenegro et al., (1999) reported that in chronically infected individuals a mixed type-1/type-2 anti-egg cytokine response can be observed.

Production of IL-4 is crucial for the development of Th2 responses and for the counter-regulation of Th1 responses. IL-4 depletion in shistosome-infected mice or intravenously egg-injected mice (a commonly used model of granulomatous response and which synchronous pulmonary granulomas are produced) demonstrated a dramatic effect in reducing egg-induced granuloma inflammation and the production of the type-2 cytokines IL-5 and IL-13 (Yamashita and Boros, 1992; Wynn et al., 1993; Cheever et al., 1994). On the otherhand, administration of recombinant IL-4 to chronically infected animals reversed the down-regulated granulomatous response typically observed in later stages of infection (Yamashita and Boros, 1992). It is clear from the above studies that IL-4 is, in part, responsible for the generation and maintenance of the type-2 immune responses.

1.5.4 How do eggs induce the Th2 response?

It was established a long time ago that the egg, rather than the adult worm, could sensitize cells to induce granuloma formation (Warren and Domingo, 1970). There has been much recent interest in trying to define the molecular basis of the interaction of egg products with cells of the innate immune response resulting in Th2 polarization of the immune response.

i- Eosinophils and Mast cells:

The ability of eggs themselves to initiate production of Th2-associated cytokines was demonstrated by Sabin et al. (1996a). Thus, intraperitoneal injection of isolated eggs into mice rapidly stimulated production by peritoneal exudate cells of IL-4 and IL-5, early peritoneal recruitment of eosinophils and a loss of mast cells. The data suggested that egg derived molecules induce IL-5 release from degranulated mast cells and this IL-5 then recruits and activates eosinophils which provide the early burst of IL-4 required for subsequent Th2 cell development. Rumbley et al., (1999) have shown that eosinophils are the major source of IL-4 in the granulomas themselves.

ii- Basophils:

Basophils have also been shown to produce IL-4 in direct response to SEA (Falcone et al., 1996). Recently, Schramm et al. (2003) have characterized a factor in *S. mansoni* eggs that could be responsible for the switch to a Th2 response. A protein, which the authors named IPSE (IL-4-inducing principle from *S. mansoni* eggs) was isolated and characterized. In response to exposure to *S. mansoni* egg antigen extract (SEA), basophils degranulate rapidly and release IL-4 and other inflammatory mediators. The results of functional *in vitro* assays in which human basophils were incubated with whole SEA, SEA fractions and recombinant IPSE, and the fact that antibodies raised against recombinant IPSE inhibited the activation of basophils by SEA, indicated that IPSE is the sole factor responsible for the basophil-activating effects of SEA. Further analysis confirmed that IPSE can bind IgE, indicating that it is the crosslinking of receptor-bound IgE that accounts for IPSE-mediated basophil activation.

iii- Dendritic cells (DC):

- <u>In Mice</u>

Much attention has been focussed in recent times on the initial interaction between microbial products and receptors on dendritic cells which are specialised for sampling foreign material and initiating appropriate responses. In an elegant series of experiments, Macdonald and Pearce (2002a; 2002b) showed that mouse derived DCs cultured with soluble *S. mansoni* egg antigen (SEA) could be primed such that they can transfer Th2 responsiveness to recipient mice i.e. splenocytes from such mice given SEA-pulsed DCs were stimulated *in vitro* by SEA to produce IL-4, IL-5, IL-13 but not IFN-γ. Carbohydrate components of the SEA have been implicated in this process because LN cells of mice that received periodate-treated SEA-pulsed DCs produced 6- to 10-fold less Th2 cytokines (IL-4, IL-5 and IL-10) upon *in vitro* stimulation with SEA (Faveeuw et al., 2002).

- <u>In Humans</u>

Studies with Human myeloid DCs have shown the upregulation of OX40L expression (a factor known to be expressed by the peripheral blood DC and involved in Th2 cell development [Flynn et al., 1998]), and production of poor levels of IL-12p70, TNF-α and IL-6 following interaction with SEA upon CD40 ligation (de Jong et al., 2002). Interestingly, glycan moieties present on egg glycoproteins or glycolipids have also been implicated in this DC priming for Th2 response.

iv- Suppressor macrophages:

Relatively recently, populations of natural suppressor cells originating from the granulocyte –macrophage lineage and expressing Gr1, CD11b and/or F4/80 have been described (Angulo et al., 1995; Maruyama et al., 1999; Salvadori et al., 2000). These fall into two categories: 'classically activated' macrophages which are IFN-γ dependent and associated with cancers and viral infections (e.g. Cauley et al., 2000) and 'alternatively activated' macrophages which are IL-4 dependent and have been demonstrated in mice in response to implanting the filarial worm *Brugia malayi* (Allen et al, 1996; MacDonald et al., 1998).

Two schistosome oligosaccharides, lacto-N-fucopentaose III (LNFPIII) and lacto-N-neotetraose (LNnT), which are components of SEA molecules were identified as Th2-biasing and capable of suppressing Th1 responses *in vivo* (Vellupillai et al., 1997; Okano, et al., 2001). Atochina et al. (2001) showed that a single injection of mice with a glycoconjugate composed of LNFPIII conjugated to dextran (LNFPIII-dex) rapidly

expanded the peritoneal CD11b+/Gr1+/F4/80 population in a T cell-independent manner suggesting a direct interaction of the CHO moieties with macrophages. These cells suppressed the *in vitro* proliferation of naïve CD4+ T cells to primary stimulation with anti-CD3 and anti-CD28 antibodies and the process of inhibition was found to be NO- and IFN-γ-dependent showing that, in contrast to the filariae (Loke et al, 2000), schistosome eggs stimulate a 'classically activated' suppressor population. Injection of the structurally related glycoconjugate lacto-N-neotetraose-dextran (LNnT-dex) has also been shown to expand such suppressor cells and these were able to imprint a Th2 phenotype on naïve CD4+ T cells, characterized by lower IFN-γ, but increased IL-13 production (Terrazas et al., 2001).

The ability of these helminth-expressed oligosaccharides to induce suppressor macrophages may also be responsible for the modulation of the host response and induction of immune anergy as the infection progresses (Atochina et al., 2001). In terms of the mechanism of action Terrazas et al. (2001) showed that the Gr1+ population that suppressed the proliferative *in vitro* response of naïve CD4+T cells required cell-to-cell contact (suggesting a receptor-mediated mechanism). Furthermore, the suppressor cells produced low levels of pro-inflammatory cytokines together with raised IL-10 and TGF-β but did not lead to CD4+ T cell apoptosis.

1.5.5 Immunopathology following a skewed Th1-immune response:

The host response to schistosome eggs is, most of the time, one that recognises the destructive nature of these agents and responds in a controlled manner to limit severe pathology. These responses can lead to fibrotic tissue lesions, but generally do not result in mortality. Deviation away from this tightly regulated, egg-associated, Th2-dominated immune response leads to pathologies that are severe and often life-threatening.

In a study undertaken by Mwatha et al. (1998), the immunological responses in *S. mansoni* infected-children and adolescents were described. There was no apparent periportal or hepatic fibrosis in either study group in which individuals were carefully matched for age, prevalence, intensity of infection and tribal origin. It was found that children/adolescents who suffered severe enlargement of liver and spleen had significantly more type-1 associated IFN-α and TNF-α production from antigen-

stimulated PBMCs in comparison to those who did not develop organ enlargement. Additionally, high plasma levels of sTNFR-I (TNF-α receptor I), sTNFR-II and ICAM-1 (Intercellular Adhesion Molecule-1) were all significantly associated with hepatosplenomegaly, whereas individuals who had less severe disease but similar intensity infections (as assessed by counting the number of eggs in faecal samples) had more of a Th2 response and low plasma levels of soluble TNFR. These observations indicated that hepatosplenomegaly, in the absence of severe fibrosis, can be associated with elevated Th1 associated immune responses.

Skewed Th1 responses have also been studied in mice. Brunet et al., (1997) showed that IL-4 deficient mice responded to schistosome infection by generating strongly polarised type-1, as opposed to type-2 inflammatory reactions. This type-1 inflammatory response was observed shortly after the onset of egg deposition and was characterised by egg-specific IFN-γ, TNF-α and inducible nitric oxide (iNO) production as well as a rapid rate of weight loss and ultimately mortality. The granulomas formed around tissue-deposited eggs in those IL-4 deficient mice were of similar size to control, infected wild-type mice but hepatic fibrosis was diminished. It was suggested that lesions seen in the gut of the $IL4^{-/-}$ mice led to bacterial invasion and it was concluded that the pronounced type-1-mediated inflammatory reactions that developed in the absence of IL-4 in combination with increased systemic endotoxin levels induced a state of cachexia that led to a rapid mortality. In another study of infected $IL4^{-/-}$ mice, the immune response was also biased to a Th1 response but the mortality in this study was less which was attributed to less gut associated pathology (Hoffmann et al, 2000).

Additional studies showed that infected IL-4 deficient mice suffering from increased mortality rates, had significantly less *ex-vivo* antigen-specific IL-10 production in comparison to control infected animals (Brunet et al., 1997). Sher at al., (1991) had earlier demonstrated that IL-10 was rapidly produced at a time concomitant with egg deposition and that this cytokine remained elevated into the chronic stages of disease. Further studies have implicated IL-10 in regulating inflammatory responses in both mice and humans. Administration of exogenous IL-10 to infected mice was found to dramatically decrease the size of granulomas during the acute stages of disease (Flores-Villaneuva et al., 1996). Later Wynn et al., (1998) demonstrated that infected IL-10-

deficient mice displayed significantly larger circumoval granulomas than control-infected WT animals. A detailed examination of the immunological characteristics in these infected IL-10-deficient mice demonstrated that they develop increases in both type-1 and type-2 immune responses and suffer enhanced mortality (Wynn et al., 1998).

Immune hyporesponsiveness characterizes the chronic stages of many helminth infections including schistosomiais, and T regulatory (Treg) cells have been implicated in this process. The factors controlling Treg as opposed to Th1 or Th2 development are comparatively poorly understood. However, recent studies have implicated TLR2 ligation in this process, as schistosome lyso-phosphatidylserine isolated from schistosome eggs or adult worms was found to condition human DCs via TLR2 to induce Treg responses (van der Kleij et al., 2002). This might account for the skewing to IL-10 production and T-cell hyporesponsiveness that is seen during chronic schistosomiasis.

Studies in humans confirmed the role of IL-10 in urinary tract morbidity during *S. haematobium* infection (King et al., 2001). IL-10 (and TNF-α) release from egg-stimulated PBMC cultures was compared in age and sex matched infected children and adolescents suffering from moderate to severe bladder wall pathology. A significantly lower ratio of egg-specific IL-10:TNF-α production from PBMC cultures obtained from the patients with severe bladder wall pathology, suggesting that low IL-10 and high TNF-α can correlate with an increased risk of developing severe disease during schistosomiasis. A recent study examining the correlates of developing severe fibrosis in adult male populations living in Uganda who were chronically infected with *S. mansoni*, also demonstrated that a deficiency in IL-10 was partly responsible (M. Booth and D.W. Dunne, cited as a personal communication in Hoffmann et al., (2002)).

In an elegant series of experiments Hoffmann et al (2000) compared the responses of knock-out mice for either IL-4, IL-10 or both cytokines. Infected, IL-4-deficient mice displayed type-1 immune responses but made similar levels of IL-10 and lived just as long as infected WT animals, whereas animals deficient in both IL-10 and IL-4 suffered 100% mortality by week 8 post-infection and showed a strongly polarized Th1 response. Rapid cachexia, abundantly produced IFN-γ and TNF-α, elevated

hepatotoxicity, minimal fibrosis and increased splenic iNO production were all detectable in the IL-10/IL-4 doubly deficient mice.

Despite the more severe pathology induced by a marked shift towards Th1 responses, it has been demonstrated in mice that promoting a Th1 response to the egg by immunizing with egg antigens plus IL-12 can result in lower Th2/Th1 cytokine response to eggs following infection with a resultant reduction in fibrosis and mortality (Wynn, 1995). It has been suggested that this might form the basis of a defined anti-pathology vaccine but clearly this is a risky strategy in a genetically diverse human population.

1.5.6 Immunopathology following a skewed Th2-immune response:

The above series of experiments (Hoffmann et al, 2000) also included mice strongly polarised to Th2 responses. Infected animals deficient in both IL-10 and IL-12, compared to WT mice, produced 10 times the amount of antigen-specific type-2-associated IL-4, IL-5 and IL-13 during the acute stages of schistosomiasis. All of the type-2 polarised, IL-10/IL-12 doubly deficient animals also lost weight at the onset of schistosome oviposition (~ 5 weeks post-infection) and over 50% succumbed to death at 12 weeks post-infection. The increased mortality was directly related to overproduction of the pro-fibrotic cytokines IL-4 (Kovacs, 1991) and IL-13 (Chiaramonte et al., 1999) as well as to diminished levels of the anti-fibrotic cytokine, IFN-γ (Czaja et al., 1989).

This increased ratio of pro-/anti-fibrotic mediators was confirmed by cDNA microarray analysis and resulted in the deposition of significantly more hepatic collagen in the livers of infected IL-10/IL-12 doubly deficient mice and ultimately death, in a high proportion of animals (Hoffmann et al., 2000; Hoffmann et al., 2001). From these investigations a link was made between the development of unregulated type-2 immune responses and severe, fibrosis-related immunopathology contributing to enhanced mortality rates during schistosomiasis.

In humans, the vast majority of all schistosome-infected humans do not develop severe hepatic morbidity, but a minority (2.5-5%) of individuals will develop quite severe hepatosplenomegaly and immunopathology related to fibrosis. These severely ill individuals are not necessarily the most heavily infected, but have usually harboured the

disease for the longest period of time. Ultrasound measurements have confirmed that the progression of hepatic and periportal fibrosis develops with age. This has been observed in *S. japonicum*-infected patients where more severe fibrosis was observed in older individuals as compared to young individuals (Olds et al., 1996). This age/fibrosis relationship was also confirmed in cross-sectional studies in Uganda and other investigators' studies in *S. mansoni* endemic areas (Hoffmann et al., 2002). These findings suggest that individuals who go on to develop severe fibrotic-related hepatosplenomegaly later in life are a subgroup of the infected population that fail to resolve their initial low-grade hepatic lesions.

In an analysis of pedigrees in the Sudan, in an area where *S. mansoni* is endemic, Dessein and colleagues found that severe hepatic fibrosis (as identified by ultrasound) was more likely to occur in certain families (Dessein et al., 1999). A codominant major gene, known as *SM2*, was shown by segregation analysis to be responsible for the observed familial distribution of hepatic fibrosis and portal hypertension. 'Informative' families, which had multiple cases of severe fibrosis, were used to map *SM2* to 6q22-q23 – a region that contains the gene that encodes IFN-γ receptor 1 (IFN-γR1) (Dessein et al., 1999). One interpretation of these data is that mutations in IFN-γR1 that lead to loss of function of the receptor are associated with a lack of effectiveness of IFN-γ in suppressing fibrogenesis. As IFN-γ is a potent anti-fibrotic cytokine (Czaja et al., 1989), segregation of 6q22-q23 among severely fibrotic individuals suggests that these infected people cannot counter-regulate the pro-fibrotic activities of IL-4 and IL-13.

A more recent study carried out in Uganda is reported to provide firm evidence linking high IL-4 and IL-13 levels with chronically infected adult male and female individuals, respectively, suffering from severe fibrosis (M. Booth and D.W. Dunne, cited as a personal communication in Hoffmann et al., (2002)). The combination of defective IFN-γ signalling with improperly balanced type-2 cytokine regulation leads to an environment suitable for collagen synthesis, hepatic and periportal fibrosis, and elevated morbidity and mortality. From existing evidence, this type of immunopathology becomes manifested fully only after years of exposure to schistosome parasites and/or abnormal host responses, which may be more often observed during the chronic stages of disease.

In summary this balanced Th2 granulomatous response to the egg is host protective despite inducing immunopathology, but a shift in the balance in either the Th1 or Th2 direction can cause even more severe disease.

1.6 The Control of Schistosomiasis:

Major factors associated with the spread and intensification of schistosomiasis are its links with water development projects, particularly construction of dams, man-made lakes and irrigation schemes, since these are often sites of population immigrations for farming and fishing. Control of schistosomiasis has been attempted for many years, and has usually been based on a mixture of measures directed against the snail hosts and chemotherapeutic treatment of man, together with environmental management and public health measures. However, in many developing countries the use of these methods and their integration within suitable control programmes is limited by economic constraints, and chemotherapy remains the mainstay of schistosomiasis control (Savioli et al., 2004).

Although Oxamniquine is still used in some areas for the treatment of *S. mansoni* and Metrifonate for the treatment of *S. haematobium*, Praziquantel, the major drug of choice, is able to eliminate all species of schistosome infections. Thus, the main problem with control programmes which rely upon chemotherapy is the cost. Although Praziquantel is a comparatively cheap drug, in areas of high transmission a high rate of reinfection after chemotherapeutic cure among young children means that re-treatment is often required at frequent intervals, thus, long-term funding needed for such control programmes can be difficult to sustain (Dunne et al., 1995). Added to this is the growing concern regarding the possible development of drug resistance which has been shown for Oxamniquine in South America (Coles et al., 1986) and experimentally in mice with praziquantel (Fallon and Doenhoff, 1994).

While highly effective drugs are available for treatment, schistosome parasites continue to cause significant morbidity and mortality in the human population. This is due in part to the failure of previously infected individuals to develop resistance against the parasite until the mid teenage years. A major focus of schistosomiasis research is to develop an

effective anti-schistosome vaccine that will be effective in children and so significantly reduce the incidence of severe disease. With this aim in view there have been extensive studies into the nature of the immune response to schistosomes in both humans and experimental animals. In addition numerous parasite antigens have been identified as potential vaccine candidates (Bergquist and Colley, 1998). In the following sections the evidence for naturally occurring immunity in man and for immunity in experimental animals is considered along with the current understanding of the status of vaccine development.

1.7 Immunity in Humans:

Two groups of individuals have been identified as manifesting immunity to schistosomiasis infection in the field. Those that are demonstrably resistant to reinfection after chemotherapeutic treatment of existing infections, and those that appear to have developed natural resistance to infection named 'Endemic Normals' (EN). The former differ from the EN in that they harbour infections of some level of intensity but their level of resistance to reinfection with schistosomes prior to treatment cannot be determined. Most work has been done on resistance after chemotherapy but reference will be made where relevant to the studies of ENs.

1.7.1 Age-intensity patterns:

In infected populations resident in schistosomiasis endemic areas, children carry the heaviest parasite burdens (Fisher, 1934). As measured by the increase in egg output (which indicates a build up in the number of established adult worms) infection intensities rise in early childhood, peak around the age of 12 years, and then rapidly decline (Mutapi, 2001). It was originally thought that this was entirely due to the fact that children, on average, tend to have more water contact than either adults or the very young (Warren, 1973). However, alternative possibilities were that adults may have lower innate susceptibility, or enhanced protective immunity either due to long-term exposure to infection or to an age-dependent ability to develop appropriate immune responses to infection (Butterworth, 1990). More recent studies of populations in which the infection had been recently introduced (reviewed by Gryseels, 1994) demonstrate that similar age-intensity profiles develop relatively soon after the start of exposure

indicating that it is age *per se* more than duration of exposure that is responsible for the lower infection intensities in the adults (Polman et al., 1995; van Dam et al., 1996).

1.7.2 Resistance after chemotherapy:

In order to investigate the relative importance of water contact and immunity several important studies have been conducted in which infected people of different ages were chemotherapeutically cured and their reinfection rates monitored along with their level of water contact. In this way water contact could be controlled for.

Such studies on *S. mansoni* in Kenya (Butterworth *et al.*, 1984; Butterworth *et al.*, 1985) identified a group of children classed as resistant because they had high water contact but low rates of reinfection and a group of susceptible children which become heavily reinfected following treatment. Comparison of the data for these two cohorts demonstrated that the average age of the resistant group was 13 and that of the susceptible 11 which indicated the operation of age dependent factors that prevent reinfection. Demeure et al. (1993) took a similar approach and concluded that although water contact was a major factor in intensity of infection it could not explain the patterns adequately.

Fulford et al., (1996) looked at data from a number of studies and showed that the peak in intensity of re-infection occurred at a very consistent age followed by a sharp decline. The data on water contact for several areas showed generally higher contact for the younger age groups but the distribution was much more spread and clearly did not mirror the age intensity pattern. Furthermore, in a study of a fishing community in Uganda, the pattern of observed contact with water known to contain infected snails was greater in adults than in children (Fulford et al., 1996). Despite this, six months after treatment, the Ugandan community showed a peak of reinfection intensity at about ten years of age, which fell away to a much lower level by the age of twenty (Kabateriene et al.,1999). In a recent study by Scott et al., (2003), of 5 recently infected villages in northern Senegal there was evidence that a person's age, sex and place of residence determines water contact behaviour, but none to suggest that exposure has an influence on the relationship between these factors and intensity of infection.

So overall water contact is obviously a major influence on infection intensity especially in the young but other factors also play a major role among which immunity has been proposed to play a role (Butterworth et al., 1992).

1.7.3 Immune Mechanisms involved in immunity *in vivo*:

1.7.3.1 Humoral responses:

A study of *S. haematobium* infection in Gambia first demonstrated that the level of IgE antibodies, mainly against adult worms, increased progressively with the age of the patient and so was related to resistance to reinfection after chemotherapy in that specific IgE was high in the adults and low in the children (Hagan et al., 1991). The levels of IgE alone however could not explain the pattern of reinfection intensity but when IgG4 and IgE were considered together a better correlation was seen. IgG4 also increased with age up to late childhood but then declined in the teenage children. The lowered reinfection intensity correlated with high IgE but lowered IgG4 and it has been suggested that the IgG4 might act to block a protective action of IgE (perhaps involving ADCC/blocking antibodies against the larval stages – see section 1.7.6.). So it is when the IgG4 production declines that the protective effect of IgE appears. The influence of IgE and the reciprocal effect of IgG4 in immunity to *S. mansoni* reinfection has also been reported in several independent investigations carried out in Africa and Brazil (Dunne et al., 1992a and 1992b; Demeure et al., 1993; Rihet et al., 1991; Caldas et al., 2000).

The influence of age itself rather than duration of infection on specific IgE levels was studied in a population of very recent immigrants to an endemic area in Kenya (Naus et al.. 1999) and it was concluded that the development of such IgE was dependent on age but also influenced by the intensity of infection. The way in which antibody isotypes are affected by age and exposure was also investigated by Naus et al., (2003). They analysed the specific antibody isotype responses (IgA, IgG1, IgG2, IgG3, IgG4, IgE, IgM) against *S. mansoni* worm and egg antigens in relation to age, sex and faecal egg-count, in a Ugandan community where adults are heavily exposed to infection through the fishing economy, but where infection intensity, as measured by egg counts, is higher

in males across all ages. Even though the duration of water contact on a daily basis is similar in the 2 sexes, they are involved in different activities that may carry different risks of infection (Kabateriene et al., 1999). Using statistical analysis, Naus et al. (2003) showed that in both sexes within this fishing Ugandan community most anti-*S. mansoni* worm antigen responses (anti-SWA) increased with age, whereas anti-SEA (anti-S. mansoni egg antigens) decreased with age, especially after puberty. IgG1, IgG4, IgE anti-*S.mansoni* worm antigen and IgG4 anti- *S. mansoni* egg antigen responses were independently higher in males, whereas IgG2-SEA responses were independently higher in females. The significant effects of sex on isotype responses to adult worm antigens may be partly because of different levels of cumulative exposure. The fishing activities of males may expose them to more cercariae than females over a period of years, and thus the cumulative exposure to schistosome antigens may vary substantially between the sexes (Naus et al., 2003).

1.7.3.2 Which antigens are recognised by "protective" antibodies?

There has been interest in trying to identify which antigens may be preferentially recognised by putatively protective antibody isotypes. IgE responses to a 22.6kDa adult worm antigen has been identified as correlating with immune status (Dunne et al., 1997, Webster et al., 1996). Although there is a focus on IgE as a potentially important effector isotype in immunity in humans there is data implicating other antibody types. Investigations in Kenya and Senegal have shown an association between IgA anti-Sm28GST and the age-dependent decrease in egg excretion in infected individuals (Grzych et al., 1993). Also, resistance to reinfection with *S. mansoni* has been associated with higher levels of IgG reactivity to a 37 kDa larval surface antigen (Dessein et al., 1988). These observations suggest that expression of immunity is not simply determined by the balance of IgE and IgG4, but might include the participation of other mechanisms.

Antibody class switching to IgE is controlled by IL-4, a key cytokine and marker of Th2 responses (Janeway et al., 2001) and so the identification of IgE as a possible mediator of resistance after chemotherapy in humans focuses attention on the Th2 component of the immune response.

1.7.4 Other evidence for Th2-mediated effector functions in resistance after chemotherapy:

1.7.4.1 IL-5 correlates with resistance to reinfection:

A longitudinal study of cellular responses of infected individuals in Kenya before and after treatment showed a clear negative association between the proliferative responses to adult worm- and schistosomulum-stage antigens and subsequent reinfection intensity in older individuals (the resistant group), who also had significantly higher levels of IL-5 in tissue culture supernatants of *in vitro*-stimulated cells than did younger individuals (the susceptible group) (Roberts et al., 1993). IL-5 is produced by Th2 cells and as it is responsible for eosinophil production/action this suggested a role for eosinophils in human immunity *in vivo.* In fact earlier studies had suggested that resistance to *S. haematobium* in humans was related to high eosinophil counts (Hagan et al., 1985).

1.7.4.2 Genetically "resistant" individuals make high Th2 responses:

The acquired immune responses that have been associated with resistance to reinfection after chemotherapy are all of the Th2 type, characterized by high secretion of IL-4, IL-5 and IgE and high numbers of eosinophils (Roberts et al, 1993; Medhat et al., 1998; Hagan et al., 1991; Dunne et al., 1992a; Rihet et al., 1991; Hagan et al., 1985).This conclusion is supported by genetic studies that demonstrated, by segregation analysis, that a major gene (*SM1*), which regulates the 'intensity of infection' phenotype, can be linked to a gene region located on chromosome 5 (5q31-q33), which encodes IL-4, IL-5, IL-9 and IL-13 cytokines involved inTh2 responses (Abel et al., 1991; Marquet et al., 1996). Most strikingly schistosome antigen-specific T cell clones derived from homozygous resistant individuals produced 10-1000 fold higher levels of IL-4 and IL-5 compared with clones from homozygous susceptible individuals but comparable levels of IFN-γ (Rodrigues et al., 1999). This clearly implicates Th2 responsiveness controlled by the *SM1* gene in natural resistance to schistosomes even in the absence of any treatment induced responses.

Apart from this clear polymorphism in relation to Th2 responsiveness there have been other studies implicating genetic diversity in relation to recognition of specific schistosome antigens. Studies in Brazilians and Kenyans infected with *S. mansoni* have suggested that there is some genetic restriction with regards to the ability of the host to recognize different epitopes, to mount a specific immune response and to produce an isotype profile against the same antigen. Thus, Bethony et al., (1999) studying *S. mansoni* egg positive siblings from different families demonstrated that there was significant familial resemblance for all IgG subclasses and IgE levels directed against adult-stage antigens (Smp20.8 and Smp50). Amongst both egg positive and egg negative siblings only IgE and IgG4 isotypes showed familial resemblance to an egg-stage antigen (Smp40). In Kenya, anti-schistosome IgE responses were restricted to only a few people whose immune system recognized a limited repertoire of antigens (Dunne et al., 1992b). Although no genetic basis has been established for the Kenyan results, they are still consistent with at least some genetic restriction of host immuno-competence and antigen recognition.

1.7.5 Immune Mechanisms demonstrated *in vitro*:

1.7.5.1 Eosinophil / IgE-mediated ADCC:

Studies performed *in vitro* have demonstrated that human effector cells: eosinophils, macrophages and platelets are able to kill freshly transformed schistosomula in the presence of complement and / or antibody (Butterworth et al., 1975; Joseph et al., 1983; Joseph et al., 1985). Regarding antibody-mediated cellular cytoxicity both the IgG (Anwar et al., 1979) and IgE (Capron et al., 1984) fractions of human infection sera have been shown to be capable of opsonising the schistosomula for killing but IgE/eosinophils is a particularly potent combination (Dunne et al., 1993).

1.7.6 Blocking antibodies:

Using blood samples taken from children deemed either resistant or susceptible to reinfection, Butterworth *et al.*, (1985) examined the antibody response to the schistosomula, adult and egg stages of the parasite as well as measuring eosinophilia. No positive correlation between resistance and the levels of antibody or number of eosinophils

was detected but negative correlations were observed between the levels of antibody raised against the egg stages and resistance to reinfection (Butterworth *et al.*, 1987) and it is now suggested that the development of human immunity requires the down regulation of antibodies which are capable of preventing the cytotoxic action of others i.e. blocking antibodies.

The existence of antibodies able to block *in vitro* ADCC were initially described by Grzych *et al.*, (1982, 1984) using monoclonal rat IgG2a, which had Fc receptors on eosinophils and IgG2c which did not. Monoclonal antibodies of the IgM isotype which are able to block *in vitro* killing of schistosomula by immune human serum and eosinophils have also been described (Dunne *et al.*, 1987). Evidence that blocking antibodies are raised during a natural infection of humans was provided by Khalife *et al.* (1986) who demonstrated that the *in vitro* killing of schistosomula by the IgG fraction of sera taken from infected individuals was abolished by prior incubation of the larvae with the IgM fraction. The levels of IgG2 in human sera have also been shown to correlate negatively with resistance to reinfection (Dunne et al., 1987; Demeure et al., 1993). As IgG2 like IgM, is raised in response to carbohydrate epitopes it has been suggested that antibodies raised against carbohydrate epitopes on the egg prevent the binding of effector antibodies directed at cross reactive epitopes upon the surface of the schistosomula and may in part account for the lack of immunity seen in younger children (Khalife et al., 1986; Dunne et al., 1987).

The killing of schistosomula with eosinophils and IgE is not affected by prior incubation with IgM (Khalife et al., 1986) and suggests that IgE recognises different epitopes to these blocking antibodies. However, as discussed above there is an *in vivo* correlation between resistance and a high IgE response coupled with a low IgG4. It has therefore been suggested that the onset of immunity may represent an immunoglobulin class switch which is caused by the involvement of different T cell cytokines (Hagan et al., 1991). Like the IgM and IgG2 isotypes which block the IgG cytotoxic killing of the schistosomula, IgG4 may block IgE mediated larval killing. Although the target antigens for any such ADCC operating *in vivo* are not known, IgG4 levels in serum of infected Kenyan schoolchildren against the recombinant glutathione-S-transferase (GST) of *S. mansoni* were found to be significantly greater in the susceptible group than in the resistant group (Auriault et al., 1990).

1.7.7 Why does intensity of infection/intensity of reinfection after treatment peak in early teens and then rapidly decline?

1.7.7.1 The influence of puberty:

In the studies of resistance to reinfection following treatment reviewed by Fulford et al. (1998), marked declines in reinfection rate occurred at a very consistent and narrow age range corresponding to the age of puberty. It is suggested (Fulford et al., 1998) that a number of physiological non-immunological responses could account for the differences in susceptibility between children and adults such as changes in skin-thickness, body fat, size of the animal, and circulating schistosomicidal factors. In addition steroid hormones such as dehydroepiandrosterone (DHEA) which rise during puberty and have been implicated in immune responsiveness, might be involved in development of the protective anti-schistosome immune response. In mice, the age-associated hormonal factors, such as testosterone and DHEA have also been implicated as putative schistosomicidal compounds (Nakazawa et al., 1997; Fallon et al., 1998). It has been recently reported that testosterone is directly toxic to larval schistosomes *in vitro* (Fantappie et al., 1999). It was shown in earlier studies that even juvenile mice, as well as hamsters, are more susceptible than adult animals to schistosome infection (Purnell et al., 1966).

1.7.7.2 History of infection:

It has also been proposed that acquired immunity develops as a function of cumulative exposure (duration and frequency) to parasite antigens (Woolhouse & Hagan, 1999). There have been several studies on the influence of infection history on immune responses before treatment (Butterworth et al., 1988; Mutapi et al., 1997). It was reported that acquired immunity develops quicker in exposed individuals in areas of high-transmission than in those areas of lower transmission because the population is exposed to a higher level and a greater diversity of antigens in a high transmission area (Mutapi et al., 1997). From studies performed in Zimbabwe, Mutapi et al., (1997) showed that the humoral responses also develop quicker and reaches higher levels in areas of intense transmission compared with those in areas of low transmission.

Inspite of this evidence of a relationship between exposure and development of resistance, the fact that the age of peak intensity does not vary much (Fulford et al, 1996) and the fact that similar age-intensity profiles become established in newly established foci (Gryseels, 1994) emphasise the overriding importance of age *per se* in relation to development of resistance.

1.7.8 Does treatment induce/enhance immunity in humans?

In recent years it has been suggested that, in the treatment/reinfection studies, rather than simply removing the worms to allow assessment of pre-existing resistant (putatively immune) status, the drug-induced killing of the worms *in vivo* resulted in antigen release and so served to boost and/or alter the immune response in a way that might enhance immunity.

1.7.8.1 Antibody responses:

A recent study of anti-schistosome Ab responses in Zimbabwean children treated with either Praziquantel (PZQ) or Oxamniquine (OXAM) by Mutapi et al., (2003) demonstrated a significant increase in the proportion producing anti-*S.mansoni* SEA IgA, IgE and IgG3 post treatment while the proportion producing IgG1 and IgG4 decreased.

There are also significant differences in the type of immune responses in children and adults following chemotherapy (Grogan et al., 1996). Two studies that compared children with adult immune responses showed that the immune response profile in treated children was similar to that in untreated adults (Grogan et al., 1996; Mutapi et al., 1998). For example, in Gabonese children (5-10 years old) infected with *S. haematobium*, the levels of IgE and IgG4 antibodies against (adult worm antigen and soluble egg antigen) schistosomes increased significantly following treatment - in contrast to the adult immune responses, which remained unchanged (Grogan et al., 1996). In Zimbabweans infected with *S. haematobium*, children produced a predominant IgA response before chemotherapy. Following treatment, all children switched from IgA to IgG1 (production against egg and worm antigens (Mutapi et al., 1998). Hence, their post-treatment Ab profile became similar to that of untreated adults.

Earlier studies on the interaction of PZQ with schistosomes reported modifications in the cell proliferative responses (Ottesen et al., 1978). These modifications have been reported to remain for >1 year (Naus et al., 1998; Satti et al., 1996) and confer some resistance to re-infection (Dunne et al., 1992a; Roberts et al., 1993).

1.7.8.2 Cellular responses:

In a re-infection study by Mduluza et al. (2003), an attempt was made to show the cytokine profiles that appear before treatment and the change in the period following treatment with PZQ. Cytokine profiles were compared, in this study, of individuals from two areas with different transmission patterns for *Schistosoma haematobium* in Zimbabwe. Before treatment, both infected and uninfected groups showed low and similar ratios, respectively, of IL-4:IFN-γ, IL-5:IFN-γ and IL-10:IFN-γ, while IFN-γ was high in the infected individuals. After treatment the non re-infected individuals had higher levels of IL-4, IL-5 and IL-10, while the infected had high levels of IFN-γ. The results indicated that the cytokine balance determines, in part, susceptibility or resistance to *S. haematobium* infection with Th-1 responses dominating during infection and the Th2 responses dominating post treatment and in uninfected individuals.

Suggestions about how the treatment might affect the immune response include increased antigen release and reduction in immunosuppression by loss of antigen stimulation. Praziquantel (PZQ) treatment has been shown to damage the adult schistosome tegument (Andrews, 1985). This treatment also exposes the antigens on the worm surface (Fallon & Doenhoff, 1995) to the host immune system, which induces specific cellular and humoral immune responses (Woolhouse & Hagan, 1999), thus resulting in the death of the parasite. Such effects may underlie the proposed enhancement of development of humoral responses to tegumental and internal antigens (Mutapi et al., 2003). Cellular studies have stressed the differences in proliferation between individuals with acute and chronic forms of schistosome infection (Colley et al., 1986; Ottesen et al., 1978). During chronic infections, the host is exposed to large quantities of antigenic material from worms and eggs of the parasite and the usual response of the host immune system to such repeated or continuous antigen exposure is

down regulating or modulating the immune response to prevent continued over-reactivity which might be harmful to the host (Barsoum et al., 1982; Ellner et al., 1985), but following treatment the antigenic stimulation of the immunosuppressive response will stop allowing other responses to increase.

Whilst it is clear that chemotherapeutic cure causes qualitative and quantitative changes in the immune response to schistosome antigens and that this seems to promote the putative immunoprotective responses e.g., specific IgE, the extent to which this is necessary for promoting the resistance to reinfection is difficult to establish. It should be pointed out that in the original observation of a correlation between resistance to reinfection and high IgE/lowered IgG4 (Hagan et al., 1991) the sera showing this correlation was taken prior to treatment.

1.7.9 Endemic Normals:

The EN individuals manifest immune responses to *S. mansoni* antigens that differ significantly from those of post-treatment-resistant and patent-infected individuals. A number of immunological studies of naturally acquired resistance in EN individuals showed the involvement of both Th1 (cellular) and Th2-type (humoral) responses in the development of protection against *S. mansoni* infection (Viana et al., 1994; Viana et al., 1995; Correa-Oliveira et al., 1989; Bahia-Oliveira et al., 1992), whereas as seen above, resistance seen following chemotherapeutic cure appears to be mediated mainly by Th2-type responses (Roberts et al., 1993).

1.7.9.1 Antibody responses:

Several studies have compared antibody responses in EN and infected/treated individuals. In the endemic area of Siqueira, longitudinal analysis of antibody responses of EN individuals demonstrated that the anti-adult worm IgE level in EN individuals was similar to that of individuals infected or resistant to reinfection after chemotherapy (identified 21 months after treatment) (Viana et al., 1995; Caldas et al., 2000). The IgE antibody response to schistosomula tegument antigens was significantly higher in EN individuals than in infected patients. After chemotherapy, the IgE response to

schistosomula tegument antigens increased only in those patients who became resistant to reinfection. This suggests that the IgE antibody response might play an important role in eliminating the parasite in EN. Regarding the response to specific antigens, Correa-Oliveira et al., (1989) reported that EN individuals had higher IgG anti-paramyosin antibody responses than did infected patients. This elevated response was associated with the lack of eggs in the stool. A significant increase in IgG anti-paramyosin antibodies was also observed in patients resistant to reinfection.

1.7.9.2 Cellular responses:

The cellular proliferative response of patients resistant to reinfection after treatment has also been compared with patients who became reinfected after chemotherapy. An increased response to soluble egg and cercarial antigen preparations has been observed in the resistant group (Colley et al., 1986). Similarly a study carried out in Brazil showed that individuals resistant to reinfection had higher cellular lymphoproliferative responses to schistosomula and adult worm antigens (Ribeiro et al., 1993). In contrast to infected individuals, peripheral blood mononuclear cells (PBMCs) from EN individuals proliferate vigorously *in vitro* after antigenic stimulation with parasite antigens. High levels of IFN-γ are secreted by PBMCs from EN individuals independent of the schistosome antigen preparation used (Viana et al., 1994; Bahia-Oliveira et al., 1992).The main cell source of IFN-γ secretion among PBMCs was found to be CD4+ T cells (Brito et al., 2000).

In EN individuals, a clear Th2 cytokine profile was not observed, as compared to resistant to reinfection individuals (Roberts et al., 1993; Medhat et al., 1998; Hagan et al., 1991). It has been suggested that EN individuals may differ genetically from those who are able to develop effective Th2 immune responses after treatment or, alternatively, that chemotherapy leads to the presentation of novel antigens that are stage specific and not recognized by cells or antibodies from EN individuals. However, the data on *SM1* resistance status and Th2 bias clearly indicates the importance of Th2 responses in resistance independent of chemotherapy (Abel et al., 1991; Marquet et al., 1996). How the Th1 responses implicated in the EN response relates to distribution of the *SM1* alleles remains to be resolved.

1.8 Co-infection with other parasites:

Many people who live in schistosome-endemic areas are also exposed to many other infectious diseases and other immunological challenges. Some of these other pathogens induce a Th1 biased immune response when present as a single infection. Given the counter-regulatory effects that are exerted by Th1 and Th2 cells on each other's development and the presence of regulatory pathways e.g. IL-10 and T-regulatory cells, studies on co-infections between schistosomes and other pathogens and effects of schistosomiasis on vaccine efficacy, allergic and autoimmune responses have been the subject of growing interest.

1.8.1 Th1 vs Th2 co-infections:

i- Experimental infections in mice

Data from experimental studies of vaccinia infections in mice, showed that mice infected with schistosomiasis are less able to mount a specific anti-viral CD8+ Th1 immune response, and are therefore less able to clear viruses (Actor et al., 1993). Infected mice also had greater susceptibility to malaria (Helmby et al., 1998), and are extremely susceptible to infection with *Toxoplasma gondii* (Marshall et al., 1999), a parasite that induces marked Th1 responses and that is lethal in mice that have defects in IFN-γ production.

ii- Human studies

Tetanus Toxoid: *In vitro* re-stimulation of PBMCs with Tetanus Toxoid antigens (TT) from TT immunized individuals, who were either not infected or had light, medium or heavy *S. mansoni* infections, showed progressively lower levels of IFN-γ and increases in Th2 cytokine mRNA. It was concluded that the schistosome-induced Th2 environment into which the TT vaccine was introduced either promoted development of TT-specific Th2 cells at the expense of Th1 cells or specifically down-regulated development of TT-specific Th1 cells, by for example stimulating the production of IL-10 (Sabin et al., 1996b).

BCG: Malhotra et al., (1999) demonstrated that children vaccinated with *Mycobacterium bacillus* Calmette-Guerin (BCG) at birth make significantly lower IFN-γ responses to purified protein derivative of *Mycobacterium bacillus* (PPD) at 10-14 months after birth if they are born of worm-infected (*S. haematobium* or *Wuchereria bancrofti*) mothers. These important observations suggest that prenatal sensitization to worm antigens results in a persistent Th2 biased immune response (producing IL-4 and IL-5). People vaccinated with BCG, on the otherhand, (before being exposed to any worms in their life) were reported to be less susceptible to intestinal nematodes (Elliott et al., 1999). This same phenomena of pre-natal exposure to worm antigens, priming the individuals immune response to a reduced Th1 response may have important implications for responses to other infections acquired early in life e.g. malaria.

AIDS/HIV-1: With regard to the possible interaction of concurrent schistosome and HIV-1 infections, Th2 cells which would be promoted by schistosome infections have, in some cases, been found to support HIV replication *in vitro* more strongly than Th1 cells (Maggi et al., 1994), which led to the hypothesis that schistosomiasis and other helminth infections contribute to the high prevalence of AIDS and HIV infection in Africa (Bentwich et al., 1999). Conversely, it was recently also found from *in vitro* studies that when comparing T cells in the peripheral blood of *S. mansoni*-infected individuals, those in schistosome and HIV co-infected individuals responded to egg antigen by making less IL-4 and IL-10, but similar (low) amounts of IFN-γ, indicating that there is a shift in the overall balance of the response from Th2 to Th1 (Mwinzi et al., 2001). On the basis of the various roles that have been established for CD4+ T cells during schistosomiasis in the mouse model, it would be anticipated that individuals with AIDS would have altered patterns of hepatic fibrosis and, perhaps, an increased risk of liver damage owing to the insufficient sequestration of egg hepatotoxins and/or the relatively increased production of pro-inflammatory cytokines.

Malaria: The possible interaction of malaria and schistosomiasis was investigated in school children from 2 areas in Kenya with contrasting prevalences of hepatosplenomegaly but similar prevalences and intensities of *Schistosoma mansoni* infection. When plasma was assayed for anti-*Plasmodium falciparum* and anti-*S.*

mansoni antibodies to test whether greater exposure to *Plasmodium* was a cofactor for hepatosplenomegaly, it was found that hepatosplenic children had significantly higher levels of anti-*P. falciparum* antibodies, compared with non-hepatosplenic children, a finding that strongly suggests that some experience of *P. falciparum* influenced the development of hepatosplenomegaly in these *S. mansoni*-infected children (Mwatha et al., 2003).

1.8.2 Th2 vs Th2 co-infections:

Ongoing Th2 responses in chronic shistosomaiasis might be beneficial (a) during co-infection with other pathogens against which Th2 responses may be host-protective (for example, the intestinal nematodes), (b) in preventing the onset of Th1-mediated autoimmunity (e.g., diabetes mellitus in genetically predisposed non-obese diabetic mice (Cooke et al., 1999) and (c) in modulating allergy.

Other Helminths:

In experimental studies of *Trichuris muris* in the mouse, in which Th2 responses are protective resulting in rapid worm expulsion in some strains of mice (Curry et al., 1995), coinfection with schistosomes was able to promote protective Th2 responses in another strain of mice which otherwise would have developed Th1 responses and persistent infection.

Allergies: In situations where helminth infections and allergy co-exist, both are associated with Th2-dominated immune responses. Allergic disease seems to be less frequent in developing countries that still have widespread helminth infection (Yazdanbakhsh et al., 2002). Several recent studies in human populations that are infected with *S. mansoni* (Araujo et al., 2000) and *S. haematobium* (van den Biggelaar, 2000b) have shown a clear inverse relationship between allergen responsiveness and schistosome infection. It has been proposed that the regulatory mechanisms that are induced as a component of the immune response to chronic helminth infection, such as the production of IL-10 and, possibly, TGF-β, may result in non-specific prevention of inflammatory sequelae during allergic responses (Yazdanbakhsh et al., 2002). For example, van den Biggelaar et al., (2000b) reported a correlation between *S. haematobium* infection and reduced levels of immediate skin test responsiveness to

house dust mite allergen. There was no correlation between this reduced level of skin test responsiveness and polyclonal IgE levels indicating that the effect was unlikely to be due to non-specific blockage of mast cell receptors by excess non-allergen IgE, which has been suggested as a mechanism of interference. However, there was an inverse correlation of skin test response with levels of IL-10 produced *in vitro* to schistosome antigen.

1.9 Immunity in Experimental Animals:

Much of the research using experimental animals has focused on *S. mansoni*, simply because it is the easiest of the three major schistosome species (*S. mansoni*, *S. haematobium* & *S. japonicum*) to maintain in the laboratory. Likewise, the ability of the mouse to manifest many pathological features of human schistosomiasis, and to develop immunity, coupled with its relatively low cost and ease of production, makes it a suitable model of study.

1.9.1 Concomitant Immunity:

Owing to the difficulties inherent in identifying and defining immune mechanisms in the human host it has proved necessary to use animal models of schistosomiasis. Progress in this area has been greatly enhanced by the fact that several laboratory animals are susceptible to the parasite, although to varying degrees depending on the host and schistosome species. Rodents, such as mice and rats and, to a lesser extent, primates such as the rhesus monkey and the baboon have all been used in experimental infections with *S.mansoni*. The baboon and the mouse are, like humans, permissive hosts, which tolerate the development of the adult worm and the onset of egg production 4-5 weeks post infection (Smithers and Doenhoff, 1982). Also the pathological consequences are similar to that in humans. In contrast, the rat is a non-permissive host in which a small proportion of stunted worms survive but do not produce viable eggs (Cioli et al., 1978) and the rhesus monkey a semi-permissive.

Studies of *S. mansoni* infections in rhesus monkeys (Smithers and Terry, 1969) showed that primary light infections could be established giving rise to egg production in the faeces, but that following a challenge infection with large numbers of cercariae, egg output was not altered although in challenge control monkeys faecal output rose

dramatically and the animals got sick. This was termed "concomitant immunity" as the primary worm burden was apparently unaffected by the response which eliminated the challenge infection. It has been suggested that a similar phenomenon may operate in naturally acquired immunity in humans. Unfortunately nothing is known of the mechanism of this resistance.

In mice, resistance to challenge arises within 8 weeks of a single unattenuated infection (Dean, 1983) and resembles concomitant immunity in that the primary infection persists but the incoming larvae appear to succumb. However, it has become apparent that the resistance seen in this model is associated with the pathological response of the host to parasite eggs deposited within the tissues (Wilson et al.,1983; McHugh et al., 1987) or with factors related to egg-associated pathology, such as the degree of portal hypertension (Harrison et al., 1982) or formation of portal shunts which allow the migrating larvae to bypass the liver and die in other organs (Wilson et al, 1983). The lack of evidence for specific anti-larval immune-mediated protection in infected mice means that this model has contributed little to our understanding of immunity to infection. This resistance, is therefore, more anatomical than immunological, and it is related to pathological changes that are more prevalent in infected mice than in infected humans. In contrast there is considerable evidence that attenuated larval vaccines do induce immunologically specific immunity.

1.9.2 Attenuated Vaccine-induced Immunity:

Most attention has been focused on resistance to reinfection which is brought about by exposure to parasites that are attenuated and die within the host during migration. In the vast majority of the studies attenuation is brought about by exposure of the larvae before infection to radiation (Irradiated Vaccine Model), but attenuation by treating with the schistosomicide Ro11-3128 1-2 days post infection has also been studied (Ro11-3128 Vaccine Model). Such attenuated vaccines are not feasible for human use but particular attention has focussed on them because crude dead antigen preparations induce poor or irreproducible levels of protection. Studying such models is aimed at understanding (i) the nature of the protective response, (ii) the characteristics of antigen presentation, and (iii) the protective antigens, so that progress can be made to develop defined antigen vaccines.

Much of this thesis is concerned with the Ro11-3128 terminated infections but the following sections describe what has been learnt about immunity from the much more extensively studied irradiated vaccine model. This is followed by a consideration of the drug terminated model, how this differs from the irradiated model and how studying it further may contribute to our understanding of the mechanisms of immunity and vaccine development.

1.9.2.1 The Irradiated Vaccine Model:

In the 1960s, protection experiments were first carried out using irradiated schistosome larvae which died out during migration and so did not reach the adult, egg producing stage (reviewed by Taylor, 1994). Later work focussed on natural schistosome species of domestic animals and Taylor et al., (1976) demonstrated that sheep could be protected against infection with *S. mattheei* by immunisation with homologous irradiated schistosomula and that both sheep and cattle could be protected against *S. bovis* under laboratory conditions (Taylor et al., 1979). The latter studies were extended into the field and it was demonstrated that immunisation with irradiated *S. bovis* schistosomula reduces worm and egg burdens and egg associated pathology (Majid et al., 1980) under conditions of natural exposure. The effectiveness of this procedure in inducing high levels of immunity (>90%) against the human schistosome species *S. haematobium* in the baboon (Harrison et al., 1990) demonstrated the potential efficacy of schistosome vaccines and the relevance of this procedure as a model for development of a human vaccine.

(A) The Effect of radiation on the parasite:

Understanding of the parasite stages responsible for, and the mechanisms of immunity have largely come from studies in rats and mice. Both species develop schistosome species-specific and long lasting immunity (>24 weeks in mice) (Bickle et al., 1985). Early studies in both mice and rats showed that the level of protection was markedly influenced by the irradiation dose used. The optimum dose of 20krad results in the majority of the attenuated parasites migrating to the lungs where they persist in significant numbers for longer than unirradiated larvae, eventually dying in that site (Ford et al., 1984a,b; Bickle et al., 1979a,b; Mastin et al., 1983). An assessment of the ultrastructure of

the attenuated schistosomula fixed *in situ* in the lungs (Mastin et al., 1985b) showed that these persistent schistosomula were normal in appearance but abnormal in location, lying in alveoli where they elicited a marked inflammatory reaction.

Wales and Kusel (1992) proposed that radiation may directly alter antigen expression so that vaccinating parasites are more immunogenic than their normal counterparts. Using electron microscopy, Harrop and Wilson (1993) revealed marked distinctions in surface morphology between normal versus irradiated larvae at the lung stage of development, artificially transformed and cultured *in vitro* for several days. The latter parasites exhibited random constrictions at intervals along the length of the body, probably resulting from overcontraction of circular muscle fibres; they were also less motile than normal larvae. The authors postulated that the disruption in neuromuscular co-ordination accounts for the limited migratory capacity of attenuated schistosomula *in vivo*.

(B) Persistence of attenuated schistosomula:

An apparent conclusion to be drawn from the above studies is that an optimum dose of radiation does not cause rapid death of schistosomula, since many are able to persist in various sites for up to 3 weeks. The persistence of attenuated schistosomula was also the subject of a subsequent autoradiographic study (Mountford et al., 1988) by paying particular attention to the lymph nodes draining organs on the parasite migration route. It was discovered that a proportion of normal (i.e. non-attenuated) schistosomula entered and rapidly migrated through lymph nodes draining the skin exposure site (SLN, skin-draining Lympn nodes), since numbers peaked at 16% of applied parasites on day 5 and declined to less than 3% on day 7. Fewer attenuated parasites were detected in SLNs (peak of 11.5% on day 5), but they persisted throughout the second week after exposure (Mountford et al., 1988). Furthermore, proportionally greater amounts of parasite-released material were detected in the nodes of vaccinated mice compared to those infected with normal cercariae (Mountford et al., 1988), which was suggested to be due to increased turnover of parasite proteins caused by irradiation. It was concluded that optimally attenuated (20krad) schistosomula are sequestered in this location and that this is vital for their optimal induction of immunity.

Earlier studies had shown that schistosomula exposed to very high doses of radiation e.g. 80krad were poorly protective and it was found that few of such irradiated larvae migrated from the skin exposure site to the SLNs and none reached the lungs (Constant et al., 1990). The importance of the sensitisation of the SLNs was investigated by surgical excision of the nodes, 5 days before vaccination and up to 10 days after. This caused a significant reduction (up to 75%) in the level of immunity compared to that in sham-operated controls (Mountford and Wilson, 1990). It was concluded that sensitisation of, and perhaps persistence of radiation-attenuated parasites in, the skin draining lymph nodes, together with the prolonged release of antigen in the latter site, are major factors in the induction of resistance.

The incomplete ablation of resistance by removal of SLN shows that a component of the response is elicited elsewhere. It was postulated by Mastin et al., (1983) that the persistence and subsequent death of irradiated schistosomula in the lungs could provide the appropriate antigenic stimulus for the induction of an immune response against challenge parasites. When optimally attenuated cercariae are applied percutaneously (Mountford at al., 1988) or attenuated schistosomula are administered intradermally (Coulson and Mountford, 1989), many migrate as far as the lungs and both can induce a high level of protection results. However, if attenuated schistosomula are introduced directly to the lungs (i.e. bypassing the lymph nodes) they induce only moderate levels (intratracheal route) or virtually no protection (intravenous route), despite the larvae remaining in this location for at least nine days (Coulson and Mountford, 1989). It therefore appears that the irradiated larvae must persist in *both* lymphatic tissue and lungs in order to generate an optimum immune response.

(C) The nature of the immune response:

Studies using athymic and μ-MT (T- and B-cell deficient animals, respectively) depleted rats and mice (Ford et al., 1987a; Sher et al.,1982) first suggested the involvement of both T and B cells in the resistance generated. In rats the immunity induced following a single or multiple exposure to irradiated cercariae is antibody (IgG2a) mediated (Ford et al., 1987b), whereas in mice a single exposure results in a mixed CMI/antibody dependent protection but with repeated vaccination antibody becomes more important.

(D) Immune Mechanisms following Single Vaccination :

Mouse strains differ widely in the extent of immunity developing after exposure to attenuated cercariae (Murrell et al., 1979). C57Bl/6 strain are consistently high responders for *S. mansoni* (Richter et al., 1995) and a single exposure to 500 optimally irradiated cercariae routinely induces 60-70% protection against a challenge with normal cercariae administered 5 weeks after vaccination. This strain has been used for the majority of studies on the irradiated vaccine and was used in the present studies also.

(i) Events in the Skin (ear Pinnae): Radiation-attenuated larvae exhibit severely retarded migration from the skin (Mastin et al., 1983; Mountford et al., 1988) and therefore would be expected to produce enhanced stimulation of accessory cells of the innate immune response, including antigen-presenting cells (APCs) (Riengrojpitak et al., 1998). Hogg et al. (2003) have recently reported significant production of IL-12p40 in the skin (pinna) after exposure to 20krd irradiated larvae, and that majority of the IL-12p40+ cells were myeloid dendritic cells (DCs) although a proportion were identified as macrophages. The attenuated larvae also induced the production of IL-10, which regulates inflammation and the production of IL-12.

(ii) Events in the Lymph Nodes: Constant et al., (1990) demonstrated that following percutaneous infection with 20 krad-irradiated parasites, there was a large fold-increase in T-cell numbers in the skin-draining (axillary) and lung-draining (mediastinal) lymph nodes. Anti-CD4 monoclonal antibody treatment largely inhibited this, implicating a major presence of T helper cells. Around day 5 post vaccination, antigen-stimulated SLN cells produced IL-2, IL-3, IL-4 and IFN-γ revealing a Th0 or mixed Th1/Th2 response (Pemberton et al., 1991). However, by days 18 and 23, IL-3 and IFN-γ were predominant and no IL-4 was seen, demonstrating a polarized Th1 response. Such a polarised cytokine response was also reported by Caulada-Benedetti et al. (1991). From 14 days after vaccination, a large and sustained expansion of the circulating leucocyte pool could be detected, mainly due to changes in lymphocyte numbers (Menson et al., 1989). This is believed to represent cells entering the circulation from the SLNs. Additionally, splenocytes (Pemberton et al., 1991) and in particular splenic CD4+ T cells (Caulada-

Benedetti et al., 1991) recovered from mice up to 4 weeks after vaccination produced substantial amounts of IFN-γ in response to schistosome antigen.

(iii) Events in the Lung: Attention has been focussed on events in the lung as this was known to be where the irradiated cercariae mainly died, because the failure of SLN removal to remove immunity suggested that stimulation of additional sites was important for induction of optimal immunity and also because the lung is the site of attrition of the challenge infection. Studies by Aitken et al. (1988) demonstrated a sustained and significant increase in the number of leucocytes present within the broncho-alveolar lavage (BAL) post vaccination. This began on day 7 post vaccination as the larvae reached the lungs in substantial numbers (Mastin et al., 1983; Mastin et al., 1985b), reached a peak fifteen fold increase in leukocyte numbers on day 21 and persisted for at least 10 weeks post vaccination. The lymphocyte influx consisted of both CD4+ and CD8+ cells and the extent of increase was shown to correlate with protection. It was proposed that the arrival of the irradiated parasites serves to attract the circulating population of sensitized T cells which then infiltrate the lung parenchyma and airways and "arm" the lung against a challenge infection.

(E) Immune Mechanisms in Multiply Vaccinated Mice:

Repeated vaccination of mice with irradiated cercariae results in development of a mixed Th1/Th2-type or Th2-biased response, with increased production of antigen-specific IL-4 and IL-5 cytokines by SLN cells and spleen cells (Caulada-Benedetti et al., 1991). Schistosome specific serum antibody levels also rise, notably IgG1 and reach levels such that significant protection can be passively transferred with serum (Mangold and Dean, 1986; Mclaren and Smithers, 1988; Wynn et al., 1996).

(F) Neither a Th1 nor a Th2 skewed response is essential for optimal immunity:

Hoffman et al., (1999) showed that in IL-10/IL-4-deficient (highly polarized type 1) or IL-10/IL-12-deficient (highly polarized type 2) mice exposure to a single vaccination with radiation-attenuated cercariae both develop immunity. Vaccinated IL-10-deficient mice, exhibit a non-polarized or mixed Th0-like phenotype (a mixed and elevated type-1 and type2-associated immune response) and developed higher anti-schistosome immunity than wild-type mice. This immunity correlated with higher parasite-specific antibody titres,

greater proliferative lymphocytes, increased frequency of IFN-gamma and IL-4 secreting cells, and elevated perivascular/peribronchial inflammatory responses in the lung. These results suggest that optimal vaccine-induced immunity even following a single vaccination does not require a highly polarized response, but both type 1- and type 2-associated immune responses can act together to optimize protection. During single vaccination of wild type mice it appears that induction of IL-10 serves to prevent the optimal stimulation of both types of response.

(G) Mechanisms of Immunity against CHALLENGE infection:

In order to determine the site of elimination of challenge parasites, recovery and autoradiographic tracking studies showed that the schistosomula of the challenge infection were also found to die predominantly in the lungs in both vaccinated mice and rats (Bickle et al., 1979a,b; Mastin et al., 1983 ; Ford et al. 1984a,b; Mangold and Dean, 1983).

(i) IFN-γ Mediated Attrition:

In one-time vaccinated mice Vignali et al. (1989a) demonstrated that the depletion of Th cells by administration of an anti-CD4+ monoclonal antibody at the time of challenge resulted in abrogation of vaccine immunity whereas depletion of CD8+ve cells was ineffective indicating a role for CMI mediated protection. Aitken et al. (1988) demonstrated that on the arrival of the challenge parasites in the lung a second influx of T lymphocytes is observed similar to that following arrival of the irradiated vaccinating parasites. The majority of these T-cells also secreted IFNγ and IL-3 in response to antigenic stimulation suggesting that an anamnestic Th1 type response occurs (Smythies et al., 1993). Upregulation of surface molecules (e.g. MHC Class II) expressed by alveolar macrophages occurs following vaccination and increases following a challenge infection (Menson and Wilson, 1990). Wynn et al. (1994) described a more mixed Th1/Th2 response after administration of a challenge infection. Neutralization of IFN-γ in these animals resulted in a decrease in IL-12 p40, TNF-α and IFN-γ mRNA expression but a significant expression of several Th2 cytokines including IL-4, IL-5 and IL-13. However, a crucial role for IFN-γ in mediating challenge attrition has been confirmed by the use of monoclonal antibody against this cytokine which reduced the development of vaccine immunity when administered at the time of challenge although there is debate about whether IFN-γ is solely responsible (Smythies et al., 1992b) or contributory (with

antibody) (Jankovic et al., 1999) to challenge attrition. In contrast, treatment with antibodies against IL-4 and IL-5 are without effect (Sher et al., 1990).

(ii) Antibody-mediated Attrition:

In B cell-deficient mice an important role for antibody even in the one-time vaccine model was suggested by Jankovic et al.,(1999). Vaccine-induced immunity in B-cell deficient (μMT) and IFN-γ knockout (GKO) animals were compared. After a single vaccination, B cell knockout (KO) mice displayed reduced protection against challenge infection, although they developed a normal IFN-γ-dominated cytokine response. This defect in resistance could be restored by passive transfer of immune sera from one-time vaccinated wild type mice confirming that the defect in host resistance in vaccinated B cell-KO mice results from the absence of humoral antibody and supporting the concept that one-time vaccine immunity involves both B cell and non-B cell-dependent mechanisms.

In multiply vaccinated mice immunity is enhanced to a degree and there is a qualitative change in the mechanisms of immunity reflecting the Th1→Th2 cytokine switch: CD4+ve T-cells (Kelly and Colley, 1988) and IFN-γ (Wynn et al., 1996) are no longer required at the time of challenge and serum is able to transfer resistance. Some experiments using fractionated sera have indicated that the IgG fraction (Mangold and Dean, 1986) or more specifically IgG1, (Delgado and McLaren, 1990) is wholly responsible for the protective capacity of sera from multiply vaccinated mice (VMS). However, the studies of Jwo and LoVerde (1989) suggested that the non-IgG fraction which contains mainly IgM and IgA, is also able to confer some protection but IgE has not been identified (Sher et al., 1983).

In singly or multiply vaccinated rats IgG2a antibody is passively protective but IgE is not involved (Ford et al., 1987b).

(iii) Mixed Cell-Mediated Immunity and Antibody response is desirable for optimal immunity :

Repeat vaccinations of mice significantly increases the level of immunity in wild-type mice, however the protection in B cell KO animals remained unchanged (Jankovic et al., 1999). In contrast, multiple vaccination resulted in increased but, nevertheless, defective resistance in GKO mice. Together these findings further support the

conclusion that optimally effective vaccination against schistosomes at least by irradiated infections, depends on the simultaneous induction of both humoral and cell-mediated immunity.

(H) How are challenge schistosomula killed in the immune lung?

Exactly how the lung stage larvae are actually killed is still unclear. Whether by IFN-γ (in singly vaccinated mice) or by antibody (in multiply vaccinated mice and vaccinated rats) there is an accelerated and enhanced inflammatory response around the larvae in vaccinated animals. Once trapped in inflammatory foci, larvae may then be killed by toxic cell products or by the effects of antibody (McLaren, 1989).

(i) Cell-mediated Effector Mechanisms:

The cellular infiltrate is rich in activated macrophages, and in mice has been shown to contain inducible NO synthase-(iNOS)-producing cells (Wynn et al,, 1994). Nitric oxide (NO) has been shown to kill skin stage schistosomula *in vitro* but not lung stage (Pearce and James, 1986), and the role of NO *in vivo* is also doubted as inhibition of NO by N-monomethyl-L-arginine (L-NMMA) fails to deplete vaccine immunity (Smythies, et al., 1993). Furthermore, although significantly reduced when compared with vaccinated wild type animals, iNOS-deficient mice maintained a marked level of vaccine-induced immunity (Coulson et al., 1998; James et al., 1998). Interestingly, the decrease in protection in iNOS-deficient mice was associated with marked increases in IFN-γ, TNF-α, and IgG antibodies. The significance of this for understanding exactly how larvae are killed is uncertain but this does show that NO serves as a potent immunoregulatory factor downregulating the Th1-type cytokine response. As suggested recently (Maclean et al., 1998) these studies indicate that iNOS-inhibitors may, in fact, be useful adjuvants for vaccination where an enhanced Th1-type response is required for protective immunity. Because direct killing of the lung stage larvae has not been shown, it has been suggested that the inflammatory focus may simply block the migratory route of the challenge infection and result in a greater number of these parasites being shunted into the alveoli from where they cannot return (Coulson and Wilson, 1988).

While lung stage attrition is widely accepted there has been some controversy about whether any killing occurs in the skin in vaccinated mice (Ward and Mclaren, 1988).

Similar foci to those seen in the lungs have been described in the skin although these contain radiosensitive leucocytes in addition to monocytes and T cells (Piper and McLaren, 1993; McLaren and Smithers, 1988) and these authors considered skin phase attrition to be important. It was suggested that trapping of parasites in these inflammatory foci may be aided by eosinophil cationic proteins which have been seen to paralyse lung worms *in vitro* (McLaren et al., 1984) and by macrophages which can mediate damage of the schistosomula musculature (McLaren and James 1985).

(ii) Antibody Mediated Effector Mechanisms:

The role of antibody in the killing of the schistosome *in vivo* is not fully understood. Following the challenge of passively immunised rats cellular reactions can be seen surrounding the dying parasites (Vignali et al., 1989b) but the lung stage schistosomula which forms the target of immunity *in vivo*, has been shown to be refractory to the antibody dependent mechanisms which kill freshly transformed schistosomula *in vitro* (Ramalho-Pinto *et al.*, 1978; Incani and McLaren, 1989).

Several suggestions have therefore been put forward regarding the role of antibody in the killing of parasites *in vivo*. Firstly, immune complexes which incorporate IgG1, IgG2a or IgE and circulating antigens could activate macrophages to release lysosomal proteins or trigger complement. The anaphylotoxins produced in this way would then attract more macrophages and eosinophils in addition to enhancing the expression of surface receptors on the latter. Products of both eosinophils and macrophages are known to damage the musculature of young schistosomula, hence parasite migration could be delayed and an inflammatory response induced which traps the parasite and prevents further migration (McLaren and Smithers, 1988). Alternatively, antibodies may bind to the lung stage larvae hence hindering their migration through capillary beds and enabling them to become good targets for an antibody mediated inflammatory response (Dean et al., 1987). Studies have demonstrated that although the lung stage parasite is refractory to antibody mediated killing *in vitro*, sera from vaccinated mice (Bickle and Ford, 1982), is able to bind to the lung stage schistosomula. However, more recently it has been shown that FcγR KO mice, which lack functional FcγRI, FcγRIII, and Fc∈RI (the major cell surface receptors involved in positive antibody/Fc-mediated triggering) showed no defects in vaccine-

induced resistance after immunization either one or three times. This indicates that antibody-mediated protection does not require FcR signalling (Jankovic et al., 1999).

1.9.2.2 The Drug-attenuated Vaccine Model:

The observation that optimal immunity with irradiated cercariae requires the larvae to survive to the lung stage whereas more highly irradiated cercariae which die at the skin stage are poorly effective suggested that the lung stage schistosomula might be uniquely antigenic and/or that death in the lungs was crucial. To investigate these possibilities Bickle and Andrews (1985) set out to immunize mice by killing developing schistosomula at specific points in migration using schistosomulicides i.e., at skin, lung or liver stages. Somewhat unexpectedly it was demonstrated that high levels of protection were induced by treatment with the benzodiazepine derivative Ro11-3128 just 24-48 hours after percutaneous exposure when the larvae would still be in the skin, external to the epidermal basement membrane (Crabtree& Wilson,1985). *In vitro*, serum from drug treated mice ("serum-derived drug", SDD) induced rapid changes to larval motility and to the larval surface resulting in death (Bickle et al., 1990). *In vivo*, very few schistosomula could be recovered by mincing and incubation of skin at 24 hours post treatment relative to untreated controls. Larval arrest predominantly in the skin was confirmed by autoradiographic tracking (Mastin et al., 1985a).

This suggested that lung stage antigens or larval death in the lungs was not crucial to development of immunity, but rather that release of protective antigens by protracted survival of the drug-damaged schistosomula in the skin was important. The levels of immunity induced by this model were as high if not higher than the irradiated cercariae but it appeared that there was a briefer and more focal exposure of the host to living larval antigen than with the irradiated vaccine. For these reasons it is considered that this model is more similar to the requirements of a defined vaccine and so may provide insight into the key antigens, antigen processing and immune responses which could help develop defined vaccines. Further work on this model is the subject of this project.

- **Ro11-3128 is superior to other drugs in inducing immunity:**

In contrast to Ro11-3128, termination of infection with other drugs such as Ro15-5458 and Oxamniquine which also kill at the larval stage induces only low or insignificant levels of

protection (Bickle et al., 1990; Bickle and Andrews, 1985). This may result from the specific effects, which this drug has on the early schistosomulum. Treatment of schistosomula *in vitro* with native Ro11-3128 or with sera from mice treated with curative doses of the drug results in the rapid formation of membraneous 'blebs'. In contrast, sera from mice given curative doses of Ro15-5458 or oxamniquine do not have these effects on the larval tegument. Smith et al., (1994) also demonstrated that, following incubation with antigen presenting cells (APC), drug-treated MS were better able to present antigen and hence stimulate the proliferation of T cells sensitised by immunisation with crude antigen. The soluble antigens and isolated 'blebs' released after Ro11-3128-treatment could be separated from the otherwise intact schistosomulum. The enhanced proliferation occurred largely in response to the isolated 'blebs', which also contained the majority of the proteinaceous material. Thus, it has been suggested that if such 'blebs' were formed following drug treatment *in vivo,* the presentation of antigen to APC in the form of membranous vesicles may well be responsible for the development of an enhanced antibody response. The antigenic composition of these blebs has been shown to be qualitatively different from that of the rest of the larval surface. For example, the membrane glycoprotein Sm16 a vaccine candidate antigen (Bickle et al., 1986) is present in the blebs. Sm16 was found to be associated with the preformed membranous bodies which give rise to the schistosomular membrane (Hockley and McLaren, 1973), as was demonstrated by IFAT labelling (indirect fluorescent antibody tests) using a mAb against surface antigen Sm16 (Bickle et al., 1990), suggesting that Ro11-3128-induced blebs may originate from this membrane reservoir. The production of membranous material may explain why Ro11-3128-abbreviated infections stimulate strong antibody responses to larval surface antigens (Bickle et al., 1990).

Apart from differential effects on the larval surface it is not known how long schistosomula treated with these other drugs survive or whether they reach the SLN so it is possible that they are rapidly killed and do not show the protracted contact with the SLN which has been suggested to be responsible for the immunity induced by Ro11-3128 treated schistosomula.

- **Ro11-3128-treated infections with 20krad-irradiated schistosomula induce poor levels of protection:**

In the early drug termination studies using doses of 150-175mg/kg an occasional worm survived to adulthood in a small proportion of mice. To try to avoid this complication experiments were carried out using 20krad cercariae so that any survivors of drug treatment would not develop to adulthood. However, it was found that when this was done poor levels of immunity were observed, significantly lower than non-irradiated cercariae (Bickle & Andrews, 1985). Such irradiated drug-treated infections were also studied by (Mountford et al., 1988; Mountford et al., 1989) who reported that such larvae survive for less time in the skin and fail to reach the SLN.

- **Current studies:**

Despite the report of the migration of a small proportion of schistosomula to the lungs following treatment on day 2 it is clear that the 24 or 48 hr Ro11-3128 attenuation protocols result in marked parasite attrition in the skin. Such focal antigen release is more similar to a defined antigen, the goal of vaccine research, than the irradiated vaccine which is required to reach and stimulate the lung in order to stimulate optimal immunity. Regarding the mechanism(s) involved, immunity was found to be abrogated following anti-CD4 treatment at the time of challenge (Vignali et al., 1989a) indicating a protective Th1 response as in the one-time irradiated vaccine. To date relatively little is known of the nature of the protective immune response induced in the Ro11-3128 model. Preliminary studies by Smith (1992, PhD thesis) showed a predominantly Th1 type response in the SLN following Ro11-3128 terminated infections but relatively little is known about the nature and kinetics of the induction of the immune response in the skin and SLN or the systemic spread of the response to the spleen in this model. One of the main objectives of this thesis was to characterise the *in vivo* immune response induced by the Ro11-3128 model focussing on the SLN and spleen cell responses and to compare this with the responses stimulated in these organs by the protective 20krad vaccine and the poorly protective Ro11-3128 treated 20krad regimen. The relevance of such observations was then investigated using anti-cytokine or KO mice to define the protective mechanisms.

Because of the proposed damage and death of drug treated parasites in the skin it was considered that skin dendritic cells would play a major role in processing of parasite antigen. Furthermore, it has been shown (Smith et al., 1994) that Ro11-3128-treated schistosomula were more effective in presenting antigen to macrophages (as antigen presenting cells) compared with untreated schistosomula. So another aspect of this work focussed on *in vitro* interactions between DCs and normal and drug-treated schistosomula, to determine if schistosomula led to DC maturation and if this was affected by drug treatment. In view of this the following section reviews the relevant literature on DCs and DC maturation.

1.10 Dendritic cells:

Dendritic cells (DCs) are a migratory group of bone marrow-derived leukocytes that are sparsely distributed, in the body tissues, and are specialized for the uptake, transport, processing and presentation of antigens to T cells (Steinman, 1991; Hart, 1997; Matzinger, 1994). At an "immature" stage of development, DCs are very efficient in antigen capture and act as sentinels in peripheral tissues, continuously sampling the antigenic environment. Encountering with Microbial products or products released by tissue damage are thought of as "Danger signals" (Matzinger, 1994) and initiate the migration of the DCs to the lymph nodes (LNs). The antigens represented at the time including any microbial products, are processed and fixed on the DC surface as peptides that are presented by upregulated major histocompatibility complex (MHC) molecules. DCs also upregulate a variety of co-stimulatory molecules that are also required for effective interaction with T cells. In the LNs, the now-'mature' DCs efficiently trigger an immune response by any naïve T cells with a receptor that is specific for the foreign-peptide-MHC complexes on the DC surface. The variety of DCs is complex as also is the nature and outcome of their interaction with pathogen molecules but this has a major bearing on the nature of the immune response that ensues e.g., an active Th1- or Th2-biased response or even a suppression of response. This is a rapidly growing field but some of the relevant literature is considered below.

1.10.1 Dendritic cell subtypes:

- **Mouse DCs:**

In normal uninfected mice, DCs are heterogeneous. The expression of certain surface markers is used to segregate DC subtypes (Iwasaki & Kelsall, 2000; Anjuere et al., 1999; Henri et al., 2001; Vremec et al., 2000). For example the T-cell markers CD4 and CD8 are expressed on mouse DCs. Others markers such as CD11b (the integrin α_M chain of Mac-1) and the interdigitating DC marker CD205 (the multilectin domain molecule DEC-205, originally known as NLDC-145) are also used in this differentiation. Using these surface markers, at least five phenotypically defined populations of DCs have been identified in the mouse (Henri et al., 2001). Three exist in the spleen: $CD11c^+CD11b^+CD4^-CD8\alpha^-$, $CD11c^+CD11b^+CD4^+CD8\alpha^-$ and $CD11c^+CD4^-CD8\alpha^+CD11b^-$ (Vremec et al., 2000). The $CD11c^+CD4^-CD8\alpha^+CD11b^-$ (which is DEC-205^+) is also found in moderate levels in LNs, but is the dominant subtype among thymic DCs (Anjuere et al., 1999), whereas the peripheral lymph nodes (LNs), Peyer's patches and mesenteric LNs contain two additional DC subtypes that are not normally found in the spleen, but which have apparently arrived in the LNs through the lymphatic system (Iwasaki & Kelsall, 2000; Anjuere et al., 1999; Henri et al., 2001). One of these, found in all LNs ($CD11c^+CD11b^{low}CD8\alpha^-CD4^-$DEC-$205^{moderate}$) is believed to be the mature form of tissue interstitial DCs. The other, found only in skin-draining LNs, express high levels of langerin, a characteristic marker of epidermal LCs ($CD11c^+$langerin$^+$ $CD8\alpha^{low}$ $CD11b^+$DEC-205^{high}) and is thought to be the mature form of this LC (Henri et al., 2001). *In vitro* bone marrow-derived (bm-DCs), as used in parts of this thesis, are $CD8\alpha^-$DEC-$205^+CD11b^+CD11c^+$ (Lutz et al., 1999).

Previously, $CD8\alpha^+$ DCs were thought to originate from the lymphoid-restricted precursors (Ardavin et al., 1993), and all $CD8\alpha^-$ DCs to derive from myeloid-restricted precursors (Izon et al., 2001; Bjorck et al., 1998), leading to the terms 'lymphoid' and 'myeloid' DCs. However this concept was found to be incorrect. Both lymphoid-restricted and myeloid-restricted precursors were isolated from BM. However, each precursor could produce all the mature splenic and thymic DC subtypes (Traver et al., 2000; Manz et al., 2001; Wu et al., 2001). Therefore, the terms 'lymphoid' and 'myeloid' DCs no longer denote haematopoietic origin but are still commonly used.

- **Human DCs:**

In contrast to the many studies on mouse DCs, there are relatively few studies on mature human DCs freshly isolated from tissues. Blood, being the only readily available source, is more a source of immature DCs and precursor DCs. Human blood DCs are heterogeneous in their expression of a range of markers, many of which reflect differences in the maturation or activation states of DC rather than separate sublineages (Hart et al., 1997). In comparison to mouse DCs, human DCs lack the expression of CD8. However, as in the mouse, human Langerhans cells comprise a separate DC subtype with distinct markers, including the presence of Birbeck granules and the expression of CD1a and langerin. Splenic and tonsillar DCs isolated from human lymphoid tissues show heterogeneity in the expression of CD4, CD11b and CD11c, (McIlroy et al., 1995). Human thymic DCs are $CD11c^{+}CD11b^{-}CD45RO^{low}$ and lack myeloid markers, so resemble mouse thymic $CD8\alpha^{+}$ DCs. A minority of human thymic DCs are $CD11c^{high}CD11b^{+}CD45RO^{high}$ and express many myeloid markers, so resemble mouse $CD8\alpha^{-}$'myeloid' DCs.

1.10.2 Dendritic Cell Activation:

(a)Antigen sampling by DCs in the peripheral tissues:

Immature DCs possess an array of mechanisms for sampling antigen. These include (a) macropinocytosis, where fluid from the extracellular environment is taken up into pinocytic vesicles and antigen is concentrated by expelling excess water via channels called aquaporins (Norbury et al., 1997), (b) receptor-mediated endocytosis, which takes place via a repertoire of receptors expressed on the DC. These receptors include FcγRII (CD32), FcγRI (CD64) (Fanger et al., 1996; Fanger et al., 1997), FcεRI (Maurer et al., 1998) and C3 bi complement receptors (CD11b) (Green et al., 1980), which increase the efficiency of immune complex endocytosis. Dendritic cells also express C-type lectin receptors such as the macrophage mannose receptor and DEC-205, which bind bacterial carbohydrates (Sallusto et al., 1995, Jiang et al., 1995), and (c) phagocytosis of particles such as latex beads (Matsuno et al., 1996), apoptotic and necrotic cell fragments (Albert et al., 1998), viruses, and bacteria including mycobacteria (Rescigno et al., 1999), as well as intracellular *Leishmania major* (Moll, 1993). The Immature DCs in the

peripheral tissues express low to modest levels of antigen presentation molecules (MHC I, II, and CD1) (Porcelli & Modlin, 1999; Mellman & Steinman, 2001) and express no costimulatory molecules. These are increased on the surface of the DCs once the DCs have stopped capturing antigen and are moving to the lymph nodes to present the antigen to the naïve T cells there.

(b)Dendritic Cell/ T-cell interactions in the Lymph Nodes:

Once triggered by antigen and 'danger signals', in the form of microbes and/or their released or breakdown products (e.g., LPS, CPG motifs in bacterial DNA, double stranded RNA) and necrotic cell products (such as heat shock proteins) (Hartmann G. et al. 1999; Sparwasser et al., 1998; Verdijk et al., 1999; Singh-Jasuja et al., 2000), immature DCs in peripheral tissues migrate and carry antigen via the lymphatic system into the T cell regions of draining lymph nodes where they stimulate primary and memory T cell responses. Chemokines such as SLC (secondary lymphoid-tissue chemokine) and MIP-3β (macrophage inflammatory protein) direct maturing peptide-laden DCs from inflamed tissues into the lymphatics towards the draining lymph node and subsequently to the para-cortical area of the lymph node, to ensure an encounter with naïve T cells (Sozzani et al., 1999; Saeki et al., 1999; Sallusto et al., 1998).

In the LNs, these primed DCs direct the fate of naïve Th cells by 3 signals. The first signal, Signal 1, determines the antigen-specificity of the response by ligation of Th cell receptors by peptides presented by MHCI, II, and CD1. An antigen-independent mechanism is required to promote efficient sampling by naïve T cells of peptide-bearing antigen-presenting cells (APCs) (Hauss et al., 1995; Flechner et al., 1988), since the naïve T cells bearing the T cell receptor (TCR) specific for a particular antigen are rare ($\leq$ 1 in 50-100 x 10^3). The multiple transient interactions of T cells with antigen-loaded APCs is described as 'clustering' or aggregate formation (Hauss et al., 1995; Flechner et al., 1988; Galkowski & Olszewski, 1992). These interactions are facilitated mainly by integrins and several members of the immunoglobulin superfamily (Starling et al., 1995; Hart, 1997). Once recognition takes place by the TCR of the correct peptide in the context of self-MHC class II, I or CD1, the DC-T cell interaction is further stabilized by increased expression of the appropriate adhesion molecules. Intercellular adhesion

molecule-1 (ICAM-1) expression, present at low levels on immature DCs, is rapidly increased as a result of the interaction with T lymphocytes (Scheeren et al., 1991). Two other adhesion molecules ICAM-2 and ICAM-3 (CD50) (expressed on both DCs and T cells) are involved in this interaction between DCs and T cells, however ICAM-2 is reported to contribute little during the initial interaction (Hart, 1997; Hart & Prickett, 1993), whereas ICAM-3 is reported to promote the initial cluster formation as it is expressed at high densities on DCs. A novel DC-specific molecule known as DC-SIGN (DC-specific ICAM-3-grabbing nonintegrin, also referred to as CD209) has been suggested to be the main adhesion receptor involved in the initial adhesive contacts with naïve T cells (Geijtenbeek et al., 2002). DC-SIGN (CD209) is a Type II transmembrane protein belonging to the C-type lectin family and has very high affinity for ICAM-2 and ICAM-3 (on T-cells). The initial clustering of DC-SIGN (on DCs) with ICAM-3 on resting T cells facilitates the formation of multiple low-avidity LFA-1 (lymphocyte function-associated antigen-1) (on T-cells)-ICAM-1 (on DCs) binding engagements and thus allows the TCR to sample MHC-peptide complexes within the contact area (Geijtenbeek et al., 2000; Engering et al., 2002).

Costimulation events involving costimulatory molecules are necessary to sustain T cell activation and provide Signal 2, and these include specific interactions between co-stimulatory molecules on the T cells and their specific ligand(s) on DCs (Schwartz, 1996). CD28, present on most T cells, is the primary costimulatory molecule that is required for delivering Signal 2 (Chambers & Allison, 1997). Both CD80 (B7.1) and CD86 (B7.2) are increased on the surface of DCs following DC activation (McLellan et al., 1996; Fagnoni et al., 1995). A number of other molecules in the CD28 family have been discovered and share common structural and functional elements with CD28 (Chambers & Allison, 1997). These include inducible costimulator (ICOS), cytotoxic T lymphocyte antigen-4 (CTLA-4), and programmed death-1 (PD-1). Despite their homology to CD28, both CTLA-4 and PD-1 function as negative regulators of T cell activation. ICOS maps to the same genetic region as CD28 and has costimulatory properties (Chambers & Allison, 1997). OX40 a member of the TNF/nerve growth factor receptor family, is another molecule that shows costimulatory properties (Calderhead et al., 1993). Accordingly, its expression is restricted to activated CD4+ and CD8+ lymphocytes, and its binding partner OX40 ligand (OX40L) which is found

on DCs and CD40L-activated B lymphocytes (Godfrey et al., 1994, Stuber et al., 1995). CD40 another member of the TNF receptor family is expressed at low levels on immature DCs, and it is rapidly induced upon DC maturation. Its ligand, CD40L is expressed on the surface of T cells during early activation (Grewal et al., 1996). The interaction of CD40 with CD40L stimulates the upregulation of both CD80 and CD86 expression on the DC surface, which results in increased engagement of CD80 and CD86 with CD28 (Yang & Wilson, 1996).

In summary, Signal 2, initiates the immune response by TCR stimulation and costimulation of naïve T cells by molecules found on the surface of DCs after ligation of pattern recognition receptors. This allows naïve Th cells to develop into protective effector cells, a process accompanied by the high level of expression and release of selective sets of cytokines. The final signal, Signal 3, is again provided by DCs and is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as the IL-12 family members IL-12, IL-23 and IL-27 (Trinchieri, 2003), Type I interferons (IFNs) (Kadowaki et al., 2000), and cell-surface expressed intercellular adhesion molecule 1 (ICAM-1) (Salomon & Bluestone, 1998), all of which polarize the Th effector cells towards the Th1 type. Th2-cell-polarizing factors include monocytic chemotactic protein 1 (MCP1, also known as CCL2) and OX40Ligand (Ohshima et al., 1998), while the regulatory T-cell polarizing factors include IL-10 and transforming growth factor-β (TGF-β) (Groux et al., 1996; Zeller et al., 1999). The conditions of activation and the degree of maturation of immature DCs are important for the way in which these 3 signals are delivered to the T cells.

(c) <u>DC+Pathogens: Toll-like receptors:</u>

Production of Signal 1 and Signal 2 depends on DC activation.

DCs recognize pathogens through a separate signal for cellular activation via pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), that recognize conserved microbe-associated molecules termed 'pathogen-associated molecular patterns' (PAMPS) (Underhill et al., 1999). Once these TLRs recognize a specific microbial structure they trigger differential signalling pathways within DCs that then controls DC maturation and ultimately the differentiation of Th cells along a specific pathway (i.e., development of Th1, Th2 or regulatory T-cell subsets). It is also reported

that pathogens will not affect DCs only directly, but indirectly through the large series of inflammation-associated tissue factors that are expressed by tissue cells in response to pathogen invasion. These include pro- or anti-inflammatory cytokines, chemokines, eicosanoids e.g. PGE2, heat-shock proteins, extracellular matrix components, necrotic cell lipids released by dying cells and others (Beg, 2002).

The exposure of immature DCs to PAMPs and their recognition via specific TLRs, and the contact with modulatory tissue factors, all direct the functional polarization of these immature DCs to mature effector DCs that express high levels of MHC and co-stimulatory molecules and migrate to the draining LNs where they present pathogen-derived antigens to naïve T cells. There they selectively bias the development of Th1, Th2 or regulatory T-cell subsets.

The increasing body of data on TLR by murine and human DCs is showing that there is significant heterogeneity of TLR expression among DC populations (Medzhitov, 2001; Kapsenberg, 2003). So far there are now ten human and nine mouse TLR-family members identified, which recognize various microbial molecules (Kapsenberg, 2003). Since different TLRs are specific for products expressed by certain classes of microbes, the DC subset that becomes activated by a pathogen or product will depend on the specific TLRs expressed by that subset.

Many TLR can recognize several, structurally unrelated ligands. Even though the actual mechanism of ligand recognition is still unknown, available evidence indicates that mammalian TLRs recognize their ligands by direct binding. For example, TLR2 has been shown to be involved in the recognition of a broad range of microbial products, including peptidoglycan from Gram-positive bacteria (Takeuchi et al., 1999), bacterial lipoproteins (Takeuchi et al., 2000), mycobacterial cell-wall lipoarabinomannan (Means et al., 1999), glycosylphosphatidylinositol lipid from *Trypanosoma cruzi* (Campos et al., 2001), a phenol-soluble modulin produced by *Staphylococcus epidermidis* (Hajjar et al., 2001), and yeast cell walls (Underhill, 1999). TLR2 is also receptor for atypical LPS produced by *Leptospira interogans* (Werts et al., 2001) and *Porphyromonas gingivitis* (Hirschfeld et al., 2001), both of which are structurally different from Gram-negative LPS. This broad range of ligands recognized by TLR2 is done, in part, by cooperation between TLR2 and at least two other TLRs: TLR1 and TLR6 (Takeuchi et al.. 2001;

Ozinsky et al., 2000). This means the formation of heterodimers between TLR2 and either TLR1 or TLR6 dictates the specificity of ligand recognition. For example. mycoplasma-derived lipoprotein triggers TLR2 that forms heterodimers with TLR-6 (Takeuchi et al., 2001), whereas bacterial lipoproteins triggers TLR2-TLR1 heterodimers (Takeuchi et al., 2002).

Recognition of PAMPS by TLRs induces the activation of signal transduction pathways within DCs leading to the induction of various genes that function in host defence, including inflammatory cytokines, chemokines, major histocompatibility complex and co-stimulatory molecules. For example, a 19 kDa lipopeptide of intracellular *Mycobacterium tuberculosis* was reported to induce only low levels of the bioactive IL-12p70 in monocyte-derived DCs (Thoma-Uszynski et al., 2000), in contrast to the high levels of IL-12p70 induced by TLR4-agonist LPS (Langenkamp et al., 2000) which primes DCs to direct the development of Th1 cells, both in human and in mice. On the otherhand, mycoplasma-derived lipopetide 2, a ligand for TLR2-TLR6 heterodimer, does not induce the production of IL-12 by human monocyte-derived DCs, but instead the production of IL-10 (Weigt et al., 2003), and the lipopeptide-primed DCs induce unpolarized T-cell responses. The yeast cell-wall component Zymosan also induces IL-10 production by classical $CD11c^{high}$ mouse DC subsets (Qi, et al., 2003). However, this IL-10 production was independent of TLR-2 triggering (Edwards et al., 2002), and instead it was found that zymosan binds concomitantly to other receptors such as the C-type lectins dectin-1 (Gantner et al., 2003) and DC-SIGN (Cambi et al., 2003) of which the latter is associated with the production of IL-10.

1.10.3 Interaction of DCs with whole Pathogens or their antigens:

1.10.3.1 DC stimulation by protozoa promoting Th1 responses:

(i) *Toxoplasma gondii*: A variety of protozoa have been shown to stimulate DC (of the myeloid lineage) function (Reis e Sousa et al., 1999). In mice exposed to *Toxoplasma gondii*, the ability to produce IL-12 is limited to CD8α+ DC subset. This response has not yet been attributed to any known Toll-like receptor (TLR) (Mason et al., 2002) but it is known to involve a synergist interaction of MyD88 (a TLR-adaptor molecule) and

chemokine receptor 5 (CCR5) signalling (Aliberti et al., 2000; Scanga et al., 2002) on the DC. The parasite molecules that trigger DC activation are present in tachyzoite extracts but are also secreted by the pathogen. One of these components has recently been identified as a parasite-derived CCR5 ligand mimic (Aliberti et al., 2003). Recently findings that *T. gondii*-infected IL-12$^{-/-}$ mice developed Th1 responses that are protective in the absence of IL-10 (Jankovic et al., 2002) questioned the view that IL-12 production by DCs is essential for Th1 response induction. However, MyD88-/- animals infected with *T. gondii* fail to make Th1 responses or survive (Scanga et al., 2002, Jankovic et al., 2002).

T. gondii infection selectively activates human DCs by increasing MHC class II, CD86 and CD83 but not CD40, CD54 (ICAM-1) or CD80 expression. However, these DCs induced apoptosis in T cells (Wei et al., 2002). Non-viable tachyzoites, or apoptotic fibroblasts loaded with tachyzoite antigens, on the otherhand, did not have this effect and activated T cells. In the same study, Wei et al., (2002), reported that parasite replication, rather than the involvement of different DC receptors for uptake, was essential to induce this form of immunosuppression. The latter studies suggests that DCs are initially activated by released parasite products (e.g., those released by *T. gondii*) or through cross-presentation from other infected cells but can become functionally impaired when directly invaded by the same pathogens. Indeed, as indicated by recent *in vivo* studies in the *T. gondii* system, co-stimulation by CD40L may be intrinsically required for continued DC activation once host infection has been initiated (Straw et al., 2003).

(ii) Leishmania spp.: Different species of *Leishmania* parasites have quite different effects on DCs and this is believed to be critical to the outcome of the infection. In human myeloid DCs, the metacyclic promastigotes of *L. major*, unlike those of *L. donovani* or *L. tropica*, efficiently primed for CD40L-induced IL-12 p70 secretion (McDowell et al., 2002). *L. major* also induced IL-12 production by murine bone marrow-derived DCs but uptake of *L. amazonensis* amastigotes or metacyclic promastigotes,while upregulating expression of MHC class II, CD40, CD80 and CD86, induced production of IL-4 but not IL-12, suggesting that the DCs are conditioned by the parasite to prime the pathogenic Th2 cells that dominate the response *in vivo* (Qi et

al., 2001). Clinically, *L. major* produces self-limiting infections that are controlled in the skin, but *L. donovani, L. tropica* and *L. amazonensis* are capable of disseminating to the viscera or to other cutaneous sites. One possibility that accounts at least in part for the differences in DC response patterns and in determining the different clinical outcomes observed, is polymorphism in the surface-associated and secreted glycan structures expressed by the different *Leishmania* species (Turco et al., 2001). A recent observation showed that DC-SIGN, is a major ligand for the amastigote stage of *L. pifanoi* (Colmenares et al., 2002) and it has also been shown to bind the mannose-capped lipophosphoglycan of *L. mexicana* (Appelmelk et al., 2003).

(iii) T*rypanosoma cruzi:* In *T. cruzi* studies, GPI anchors and glycoinositolphospholipids induce TLR2-dependent IL-12 production (Campos et al., 2001). Further studies into the same parasite now show that a member of the glutathione-S-transferase superfamily, Tc52, can also induce TLR2-dependent signalling, NFkB activation and cytokine production (IL-12) (Ouaissi et al., 2002).Tc52 binds to human monocyte-derived DCs and signals through TLR2. Inhibition studies suggest that this response requires TLR2 recognition of the active glutathione binding site present in Tc52. *T cruzi* HSP-70 fusions also induces DC maturation (Planelles et al., 2002).

(iv) *Malaria:* In malaria, Seixas et al., (2001) reported that bm-DCs cultured with *Plasmodium.c.chabaudi* schizonts were induced to produce TNF-α, IL-6 and IL-12p40 and p70, and the upregulation of MHCII, CD40, and CD86 but not CD80. In addition, these *P.c.chabaudi* did not inhibit the upregulation of MHCII, CD86 and CD40 induced by LPS-stimulated bm-DCs. Therefore, the erythrocytic stages of *P.c.chabaudi* are able to activate DCs directly.

1.10.3.2 DC stimulation by helminths promoting Th2 responses:

It has become increasingly clear that DCs are able to interpret information from helminth antigens to induce antigen-specific Th2 responses (MacDonald et al., 2002 a; de Jong et al., 2002; Faveeuw, 2002; MacDonald et al., 2002 b). As yet, however, there is no clear explanation of how this is accomplished.

A study carried out by Whelan et al., (2000) showed that a phosphorylcholine-containing glycoprotein ES-62, of the filarial nematode *Acanthocheilonema vitae*. which was known to be associated with Th2 cell responses *in vivo*, induced the *in vitro* maturation of DCs that induced Th2 cells. So far the binding sites of this type 2 PAMP are unknown. It is anticipated that there are many undiscovered PRRs capable of recognizing unique molecular features in helminth antigens to induce Th2 maturation pathways in DCs (Barton & Medzhitov, 2002) and there is some evidence for these from studies on interactions of human and mouse DCs with schistosome egg antigens (SEA) which are associated with induction of Th2 responses *in vivo.*

- **Human DC:**

Human myeloid DCs conditioned by SEA to induce Th2 responses were found to express the surface marker OX40L, whereas DCs that were conditioned by microbial stimuli, e.g., toxin from the intracellular bacterium *Bordetella pertussis*, did not but rather induced the development of DC1s with enhanced IL-12 production, which promotes a Th1 cell development (de Jong EC et al., 2002). Regarding the nature of the parasite molecules responsible for this induction, there has been a particular interest in sugar moieties as ligands for hypothetical Th2-response-associated PRRs, as carbohydrates in SEA have been shown to be essential for Th2 response induction by this antigen (Okano et al., 2001). Although currently no DC lectin was identified that is acting as a PRR to condition DC to promote Th2 immunity, recent studies have shown that an extract of *S. mansoni* eggs (a glycoprotein found in the eggs called Lewis X antigen [Le^x]) binds to DC-SIGN (DC specific ICAM-3-grabbing nonintegrin) (Appelmelk et al., 2003).

- **Mouse DC:**

In spite of the evidence from human DC/SEA interactions of DC surface marker upregulation studies using murine bm-DC/SEA have failed to show surface marker activation but nevertheless the resulting DCs were able to confer Th2 responsiveness (MacDonald et al., 2001). Thus, there was no upregulation of MHC II, CD86 or CD80. CD40, CD54 or OX40L following culture of bm-DCs with SEA. Similarly there was no production of IL-12, IL-4, or IL-10. However, when the SEA-pulsed DCs were injected

to naïve mice, spleen cells recovered at 7 days and treated *in vitro* with SEA induced splenocytes from mice injected with SEA-pulsed DCs to make IL-4, IL-5 and IL-13, but no IFN-γ. In contrast, splenocytes from mice injected with DCs pulsed with the Th1 inducing antigen *Propionebacterium acnes* produced IFN-γ, but no IL-4, IL-5 or IL-13 in response to stimulation with *P.acnes*. MacDonald and Pearce (2002b), demonstrated that Th2 induction by DC occurs independently of DC IL-4 , as was shown when the splenocytes from naive mice that had been injected 1 week before with SEA-pulsed IL-4-/- DCs were still able to mount a marked SEA-specific Th2 response (high levels of IL-4, IL-5, IL-13 and no IFN-γ). However, recipient derived IL-4 is vital for DC-induced Th2 development, as was shown when splenocytes from IL-4-/- recipients of SEA-pulsed wild type DCs failed to produce IL-4, IL-5 or IL-13 in response to SEA restimulation. It was suggested that the production of polarizing cytokines by cells other than transferred DC was vital for expansion/amplification of the T cell response initiated by DC. DCs could initiate Th polarization but selection, survival and expansion of such cells then becomes reliant upon cytokines from other sources. The source of recipient IL-4 is not yet known.

The fact that conventional DC maturation was not induced during schistosome infection raised the question of how these cells are able to activate naïve antigen-specific Th cells, especially as immature DCs are usually thought of as good inducers of tolerance rather than of productive Th responses. A clue was provided by the observation that $CD40L^{-/-}$ mice do not mount Th2 responses when infected with schistosomes (MacDonald et al., 2002c). Studies on $CD40^{-/-}$ bone-marrow-derived DCs revealed that these cells are incapable of initiating SEA-specific Th2 responses when injected into wild-type mice, raising the possibility that CD40 ligation by CD40L is an essential endogenous signal for DC maturation during Th2 response intiation. That this is the case was suggested by the finding that DCs from schistosome-infected $CD40L^{-/-}$ mice exhibit a phenotype equivalent to that of DCs from uninfected animals, which contrasts with the more activated phenotype of splenic DCs in infected wild-type mice (Straw et al., 2003).

Regarding the molecular nature of the SEA stimulus to DCs , in contrast to DCs pulsed with SEA, DCs pulsed with periodate-treated SEA were recently reported to be incapable of inducing Th2 responses (Faveeuw et al., 2002). However, the finding in

the same study that CD1d is essential for DCs to prime SEA-specific Th2 responses suggests that the important polarizing glycoconjugates in SEA might be glycolipid(s) that are presented on CD1d to NK T cells, since in mice glycolipids or lipids are preferentially presented via the CD1d molecule expressed on DCs to receptors on CD1d-restricted NK T cells (Godfrey et al., 2000). NK T cells have been previously reported to be an early source of the IL-4 essential for polarizing Th-cell responses (Miyamoto et al., 2001).

1.10.3.3 Impaired DC function during parasitic infection:

It is important for both host and parasite that immune responses are carefully regulated in time and magnitude. For the host, excessive, potentially lethal, pathology of chronic Th1- or Th2-cell-mediated inflammation needs to be avoided. As for parasites the need to survive within the host is the ultimate aim. Some parasites appear to avoid activating DCs in the first place, and there is growing evidence for more selective forms of functional impairment.

***S. mansoni*:** Langerhans cells in the skin of mice percutaneously infected with *S. mansoni* become activated but are unable to migrate from the epidermis. This inhibition appears to be mediated by prostaglandin D2 produced by the parasite and may help delay the subsequent adaptive response (Angeli et al., 2001). It was observed that patients with chronic schistosomiasis have profound *in vitro* hyporesponsiveness to specific antigen restimulation. Although it was initially proposed that this might be due to loss of T cells from the periphery, a recent study showed that *in vitro* responses could be restored by the addition of autologous monocyte-derived DC, implicating that the DC function *in vivo* is defective (van den Biggelaar et al., 2000a).

Malaria: In the case of the malaria parasite *Plasmodium falciparum* infections immune response is not just delayed, but chronically compromised, involving the impaired maturation and function of the appropriate DC subpopulations (Urban & Roberts, 2002). Malaria-infected erythrocytes were found to bind to the surface of human monocyte-derived DCs *in vitro* and to markedly suppress the LPS-mediated upregulation of MHC class II, adhesion molecules (e.g., ICAM-1) and co-stimulatory molecules (CD40, CD80, CD83 and CD86) on DCs. Those resulting DCs are severely

impaired in their capacity to induce allogeneic as well as antigen-specific primary and secondary T-cell responses (Urban et al., 1999). The parasite subsequently paralyse DCs by binding to receptors on the surface of DCs, mediating this inhibitory effect, the integrins CD36 and CD51 (Urban et al., 2001). The major parasite ligand *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a conserved domain of a molecule that undergoes antigenic variation, appeared to be involved in this DC interaction. In addition, DCs exposed to blood-stage malaria parasites also suppress CD8+ T cell responses against the liver stages of this parasite, and inhibit CD8+ T-cell priming following vaccination with irradiated sporozoites. This method of immunosuppression may benefit the parasite by leaving individuals open to constant reinfection (Ocana-Morgner et al., 2003).

Leishmania: In visceral leishmaniasis, chronic immunosuppression is also an important clinical outcome. In recent studies in an experimental model it was suggested that this impaired responsiveness may stem in part from a parasite-induced defect in DC-T cell contact. DC from mice chronically infected with *L. donovani* failed to migrate from the marginal zone to the T cell areas of the spleen due to TNF-dependent IL-10-mediated inhibition of CCR7 expression on the DC (Ato, et al., 2002). Poor DC migration may also explain why individuals with visceral leishmaniasis tend to succumb to secondary infection.

Other Pathogens: There are pathogens that affect DC activation in such a way that those DCs induce the development of regulatory T-cells, i.e., T cells that neither bias the immune response towards a specific Th1- nor Th2-cell response but instead will develop some form of tolerance. Some microbes may use DC-SIGN or similar ligands to subvert the immune system, for example *Mycobacterium* species induce regulatory DCs with an immature phenotype by binding to DC-SIGN with their mannosylated lipoarabinomannan (Geijtenbeek et al., 2003). Viruses such as Hepatitis C virus which bind by means of its core protein and non-structural protein 3 (Dolganiuc et al., 2003), herpes simplex virus (Salio et al., 1999) and cytomegalovirus (Moutaftsi et al., 2002) are all reported to inhibit DC function and delay or prevent the generation of virus-specific cytotoxic T cells. Amongst the pathogens that prime for regulatory DCs with a mature phenotype are *Bordetella pertussis* that uses filamentous haemagglutinin

(McGuirk et al., 2002), and *S. mansoni* that uses lysophosphatidylserine to ligate TLR2 (van der Kleij et al., 2002).

1.10.3.4 Dendritic cells and attenuated schistosomula in the Skin and Skin draining Lymph Nodes (SLN):

Because exposure to attenuated larvae is the most effective way of inducing immunity in schistosomiasis there is interest trying to understand how this protection is initiated. The retarded migration of optimally irradiated (20krad) larvae from the skin and persistence in the skin draining lymph nodes produce a Th1 dominated response in the SLN which is crucial for immunity (Coulson, 1997). More highly irradiated cercariae (80krad) all die in the skin but fail to induce protection (Mountford et al., 1992) whereas infections drug terminated in the skin can stimulate high levels of immunity (Bickle and Andrews, 1985). So how does theTh1 response develop and why do the different protocols differ so much in protection? The following section considers what little is known about the reaction to parasites in the skin and the involvement of DC in the skin phase.

In contrast to normal cercariae, irradiated larvae were observed in the dermis for >10 days often surrounded by aggregates of macrophages and dendritic cells. This results in inflammation and skin thickening up to 75% increase on day 4 (Hogg et al,, 2003). Riengrojpitak et al., (1998) investigated the interactions of vaccinating parasites with host leucocytes in both the skin and/or draining lymph nodes of C57BL/6 mice. They observed extensive lateral spreading of cercarial secretions along layers of the stratum corneum but the contents of the pre- and post-acetabular glands, potentially rich in antigenic proteins (Harrop & Wilson, 1993), remains superficial and is sloughed off rather than remain a major stimulus for the immune system. Parasites migrated into the stratified epidermis and there was little direct contact with host leucocytes during the first 1-2 days when the parasites lay at the base of the epidermis, but cells accumulated in the underlying dermis. The lack of leucocytes adhering to both attenuated and normal parasites at this stage of penetration suggested that they are not 'leaking' significant quantities of antigen. Cells bearing MHC II, CD11b or CD11c markers were present in

the lymph nodes, particularly in the periphery and paracortical areas, no obvious redistribution was seen as a result of parasite residence there for 5-15 days (Riengrojpitak et al. 1998). Interactions between parasites and potential antigen-presenting cells in the SLN's were much less marked than in the skin. Ultrastructural studies did reveal cells with characteristics of DC/macrophage lineage in the vicinity of the parasites but there were no qualitative differences in the interactions of normal or irradiated larvae with constituent leucocytes and again the conclusion was just the protracted exposure of living larvae with the SLN.

Hogg et al., (2003b) analyzed the innate response to radiation-attenuated schistosome larvae in order to define the cellular constituents and cytokines in the skin which are likely to stimulate a Th1-mediated protective response at the skin infection site. At 24hr post infection the cytokines IL-1β, IL-6, IL-10 and IL-12 p40 and various chemokines were all significantly increased. IL-12 p40 was also elevated in mice given unirradiated cercariae at day 1 but the levels then dropped whereas they remained high until at least day 14 in mice given irradiated cercariae. A similar pattern was found in the SLN. IL-12 plays a key role in differentiation of Th1 cells (O'Garra, 1998) and is necessary for the Th1 mediated protection in the irradiated cercaria model (Anderson et al, 1998). Compared with non-protective non-irradiated larvae, the irradiated larvae induced the production of high levels of IL-12 and IL-18 until at least day 14 after the infection. IL-10 was produced from both vaccinated and infected mice, and it was shown to regulate IL-12-associated dermal inflammation, since in the ear pinna thickness of vaccinated IL-10-/- mice was greatly increased concurrent with elevated levels of IL-12p40 (Hogg et al., 2003b). Regarding the cell source of the IL-12p40, the majority of the IL-12p40+ cells in the skin infection site were found to be myeloid DCs, although a proportion were $CD11c^{-}$ F4/80+, suggesting that macrophages were an additional source of IL-12 in the skin.

1.11 Focus of the present study:

As described above after inducing priming in the skin, the migration of the majority of the irradiated larvae to the lungs is necessary in order to attract circulating cells and so arm the lung tissue in order to arrest larvae of a challenge infection. As a model for development of a defined antigen vaccine this requirement for priming in the skin

followed by inflammation in the lungs is rather complex. Infections treated at the skin stage with the drug Ro11-3128 seem to arrest predominantly in the skin raising the question as to the nature and level of response in the skin to this regimen compared to the irradiated infection and the nature of the protective mechanisms. This was the starting point of the studies in this thesis (Chapter 3). Earlier work had shown that termination of infection with Ro11-3128 could lead to much higher levels of protection than other drugs tested and so the studies in Chapter 4 were aimed at trying to explain the individual effects of the drug e.g. enhanced antigen presentation or adjuvanticity. The demonstration of a Th1 dominated response in the studies in Chapter 3 led to studies aimed at defining if and how DCs are activated by interaction with schistosomula (Chapter 5).

1.12 Aims of the Project

1) To determine the sites of antigen presentation/stimulation and the key features of the induction and operation of immunity induced by Ro11-3128 attenuated larval infections by:

(i) Defining the nature and kinetics of the cytokine responses to larval antigens in the SLN, at intervals post vaccination with Ro11-3128-terminated infections compared to radiation-attenuated infections (both of which elicit high levels of protection) and to Ro11-3128-terminated irradiated infections (which elicit poor levels of protection).

(ii) Determining the nature and level of the systemic spread of this response as judged by responses in the spleen following these different immunizing regimens.

2) To establish the *in vivo* relevance of the protective mechanism(s) predicted from the work in (1) by:

(i) immunization of immunodeficient, targeted knockout mice.

(ii) immunization followed by cytokine neutralization with monoclonal antibodies.

3) To investigate the properties and effects of Ro11-3128 in *in vitro* cultures:

(i) To compare the effects of Ro11-3128 on normal and irradiated schistosomula.

(ii) To test if Ro11-3128 has adjuvant properties.

(iii)To test if Ro11-3128 induces enhanced antigen presentation.

(4) To investigate the interaction of normal and drug-treated worms with DCs by:

(i) Establishing an *in vitro* system of interacting DCs and living normal/drug-treated schistosomula.

(ii) Defining the effects on DC maturation of co-culture with live normal/drug-treated schistosomula on surface maturation markers (CD40, CD86, MHCII).

(iii) Defining the effects on DC maturation of co-culture with live normal/drug-treated schistosomula on cytokine production (IL-12p70, IL-10, TNF-α, IL-6).

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice:

Outbred, CD-1 strain, Swiss albino mice were used to maintain the Puerto Rican strain of *Schistosoma mansoni* life cycle. Five to eight-week-old C57BL/6 wild type mice were used in most of the experiments. Other strains used were BALB/c mice and C3H/HeJ and C3H/OuJ mice (non- and responders to LPS, respectively). The mice were either bred and maintained at the London School of Hygiene and Tropical Medicine (LSHTM) Biological Services Facilities according to the UK Home Office guidelines, or purchased from Charles River, UK. B6.RAG1-/- (Recombinase activation gene knockout) (RAG on C57BL/6 background) (deficient in both T & B cells), mice with a targeted disruption of the IFN-γ gene on the C57BL/6 background (B6.IFN-γ-/-) also called C57BL/6.12957, and B-cell knock out mice on the same background (C57BL6/μMT) were bred and maintained in the Biological Services Facilities of LSHTM. All experiments were performed under the regulations of the Home Office Scientific Procedures Act (1986).

2.2 Production of Cercariae:

As part of the life cycle, the Puerto Rican strain of *Schistosoma mansoni* was maintained in laboratory-reared *Biomphalaria glabrata* snails. The snails were kept in clean tap water at 24-26°C. Cercariae were shed in a minimum volume of clean tap water at 28-30°C under direct illumination for 1-2 hours. Where sterile parasites were required for tissue culture, snails were washed several times in clean tap water before shedding, and snail faeces were allowed to settle before decanting the cercarial suspension. These *S. mansoni* cercariae were either used for infecting mice within a few hours of collection either directly or after exposure to radiation, or transformed to the equivalent of skin-stage schistosomula for tissue culture experiments.

2.3 Irradiation of Cercariae:

Freshly shed cercariae were poured into a 30ml 'Universal' tube (Sterilin. UK) and exposed to a Cesium 137 source at the LSHTM (Gammacell 1000 Elite). The irradiation dose used was 20 krad γ-radiation (Bickle et al.. 1979a).

2.4 Preparation of Schistosomula:

2.4.1 Mechanical Transformation of Cercariae to Schistosomula:

The method of transformation of schistosomula from *S.mansoni* cercariae was modified from methods previously described by Ramalho-Pinto et al., (1974), Basch (1981), and James and Taylor (1976). Cercariae were collected and concentrated in clean tap water, as described in section 2.2. 10ml aliquots of the cercarial suspension were then pipetted into 15ml glass conical centrifuge tubes and cooled in an ice bath for 10 minutes to reduce motility of the organisms and facilitate concentration of cercariae (Ramalho-Pinto et al., 1974). These were centrifuged at 400 xg for 2 minutes at 4°C and the upper 90% of water was carefully removed leaving behind packed cercariae (Basch, 1981). They were then quickly resuspended in 2ml cold E/LAC medium (see Appendix A.1), and pooled from all the glass tubes into a conical-based plastic 30ml 'Universal' tube (Sterilin, UK). E/LAC was supplemented with 300U/ml Penicillin (Gibco, UK), 300µg/ml Streptomycin (Gibco, UK) and 160µg/ml Gentamicin (Sigma, UK).

Mechanical transformation of cercariae into schistosomula (MS) was done by the disruption of cercarial heads from tails by the 'Syringe method' (James and Taylor, 1976). Briefly, cercariae were forced through a 21-guage needle (0.8mm x 40mm, Becton Dickinson, Microlance™ 3, UK) attached to a Luer Lock Syringe (Becton Dickinson, UK) a total of 20-22 times. The cercarial heads were left to settle by sedimentation in a 15ml plastic tube (Scientific Laboratory Supplies [SLS], UK) at 37°C and 5% CO_2 for 10-15 minutes. After this, the tail-rich supernatant was carefully removed, and the remaining 1-2mls of the heads-rich supernatant were carefully pipetted on the top of a sterile Percoll gradient which consisted of 3 layers of 50%, 60% and 70% densities, in a 15 ml plastic tube (procedure modified from Lazdins et al., 1982). The tube was spun for 10 minutes at 350 xg and 4°C. After centrifugation, each of the 3 layers was carefully removed, under sterile conditions, and placed in a 60mm diameter petri-dish (Nunc, UK). Heads were mostly observed in the 60% gradient (as was seen under the microscope), and were free of tails. This portion of the gradient was then transferred into a 50ml plastic tube (SLS, UK) and washed 2x in 20-25mls of E/LAC supplemented with 300U/ml Penicillin, 300µg/ml Streptomycin and 160µg/ml Gentamicin at 400 xg and 4°C for 2 minutes. The medium was carefully removed leaving behind about 2mls containing a pellet of 'heads'. This was transferred to a new sterile 15ml plastic tube and topped up with 10ml of fresh medium. Heads were then

left to sediment at 37°C and 5% CO_2 for 10 minutes. This washing step was repeated 3x after which heads were washed 1-2x in exactly the same way but with either M169S (Basch, 1981, see section 2.8.1) or cDMEM (complete Dulbecco's Modified Eagle's Medium, Sigma, UK) (see section 2.8.1) depending on the experiment. Then the heads were counted and resuspended in an appropriate volume of medium to obtain required concentration of parasites. Transformation and washing were all done at room temperature and under sterile conditions.

2.4.2 Preparation of Density Gradients:

Solutions of Percoll (polyvinylpyrolidone-coated colloidal silica particles, Sigma, UK) of different densities were prepared by diluting the stock suspension with the appropriate volumes of 10x concentrate Minimal Essential Medium (MEM) (Sigma, UK) and sterile double processed tissue culture water (Sigma, UK). The solutions were then buffered with 1M HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid] to a final concentration of 25mM and the pH adjusted to 7.0-7.2 with 1N NaOH. Three density gradients were prepared at 50%, 60% and 70% of original Percoll density. These sterile solutions were prepared fresh and kept at 4°C until use on the same day. To achieve sharp interfaces, 3 ml of each of these solutions was layered from the bottom of sterile 15ml plastic conical centrifuge tubes using a 19-gauge, 5-inch-long (spinal) needle (Becton Dickinson, UK) placed in the tube. Care was taken not to introduce air bubbles into the gradients. This needle was connected to a 10 ml syringe via a two-way stopcock that allowed regulation of the flow rate of the Percoll solutions.

2.4.3 Assessing schistosomula viability:

The viability of schistosomula in cultures was assessed by direct microscopic examination of parasite motility and appearance. Parasites were scored as dead if they were immotile and had become granular and opaque in appearance.

2.5 Preparation of Antigens:

Soluble cercarial antigen preparation (SCAP) and deoxycholate cercarial antigen preparation (DOC-SCAP) were prepared from *S.mansoni* cercariae (as described previously for preparing adult worm antigens in Zhang et al., 1999). Care was taken to prevent the cercarial suspension being contaminated by snail faeces and the cercariae washed by sedimentation on ice. The eventual pellets of cercariae were homogenized on

ice in PBS in the presence of 1mM phenylmethanesulfonyl fluoride, 0.2mM tosyl-L-phenylalaninechloromethyl ketone, 0.1μM aprotonin and 1μM leupeptin. The homogenate was centrifuged at 100,000 xg for 1 hour at 4°C using a Beckman L8-80 ultracentrifuge. The supernatant (SCAP) was filter sterilised through a 0.22μM filter (Sartorius, UK) and aliquoted and stored in -70°C. The pellet was resuspended in PBS containing 4mM sodium deoxycholate (DOC) and protease inhibitors. The resuspension was sonicated and then left on ice for 1 hour. This was then centrifuged as for SCAP. The supernatant (DOC-SCAP) was, filter sterilized, aliquoted and stored at -70°C. The protein concentration was determined using a Biorad protein estimation kit.

For the initial experiments in section 5.2.1 (Chapter 5) two antigen preparations kindly supplied by Dr Mike Doenhoff, (University of Bangor) were used: SCA (soluble cercarial antigen) which had been prepared from pelleted cercariae in a similar way to the preparation of SCAP; Cercarial transformation fluid (CTF) was prepared by resuspending pelleted cercariae in RPMI-1640, mechanically transforming them as described above (section 2.4.1) and collection of the medium used for transformation after centrifugation of the parasites at 1000 xg for 5 minutes.

2.6 Preparation of Ro11-3128:

2.6.1 For Mouse administration:

Ro11-3128 powder had kindly been provided by Dr H Stohler (Hoffman-La Roche, Basle, Switzerland). It was dispersed by sonication in distilled water containing 2.5% Cremaphor El (Sigma, UK) and administered orally 2 days after infection (Bickle and Andrews, 1985) at a dose of 200mg/kg body weight. This dose was increased from that previously used (Bickle &Andrews, 1985; Mountford et al., 1989; Bickle, Sacko & Vignali, 1990) in order to enhance skin phase arrest.

2.6.2 For *in vitro* culture assays:

Since Ro11-3218 is not soluble in medium, the drug was first dissolved in DMSO (Dimethyl Sulfoxide) (Sigma, UK) and then added to medium in *in vitro* cell cultures. In some experiments absolute ethanol was used to dissolve Ro11-3128.

2.7 Mouse infections and measurement of resistance:

2.7.1 Immunizations and Challenge Infections:

Mice were first anaesthetized with sodium pentobarbitone (0.06 mg/g body weight *Sagatal*; May and Baker Ltd., Dagenham, England) and then percutaneously infected on the shaved belly by the 'Ring Method' of Smithers and Terry (1965) as adapted by Doenhoff et al., (1978). A suspension (0.2-0.5ml) of normal or irradiated *S. mansoni* cercariae was placed in a plastic ring retained on the skin of the belly region with the aid of transparent self-adhesive tape. This was left on the skin for about 20 minutes, at which time fluid and rings were removed and the animals allowed to recover over gentle warmth. Mice that were infected with a primary infection of 500 cercariae were then given an oral dose of 200mg/kg of Ro11-3128 2 days (40 h) post-infection. Those mice to be challenged were exposed again at 5-6 weeks to another infection of 100-200 cercariae in the same way as above but on a different site, the flank of the mouse, and perfused 3 weeks later.

2.7.2 Immunization and drug administration regimes:

For the different vaccination/infection regimes mice were infected percutaneously as follows: NI, 500 normal *S. mansoni* cercariae on day 0; RoNI, 500 normal *S. mansoni* cercariae on day 0 and treatment with Ro11-3128 on day 2 (40 h pi); GI, 500 γ-irradiated cercariae on day 0; RoGI, 500 γ-irradiated cercariae on day 0 and treatment with Ro11-3128 on day 2 (40 h p.i). Controls were naïve mice (CC) for NI and GI or Ro11-3128 treated naïve mice (RoCC) for RoNI and RoGI.

2.7.3 Perfusion technique:

Challenged mice were perfused as described by Doenhoff et al., (1978). Mice were killed with an overdose of sodium pentobarbitone (100µl *Sagatal* containing 10 units of sodium heparin [Sigma, UK]/mouse) given intraperitonally. The thoracic and peritoneal cavities were opened, the mouse suspended over a conical funnel leading into a 30ml Universal tube, the hepatic portal vein cut and 25ml of citrate/saline perfusion fluid (8.6g NaCl and 15.0g trisodium citrate/litre) was injected into the left ventricle, the liver and intestines were gently massaged and the perfuseate emerged from the cut hepatic portal vein and was collected in the tube. Parasites were allowed to settle and excess solution was removed by suction, leaving behind 1-1.5ml. More perfusion buffer was

added, allowing 5–10 minutes for worms to settle and the supernatant similarly removed. This was repeated to remove most of the erythrocytes. A drop of 1% aqueous saponin (Sigma, UK) solution was added to lyse the remaining erythrocytes and the solution poured into a 6cm Petri dish. The tube was rinsed with perfusion fluid and this poured into the Petri dish. The worms were counted under a dissecting microscope.

2.7.4 Measure of Level of Resistance:

The level of resistance for vaccinated mice was calculated from the worm burdens recovered using the following formula:

$$\% \text{ Resistance} = \frac{\text{Control Worm Recovery} - \text{Vaccinated Worm Recovery}}{\text{Control Worm Recovery}} \times 100\ \%$$

2.7.5 Statistical Analysis:

Data for worm burdens and cytokines were compared using two-tailed Student's t-test or the Mann-Whitney U test. P-value of ≤ 0.05 was considered statistically significant.

2.8 *In vitro* cultures of parasites and cells:

2.8.1 *In vitro* culture of Schistosomula:

(A) Preparation of M169:

Medium 169 (M169), a medium specially developed for culturing schistosomula, was prepared as defined by Basch (1981) and as shown in Appendix A.2. The freshly prepared medium was filter sterilized and stored at 4°C.

(B) Culturing of schistosomula:

Sedimented schistosomula were cultured in either pre-warmed complete schistosome culture medium M169 supplemented with 300U/ml Penicillin (Gibco, UK), 300µg/ml Streptomycin (Gibco, UK), 160µg/ml Gentamicin (Sigma, UK) and 5% Foetal Calf Serum (FCS) (Sigma, UK), or cultured in Dulbecco's Modified Eagle's Medium [DMEM] (Sigma, UK) supplemented with 10% FCS (Sigma, UK), 1mM Sodium Pyruvate (Sigma, UK), 2mM L-glutamine (Sigma, UK), 50µM 2-Mercaptoethanol (Gibco, UK), 100U/ml Penicillin and 100µg/ml Streptomycin (Gibco, UK) and 160µ g/ml Gentamicin (Sigma, UK). Complete media containing FCS were referred to as

cDMEM and M169S. In cultures where parasites were required to develop for 4 days, mouse red blood cells were added to the cDMEM. For this purpose, a mouse was killed by cervical dislocation and blood withdrawn from the heart into a 1ml syringe containing 20units sodium heparin. The cells were washed by adding to 10ml DMEM containing 5units/ml sodium heparin and centrifuging at 1500 xg for 5 minutes and then resuspending in 1ml DMEM.

Cultures were incubated at 37°C in 5% CO_2 for 1-4 days, according to the conditions of the specific experiment. When the parasites were to be cultured ultimately with splenocytes or lymph node cells the medium in which the schistosomula were pre-cultured in was cDMEM.

2.8.2 Preparation and culture of lymphocytes in *in vitro* culture assays:

(A) Cell Culture Media:

The medium used for experiments involving cultures of cells with schistosomula was Dulbecco's Modified Eagle's Medium (Sigma, UK). For other experiments RPMI-1640 medium (Gibco, UK) was used. Both were supplemented with 10% heat-inactivated FCS (Sigma, UK), 1mM Sodium Pyruvate (Sigma, UK), 2mM L-glutamine (Sigma, UK), 50μM 2-Mercaptoethanol (Gibco, UK), 100U/ml penicillin and 100μg/ml streptomycin (Gibco, UK).

(B) Spleen and Lymph node cell preparation:

Spleens or lymph nodes from mice were removed aseptically on days 7 and/or 21 post-infection (pi). For spleen cell preparations, spleens were passed gently through a 100-μm sieve (Becton Dickinson-Falcon, UK), using a 5ml syringe plunger to disrupt the tissue. The cell suspension was centrifuged once in complete medium, at 1200 revolutions per minute (rpm), for 10 minutes at 4°C, and the supernatant discarded. Red blood cells were lysed by the addition of cold 1x Gey's Solution (see Appendix A4) for 7-8 minutes at room temperature (9ml of 1x Gey's solution:1ml of cell suspension). The cell suspension was then diluted in complete cell culture medium and centrifuged once at 1200 rpm, for 10 minutes at 4°C. After discarding the supernatant the cell pellet was resuspended in fresh complete medium and washed twice at 1200 rpm, for 10 minutes at 4°C. Viable cell counts were made using Trypan Blue solution (Sigma, UK), where

Trypan Blue negative cells were counted as viable under the microscope in a Haemocytometer.

The axillary and inguinal, skin-draining lymph nodes (SLN) and popliteal lymph nodes were passed gently through a 100-μm sieve as above and the cell suspension was centrifuged once in complete cell culture medium, at 1200 rpm for 10 minutes at 4°C. Lymph node cells were not resuspended in 1x Gey's solution. Cells were counted in Trypan Blue as above.

(C) Cell cultures:

Single cell suspensions of either spleen or lymph node cells were seeded in 96-well flat-bottomed tissue culture plates (Nunc, UK) at either 3 or $5x10^5$ and 2.5 or $5x10^5$ cells/well, respectively, in 200μl final volumes/well, depending on the experiments. In some experiments immature dendritic cells (DCs) were added to lymph node cells at a concentration of $2.5x10^4$ DCs:$2.5x10^5$ LN cells, such that the DC:T cell ratio was 1:10 (Semnani et al., 2001; Whelan et al., 2000). Cells were either stimulated with *Mycobacterium bovis* Bacillus-Calmette Guerin (BCG), the mitogen Concanavalin A (ConA), antigens e.g., SCAP or DOC-SCAP, or live schistosomulae (±Ro11-3128). All cultures were incubated at 37°C in the presence of 5%CO_2. Cell supernatant fluids were collected at 24 hours for IL-4 assays and 72 hours for IFN-γ, IL-5 and IL-10 assays as in other similar studies (Grzych et al., 1991; Pearce et al., 1991; Mountford et al., 1999). IL-4, IL-5, IFN-γ and IL-10 levels in the cell culture supernatant were measured using PharMingen OptEIA™ ELISA kits according to manufacturer's instructions (section 2.11).

2.9 Preparation of mouse bone marrow derived Dendritic Cells (bm-DCs):

2.9.1 Producing GM-CSF from a GM-CSF secreting cell line (x63-GM-CSF):

The mouse gene for GM-CSF (PCR derived) has been cloned into a mammalian expression vector (BCMGSNeo; Karasuyama et al., 1990) and transfected into the plasmacytoma line X63-Ag8 (Stockinger et al.,1996). This x63-GM-CSF cell line was obtained from Prof. D. Gray, University of Edinburgh.

The cells were grown in selection medium, made of cDMEM containing 1mg/ml of the selective antibiotic G418/Geniticin Sulphate (Sigma, UK), until the cells had grown to

'medium' density. These were harvested, washed twice in cDMEM and returned to culture for 2-4 days in medium without G418. The supernatant was then harvested by centrifugation and aliquoted to be stored at -80°C. Filtration was not used as filtering causes loss of GM-CSF activity (Professor Gray – personal communication). Specific GM-CSF activity of each batch was checked by titrating the collected GM-CSF supernatant into bone marrow cultures and assessing generation of dendritic cells. Initially a supernatant dilution of 1:40 dilution of GM-CSF was used, but this was increased to 1:10 GM-CSF as recommended by Lutz M. et al., (1999).

2.9.2 Bone marrow preparation :

The principle for culturing bm-DC with GM-CSF was according to Lutz et al., (1999). Under sterile conditions the femurs of female C57BL/6 mice were removed and surrounding muscle tissue cut away. Both ends of the femur were then cut with scissors and the marrow flushed by inserting a 1ml syringe with a 0.5mm diameter needle containing warm cDMEM. The flushed marrow was collected in a 90 mm diameter Sterilin grade petri-dish (Sterilin, UK). Clusters within the marrow suspension were dispersed by vigorous pipetting. The bone marrow cells were washed once in cDMEM at 1200 rpm for 10 minutes at 4°C, and cell counts were obtained by staining an aliquot volume of cells mixed 1:1 in Trypan blue solution (Sigma, UK). Erythrocytes were excluded by size and shape, and viable cells (i.e. not staining) were counted under the microscope in a Neubauer haemocytometer. About 1-1.5x10^7 cells were obtained per femur.

2.9.3 Bone marrow cell culture with GM-CSF:

At day 0 bone marrow leukocytes were seeded at 2 x 10^6 cells per 90mm bacterial grade petri-dishes Triple Vent (Sterilin, UK) in 10 ml cDMEM containing 10% GM-CSF supernatant. These were incubated at 37°C and 5% CO_2. At day 3 another 10 ml cDMEM containing 10% GM-CSF supernatant was added to the plates. At days 6 and 8 half of the culture supernatant was carefully aspirated from the surface of the liquid using a pastette with a 200µl Gilson pipette tip (Sarstedt, UK) attached. Another 10 ml of fresh cDMEM containing 10% GM-CSF supernatant was gently added dropwise. Care was taken not to aspirate non-adherent cells, and not to move plates to avoid stimulation and therefore maturation of DCs in culture. On day 10 all culture

supernatants containing the non-adherent cells were gently transferred to a 50ml Falcon tube and centrifuged at 1200rpm for 10 minutes at room temperature to pellet the cells. Pelleted bm-DCs were then quickly resuspended and washed once in fresh cDMEM containing 5% GM-CSF (at 1200rpm for 10minutes, at room temperature), counted in a haemocytometer as described above, and used as immature bm-DCs in *in vitro* experiments. DCs were identified as granular, irregularly shaped cells with small protrusions. In cultures containing DCs ± parasites ± other antigens, supernatants were collected 19-24 hours after culture and tested for the presence of IL-12p70, IL-6, IL-10 and TNF-α using standard ELISA kits as explained in section 2.11.

2.10 *Ex-vivo* Surface Cytokine Staining for Flow Cytometry Analysis :

Bm-DCs were carefully collected from the culture wells and transferred to 50 ml Falcon tubes. If schistosomula were present in the cell cultures, these were first separated from the cells by passing the parasite-cell mix carefully through a 25μm filter mesh (Allied Filter Products Ltd., UK) attached to the top of 50ml Falcon plastic tubes via autoclave tape. Preliminary experiments were carried out to determine the optimal mesh size for this purpose. The collected DCs were then washed once in ice-cold FACS-EDTA buffer (Appendix A.3) at 1200 rpm and 4°C for 10 minutes. Supernatants were discarded and the cells, at the bottom of the tube, were gently flicked and then removed and placed into FACS tubes (Becton Dickinson-Falcon, No. 352052, UK) at 1-5x10^5 cells/tube. These tubes were spun again at 1200 rpm and 4°C for 10 minutes, and supernatants were poured off. Non-specific binding of the cells was blocked by adding 5μl of Blocking Buffer (1:1 Horse serum [Sigma, UK]:24G2 [Fc blocking antibody, supernatant from a negative control rat cell hybridoma grown in-house, obtained from C. Meier, LSHTM], filtered before use) to 100μl of cell suspension in a 1:20 dilution, and cells were left to incubate for 15 minutes on ice. Anti-cell-surface marker antibodies or isotype controls were then added to the cells and tubes gently flicked to ensure good mixing of cells with antibodies. Staining was done in the dark. See Table 2.1 for details of anti-cell-surface monoclonal antibodies and isotype controls used, and the dilutions they were used in. Tubes were incubated for 30 minutes at 4°C in the dark. Cells were then washed once in 3ml cold FACS-EDTA buffer at 1200rpm for 10 minutes at 4°C and the supernatant discarded. To those cells stained with biotinylated antibodies, Streptavidin-Quantum Red (SA-QR) was added at 1μl:100μl/sample and the tubes gently flicked and incubated in the dark for another 30 minutes at 4°C, after which these

cells were washed once more in FACS-EDTA buffer. All the cells were fixed with 1% cold Paraformaldehyde and stored at 4˚C until analysis, which was done within 1 week of staining. Cells were analysed on a FACScan (Fluorescence-Activated Cell Sorter) flow cytometer with CELLQuest software (Becton Dickinson, USA).

2.11 Cytokine detection using Enzyme linked Immunosorbent Assays (ELISA):

The cyokines IFN-γ, IL-4, IL-5, IL-10, IL-12p70 and IL-6 were detected using PharMingen OptEIA^{TM} ELISA kits (PharMingen, UK), and TNF-α using an R&D kit (R&D systems, UK) according to manufacturers instructions. Briefly, 96-well Nunc Maxisorp plates were coated with 50µl/well of anti-mouse capture antibody to the specific cytokine being tested. Plates were sealed and incubated overnight at 4^{o}C. The following day, plates were aspirated and washed as required with freshly prepared Wash Buffer (0.05 % Tween-20 in 1x PBS, pH 7.4). Plates were then blocked with Assay Diluent (10% FCS in PBS, pH 7.0) for one hour, or for overnight, at room temperature. Wells were then aspirated and washed with Wash Buffer as above. 50µl of appropriately diluted supernatant specimens were added in duplicates into the wells, alongside serial dilutions of standards (i.e., recombinant mouse-IFN-γ, IL-4, IL-5, IL-10, IL-12p70, IL-6, or TNF-α) run also in duplicates. The plates were then sealed and left to incubate for 2 hours at room temperature, after which they were aspirated and washed as required. 50µl of Working Detector, which is a combination of biotinylated anti-mouse-detection antibody to the specific cytokine tested and avidin-horseradish peroxidase conjugate enzyme reagent, were added to each well and the plates sealed and incubated for 1 hour at room temperature. Finally the wells were aspirated/washed as required. 100µl of TMB-ELISA solution (Gibco, UK) was used as substrate and added to each well, and plates incubated for 10-30 minutes at room temperature in the dark. The reaction was stopped by adding 50µl/well of Stop Solution (2N H_2SO_4). The optical density was read at an absorbance of 450 nm using an ELISA reader (MRX microplate reader, Dynex Technologies, UK) within 30 minutes of stopping the reaction. The cytokine levels in the samples were calculated according to the standard curve obtained for each ELISA plate.

2.12 Measuring Apoptosis and Necrosis by Flow Cytometry:

Annexin V-FITC is used to quantitatively determine the percentage of cells undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early

phases of apoptosis. In these cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS. Propidium iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable cells from nonviable cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead or damaged cells are permeable to PI. Cells that stain positive for Annexin V-FITC and negative for Propidium iodide (PI) are in the early stages of apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, are undergoing necrosis or are already dead. Cells that are negative for both Annexin V-FITC and PI staining are alive and not undergoing measurable apoptosis.

The procedure and reagents required for measuring apoptosis in cells were all obtained from the Annexin V-FITC Apoptosis Detection Kit I (PharMingen, UK). Bm-DCs and bm-DCs separated from parasites via the 25μm filter mesh were washed twice in cold sterile 1x PBS (Dulbecco's Phosphate Buffered Saline solution, Sigma, UK) by spinning at 1200 rpm for 10 minutes at 4°C. These cells were then resuspended in 100μl of 1x Binding Buffer (Appendix A.6) at a concentration of 1-5 x 10^5 cells on ice. 5μl of Annexin V-FITC and 10μl of Propidium Iodide (Appendix A.6) were added and the tubes were gently flicked to mix the cells and stains, and incubated for 15 minutes at room temperature in the dark. 200μl of 1x Binding Buffer were then added to each tube and cells were analysed by flow cytometry within 1 hr of staining. For setting up compensation and quadrants for staining controls were set up: (i) unstained cells, (ii) cells stained with Annexin V-FITC alone (no PI), and (iii) cells stained with PI alone (no Annexin V-FITC).

2.13 Preparation of anti-IFN-γ monoclonal antibody:

2.13.1 Culture of anti-IFN-γ monoclonal antibody secreting cell line:

The rat cell line (XMG 1.6, secreting IgG1 anti-mouse IFN-γ; initially from ATCC (American Type Culture Collection, Bethseda, MD, USA) and kindly provided by Dr C. Engwerda, (LSHTM) was cultured in RPMI-1640, 5% CO_2 and 37°C. For collection of tissue culture supernatants, cell lines were harvested in log phase growth, cells removed by centrifugation, and the supernatant collected. 0.01% sodium azide (NaN_3) was added

to the supernatant. The supernatants were then stored in 4°C until purification by affinity chromatography using a protein G column.

2.13.2 Purification using Protein G affinity Chromatography:

Cell culture supernatant was filter sterilized. Protein G Sepharose Fast Flow gel (Pharmacia Biotech, UK) was packed in a plastic column (Sigma, UK). The column was connected to a pump and equilibrated by running 5 column volumes of Running Buffer (0.02 M phosphate buffer, pH 7.0) through it. The supernatant was then passed directly over the column at a slow rate, at 4°C. To dissociate antibody that had attached to Protein G, the column was washed with Running buffer until non-specific protein ceased to be washed from the column. At this point Elution Buffer was run through the column (0.1 M glycine-HCl, pH 3.0-2.7) to dissociate specific IgG. The antibody was collected. To preserve the activity of acid labile IgG 50µl of 1M Tris-HCl, pH 9.0 (Neutralizing Buffer) was added to every 1ml of eluate collected to bring back its pH to 7.0. This antibody was then dialysed against 0.09% NaCl for 72 hours at 4°C, freeze-dried and then resuspended in $1/10^{th}$ the original volume with sterile distilled water. This was mixed well and then filter sterilized using 0.2µm filter (Pall Gelman Lab., UK). The concentration of the antibody was calculated by diluting antibody in distilled water, measuring the optical density at 280nm using a spectrophotometer (UV-1201, spectrophotometer, Shimadzu, UK) and then using the following formula:

$$\text{Concentration of sample} = \frac{\text{Optical Density at 280nm}}{\text{Extinction Coefficient at 280nm}} \times 10 \text{ mg/ml} \times \text{dilution factor.}$$

The antibody was kept at -20°C, until required.

Table 2.1: List of the anti-mouse monoclonal antibodies used to stain surface markers on bm-DCs for FACScan analysis.

Monoclonal Antibody	Clone	Company	Dilution	Isotype Control	Clone	Company	Dilution
PE-conjugated hamster anti-mouse CD11c	HL3 (Hamster IgG)	PharMingen, UK	1:100	Hamster IgG-PE	-	Serotec, UK	1:100
FITC-conjugated rat anti-mouse I-A/I-E (MHCII)	2G9 (Rat IgG2a)	PharMingen, UK	1:100	FITC-conjugated Rat IgG2a	R35-95	PharMingen, UK	1:100
Biotin-conjugated rat anti-mouse CD86 (B7-2)	GL1 (Rat IgG2a)	PharMingen, UK	1:200	Biotin-conjugated Rat IgG2a	MAC4	Grown in-house (Hybridoma culture supernatants)	1:200
Biotin-conjugated rat anti-mouse CD40	3/23 (Rat IgG2a)	PharMingen, UK	1:100	Biotin-conjugated Rat IgG2a	MAC 4	Grown in- house (Hybridoma culture supernatants)	1:100
Streptavidin-Quantum Red	-	Sigma, UK	1:100	-	-	-	-

PE = R-Phycoerythrin

FITC= Flourescein isothiocyanate

CHAPTER 3

IN VIVO AND *IN VITRO* STUDIES ON THE MECHANISMS OF IMMUNITY IN DRUG-TERMINATED INFECTIONS.

3.1. Introduction:

High levels of immunity against schistosomes can be induced in experimental animals by exposure to radiation attenuated infections (Taylor, 1994). Although such live vaccines are not feasible for field use, studies of attenuated *Schistosoma mansoni* vaccines have provided some insight into the mechanisms of immunity that are effective against infection. Both antibody (Ford et al, 1984 b; Mangold and Dean, 1986; McLaren and Smithers, 1988; Jankovic et al., 1999) and $CD4^+$ T-cell mediated IFN-γ-dependent effector mechanisms (Jankovic et al, 1999; Kelly and Colley, 1988; Vignali et al., 1989a; Sher et al., 1990; Smythies et al., 1992a; Wynn et al., 1994) have been demonstrated, depending on the host species and the number of vaccinations. However, relatively little is known about how living irradiated or drug-terminated infections are so effective at inducing immunity. The relatively poor results obtained to date with dead parasite antigens or defined candidate vaccine antigens (Dean, 1983; Bergquist and Colley, 1998) demonstrate that a better understanding of the induction of protective immunity by such attenuated vaccines may be needed to allow development of effective defined vaccines.

Studies in mice have shown that the optimum doses of radiation for induction of immunity are of the order of 15-20 krad (Bickle et al., 1979b; Reynolds and Harn, 1992) which produce a slight delay in the skin, but then arrest and death of many of the larvae takes place in the lungs around 21days post infection (Mastin et al., 1983; Mangold and Dean, 1984). Significantly lower levels of immunity are induced when either lower doses of irradiation, that resulted in the larvae reaching the liver and producing sterile stunted worms, or higher doses of irradiation which arrested the larvae in the skin were tested (Minard et al., 1978; Bickle et al., 1979b; Mountford et al., 1992). The predominant death in the lungs of 20krad infections, which is thought to be due to radiation-induced damage to

the neuromuscular coordination of the parasite preventing migration (Harrop and Wilson, 1993), raised the question whether death in the lungs or exposure to lung stage-specific antigens released by death of the lung stage schistosomula were crucial features of inducing protection. So experiments were carried out to drug attenuate the infection in either the skin, lungs or liver to see if the level of immunity also correlated with the site of death. The drug, Ro11-3218, was found to be able to kill all of the larvae when given at the skin, lung and/or liver stages (Bickle and Andrews, 1985). High levels of immunity could be induced when the drug was given at the lung stage and poor levels of immunity occurred when the drug was given at the liver stage. Unexpectedly though, high levels of immunity could also be induced when the drug was given at day +1 or +2 post infection when the larvae were in the skin. Mincing and incubation (Bickle and Andrews, 1985) and quantitative histology (Mastin et al., 1983) showed that following day +1 treatments the larvae were retained in the skin. Similar experiments with other drugs (Oxamniquine - Bickle and Andrews, 1985, or Ro15-5458 - Bickle et al., 1990) failed to induce such high levels of immunity. Ro15-5458, like Ro11-3128 seemed to arrest the larvae in the skin (Bickle et al., 1990) but the quantitative histology indicated that following oxamniquine treatment a significant proportion of parasites reached the lungs. Exposure of cercariae to a high dose of radiation, 80krad, also arrests the larvae in the skin but fails to induce significant protection (Mountford et al., 1992). These studies indicated that arrest of the larvae in the skin could induce levels of immunity comparable to the optimal irradiated infections which reached the lungs, but that there were unexplained differences between the potential of infections terminated with different drugs or with high doses of radiation to induce immunity.

The optimally irradiated (20krad) larvae show a protracted migration in the skin-draining lymph nodes (SLN) compared with a normal infection and release greater amounts of parasite antigen in the nodes (Mountford et al., 1988). This is believed to result in the increased level of priming of responses in the SLN compared with infections with unirradiated cercariae, but in both cases the response which develops following single exposure to irradiated cercariae is a Th1 dominated response with IFN-γ the dominant cytokine (Mountford et al., 1988; Constant et al., 1990; Pemberton et al., 1991).

Surgical removal of the SLN before vaccination showed that priming in the SLN plays a major role in inducing immunity (Mountford and Wilson, 1990). The subsequent migration of the majority of the optimally irradiated larvae to the lungs (Bickle et al, 1979b; Mastin et al., 1983; Mangold and Dean, 1984) induces marked and persistent cellular infiltration and elevated expression of various cytokines notably antigen-specific IFN-γ production (Aitken et al., 1988; Smythies et al., 1992b; Wynn et al., 1995). The larval stimulation of both the skin and the lungs are features considered crucial for the optimal efficacy of the 20krad irradiated vaccine (Coulson, 1997). This conclusion was supported by the more recent demonstration of significantly lower protection in the non-vaccinated partners of vaccinated parabiotic pairs compared with the vaccinated partner (Coulson and Wilson, 1997). The data indicate that the inflammatory responses induced by the death of the irradiated larvae in the lung leads to recruitment of a persistent population of sensitized Th1 cells which can react quickly to larvae from a challenge infection and produced focal inflammatory responses which trap the migrating larvae (Crabtree and Wilson, 1986; Coulson, 1997).

Although the work on the optimally irradiated vaccine has supported the feasibility of developing a schistosome vaccine for humans, this requirement for priming in the skin followed by inflammation in the lungs in order to "arm" the lungs is a complicated model on which to base a defined antigen vaccine. In contrast the Ro11-3128 terminated infections seem to provide a focus of antigen presentation in the skin, i.e., more similar to what a defined antigen vaccine could produce. For this reason it was thought that studies of the mechanisms of immunity in this model might prove valuable. So this Chapter contains *in vitro* and *in vivo* experiments on the type of immune responses stimulated in the skin draining lymph nodes and the spleen and on the mechanisms of immunity in Ro11-328 terminated infections.

Comparisons are made with the 20 krad irradiated vaccine and with 20krad infections which were also drug treated which are interesting because they induce markedly less protection than drug treated unirradiated infections (Bickle and Andrews, 1985; Mountford et al., 1989; Bickle et al., 1990). Such drug-arrested unirradiated and irradiated infections would both be expected to induce marked responses in the skin draining lymph nodes and

so it was hoped that a comparison of the immune responses induced would show some differences which would shed light on the key requirements for immunity.

At the start of this study all that was known about the immunity induced by the drug terminated infections was that depletion of CD4+ve but not CD8+ve cells during the challenge phase of immunized mice led to a marked reduction in protection (Vignali et al, 1989a) suggesting the involvement of CMI effector mechanisms in the immunity.

The following abbreviations have been used for the different vaccination protocols:

- **NI** – (Normal Infection) i.e. percutaneous exposure to 500 unirradiated cercariae.
- **RoNI** – (Ro11-3128 treated Normal Infection) i.e. percutaneous exposure to 500 unirradiated cercariae followed by treatment with 200mg/kg Ro11-3128.
- **GI** – (Gamma irradiated infection) i.e. percutaneous exposure to 500 cercariae irradiated with 20krad.
- **RoGI** – (Ro11-3128 treated Gamma irradiated infection) i.e. percutaneous exposure to 500 cercariae irradiated with 20krad followed by treatment with 200mg/kg Ro11-3128.

The control groups are:

- **RoCC** – (Ro11-3128 treated Challenge Controls) i.e. naïve mice used as controls for mice given drug-terminated infections and so they are treated with 200mg/kg Ro11-3128 at the same time as the immunized groups.
- **CC** – (Challenge Controls). Naïve mice.

3.2 Results

3.2.1 RoNI induces very high levels of protection.

In previous studies using Ro11-3128 terminated infections to induce immunity (Bickle and Andrews, 1985; Mountford et al., 1989; Bickle et al., 1990) 150-175mg/kg of drug were used. Using radio-labelling of the parasites and autoradiography, Mountford et al. (1989) reported that following treatment with 175mg/kg at 48hr a significant minority of the

applied cercariae showed a very delayed migration to the lungs. Because we wanted to focus on the skin responses the dose of RoNI was increased to 200mg/kg in order to maximise the attenuation possible with this drug (200mg/kg is close to a dose which would cause mortality to the mice due to the sedative side effects of this benzodiazepine). Several experiments were carried out to assess the use of the 200mg/kg RoNI protocol and to compare this with the RoGI one (Table 3.1). Three separate experiments showed very high levels of protection. An average of almost 90% worm reductions, were seen with RoNI.

In a direct comparison using 200mg/kg Ro11-3128 (Experiment 3) RoNI induced 84.5% protection compared with only 27.9% with RoGI. This shows that the superiority of RoNI over RoGI referred to above also holds for the use of the higher drug dose. Although not tested in direct comparison the 20krad, GI induced rather lower levels of protection than RoNI (Experiments 4 and 5).

Table 3.1. Levels of resistance in C57BL/6 mice

Experiment No.	Regimen	Worm burden ± SD		Worm reduction (%)	*P*
		Vaccinated	Control		
1	RoNI	8.5 ± 3.9	64.8 ± 15.4	86.9	<0.01
2	RoNI	4.0 ± 1.9	51.0 ± 6.5	92.2	<0.01
3	RoNI	6.5 ± 2.7	42.0 ± 8.7	84.5	<0.01
	RoGI	30.3 ± 7.0	42.0 ± 8.7	27.9*	<0.05
4	GI	29.2 ± 8.7	88.7 ± 11.5	67.1	<0.01
5	GI	30.9 ± 11.7	83.9 ± 10.4	63.2	<0.01

Table 3.1. C57BL/6 mice (n = 4-8) were vaccinated with 500 cercariae as described in Materials and Methods. They were challenged percutaneously with 100 (Experiments 1-3) or 150 (Experiments 4 and 5) normal cercariae on day 35 and perfused on day 56. Significant levels of protection were achieved in vaccinated mice compared with controls. *Significantly lower than in RoNI-vaccinated mice ($P<0.001$).

3.2.2 RoNI: Double treatment with Ro11-3128 (at days +2 and +4) also results in very high levels of protection.

Apart from increasing the dose of drug to 200mg/kg to increase attenuation at the skin stage experiments were also carried out giving either one dose of Ro11-3128 at day +2 or two doses, one at day +2 and the other at day +4. Treatment of mice with Ro11-3128 at each of these times has been shown to kill all larval stages (Bickle & Andrews, 1985) and so it was considered that fewer if any larvae would have migrated beyond the skin in the double treatment regimen. The results are shown in Figure 3.1.

Figure 3.1

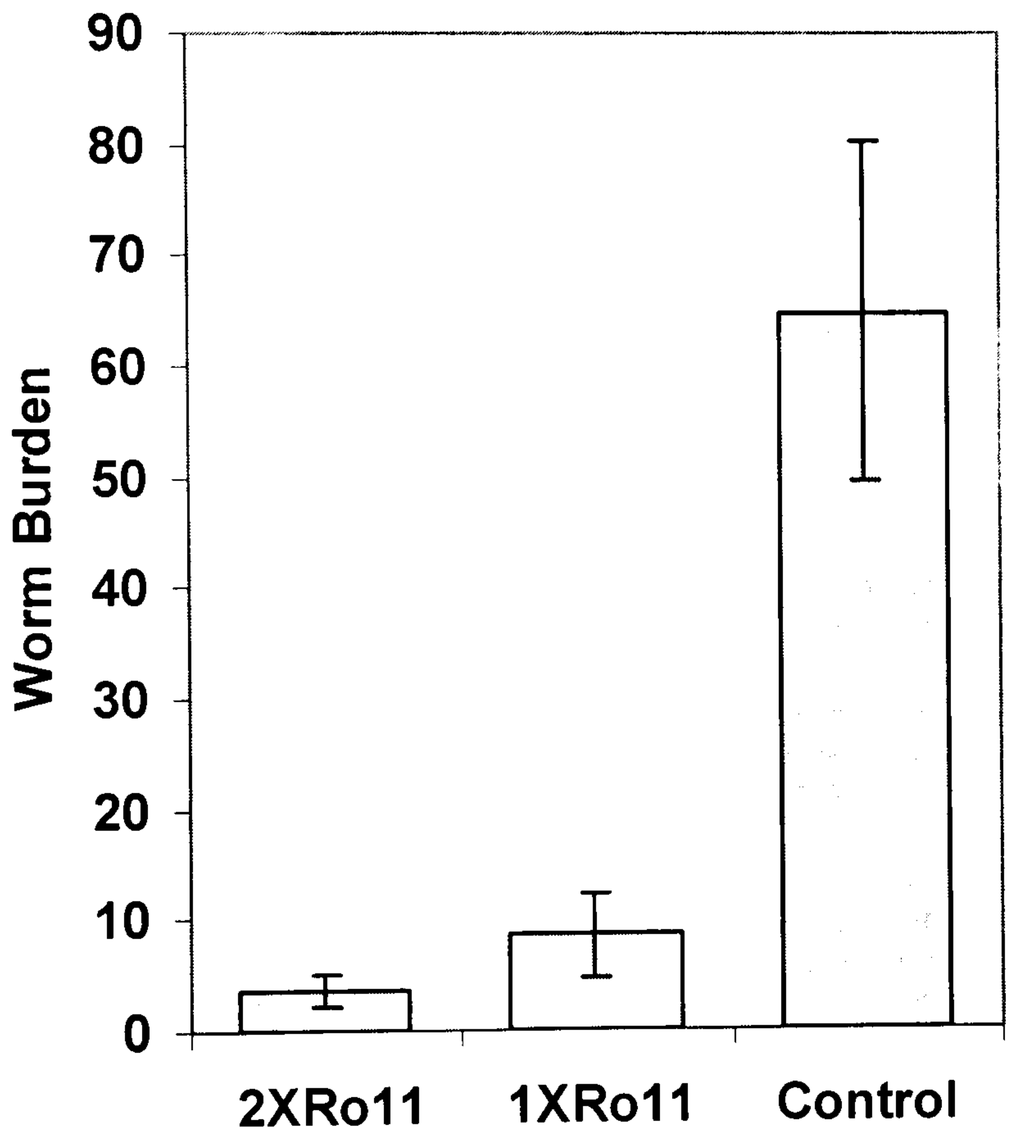


Figure 3.1 C57BL/6 mice (6/group) were infected with 500 normal cercariae and drug treated orally with 200mg/kg Ro11-3128 40 hr later (1x Ro11) or at 40 hr and then again at 96hr (2x Ro11). The mice were challenged with 150 cercariae 5 weeks later and perfused 3 weeks post challenge. The control mice were given the double drug treatment. The bars show mean ± SD.

High levels of protection were seen with both a single and double treatment but the double treatment was even more effective than the single (94.3 compared with 86.9% protection respectively, both $P<0.0001$).

3.2.3. Comparison of cytokine responses in skin draining lymph nodes (SLN) and spleen of mice given normal cercariae or γ-irradiated cercariae with or without Ro11-3128 treatment (NI, RoNI, GI or RoGI).

3.2.3.1 Comparison of the responses in different SLNs following different routes of infection:

An initial experiment was carried out to compare the response and cell yield from either the popliteal or the axillary and inguinal lymph nodes (LN) after RoNI treatment in order to decide on the best source of cells for later experiments. Following percutaneous infection on the mouse foot the popliteal node is the sole draining lymph node, but following infection on the mouse belly the axillary and inguinal nodes on each side of the body respond and so there is potentially a larger cell source, but it was unknown how the levels of cellular stimulation would compare. Two antigen preparations were used, SCAP, (Soluble Cercarial Antigen Preparation – an aqueous extract of cercariae) and DOC-SCAP (a Deoxycholate extract of cercariae made following the SCAP extraction) [see Materials and Methods, section 2.5]. These were used to restimulate the LN cells *in vitro*. IL-4 and IFN-γ were both measured but low levels of IL-4 were produced and only the IFN-γ is shown (Figure 3.2).

Figure 3.2

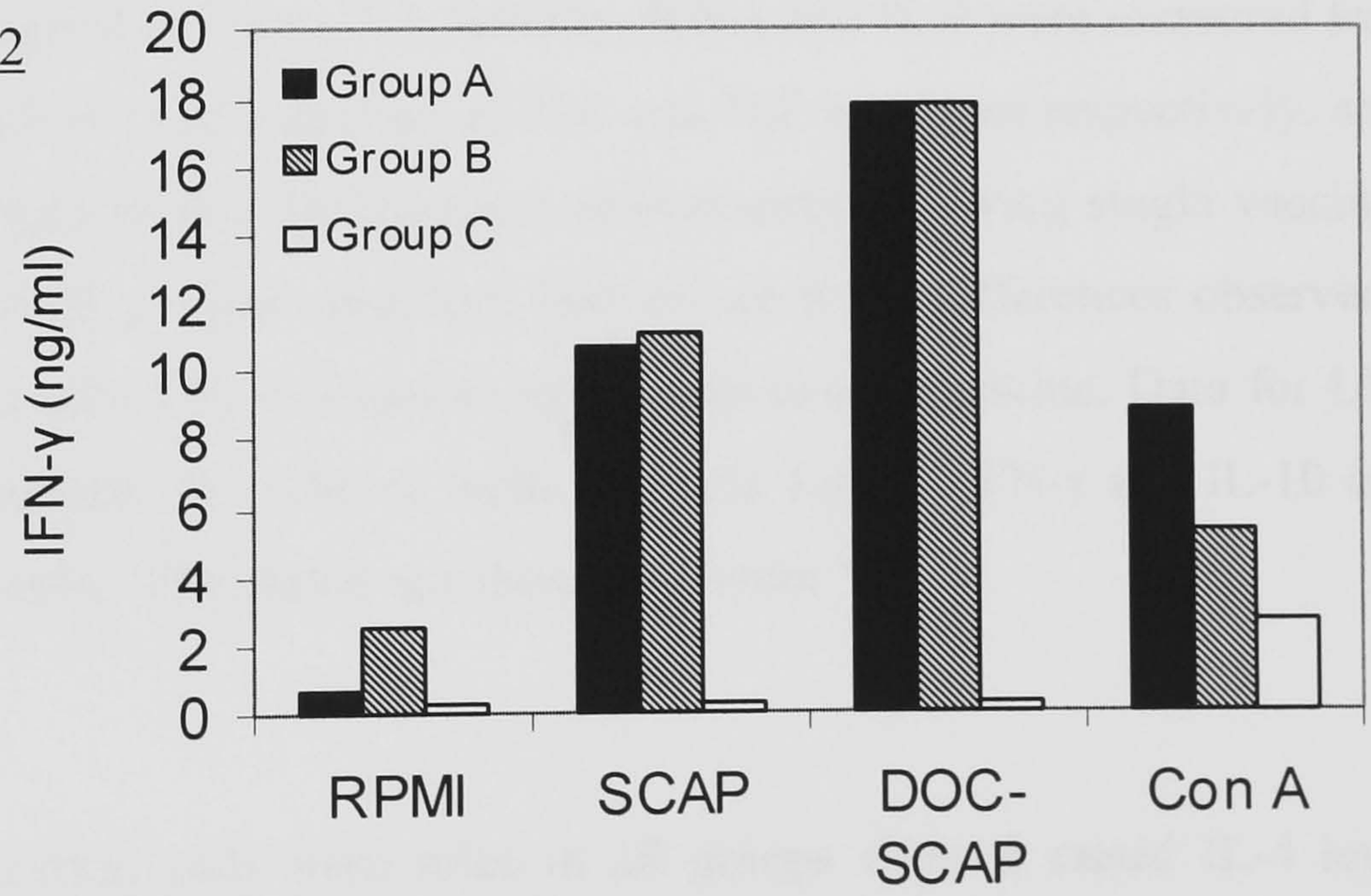


Figure 3.2 Three mice (C57BL/6) were infected on the foot or the belly skin with 500 cercariae and treated with 200mg/kg Ro11-3128 40 hr later. Lymph nodes were removed 7 days post infection. Culture wells contained $5x10^5$ cells/well lymph node cells from different sites (A=Inguinal+Axillary LNs [from RoNI mice], B=Popliteal LN [from RoNI mice], C=Inguinal+Axillary LNs [from Control mice]). Cells were stimulated with Con A (2.5μg/ml), SCAP (10μg/ml) and DOC-SCAP (5μg/ml), respectively.

Comparable and high levels of IFN-γ were produced by the Inguinal+Axillary and popliteal lymph node cells from RoNI mice to both antigen preparations. Control mice made negligible amounts of IFN-γ with antigen. All cell types responded to Con A and this was highest with the Inguinal+Axillary cells. All of the nodes were greatly increased in size following RoNI and given the much higher yield of cells from the inguinal and axillary nodes these were chosen for all later studies.

3.2.3.2. Experiment 1: responses in the Skin draining lymph nodes (SLN):

This first experiment was carried out to compare the cytokine responses in the SLN of mice given different infection/immunisation regimes. The experiment was set up as follows: Group 1, normal cercarial infection (NI); Group 2, normal cercarial infection with Ro11-3128 treatment (RoNI) [induces high levels of protection]; Group 3, gamma-irradiated cercarial infection (GI) [induces high levels of protection]; Group 4, gamma-irradiated cercarial infection with Ro11-3128 treatment (RoGI) [induces poor levels of protection]; Group 5, normal control mice (CC); and Group 6, normal control mice given Ro11-3128 treatment control (RoCC). Lymph nodes were removed at days 7 or 21 and cultured as described in the Legend to Figure 3.3. Initially, IFN-γ and IL-4 were measured from culture supernatants as indicators of induction of Th1 and Th2 responses respectively, and because IFN-γ has been implicated as the mediator of immunity following single vaccination with irradiated cercariae. IL-10 was measured later to see if the differences observed in IFN-γ levels could be explained by differences in this regulatory cytokine. Data for LN IL-4 are from pooled supernatant of triplicate wells and data for LN IFN-γ and IL-10 are mean ± SEM of triplicate wells. The results are shown in Figure 3.3.

IL-4 levels

At day 7 post-infection cells from mice in all groups showed raised IL-4 levels in the presence of Con A and this was highest in the different infected groups compared with controls. A similar difference between infected and control groups was seen at day 21 with Con A, but the levels were very low. When cells were stimulated with specific antigen (SCAP or DOC-SCAP) there was no difference between infected and control groups but

Figure 3.3.

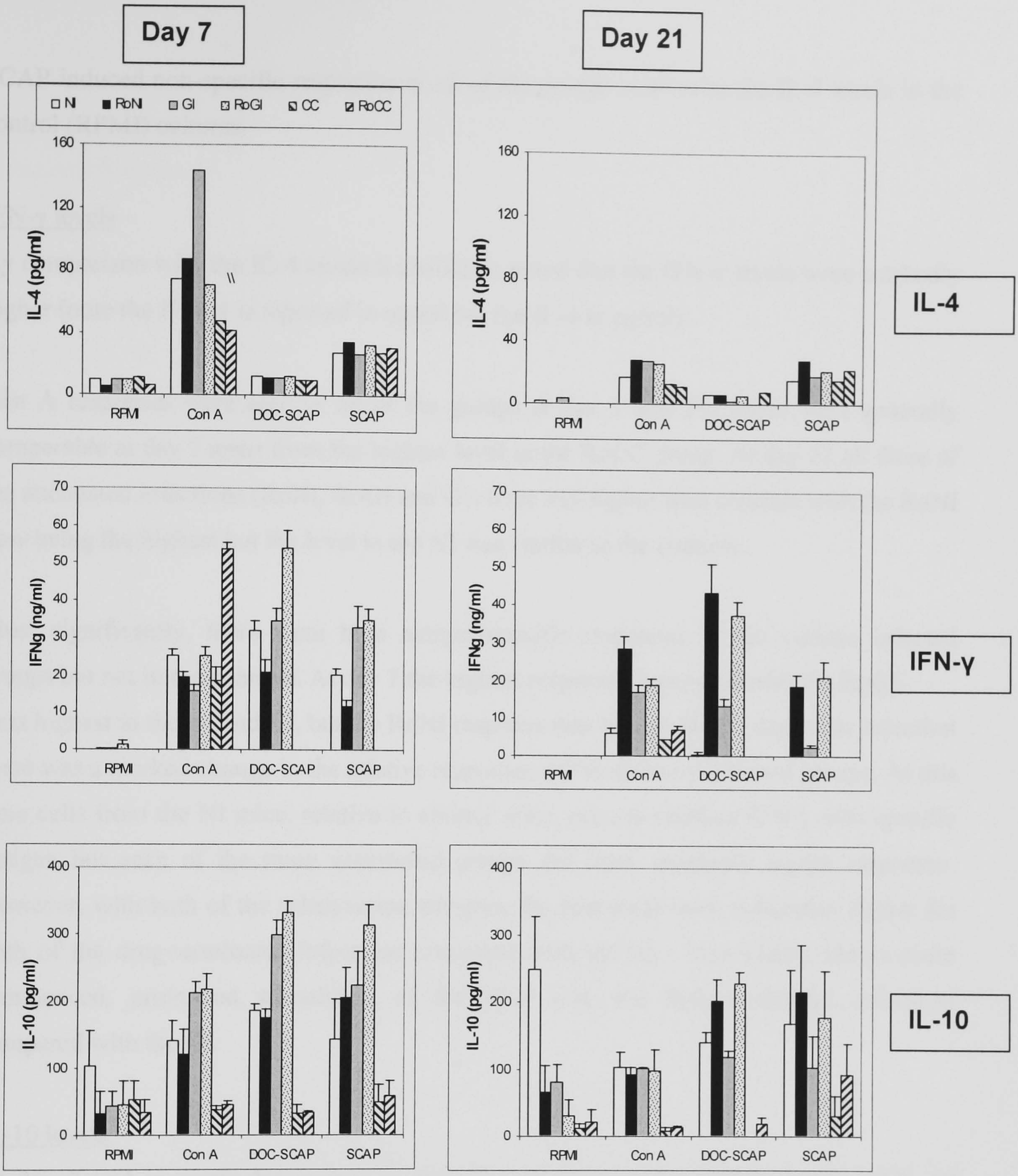


Figure 3.3. C57BL/6 mice (3/group) were infected percutaneously with:- 500 normal cercariae (NI); 500 normal cercariae and drug treated orally with 200mg/kg Ro11-3128 40 hr later (RoNI); 500 gamma irradiated cercariae (GI): 500 gamma irradiated cercariae and drug treated as above (RoGI). CC were normal control mice and RoCC were normal mice treated with Ro11-3128 as for RoNI. Cells prepared from pooled lymph nodes removed at 7 or 21 days post infection were cultured in triplicate at 5×10^5/well in 96-well tissue culture plates, and stimulated with Con A (2.5µg/ml), SCAP (10µg/ml) and DOC-SCAP (5µg/ml), respectively. IL-4 was tested by ELISA using 48h supernatant and IFN-γ and IL-10 using 72h supernatant.

SCAP induced non-specific responses in all of the groups relative to the IL-4 levels in the control (RPMI) cultures.

IFN-γ levels

By comparison with the IL-4 levels it should be noted that the IFN-γ levels were markedly higher (note the IFN- γ is reported in ng/ml but the IL-4 in pg/ml).

Con A responses were seen in all of the groups at day 7 and 21. These were generally comparable at day 7 apart from the highest level in the RoCC group. At day 21 all three of the attenuated infections (RoNI, RoGI and GI) were still higher than controls with the RoNI now being the highest but the level in the NI was similar to the controls.

Most significantly, there were high antigen-specific responses in the various infected groups but not in the controls. At day 7 the highest responses were seen with the RoGI, next highest in the NI and GI, but the RoNI response was lowest. By 21 days after infection there was a marked change in the relative responses of the different infected groups. At this time cells from the NI mice, relative to control mice, did not produce IFN-γ with specific antigen but each of the three vaccinated groups did show markedly higher responses. However, with both of the schistosome antigens, the responses were noticeably higher for both of the drug-terminated infections compared with the GI. This clearly shows more pronounced, protracted stimulation of the SLN with the drug-terminated infections compared with the GI.

IL-10 levels

At day 7 and in particular at day 21 the NI cells showed the highest levels of IL-10 production relative to the other groups in wells without stimulation, i.e., RPMI alone. At day 21 this was higher than following Con A or specific antigens. The reason for this is unknown. This does give some uncertainty about the interpretation of the 21 day results but below the results are basically considered relative to the other groups given the same stimulus, Con A or antigen.

With Con A, IL-10 production was higher and similar in all infected groups compared with control responses at both day 7 and day 21 but responses were somewhat higher at day 7 compared with day 21.

The antigen-specific responses showed a rather similar pattern to the IFN-γ response especially at day 7. As with IFN-γ, the antigen specific responses at day 7 were highest in the RoGI group, a little lower in the GI group and again somewhat lower in the RoNI and NI. The specific antigen IL-10 response was also similar to IFN-γ at day 21 showing the highest response with RoGI and RoNI and lower levels with GI. The NI cells also made highish IL-10 levels at day 21 and this was in contrast to an absence of IFN-γ production by NI (see above).

3.2.3.3. Experiment 2: responses in the Skin draining lymph nodes (SLN) and spleen:

This last experiment was repeated essentially as before but this time extending the observations to the spleen also (see Figures 3.4. and 3.5.).

Lymph nodes

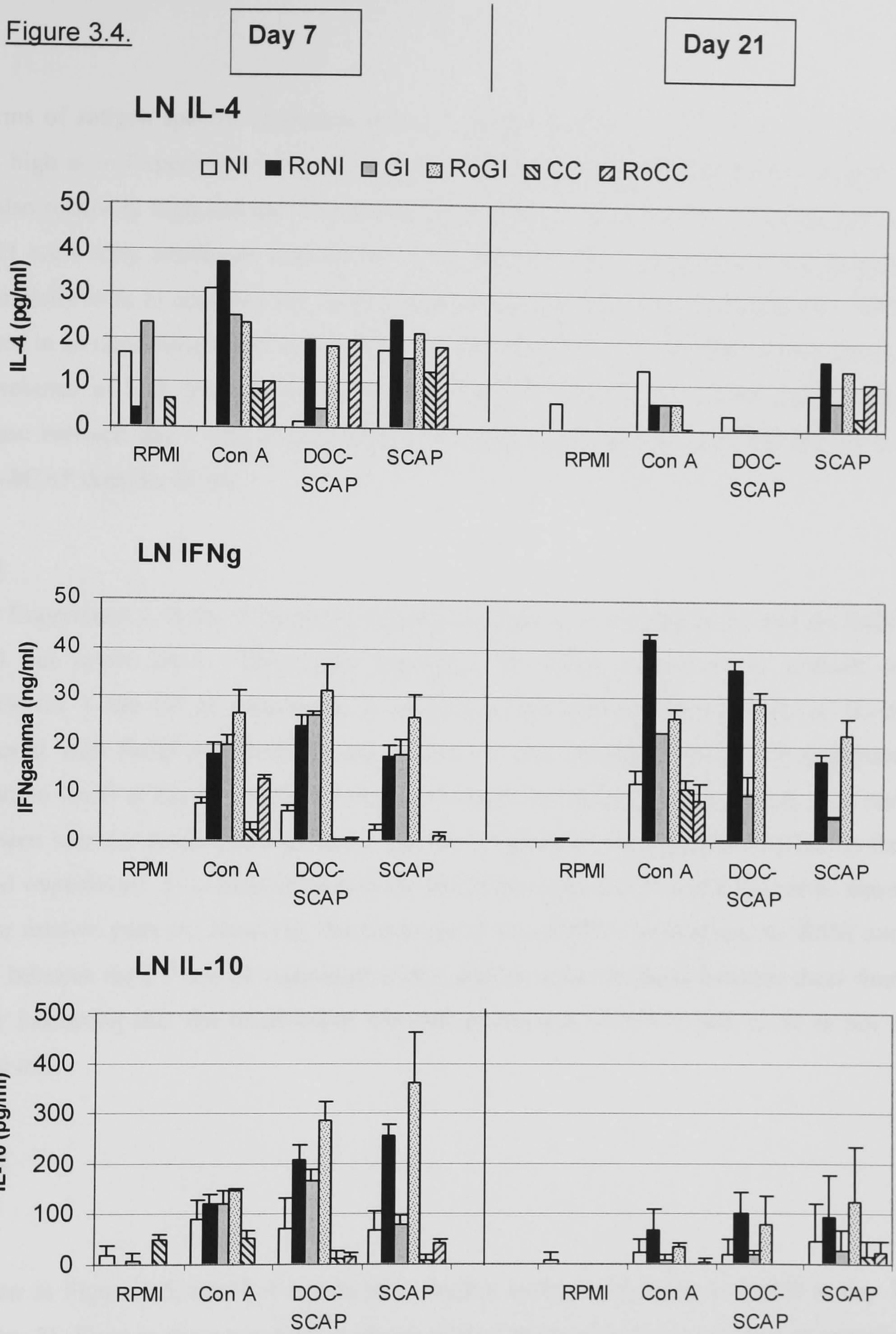
IL-4 levels

As in Experiment 1 absolute levels of IL-4 were low throughout. With medium alone and with Con A there was an indication that exposure to unattenuated or attenuated worms induced increased IL- 4 production and this was greater at day 7 than day 21. But with the schistosome antigens there was no evidence of antigen specific IL-4 production in any of the groups.

IFN-γ levels

With Con A stimulation there was, as in Experiment 1, greater production of IFN-γ in RoCC compared with CC which could indicate an immunomodulatory role for Ro11-3128 (See Chapter 4) at day 7. At day 7 each of the groups exposed to attenuated infections showed markedly higher IFN-γ production compared with the control mice or the NI. By day 21 a pattern very similar to Experiment 1 was clear – RoNI showing the highest response, RoGI and GI lower and similar responses to each other, and NI lower again.

Figure 3.4.



Figures 3.4. C57BL/6 mice (3/group) were treated (NI, RoNI, GI, RoGI, CC and RoCC) as described in legend to Figure 3.3. Cells prepared from pooled lymph nodes removed at 7 or 21 days post infection were cultured in triplicate at 5×10^5/well in 96-well tissue culture plates, and stimulated with Con A (2.5µg/ml), SCAP (10µg/ml) and DOC-SCAP (5µg/ml), respectively. IL-4 was tested by ELISA using 48h supernatant and IFN-γ and IL-10 using 72h supernatant.

In terms of antigen specific responses at day 7, IFN-γ production with RoGI and GI was again high as in Experiment 1 but in contrast to this experiment the RoNI IFN-γ response was also relatively high and the NI response relatively very much lower. The responses at day 21 were very similar to Experiment 1 with the GI IFN-γ response having dropped substantially both in absolute and relative terms compared with day 7, whereas the RoNI resulted in an interesting increase in the absolute and relative levels of IFN-γ production in the presence of both antigens notably to DOC-SCAP. In both experiments this relative increase between day 7 and 21 for IFN-γ production by RoNI was more marked for the DOC-SCAP than the SCAP.

IL-10

As in Experiment 1 at day 7 the RoGI induced the highest IL-10 production and the RoNI raised but lower levels. This again paralleled the IFN-γ responses. In contrast to Experiment 1 the GI in experiment 2 resulted in substantially lower levels of IL-10 compared with RoNI and RoGI at day 7. The NI also induced lower IL-10 responses relative to RoNI at day 7. By day 21 the IL-10 levels had dropped in all groups. This had also been seen for most of the groups in the first experiment but was more marked in the second experiment. It is interesting to note that IFN-γ and IL-10 levels tended to show similar relative patterns. However, the persistent or raised IFN-γ production for RoNI and RoGI between days 7 and 21 contrasted with a decline in IL-10 levels between these time points indicating that the relationship between production of IFN-γ and IL-10 is not a simple one.

Spleen

IL-4

As seen in Figure 3.5. the IL-4 results show higher background levels in RPMI at day 7 than day 21. Even so there was little evidence of IL-4 production either to ConA or specific antigen at either day 7 or day 21.

Figure 3.5.

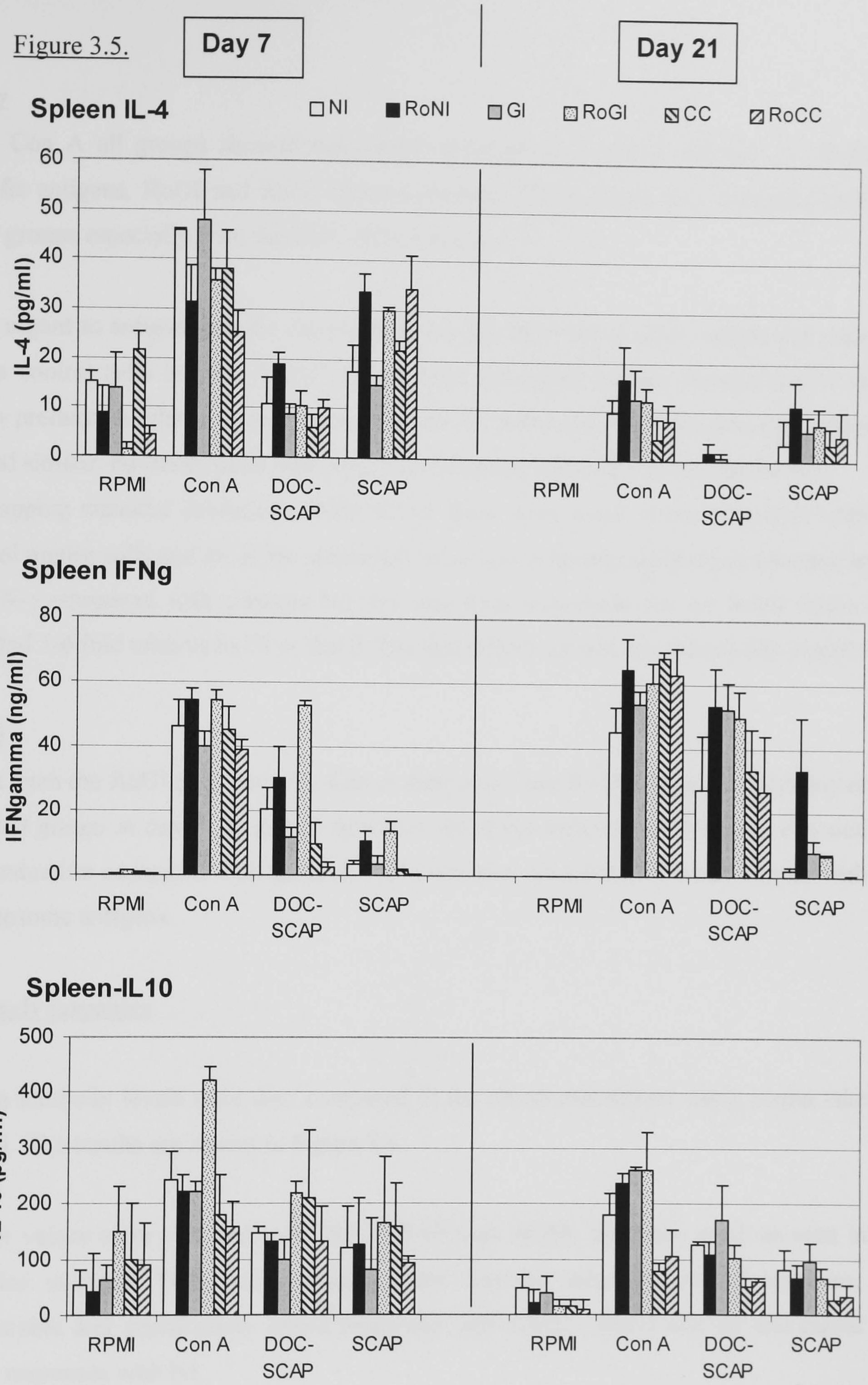


Figure 3.5. See legend to Figure 3.4. Cells prepared from individual spleens removed at 7 or 21 days post infection were cultured in triplicate at 5×10^5/well in 96-well tissue culture plates, and stimulated with Con A (2.5µg/ml), SCAP (10µg/ml) and DOC-SCAP (5µg/ml), respectively. IL-4 was tested by ELISA using 48h supernatant and IFN-γ and IL-10 using 72h supernatant.

IFN-γ

With Con A all groups showed comparable production at day 7 and day 21. With the specific antigens, RoGI and RoNI showed elevated IFN-γ production compared with the other groups especially with the DOC-SCAP at day 7.

With regard to antigen specific responses at day 21, there was a high background response in the control cells in DOC-SCAP but all three attenuated groups showed similar raised IFN-γ production relative to the controls and to NI. Although all three attenuated infections looked similar, however, there was quite high variation within the group results and overlapping standard deviations. With SCAP there were much lower responses with the control mouse cells and all of the attenuated infections induced significantly elevated levels of IFN-γ compared with controls but this was most noticeable for the RoNI which was elevated 5-6 fold relative to GI or RoGI. But this difference was not statistically significant.

IL-10

Apart from the RoGI response with Con A there was little IL-10 production with any of the infected groups at day 7. At day 21 however, all of the infected mice showed elevated IL-10 production compared with controls. This was also seen but to a lesser degree with the schistosome antigens.

Antibody responses

Serum antibody levels were also compared in the above experiment using serum taken at day 21. The results are shown in Figure 3.6.

Higher values were obtained with DOC-SCAP than SCAP, the same result as seen in the cytokine cultures. With both antigens there was the same pattern of response, i.e. comparable and significantly raised responses with RoGI , RoNI and GI and raised but lower responses with NI.

Figure 3.6

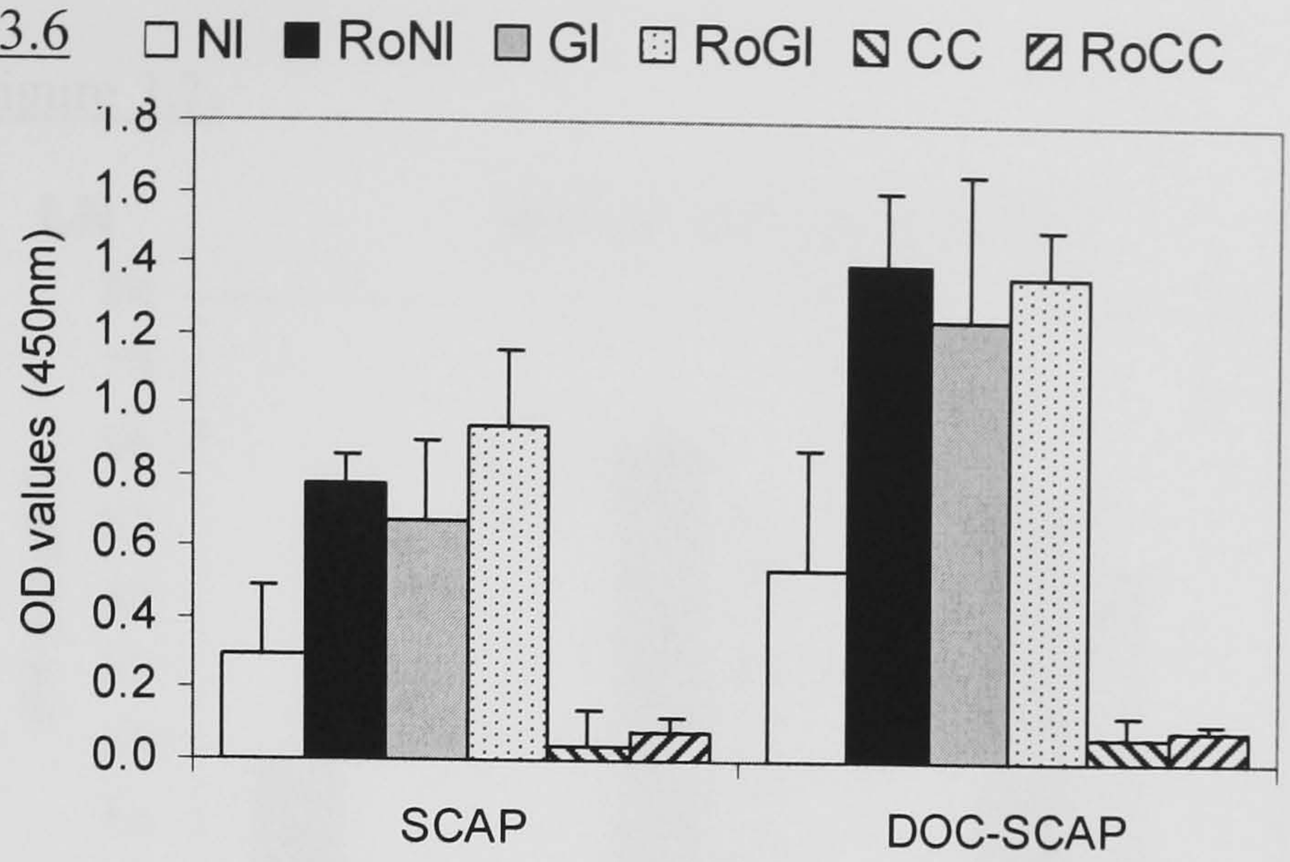


Figure 3.6 Optical density values for IgG measured in serum taken at day 21 after treatment of mice as described in legend to Figure 3.3. (NI, RoNI, GI, RoGI, CC, RoCC). SCAP or DOC-SCAP were coated at 2μg/ml. Sera were tested at 1:100 dilution. Mean plus SD for data from three mice is presented. (RoNI, GI and RoGI vs CC or RoCC, $P<0.01$).

The major distinguishing feature between the highly protective RoNI and the poorly protective RoGI in the above experiment was the difference seen in the spleen IFN-γ response at day 21. It was thought that this might indicate that the RoNI caused a more protracted stimulation of the host leading to more systemic spread of antigen-specific IFN-γ effector/memory Th1 cells. The RoNI splenic IFN-γ response was also higher in RoNI than GI so this was compared again also.

3.2.4. Further comparison of the IFN-γ responses in spleen between RoNI and GI or RoGI.

3.2.4.1 Comparison of the splenic IFN-γ response in RoNI and RoGI.

This was carried out exactly as above and the LN and spleen removed for culture on day 21. The results are shown in Figure 3.7.

Figure 3.7.

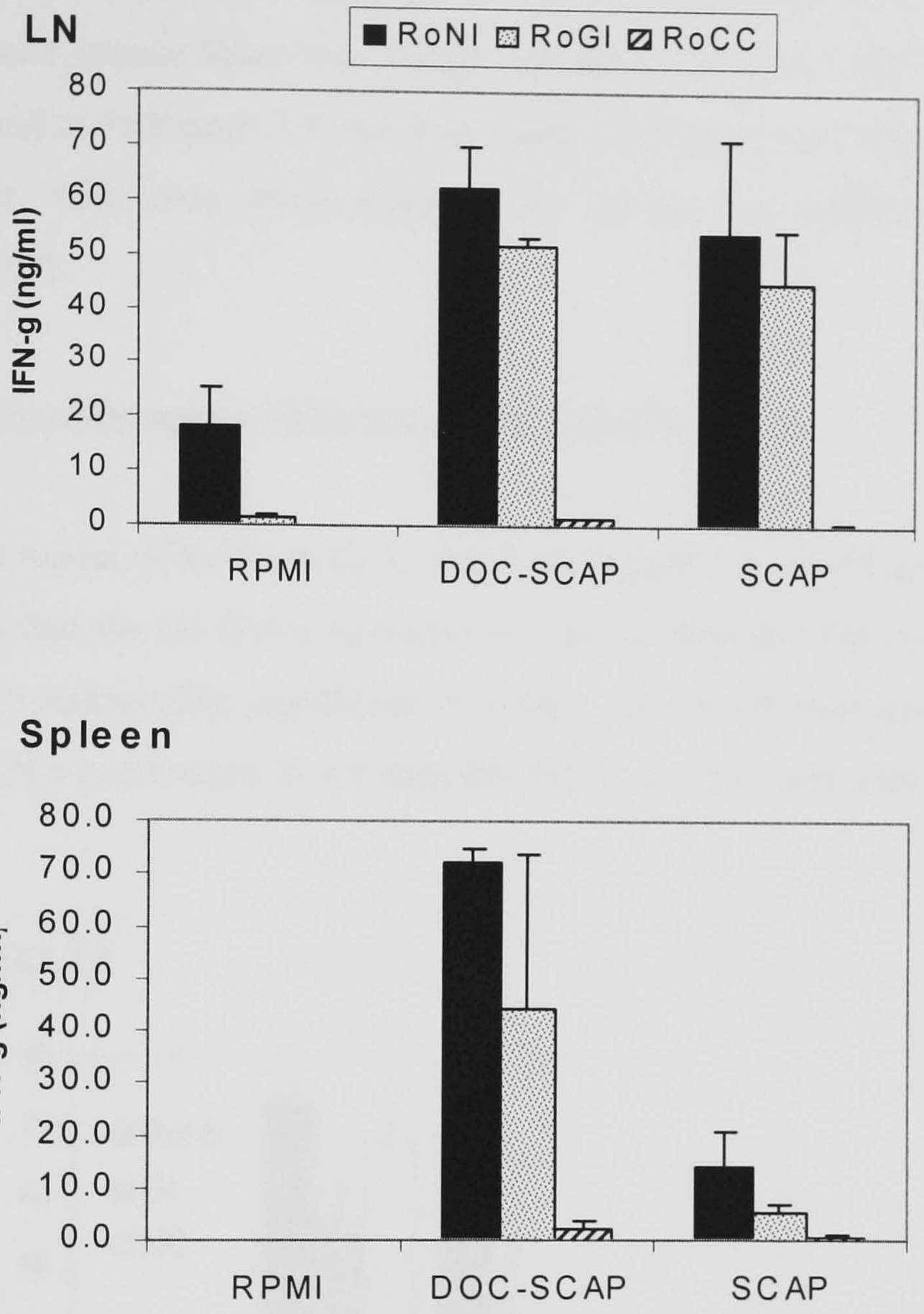


Figure 3.7. C57BL/6 mice (3/group) were treated with RoNI, RoGI or RoCC as described in legend to Figure 3.3. Cells prepared from pooled lymph nodes (top figure) or spleens (bottom figure) removed at 21 days post infection were cultured in triplicate at 5×10^5/well in 96-well tissue culture plates, and stimulated with SCAP (10μg/ml) and DOC-SCAP (5μg/ml). IFN-γ was tested by ELISA using 72h supernatant. Results represent the mean plus SD from the three individual mice.

The lymph node cells from both RoNI and RoGI responded as in Figure 3.4., both showing high and comparable levels of IFN-γ production with both SCAP and DOC-SCAP.

The spleen cells also showed a similar pattern of response to Figure 3.5. With DOC-SCAP there was a generally lower IFN-γ response in RoGI compared with RoNI but owing to a high response in one mouse there was a high standard deviation. The response to SCAP was again lower and as in Figure 3.5. the RoNI response was lower. The variation was less in this group but, with only three mice in the group, the difference did not reach significance (P=0.07).

3.2.4.2 Comparison of the splenic IFN-γ response in RoNI and GI.

The data from this repeat of RoNI vs GI is shown in Figure 3.8., and this is again similar to Figure 3.5. except that the GI IFN-γ response was lower than the RoNI in this experiment and this was nearly statistically significant (P=0.06). The SCAP response was as in Figure 3.5., i.e., the GI IFN-γ production lower than the RoNI, and this was statistically significant (P<0.006).

Figure 3.8

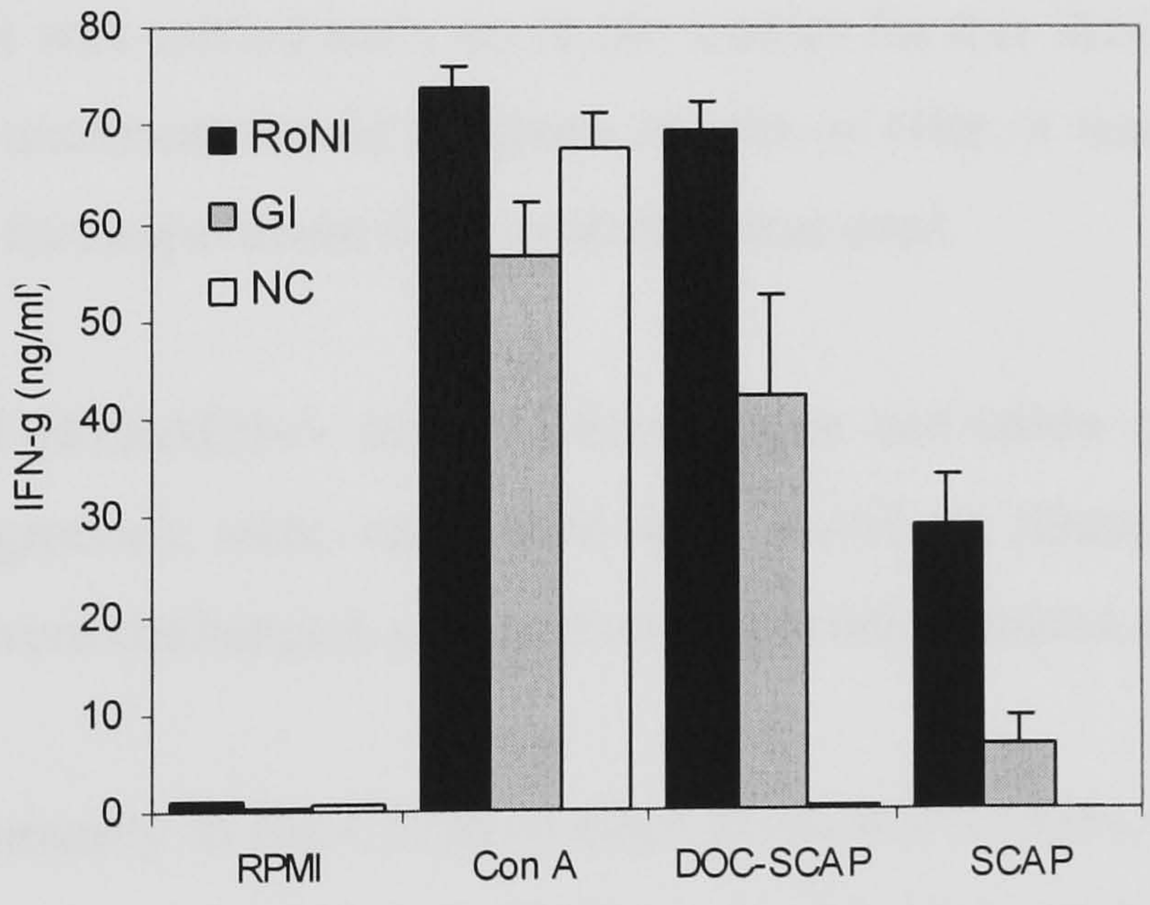


Figure 3.8 C57BL/6 mice (3/group) were treated with RoNI, GI or RoCC as described in legend to Figure 3.3., or NC (Normal Control mice). Cells prepared from individual spleens removed at 21 days post infection were cultured in triplicate at 5×10^5/well in 96-well tissue culture plates, and stimulated with Con A (2.5μg/ml), DOC-SCAP (5μg/ml) or SCAP (10μg/ml). IFN-γ was tested by ELISA using 72h supernatant. Results represent the mean plus SD from the three individual mice.

3.2.5. *In vivo* experiments on the mechanism of RoNI immunity.

The above experiments had shown that all of the attenuated infections are associated with induction of Th1 type responses as seen by high IFN-γ production in the SLN but low IL-4. This remains high in both drug treated infections but declines in the GI which is assumed to be due to the migration away by the GI. The SLN IFN-γ production by highly protective RoNI and poorly protective RoGI could not be distinguished but responses in the spleen were elevated for longer indicating that the RoNI induces a Th1 response that spreads systemically more effectively than in RoGI. Further experiments were aimed at trying to confirm that the IFN-γ responses seen were involved in protection.

3.2.5.1 Vaccination of B6.RAG1-/-mice shows the specific immune requirement for RoNI induced resistance

RAG1-/- mice are deficient in both T and B cell responses (Mombaerts et al., 1992) and so this experiment was carried out to show that the protection was fully dependent on specific immunological responses.

This experiment was carried early on in the studies for this thesis when it was not clear if the Ro11-3128 treatment would be given at 24hr or 40hr. It was eventually decided to go for 40hr but for this experiment 24hr treatment was used.

Two groups of B6.RAG1-/- strain (Recombinase activation gene knockout) (RAG on C57BL/6 background) were vaccinated with RoNI or Sham treated with Ro11-3128 (RoCC). They were challenged, perfused and the worm burdens are shown in Figure 3.9.

The level of immunity in the C57BL/6 mice given RoNI (53%, P =0.001) was lower than when treatment was delayed until 40 hr as in the other protection experiments in this Chapter. This difference between protection induced by Ro11-3128 treatment at either 1 or 2 days post infection is consistent with previous work (Bickle & Andrews, 1985). It is clear that the RoNI vaccinated B6.RAG1-/- mice failed to develop any resistance, and actually having slightly higher worm burdens than controls although not significantly so.

Figure 3.9

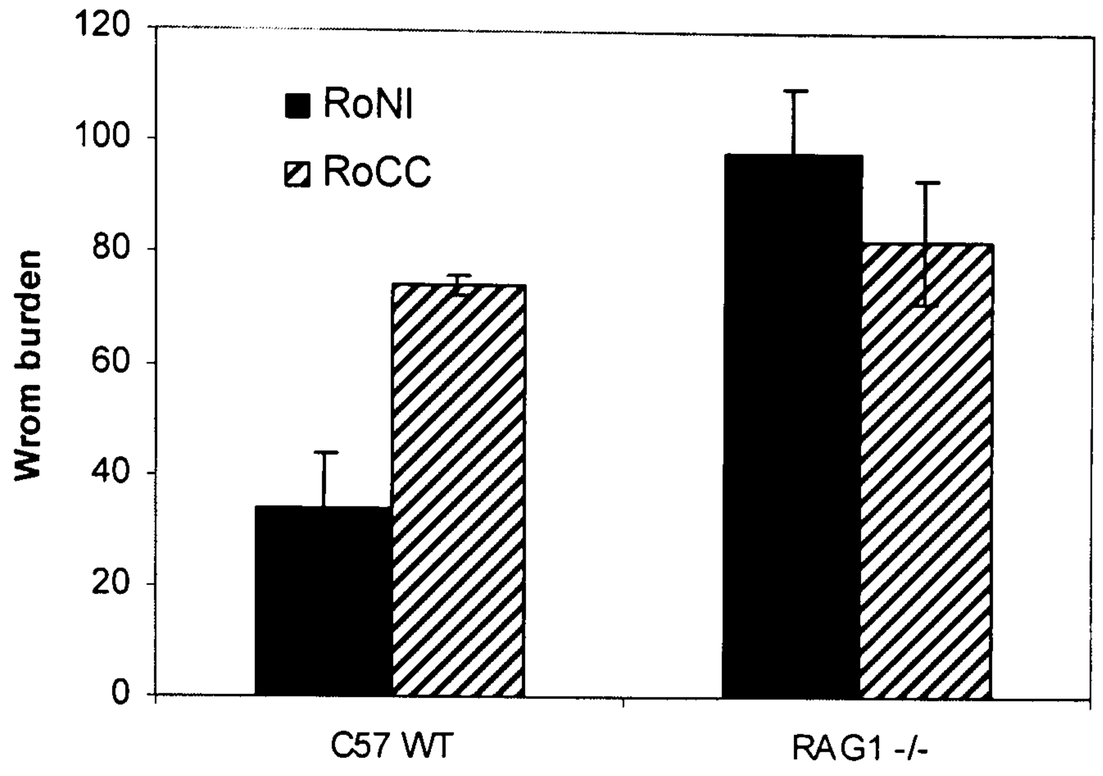


Figure 3.9 C57BL/6 (Wild Type) or B6.RAG1-/- mice (6/group) were treated (RoNI or RoCC) as described in the legend to Figure 3.3. The mice were challenged with 200 cercariae 4 weeks later and perfused 3 weeks post challenge. The bars show mean ± SEM worm burden.

3.2.5.2. RoNI vaccination of IFN-$\gamma^{-/-}$ (IFN-γ KO mice).

The above *in vitro* studies have implicated Th1 responses in the immunity induced by RoNI by showing that there is a prolonged Th1 biased response in the SLN and spleen following exposure. The idea that cell mediated responses are involved in RoNI protection is supported by the earlier demonstration (Vignali et al., 1989a) that depletion of CD4+ve cells at the time of challenge depletes immunity. To directly test if IFN-γ is involved in RoNI protection, experiments were carried out in IFN-γ KO mice based on the C57BL/6 background. In this first experiment groups of 6 male mice were used. The protection data is shown in Figure 3.10.

The mean worm burdens in the control mice were nearly identical (42.0 and 42.5 for the WT and KO respectively) so that statistical comparison between the vaccinated mice was possible. The WT RoNI vaccinated mice showed 85% reduction in worm recoveries compared with RoCC ($P < 0.0001$) and the KO mice 39% ($P < 0.05$). So the KO mice

showed a 54% reduction in protection compared with the WT and the worm recoveries from the vaccinated mice were significantly different ($P<0.01$).

Figure 3.10

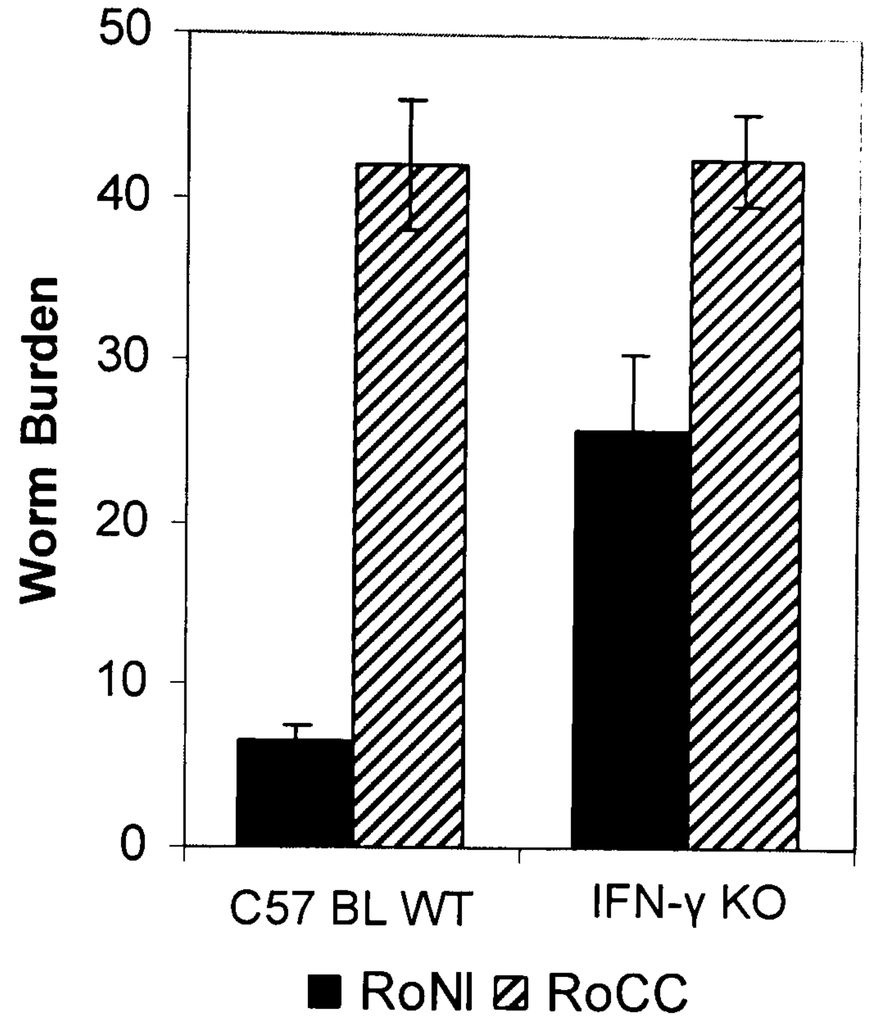


Figure 3.10 Male C57BL/6 (Wild Type) C57BL/6.12957 (IFN-γ KO) mice (6/group) were treated (RoNI or RoCC) as described in the legend to Figure 3.3. The mice were challenged with 200 cercariae 6 weeks later and perfused 3 weeks post challenge. The bars show mean ± SEM worm burden.

3.2.5.3. Repeat of vaccination of IFN-$\gamma^{-/-}$ mice

The above experiment was repeated and because nearly all the other work in this thesis had used females mice these were used in this repeat experiment. The data is shown in Figure 3.11. In this experiment there was a difference between the mean worm burdens from the WT and KO RoCC mice but this was not significant. The percentage protection in the WT RoNI compared with the WT RoCC was 93.7% ($P<0.0001$) and in the KO mice the protection was 43.8%. ($P<0.01$). So, very similar to the previous experiment, the percentage difference between WT and KO was 53%.

Figure 3.11

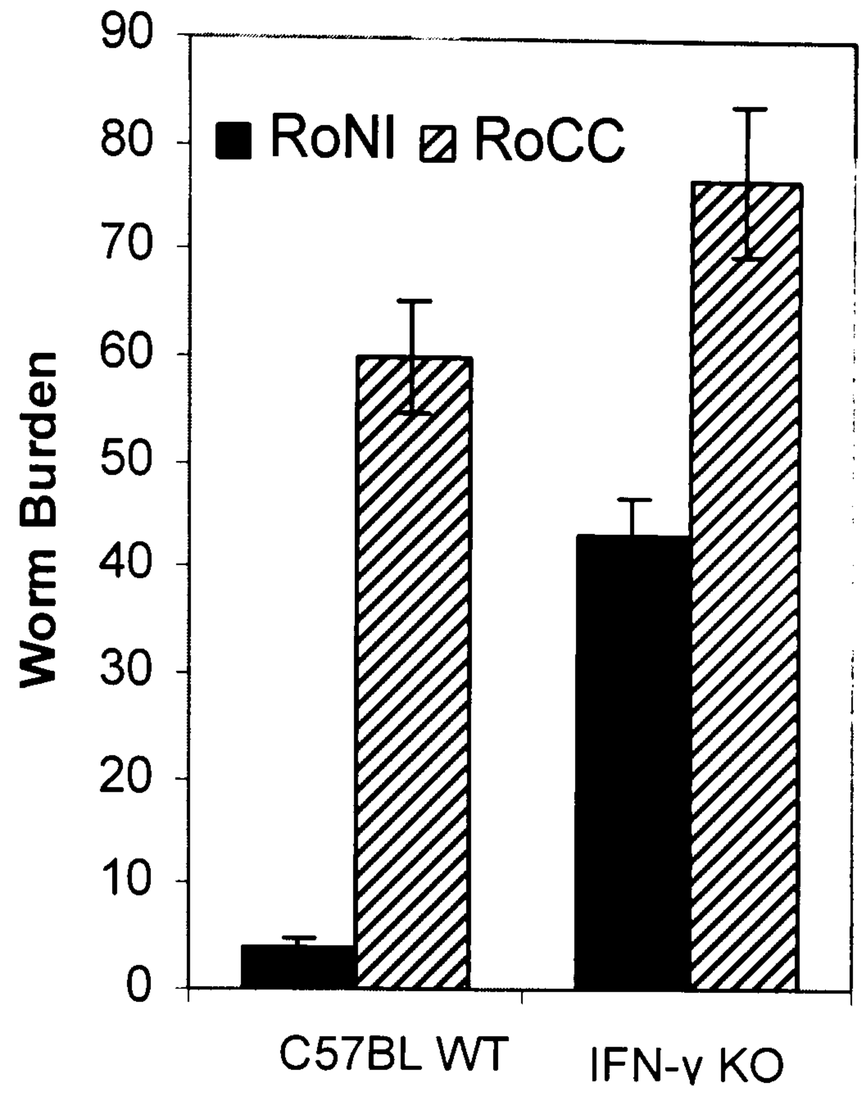


Figure 3.11 Female C57BL/6 (Wild Type) C57BL/6.12957 (IFN-γ KO) mice (6/group) were treated (RoNI or RoCC) as described in the legend to Figure 3.3. The mice were challenged with 200 cercariae 6 weeks later and perfused 3 weeks post challenge. The bars show mean±SEM worm burden.

3.2.5.4. Effect of depletion of IFN-γ at the time of challenge following RoNI.

IFN-γ KO mice lack IFN-γ throughout the generation and effector components of the immune response and so in order to find out if the immune effector mechanisms which actually kill the challenge infection depend on IFN-γ an experiment was carried out in which RoNI vaccinated mice were depleted of IFN-γ by injection of the neutralizing anti-mouse IFN-γ monoclonal antibody (mAb), XMG 1.6, during the challenge period only (day-1 to +15 relative to the challenge infection). The cell line for this antibody was a gift from Prof. Paul Kaye and the antibody was purified from cell culture supernatant as described in section 2.13. To test the *in vivo* effectiveness of the antibody, 1mg was injected i.p. into each of three BALB/c mice one day before injection of virulent *Berkholderia pseudomelliae* which is normally lethal to mice in a few weeks but is rapidly fatal in the absence of IFN-γ. This experiment was carried out in Category III conditions in

collaboration with Dr Debbie Smith. Two of the mice injected with the XMG 1.6 died on day 2 post infection and the third on day 3. Three control mice were still alive 3 weeks later and were then culled. This result demonstrated the potent *in vivo* neutralizing effect of the purified antibody.

The results of the neutralization experiment in RoNI mice is shown in Figure 3.12.

Figure 3.12

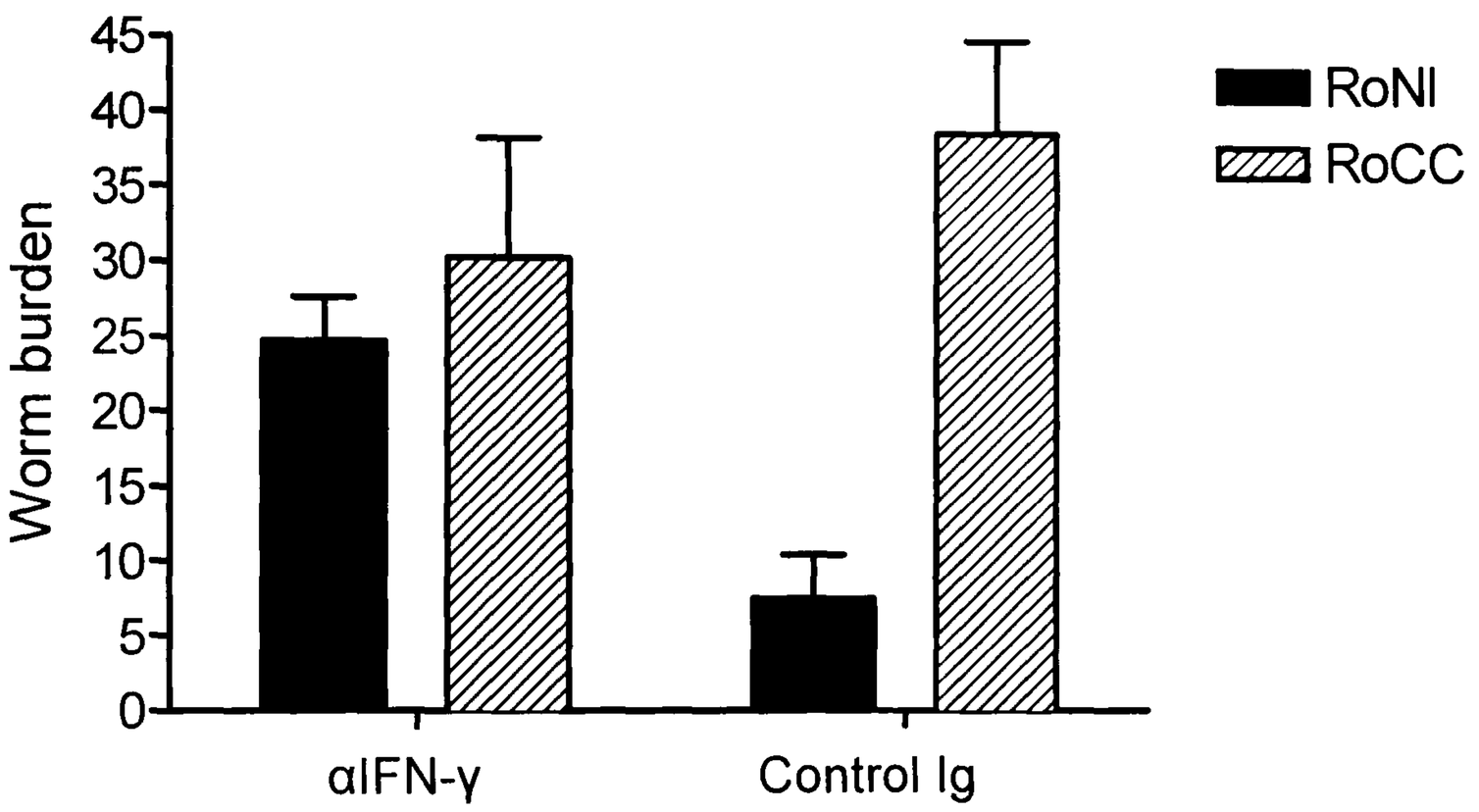


Figure. 3.12 Two groups of C57BL/6 mice (6/group) were treated with RoNI and two others with RoCC, as described in the legend to Figure 3.3. On day 34, 38, 42, 46 and 50 post vaccination one of the RoNI and one of the RoCC groups were injected i.p with 1mg of purified rat anti-mouse IFN-γ mAb (XMG 1.6) (αIFN-γ). The other RoNI and RoCC groups were similarly injected but with 1mg purified rat immunoglobulin (Control Ig). The mice were challenged with 200 cercariae on day 35 post vaccination and perfused 3 weeks post challenge. The bars show mean + SEM worm burden

The worm recoveries were more variable than usual in this experiment particularly in the RoCC treated with rat anti-IFN-γ. Despite this, when compared to the respective RoCC controls, the percentage reduction in worm burden in the mice given the anti-IFN-γ was 18.7% which was not statistically significant and that in the group given control rat Ig the

protection was 80.6% (P<0.001). So it is clear from this that IFN-γ is required for the immune response which eliminates the challenge larvae.

The two experiments in IFN-γ KO mice showed protection reduced by 53 and 54%. The fact that the immunity was not reduced completely could be due to the development of protective Th2 responses to vaccination as a result of the absence of the Th1 promoting IFN-γ during the vaccination as suggested for IFN-γ receptor KO mice vaccinated with RoNI. The low level of protection (18.7%, NS) in the RoNI mice depleted of IFN-γ at the time of challenge suggested that the immunity was almost exclusively dependent on CMI mechanisms of attrition. But in view of the residual immunity in the treated mice and the variability seen in the worm recoveries in this depletion experiment, experiments were carried out to test if antibody might contribute in any way to the protection in RoNI as was shown in some of the studies on single exposure to GI (Jankovic et al., 1999).

3.2.5.5. Vaccination of B cell deficient μMT mice

Wild type C57BL/6 and μMT mice (C57BL/6 background) were exposed to RoNI and challenged along with appropriate controls. The results of two experiments are shown in Figures 3.13 and 3.14. There were no significant differences between the worm recoveries from the μMT and WT RoCC mice. In both experiments high levels of protection were seen in the RoNI WT mice (94.3 and 96.2% in Figures 3.13 and 3.14 respectively). High levels of protection were also seen in the μMT RoNI mice (78.7 and 86.1% respectively). In both experiments this was lower than the WT mice (16.5 and 10.5% respectively in the two experiments) and the worm burden differences in the RoNI groups were significantly different in the first experiment (P=0.036) but not in the second.

Figure 3.13

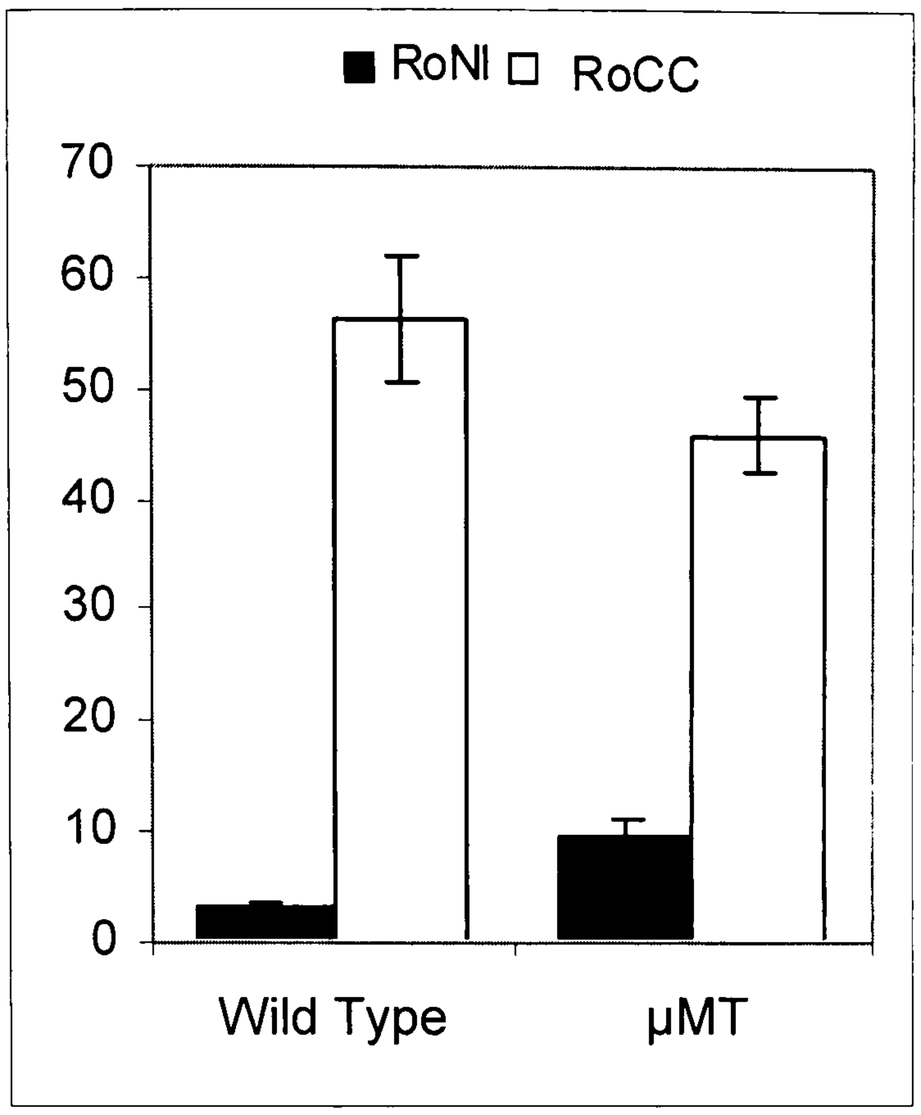


Figure 3.13 C57BL/6 (Wild Type) and µMT (B-cell KO) mice (5/group) were treated (RoNI, black bars or RoCC, open bars) as described in the legend to Figure 3.3. The mice were challenged with 200 cercariae 6 weeks later and perfused 3 weeks post challenge. The bars show mean ± SD worm burden.

Figure 3.14.

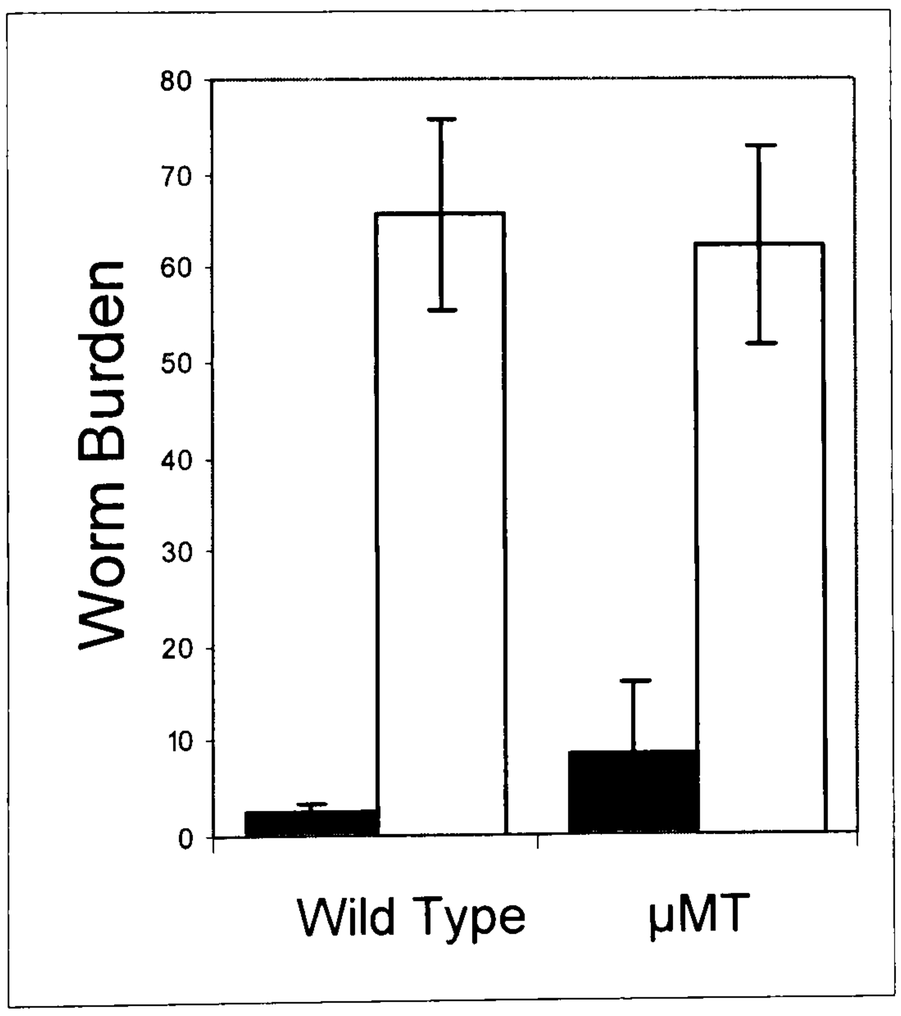


Figure 3.14 C57BL/6 (Wild Type) and µMT (B-cell KO) mice (6/group) were treated (RoNI, black bars or RoCC, open bars) as described in the legend to Figure 3.3. The mice were challenged with 200 cercariae 6 weeks later and perfused 3 weeks post challenge. The bars show mean ± SD worm burden.

3.3. Discussion

RoNI induces very high levels of protection

Very high levels of protection were shown to be produced by RoNI using the dose of 200mg/kg, higher than used in earlier studies (Table 3.1). The mean percentage reduction from 8 experiments in this chapter is 89.9%. This was higher than the 63 and 65% protection seen in two GI experiments (Table 3.1). Similarly Mountford et al. (1992), reported a mean level of protection of 61% in 5 separate experiments using GI in the same C57BL/6 strain of mice. Levels of protection comparable to the RoNI (~90%) can be achieved with GI but only by repeated exposure or simultaneous administration of rIL-12 (Jankovic, et al., 1999; Wynn et al., 1995). So for a single vaccination in mice the RoNI is consistently more effective than any other reported vaccination regimen. Since both the GI and the RoNI induce high levels of protection and the RoGI poor levels of protection (Table 3.1) the focus of interest was to try to understand what the key features of the protective regimens were.

Induction of the T cell response in the skin-draining lymph nodes (SLN):

The importance of the SLN for the induction of protective responses in the GI was shown by the 70% reduction in protection by regional lymphadenectomy before vaccination with GI (Mountford and Wilson, 1990). So in the present studies immune responses in the SLN were compared in RoNI, RoGI, GI and NI. NI is considered not to be able to induce protection. It is not possible to formally demonstrate this as the unattenuated NI would proceed to develop into a huge worm and egg burden, which would kill the mice in 5-6 weeks. However, when equally large infections with 500 unirradiated cercariae were given and treated with Ro11-3128 on day 15 after which they had undergone a normal migration through skin and lungs the immunity seen was poor (Bickle and Andrews, 1985). This strongly suggests that the immune responses demonstrated following the skin and lung migration of normal parasites would not be protective especially compared with the RoNI and GI.

Considering cytokine production in the SLN no schistosome specific IL-4 was produced by any of the infected groups at either 7 and 21 days post vaccination, although there was some evidence of generally raised IL-4 production in all groups of infected mice as seen by the Con A responses in infected compared with control mice especially at day 7. Pemberton et al. (1991) reported some antigen specific IL-4 production with GI at day 5 but this had declined by 18 days.

All of the attenuated infections (RoNI, RoGI and GI) induced highly elevated levels of antigen-specific IFN-γ at day 7, and there was a tendency for the irradiated infections GI and RoGI to induce higher levels than the corresponding unirradiated infections (NI and RoNI). This supports the idea that irradiation can enhance/alter antigen presentation at least by the early schistosomula. Wales et al. (1993), for example, have suggested that irradiation can change the larval surface carbohydrate antigens making them more immunogenic.

It is interesting that of the three attenuated infections, RoNI produced the lowest levels of IFN-γ at day 7 and in fact in one of the two experiments the NI induced comparable IFN-γ levels at this time (Fig 3.3.). By day 21 there was a significant change in IFN-γ levels. Of particular note is the fact that the drug treated infections (RoNI and RoGI) showed markedly higher IFN-γ responses than the GI and NI. The antigen specific NI response had disappeared and there was a substantial drop in the level of IFN-γ produced by GI compared with day 7 which can be explained by the fact that the vast majority of the NI and GI larvae would have left the skin and migrated to the lungs long before day 21 (Mangold and Dean, 1983). IFN-γ production by GI was still substantially higher than by the NI as emphasized by Pemberton et al. (1991), but clearly substantially lower than either RoGI or RoNI. The RoGI response remained at a similar high level as at day 7 but notably the RoNI response was increased substantially at day 21 compared with day 7, so that at day 21 the IFN-γ levels following Con A or DOC-SCAP stimulation were higher than in any group. This pattern of higher IFN-γ production with RoGI than RoNI at day 7 and the reversal at day 21 was consistent in both experiments and must reflect important differences in the survival/antigen presentation of these two types of protocols.

Do attenuated infections inhibit IL-10 in the skin/SLNs and does this result in increased IFN-γ production?

In both of the SLN cytokine experiments, NI induced much lower IFN-γ production both to Con A and antigen compared with attenuated parasites at day 21. The NI responses were also lower than the GI and RoGI at day 7. Ramaswamy et al. (2000) has suggested that normal schistosomula (as in NI)produce prostaglandin E2 which results in IL-10 production (Harizi et al., 2002) which in turn inhibits IL-12 production (van der Pouw Kraan et al, 1995) and so development of Th1 responses and inflammation, which may facilitate parasite survival. Furthermore, they suggest that the reduced capability of gamma-irradiated larvae (as in GI) to induce prostaglandin E2 results in lower IL-10 and so more pro-inflammatory IL-12. This may be true early post vaccination as Hogg et al. (2003b) also showed highly elevated IL-10 at day 4 in the skin of NI mice and lower although still raised levels in GI mice. However, by days 8 and 14, the IL-10 produced by skin was actually higher in the GI than the NI (Hogg et al., 2003b). So it may be true that during the skin migration of normal schistosomula IL-10 stimulation inhibits inflammation which may help parasite survival and that irradiation reduces this immunomodulation resulting in greater inflammation. The present studies show that by the time they reach the SLN this pattern no longer holds as both GI and RoGI induced higher antigen specific IL-10 than the NI in the SLN at day 7. It is interesting that although IL-10 is known to be anti-inflammatory (e.g. De Smedt et al., 1997) there was generally a positive correlation between IL-10 levels and IFN-γ levels at day 7. Perhaps as suggested by others (Wynn et al., 1994) IL-10 rises along with IFN-γ until it is able to terminate the IFN-γ response. For the NI and GI this may be what happens in the SLN between day 7 and day 21 as the antigenic stimulus declines but for the RoNI which induces high levels of IL-10 at day 7, the IFN-γ levels continue to rise between days 7 and 21 showing that some feature of this protocol overcomes any such IL-10 mediated suppression. A possible reason for the more rapid decline in IFN-γ responses between days 7 and 21 in RoGI is that, along with the high IFN-γ levels at day 7 there were also higher levels of IL-10, perhaps again related to higher early antigen release, which could result in more rapid down-modulation of the IFN-γ response.

Normal schistosomula have also been shown to stimulate production of prostaglandin D2 (Angeli et al, 2001) and IL-7 (Wolowczuk et al., 1997) which inhibit immune responses in the skin/SLN and, as suggested prostaglandin E2 above, drug-treated parasites may be less effective at this inhibition and so induce more inflammation and more sensitization.

The potential immunomodulatory role of IL-10 in vaccine efficacy has been demonstrated for the GI. GI vaccinated IL-10-/- mice show a highly polarized Th1 response and high levels of protection and it has been suggested that effective vaccines may need to avoid stimulating regulatory IL-10 (Hoffmann et al, 1999).

<u>What features of the parasite migration/survival may underly the pattern of cytokines?</u>

GI parasites have been reported to undergo slower migration away from the skin infection site than NI such that even at day 14 post infection there were 23% of parasites remaining in the skin of GI compared with 4% in the NI (Mountford et al., 1988). A proportion (10-15 %) of both NI and GI parasites were found in SLN and again GI parasites were present for longer. Material released from radiolabelled parasites was found in the SLN in somewhat greater amounts in the GI cf NI on days 5 and 7. So it was suggested that: "The attenuated parasites deliver themselves to a site where antigen processing and presentation is most intense. Then, by releasing macromolecules over an extended period, they provide a continuing stimulus for lymphocyte proliferation". This idea was supported by the demonstration that very highly irradiated, 80krad, parasites which die before day 14 in the skin and do not reach the lymph nodes (Constant et al., 1990), initially induced comparable levels of IFN-γ to the 20krad GI at day 4 post vaccination but then the levels in the 80krad dropped such that at day 11 the levels were ten times lower than in the 20 krad GI. Also there were much lower increases in cell content in the SLN (Mountford et al., 1988). So it is concluded that the schistosomula antigens released into the lymphatics or processed in the skin by DCs is less effective in sustaining the response than the protracted survival living parasites in the SLN.

As discussed above both of the drug attenuation regimens, RoGI and RoNI, induce markedly higher IFN-γ responses in the SLN than both the NI and the GI and so what is

known about where they reach and survive? Mountford et al (1989) carried out tracking studies on the RoNI infection. The larvae were clearly retained in the skin with nearly 40% still present at day 7 (when the NI was around 5%) and 25% still present at day 10. This was very similar to the numbers of GI parasites reported to be in the skin in an earlier study (Mountford, et al., 1988). However, there seemed to be a delay in the arrival of the RoNI parasites in the SLN because at day 5 post infection the GI parasites peaked at around 11% of the applied larvae but few RoNI larvae were found in the SLN. By day 7 however, GI numbers were similar and RoNI numbers had increased to around 8%. Interestingly, in the RoNI this number was still maintained at day 14 but had dropped to around 2% in the GI. Although this is comparing single experiments carried out at different times it does suggest that the increase in IFN-γ production by SLN cells in RoNI between day 7 and day 21 to a level notably higher than the GI is due to persistent residence in and stimulation of the SLNs.

The pattern of higher IFN-γ production with RoGI than RoNI at day 7 and the reversal at day 21 is consistent with the larvae in RoGI dying more rapidly after infection than in RoNI leading to higher initial antigen production and cytokine responses but less persistent responses. In the above tracking studies RoGI involving treatment at 48hr [RoGI(48)] was not studied but a similar regimen in which treatment was given at 24hr [RoGI(24)] was studied. It was concluded that with the RoGI(24) the majority of parasites were dead or dying in the skin infection site rather than the SLN, and Mountford et al., (1988) also demonstrated that large quantities of antigenic material are released from dying parasites in the skin, bloodstream and liver of RoGI(24) mice, but negligible levels in the draining lymph nodes. So a similar pattern may be involved in the RoGI(48) as studied here, i.e., more death of the RoGI compared with the RoNI in the skin and less protracted persistence in the SLNs. However, this does not explain the fact that the RoGI induced the higher levels of IFN-γ compared with NI, GI or RoNI at day 7 post vaccination and substantially higher levels than NI and GI at day 21. But these drug terminated infections involve more than just the SLN. Studies on cytokine expression in the skin following RoNI (Dr Yaobi Zhang – personal communication) show marked expression of pro-inflammatory IL-1β (elevated 25 fold and 10 fold higher than GI or NI), IL-6 (elevated 5 fold compared with NI

or GI) and IFN-γ (elevated 10 fold compared with NI or GI). Additional comparative studies on tracking of these larvae in the SLN and histology of the skin and SLN may give some explanations for these observations.

The SLN cytokine data on RoGI is important because it shows that high and persistent levels of antigen specific IFN-γ production by sensitised SLN cells (higher than GI) is not sufficient to induce high levels of protection, i.e., antigen specific SLN IFN-γ production does not predict immunity. So what other components are involved?

The importance of the lung in the GI model

Following the delay in migration through the skin and SLNs a large proportion of the 20krad schistosomula, as in GI, reach the lungs (57% of the applied larvae on day 7) and persist there until at least day 21 post infection (Mastin et al., 1983). Unlike the unirradiated infection none reach the liver (Mountford et al., 1988). Arrival of the parasites in the lungs induces inflammation with a 4-5 fold increase in lymphocytes, macrophages and eosinophils present in broncho-alveolar lavage (BAL). T lymphocyte numbers in the airways peak on day 21 and remain elevated for up to 10 weeks (Aitken et al., 1988). As judged by oxidation index (10 times normal levels) activated alveolar macrophages also peak at day 21 but remain in an activated state for weeks (Menson and Wilson, 1989). In addition to the influx of lymphocytes into the lung there was a marked increase in production of IL-3 and IFN-γ by BAL cells T at day 21.

That this lung migration was important in recruiting cells sensitized in the skin to the lungs was suggested by the fact that the 80krad irradiated infections which did not stimulate protection did not reach the lungs (Mountford et al., 1992) but if mice were given an 80krad infection and then also injected intravenously with irradiated lung stage parasites (which on their own do not stimulate immunity) the mice were immune (Mountford et al, 1992). So Coulson (1997) concludes: "It would appear that irradiated larvae must persist in both the (skin-draining) lymphatic tissue and lungs in order to generate an optimum immune response. The lung phase was considered to be important either (i) to present antigens in the

pulmonary tissues and/or (ii) for the inflammatory response induced by the larvae to simply act as a stimulus for the recruitment of reactive cells from the circulation."

Although the majority of the GI parasites reach and die in the lungs, Mountford et al., (1989) dismiss the possibility that the lungs are an important site for the induction of immunity based on the observations that (i) they did not find parasites or released material in the lung draining LNs, (ii) a significant proportion of normal (unirradiated) schistosomula die in the lungs but as discussed above the NI is assumed not to stimulate high protective responses. Also it has been shown that irradiated LS (Lung Stage) parasites injected into the lungs fail to stimulate significant immunity (Coulson and Mountford, 1989). Interestingly, however, in GI the IFN-γ levels in the SLN follow a decline between days 4 and 11 post vaccination but then there was 2.5 fold increase in IFN-γ production by day 15 which was retained at day 20. This did not happen with the 80krad infection and it seems possible that this increase is associated with boosting of the immune response by death of the 20krad larvae in the lungs which coincides with this increase in the SLN response. Nevertheless, the favoured idea is that the irradiated larvae embolising in the lung act as a stimulus for recruiting sensitized cells.

The crucial importance of the death of the irradiated larvae in the lungs was shown by experiments in which a mouse vaccinated with GI was joined in parabiotic union two days post vaccination to a naïve mouse (Coulson and Wilson, 1997). After 28 days the mice were separated and then challenged. In this way the systemic response generated by the response in the skin and SLN would be shared by the mice but that only the vaccinated partner would have the irradiated larvae reaching and dying in the lungs and so recruiting cells to the airways. In four such experiments the mean level of protection in the vaccinated partner was 61% and in the non-vaccinated partner only 41%. It was assumed that the protection in the non-vaccinated partner arose from the stimulation of secondary responses prior to arrival of the challenge larvae in the lungs and when this was avoided by intravenous injection of challenge larvae directly into the lungs the vaccinated partners showed 47% immunity and the non-vaccinated partners no protection. These elegant experiments clearly show that in the GI the systemic response stimulated is only able to

confer around 40% protection and the physical presence of the irradiated larvae in the lungs is necessary to stimulate recruitment of sensitized cells to the lungs so arming them. Only then can high levels of protection be stimulated.

In the vast majority of studies on this mouse model the vaccinated mice are challenged at 4-5 weeks post vaccination i.e., at a time when significant numbers of sensitized T cells and activated alveolar macrophages are persisting in the lung airways after the inflammation induced by the death of the irradiated larvae. The challenge larvae are eliminated predominantly in the lungs due to focal inflammatory reactions (Crabtree and Wilson 1986) which as discussed later depend on IFN-γ. It is believed that the recruited schistosome-specific lymphocytes "pre-arms" the lung so that there is a rapid recall response (Wilson and Coulson, 1989). Following challenge, one study (Aitken et al, 1987) reported a significant rise in total leucocyte and T lymphocyte recoveries from the lungs at day 14 post challenge, but another study showed no evidence of further cellular recruitment above that persisting post vaccination. However, there was an increase in the oxidation index of the alveolar macrophages (increased nearly three fold in vaccinated mice compared with control challenged mice) (Menson and Wilson, 1989). So this protection is rather different from recall of a resting memory cell response from secondary lymphoid organs (spleen and lymph nodes).

Is the lung involved in the RoNI?

In contrast to the GI there is strong evidence that the RoNI does not rely on this arming of the lung by death of attenuated larvae. Early studies using mincing and incubation techniques indicated that the effect of the drug on early infections was rapid. Following treatment of a 24-h-old infections no lung stage parasites were recovered by the lung-chop method 5 days post-infection and following 72 hr treatment only 8% of the number reaching the lungs of control animals was found (Bickle et al, 1985). Autoradiographic-tracking studies by Mastin et al., (1985) confirmed that 24 hr treatment prevented parasites reaching the lungs. However, by autoradiographic tracking Mountford et al (1989) reported that, in RoNI treated at 48 hr as in our studies, a significant proportion of the drug treated larvae also undergo a very delayed migration to the lungs following treatment with

175mg/kg Ro11-3128 (peaking at 20% of the infective larvae on day 14). This compares with 30-35% reported to reach the lungs in the GI (Mountford et al., 1988). However, we do not believe that, in our current RoNI regimen using the increased dose of 200mg/kg Ro11-3128, there is significant lung involvement for the following reasons. Firstly, extensive histology of the lung has failed to find either parasites or reactions at day 14 or 21 after RoNI in contrast to a parallel study using GI (Quentin Bickle- personal communication). Secondly, there was no evidence of inflammatory responses in the lungs at any time following RoNI, as judged by extensive studies of cytokine and chemokine mRNA production or presence of antigen specific or non–specific IFN-γ producing cells in the lung tissue or in broncho-alveolar lavage during the time the schistosomula might be expected to reach and die in the lungs, i.e., up to day 21 (Dr Yaobi Zang, personal communication). This was in contrast to highly elevated responses in control animals given GI as reported earlier (Smythies et al, 1992a; Smythies et al, 1993).

This data strongly suggests that the RoNI does not involve the migration and death of vaccinating larvae in the lungs and the inflammation this induces. But the presence of antigen specific Th1 cells has been demonstrated in the lungs/ lung lymph nodes at day 35 (Zhang – personal communication) which are believed to represent systemic spread of the responses stimulated in the skin as was also demonstrated in the spleen. That recruitment of sensitised cells to the lungs does not depend on antigen presentation there as has been shown for other systems e.g. following i.p. administraion of allo-antigens (Liu et al, 1982).

Further evidence that the lung is not involved in the RoNI protection came from the demonstration of the double Ro11-3128 treatment (on days +2 and +4). This was considered likely to cause even more restriction on the survival of the RoNI, and again histology failed to demonstrate any parasites or parasite reactions in the lungs at day 14 post infection, in contrast to the GI (Quentin Bickle- personal communication). But this regimen induced even higher levels of protection than the single treatment RoNI. As this regimen is thought likely to truncate the larval survival even more than the single treatment this raises some doubts about the explanation for the lack of resistance in the RoGI being due to shorter persistence compared to RoNI. Further work on the survival/migration and

SLN immune response following the double treatment protocol would be of particular interest.

Why does the RoGI fail to stimulate protection?

It is clear from the present studies that low stimulation of the SLN does not on its own explain the poor protection because RoGI induced stronger and more persistent Th1 responses in the SLN compared with the protective GI. This also shows that stimulation of high Th1 cell responses in the SLN is not sufficient to lead to immunity because the RoGI induces only poor levels of protection.

The major distinguishing features between the highly protective RoNI and the poorly protective RoGI in the above experiment were: (i) the fact that the IFN-γ levels were highest with the RoGI at day 7 and tended to decline by day 21 whereas in the RoNI the levels at day 7 were lower but increased significantly to day 21 (ii) the higher IFN-γ response in the spleen at day 21. It may be that the combination of irradiation and drug results in earlier death and so higher early but less persistent stimulation. The fact that the double treatment of RoNI at day +2 and +4 led to even higher protection than the single treatment raises some doubts about whether persistence itself is the key factor. It would be valuable to compare the persistence in the skin/SLN of the RoNI single and double treatment and the RoGI and also the SLN and spleen IFN-γ responses.

However, it may be too simple to just look at levels of antigen specific IFN-γ production as an indicator of protection. In studies on the P strain of mouse which develops poor levels of immunity following irradiated cercarial vaccination it was shown using spleen cells taken 4 weeks after vaccination and stimulated with F/T schistosomula, ConA or soluble SWAP that P strain mice did not make less macrophage activating IFN-γ than the highly protective C57BL/6. But interestingly they did make nearly 3x more IL-10 and >2x IL-4 than C57BL/6. Depletion studies showed that both the Th1 (IFN-γ and IL-2) and Th2-related (IL-4 and IL-10) cytokines were totally dependent on CD4+ve T cells (Oswald et al., 1998). It was concluded that the skewed balance of counter-regulatory cytokine

production in P strain mice happens early post vaccination with raised IL-10 inhibiting IL-12 synthesis (D'Andrea et al., 1993) and thus promoting the raised IL-4 production. So the determining immunological abnormality in P strain mice is the production of high IL-10 early on in infection. It is interesting to note that this does not result in lower IFN-γ and the deficiency in immunity is put down to the inhibitory effects of the raised IL-10 and IL-4 on IFN-γ mediated macrophage activation.

In the present studies, antigen specific IL-10 production by SLN cells from RoGI was higher than in the RoNI at day7 post infection in both the experiments in Figure 3.3. and 3.4. especially in Figure 3.3. But by day 21 post vaccination when the levels of IL-10 had dropped there was no significant difference between RoNI and RoGI IL-10 levels. As described for the P strain mouse above, this higher IL-10 at day 7 did not cause lower IFN-γ responses but rather correlated with higher levels. Whether the presence of the higher IL-10 responses only during the early part of the RoGI results in more rapid attenuation of the IFN-γ response or could inhibit later macrophage mediated inflammatory responses to challenge larvae is not known.

Mechanisms of RoNI.

Before these studies little was know about the mechanisms of immunity in the RoNI model. It had been shown that immuity in both the GI and the RoNI models could be ablated/reduced by treatment during the challenge phase with anti-CD4 antibody but not with anti-CD8 antibody (Vignali et al., 1989a). Any antibody responses would not have been affected by this treatment and so this strongly suggested that the effector mechanism of elimination of the challenge parasites was due to CD4 cytokine mediated CMI responses.

The demonstration that IFN-γ KO mice show a 53-54% reduction in immunity clearly shows that IFN-γ plays a major role in RoNI immunity but this did not prove that IFN-γ was involved in the protective mechanism. This was demonstrated by the failure of mice treated with neutralising mAb to IFN-γ to develop specific immunity. The fact that immunity was not completely ablated in IFN-γ KO mice could be due to a switch towards

Th2 protective mechanisms as has been suggested to occur with the GI in such deficient mice (Wynn et al., 1994; Wilson et al., 1996; Hoffmann et al, 1999). The fact that the antibody deficient μMT mice (Kitamura et al, 1991) showed no defect in immunity, shows that antibody is not involved in this model and so the data is consistent with the high level of immunity in RoNI (~90%) being entirely IFN-γ mediated.

The single GI vaccine induces 60-70% immunity and a key role for IFN-γ in immunity has also been demonstrated (Coulson, 1997; Jankovic et al., 1999) but even in the same C57BL/6 strain of mice two different studies differ about the relative importance of IFN-γ and antibody. One concludes that only IFN-γ is involved because neutralisation of IFN-γ ablates immunity (Smythies et al, 1992a) but another study shows that the immunity drops to 40% in B cell deficient mice and that this deficiency can be replaced by serum transfer from WT GI mice so they conclude that both IFN-γ and antibody plays a role after single vaccination (Jankovic et al., 1999). The reasons for the difference in these different studies is not certain, although different doses of radiation were used (20krad in Smythies et al., 1992a and 50krad in Jankovic et al., 1999). Following repeated GI vaccination the levels of immunity increases a little (e.g. up to 85%) (Jankovic et al., 1999). B cell deficient mice do not show an increase in resistance following multiple vaccination but IFN-γ KO mice do, which indicates that the increased immunity is not due to boosting of the IFN-γ response but rather to the boosting of the antibody response and indeed sera from mice given repeat vaccination becomes passively protective (Mangold and Dean, 1986).

So in conclusion, exposure to RoNI induces greater immunity than a single exposure to GI and equal to or greater immunity than repeated GI vaccination. This immunity seems to depend exclusively on IFN-γ and to be generated by a high and persistent Th1 response in the skin/SLN resulting in systemic spread of the sensitized memory/effector cells as seen by persistent responses in the spleen but which does not seem to require parasite induced inflammation in the lungs as is needed for high levels of immunity in the GI. The conclusion that very high levels of immunity can be induced by vaccination restricted to the skin focuses interest on possible ways of delivering defined antigens so as to mimic this

mechanism of immunity. It is interesting to note that the most effective non-living vaccine against *S. mansoni* (James et al., 1985) has important features in common with the RoNI. This vaccine consisted of i.d. injection of large numbers of freeze/thawed schistosomula plus BCG (FT/BCG) and was designed to produce CMI responses like the GI. It is of particular interest that the same vaccine given by different routes is far less effective than the i.d. route and was associated with induction of immunomodulatory cytokine, TGF-β (Williams et al., 1995). The ability of the i.d. FT vaccine to induce significant protection shows that it is not vital that living parasites migrate to the SLN in order to induce significant protection but in this case 10,000 larvae were administered compared with the 500 in the RoNI which also stimulated much higher levels of protection. So it would still be of great interest to know more about the nature of the persistence of antigen presentation in the skin/SLN in the RoNI and a more complete understanding of the cytokine response which may promote the Th1 response e.g. IL-12, IL-23 and IL-27. Ultimately it may be possible to simulate the features of the RoNI e.g., by prime-boost vaccine strategies based on T cell priming with DNA vaccines or recombinant virus followed by boosting with a heterologous recombinant virus expressing the same antigen(s). Such strategies induce pronounced T cell responses and have recently been shown to induce protection against a number of infections including human immunodeficiency virus (Amara et al., 2001), Ebola virus in subhuman primates (Sullivan et al., 2000) and malaria (Bruna-Romero et al., 2001; Gilbert et al., 2002; Rogers et al., 2002).

CHAPTER 4

IN VIVO AND *IN VITRO* INVESTIGATIONS INTO THE EFFECTS OF Ro11-3128 ON ANTIGEN PRESENTATION AND AS AN IMMUNOMODULATOR

4.1. Introduction:

Earlier studies (Bickle & Andrews, 1985; Mountford et al, 1989) and those in Chapter 3 demonstrated that RoNI and GI are highly protective regimens. The conclusions from Chapter 3 were that GI can prime a response during protracted survival in the SLN followed by boosting of this response and attraction of effector cells to the lung by death of the irradiated larvae there, whereas the RoNI induced a stronger response in the skin which resulted in a strong systemic response.

However, it is also clear from various studies that death of large numbers of schistosomula in the skin is not guaranteed to induce optimal levels of protection i.e. exposure of cercariae to very high doses of radiation such that they are arrested in the skin infection site induces poor levels of protection. For example, cercariae irradiated with 80krad produces similar skin retention as the RoNI but poor levels of resistance (Mountford et al, 1992). Also in baboons vaccinated with irradiated *S. haematobium* (Harrison et al., 1990) exposure to 60krad resulted in markedly lower protection than 20krad. By comparison with studies on migration of irradiated *S. mansoni*, the majority of the 20krad larvae would be expected to reach the lungs (Mastin et al., 1985b) but a large proportion of the 60krad cercariae would be expected to die in the skin (Mountford et al, 1992). These observations, together with the fact that 20krad infections treated with Ro11-3128 at the skin stage (RoGI) are poorly protective, indicate that there is something special about the immunogencity of Ro11-3128 treated non-irradiated infections.

It has also been shown that Ro11-3128 is particularly effective compared with other drugs in inducing protection after treatment of skin phase infections. Pellegrino and Katz (1974) failed to induce immunity by therapeutically killing developing schistosomula with the tetrahydroquinoline compound, UK 3883 (Pfizer), administered

around the time of infection. This produced efficient killing of parasites but induced no resistance to a challenge. Bickle and Andrews (1985) found that Oxamniquine (Pfizer) administered at 24hr post infection was able to kill infecting larvae but did not induce resistance. However, Mastin et al. (1985a) using autoradiographic tracking showed this treatment did not kill the parasites in the skin but allowed them to develop at least to the lung stage. But later studies showed that another drug, Ro15-5458, which functioned similarly to Ro11-3128 in arresting parasites in the skin was significantly less effective in stimulating immunity than Ro11-3128 (Bickle et al., 1990). This again points to the need to understand the effects that Ro11-3128 in particular has on the immunizing infections. The experiments in this chapter are concerned with investigating different possibilities concerning the way in which Ro11-3128 might contribute to the high levels of immunity induced and they were carried out in parallel with the studies described in the other two chapters.

One possibility which has been suggested is that the better protective effect of Ro11-3128 compared to other drugs and very highly irradiated parasites which die in the skin, may result from unique effects which Ro11-3128 has on antigen release by the early schistosomulum. Bickle et al. (1990) showed that *in vitro* the drug caused the production of membraneous vesicles and other exudates at the surface of the skin stage parasites. It was suggested that this altered antigen release may be involved in the induction of immunity in this model.

The poor efficacy of Ro11-3128 treated irradiated larvae (RoGI) in inducing immunity (Bickle & Andrews, 1985 and Chapter 3) seems to pose a problem for the idea that Ro11-3128 is effective because of uniquely altered antigen expression by the drug-affected schistosomula. But it was considered possible that unirradiated parasites may respond differently to the effects of the drug compared with the irradiated ones. Perhaps, in attempting to repair the drug induced damage, normal schistosomula may synthesize and release antigenic material including protective antigens but irradiation-induced damage may interfere with such synthesis and repair mechanisms resulting in more rapid death or reduced production of protective antigens. Firstly the irradiated larvae may simply produce less of the released material including the membraneous blebs. Secondly they may fail to synthesize and release other soluble molecules including protective antigens. The first of these possibilities was assessed in this chapter

by comparing 'blebbing' by normal and irradiated larvae *in vitro*. These studies also had another purpose. In the previous tests of the effects of Ro11-3128 on schistosomula Bickle et al. (1990) and Smith et al. (1994) used 50% serum taken from mice given curative doses of Ro11-3128 (serum-derived drug [SDD]) in order to induce parasite 'blebbing'. In other studies in this Chapter and in Chapter 5 drug cultures were set up using live schistosomula, drug and responding sensitized T cells. For such cultures it was thought that the use of SDD was undesirable as high concentrations of mouse serum are not optimal for cell culture and also inconvenient to produce. So the effect of different concentrations of native drug on the schistosomula was investigated.

The possibility that Ro11-3128 is effective because it simply promotes production/release of protective antigens was also investigated by drug treating schistosomula *in vitro* and then vaccinating mice intradermally with the killed (by freezing) schistosomula and released products.

The possibility that the Ro11-3128 treated schistosomula were able to present antigen differently from non-treated larvae was tested in a different and more sensitive way by Smith et al., (1994). They reported that, *in vitro*, Ro11-3128 treated schistosomula resulted in triggering T-cell proliferation (as measured by [^{3}H] thymidine uptake) by primed T cells from the skin draining LNs of mice sensitized with crude schistosomula antigen plus Complete Freund's Adjuvant (CFA), in the presence of fixed macrophages (pre-exposed to the drug-treated parasites before fixation) than normal larvae. Since that time the critical key role of the dendritic cell in antigen presentation leading to the initiation and regulation of immune responses has been recognised (Reis e Sousa et al., 1999). The focal and synchronised release of larval antigen occurring in the skin and/or in the draining lymph nodes following Ro11-3128 treatment provides a model for studying the release of protective antigen and its early processing and presentation, notably by dendritic cells. So a series of experiments were designed to extend the studies of Smith et al., (1994) by using a culture system that was closer to the *in vivo* environment involving the co-culture of (i) sensitized SLN cells from mice exposed to the protective regimen, RoNI, (ii) living schistosomula with or without drug (iii) dendritic cells.

Since the work in Chapter 3 demonstrated the importance of Th1 responses in RoNI immunity another possibility considered was that Ro11-3128 can act as an immunostimulant or adjuvant promoting the Th1 arm of the immune response to antigens produced by the drug arrested larvae. In the present Chapter some studies addressed the possibility that the early induction of Th1 responses might be induced by the drug non-specifically enhancing the immune response. For this purpose an *in vitro* system known to generate Th1 responses was used, i.e., generation of an IFN-γ response to BCG cultured with spleen cells. In this system BCG is taken up by splenic macrophages or dendritic cells and this has been shown to result in production of IL-12 and TNF-α which act on NK cells to produce IFN-γ (Bancroft, 1993). This in turn acts on developing Th1 cells promoting production by them of IFN-γ after they have been presented with antigen by the infected macrophages. In this system cultures were looked at 5 days after being set up which is time for the Th1 cell response to have developed. So any effect that Ro11-3128 would have on any of these early steps i.e., activation of macrophages, NK cells, specific T cells should be identified in this model.

4.2 Results:

4.2.1 *In vitro* effects of Ro11-3128 (dissolved in DMSO) on irradiated and non-irradiated schistosomula.

As was mentioned earlier, Bickle and Andrews (1985) had shown that if irradiated cercariae were used drug-terminated infections were significantly less protective than when unirradiated larvae were used. Possible explanations included that irradiated cercariae may respond differently to Ro11-3128 by either producing fewer membranous blebs/secrete less soluble antigen, or that the combination of irradiation and drug treatment may either reduce the overall viability of the parasites so that they survive for a shorter time and so provide less antigenic stimulation or alter migration of the parasites thus changing the site of antigen presentation and priming. To investigate the first of these possibilities the effects of native Ro11-3128 on production of the characteristic membraneous blebs by irradiated and normal schistosomula were compared.

Both 20krad-irradiated and non-irradiated cercariae were transformed and cultured for 2 days in M169S to simulate the skin parasites present at the time of the 40hr treatments

employed in the RoNI protocols in this thesis. The larvae were then cultured for 24 hours in M169S containing 0-50μg/ml Ro11-3128 (stock solutions were made in the organic solvent DMSO). Parasites were added at 50-70 parasites/well and percentage blebbing and death were assessed at 4 and 24 hours. Parasite viability was judged by their body shape, granularity and movement.

As shown in Figure 4.1.A, at 4 hours, both irradiated and non-irradiated parasites started producing blebs at 0.625μg/ml and produced a high and comparable percentage of vesicular blebs at 1.25μg/ml-10.0μg/ml drug. Both the percentage parasites blebbing and the degree of blebbing were unaltered between 4 and 24 hours (Figure 4.1.B) for both irradiated and non-irradiated parasites. However, as can be seen from both Figures 4.1.A and B parasites treated with 50μg/ml produced few blebs.

Parasite mortality at 4 hours among non-irradiated parasites (Figure 4.1.C) reached 100% among parasites treated with high drug concentrations from 2.5-50.0μg/ml. However, at 1.25μg/ml only 5% were dead. The irradiated parasites showed lower mortality in 2.5μg/ml but 100% mortality with higher drug concentrations. Low mortality occurred at low drug concentrations (0.039 -1.25μg/ml) among both irradiated and non-irradiated parasites. By 24 hours (Figure 4.1.D) all irradiated and non-irradiated parasites treated at 1.25μg/ml were dead but below this there was little effect. Control non-irradiated and irradiated parasites cultured in either M169S or DMSO alone were alive and healthy throughout the experiment.

The observed effects of native drug on the schistosomula were comparable in irradiated and non-irradiated schistosomula and comparable to those seen with SDD in previous studies (Bickle et al., 1990) i.e., parasites produced thin-walled large vesicles which generally occurred first and most frequently at the anterior end of the larvae and with longer incubations at other points along the body. Many of the vesicles were compound, comprising/containing from one or two up to many smaller vesicles (multivesicular blebs). Some of the larvae exhibited both single and multivesicular blebs at any one time. Another type of exudates was produced from the posterior end of the larvae and was made up of small spherical, thick-walled structures, often

Figure 4.1. (A and B): Comparison of Ro11-3128-induced blebbing by irradiated or non-irradiated schistosomula

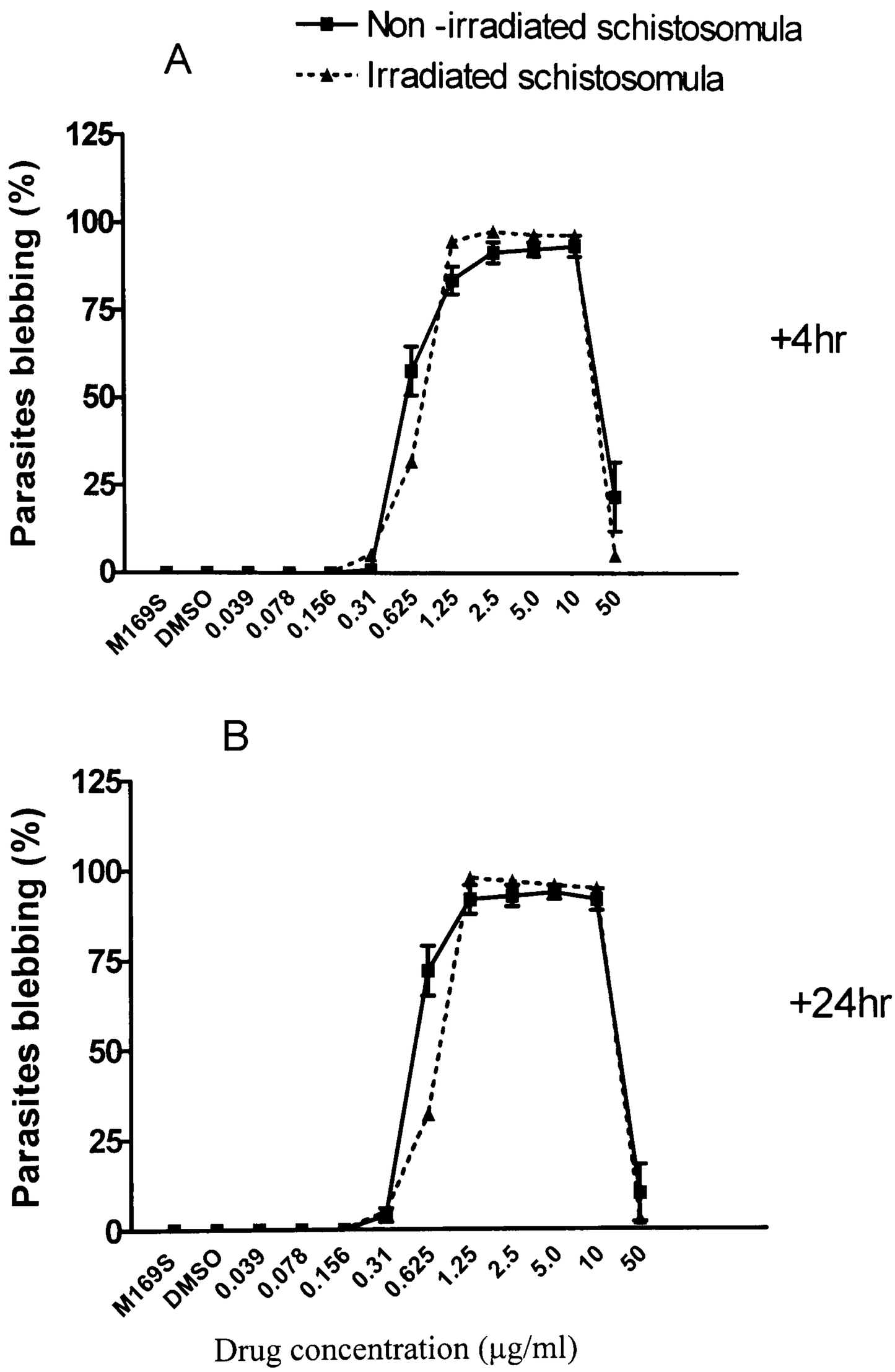
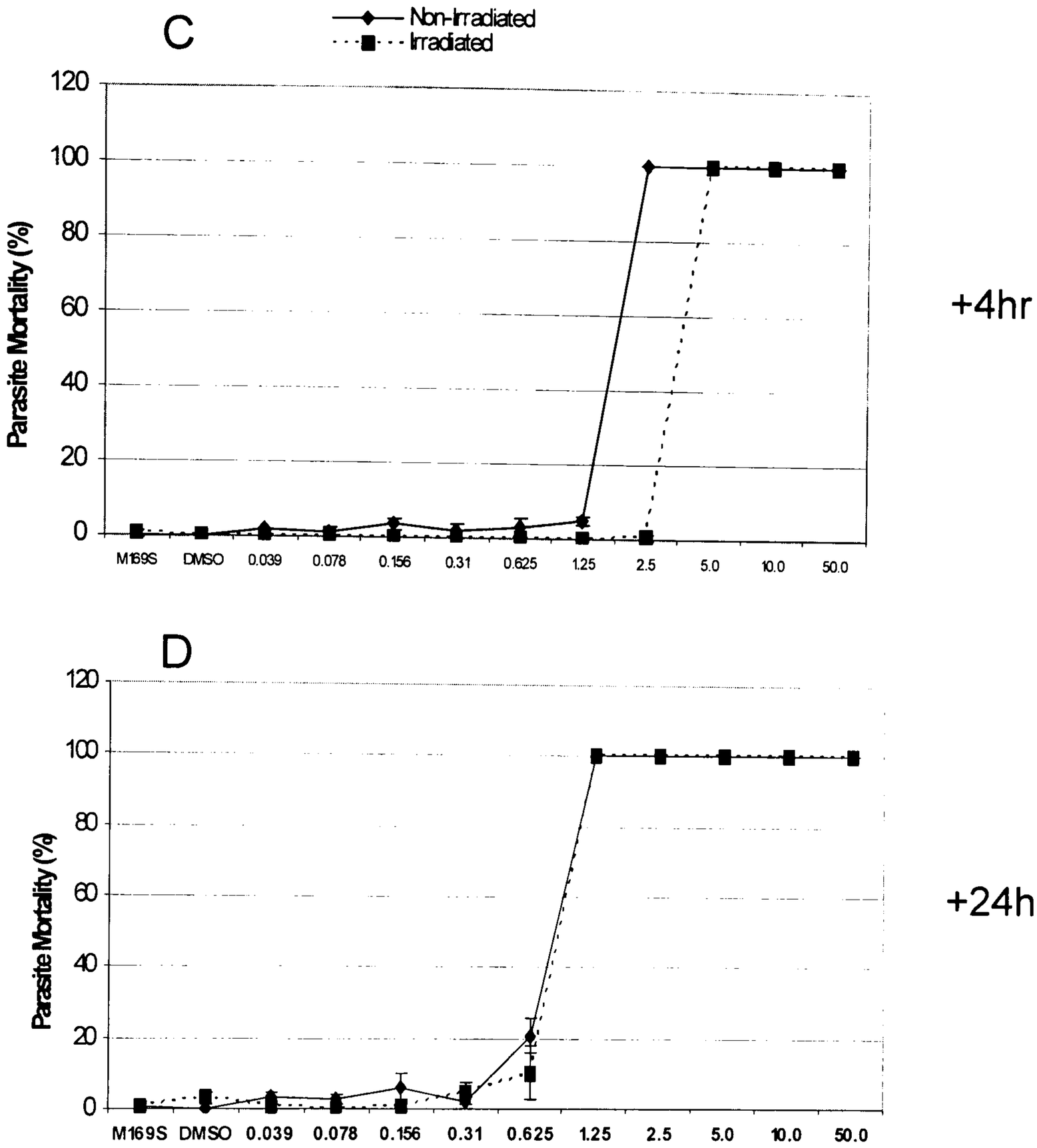


Figure 4.1. **A** and **B** above, **C** and **D** below. Non-irradiated and 20krad-irradiated schistosomula cultured in M169S for 2 days were added at 50-70 parasites/well to Ro11-3128 concentrations ranging from 0.039-50.0µg/ml. Controls consisted of parasites cultured in either M169S or M169S containing DMSO as in the wells containing drug. Cultures were incubated at 37°C and 5% CO_2. Percentage blebbing was assessed at 4 hours **(A)** and 24 hours **(B)**. Percentage death of Non-irradiated and Irradiated parasites was assessed at 4 hours **(C)** and 24 hours **(D)** (see following page). Results are representative of two separate experiments. Data is presented as the mean ± SD for triplicate well observations.

Figure 4.1. (C and D): Comparison of Ro11-3128-induced blebbing by irradiated or non-irradiated schistosomula



giving the appearance of a bunch of grapes. Constrictions occurred at one or more points along the body length, for both irradiated and non-irradiated parasites. A dose of 1.25µg/ml appeared to have comparable effects on morphology and viability to SDD (Smith et al., 1994) and so this dose was used in later studies (4.2.3.1).

The fact that both non-irradiated and irradiated parasites behaved similarly indicated that the production of blebs *per se* is not the only requirement for the induction of optimal immunity by Ro11-3128-terminated unirradiated infections and that the difference in protective potential of the irradiated and normal drug-treated parasites might relate to the antigenic composition of the material released or the length of survival of the parasites in RoNI and RoGI *in vivo*.

4.2.2 Vaccination with killed Ro11-3128-treated schistosomula

One possible explanation for efficacy of Ro11-3128 terminated infections relative to other drugs was that the drug caused damage to the parasites and that they were promoted to synthesize and/or release material including protective larval antigens. Although the above experiments showed that *in vitro* the drug-treated normal and irradiated schistosomula both produced similar amounts of blebs it is possible that the radiation-induced damage prevented the larvae synthesizing the same repertoire of new antigens compared with the unirradiated larvae. The following experiment was designed to simply test if injection of drug-treated parasites and their released products could induce protection. Three groups of 9 C57BL/6 mice were set up: Group A was vaccinated intradermally (i.d.) with the drug-treated parasites; Group B were given RoNI and group C were control untreated mice. For the antigen preparation, transformed and purified schistosomula were cultured in M169 for two days, washed and then cultured at 10,000 parasites/ml in 1.25μg/ml Ro11-3128 for two days.

Figure 4.2 IFN-γ response following vaccination with killed Ro11-3128 treated schistosomula or RoNI

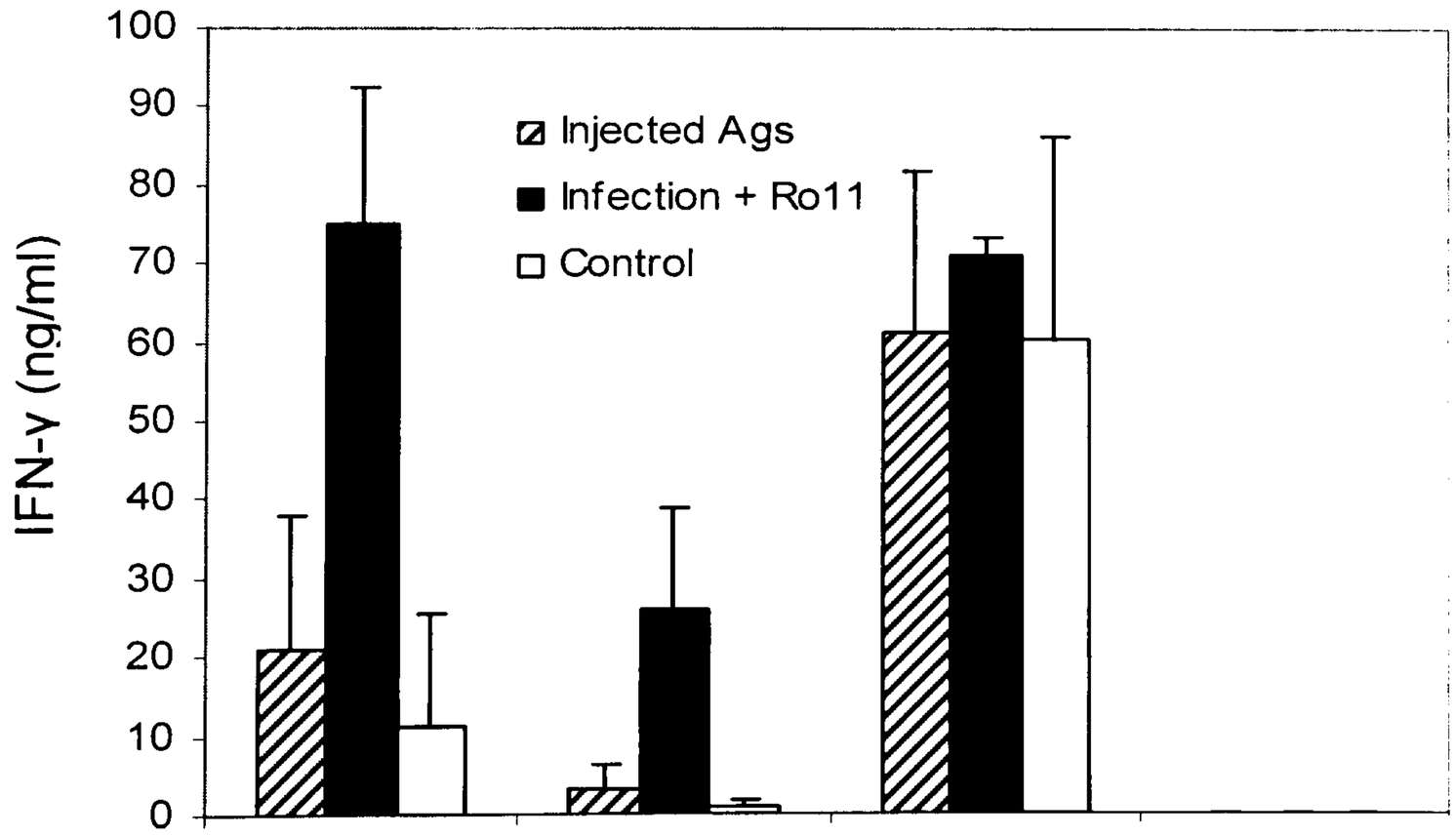


Figure 4.2 IFNγ response by spleen cells of mice vaccinated by: i.d. injection of 500 freeze/thawed, Ro11-3128 treated schistosomula (Injected Ags); infection with 500 cercariae followed by 200mg/kg Ro11-3128 on day +2 (infection + Ro11 [RoNI]) or left untreated (Control). Day 21 spleen cells were cultured at a concentration of $5x10^5$/well and stimulated with Con A (2.5μg/ml), SCAP (10μg/ml) and DOC-SCAP (5μg/ml). Data for spleen cytokines were mean+SEM from separate cultures from 3 individual mice.

The whole culture was removed and frozen at -20°C. For the injection, the culture was thawed, diluted 1:1 with sterile PBS and 100µl of carefully mixed suspension injected intradermally at two sites (i.e. 50µl/site) on the flank of the mice. 21 days post infection/injection three mice were sacrificed and spleen cell cultures set up. The results (Figure 4.2) showed that RoNI induced significant ($P<0.01$) IFN-γ production relative to the control mice, but although the mean level was also higher than the controls for the Antigen injected mice the difference was not significant. The mice were then bled 5 weeks post vaccination and challenged two days later. The serum was tested for antibody against schistosomula antigens. The results in Figure 4.3 showed that there

Figure 4.3: Antibody response to schistosomula antigens in serum of mice vaccinated with killed drug treated schistosomula or by RoNI.

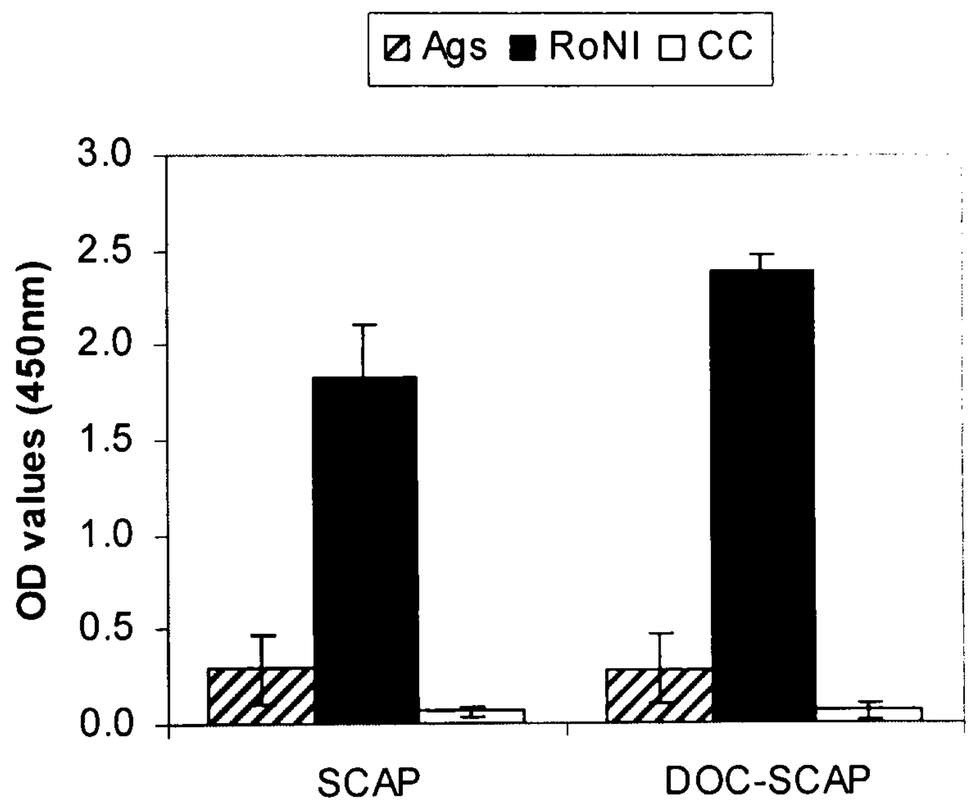


Figure 4.3 : O.D. values from ELISA using serum from mice bled 35 days post vaccination. Sera were tested at 1:100 dilution.

was a highly significant antibody response in the RoNI group compared with the Controls ($P<0.0001$). The antigen injected mice also showed a statistically significant response ($P<0.05$) but this was low, and significantly lower than the RoNI response ($P<0.001$).

At perfusion 3 weeks post challenge the worm recoveries were as shown in Figure 4.4. There was a highly significant (92.2%, $P<0.0001$) reduction in worm recovery from the RoNI relative to the Controls. The worm recoveries from the mice injected with killed schistosomula were very variable (1, 10, 13, 47, 53, 63) with three mice showing low

and three higher counts. Overall, however, there was no statistically significant difference between this vaccinated group and the controls.

Figure 4.4 : Worm recovery from mice vaccinated with killed drug-treated schistosomula or by RoNI.

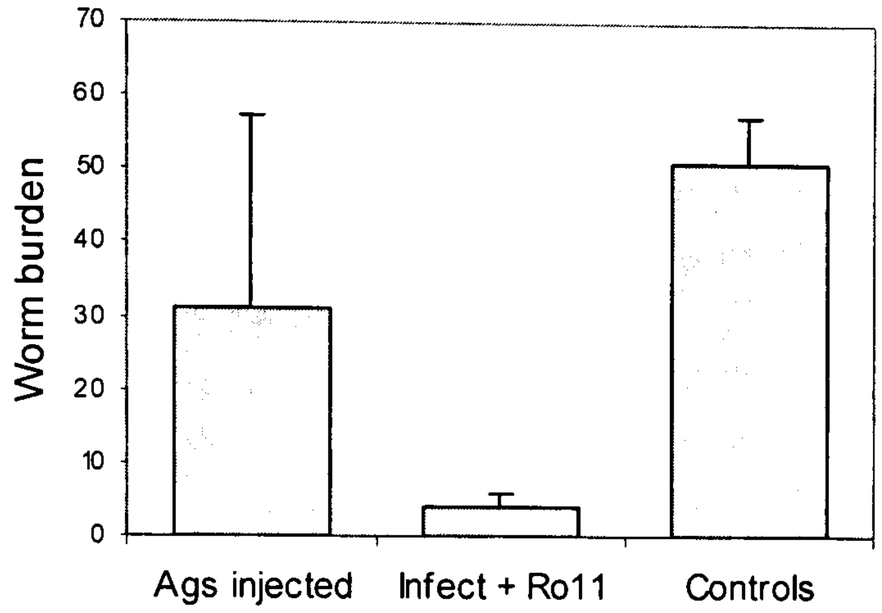


Figure 4.4 : Mean worm recovery (+SD) from vaccinated mice perfused 21days post challenge of mice exposed to RoNI or vaccinated with killed schistosomula 5 weeks before challenge.

This simple *in vivo* experiment failed to demonstrate that the efficacy of Ro11-3128 treated parasites was simply due to production of increased protective antigens able to protect mice when injected intradermally. Later experiments in this Chapter were aimed at a more sensitive assay of antigen production, i.e., the ability of normal and drug-treated parasites to produce antigen *in vitro* which could induce a recall response in SLN cells from mice given RoNI. In these experiments it was planned that native drug was to be added to the cultures, and so it was necessary to be sure that the drug would not damage the SLN cells. So experiments were first planned to assess this using an established *in vitro* system. For this purpose the *in vitro* production of IFN-γ by co-cultures of BCG and naïve spleen cells were used (Bancroft, 1993). This system, which is known to promote a strong Th1 response, was also of value to assess if Ro11-3128, rather than being damaging to the immune cells, could act as an adjuvant for the generation of a Th1 response such as develops after the RoNI (Chapter 3).

4.2.3 *In vitro* effects of Ro11-3128 on BCG/spleen cell co-cultures.

4.2.3.1 Ro11-3128 dissolved in DMSO:

Dimtheyl sulphoxide (DMSO) is a commonly used solvent for drugs in order to solubilize them for dilution in aqueous solutions, and had been found to be an effective solvent for Ro11-3128 in the studies in section 4.2.1. So DMSO was used initially in these experiments. Naïve spleen cells obtained from BALB/c mice were cultured with live *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) (Glaxo-Wellcome- Glaxo strain, supplied by Evans Medical Ltd., Horsham, UK) and kindly provided by Dr D. Smith, LSHTM (at a Stock of $1x10^9$/ml). Two BCG:spleen cells ratios were chosen, $15x10^5$ BCG:$3x10^5$ spleen cells/well (BCG:cells 5:1) and $30x10^5$ BCG: $3x10^5$ spleen cells/well (BCG:cells 10:1). Ro11-3128 was added to cultures to give 10, 5, 2.5, 1.25, 0.625 and 0.31μg/ml concentrations. This range of drug doses was chosen because it was based around the 1.25μg/ml value which gave similar effects on the parasite in terms of blebbing and survival to those caused by serum from mice given the doses of Ro11-3128 (200μg/ml) used to terminate infections in the RoNI system. So it was reasoned that this range of doses for *in vitro* use would include the concentration of drug which the immune cells of *in vivo* treated mice would be exposed to during the RoNI. In the first experiment described, the stock concentrations of Ro11-3128 were dissolved in DMSO such that the final concentration of DMSO in the cell cultures was 1:1,000 (0.1%) (Figure 4.5). Culture plates were incubated at 37°C and 5%CO_2. Supernatants were harvested at 48 hours for IL-4 detection and on day 5 for IFN-γ detection.

No IL-4 was produced in any of the cultures (data not shown). As for IFN-γ, Figure 4.5 showed that in the absence of the BCG stimulant no IFN-γ was detected from cultures containing DMSO alone or Ro11-3128. BCG did promote production of IFN-γ and there was a marked increase in IFN-γ production with 10:1 BCG:cells inducing twice the level of IFN-γ compared with 5:1. The presence of 0.1% DMSO appeared to markedly reduce the IFN-γ production. However, the presence of Ro11-3128 in the cultures appeared to overcome this DMSO effect in a dose dependent manner up to 1.25μg/ml at which concentration the IFN-γ production was comparable to the non-

DMSO treated cultures with both 5:1 and 10:1 BCG/cell ratios (NB the Ro11-3128 cultures all had 0.1% DMSO in as well as drug).

Figure 4.5 Observations on the immunomodulatory effect of Ro11-3128 dissolved in DMSO on cells in *in vitro* cultures as judged by IFN-γ production.

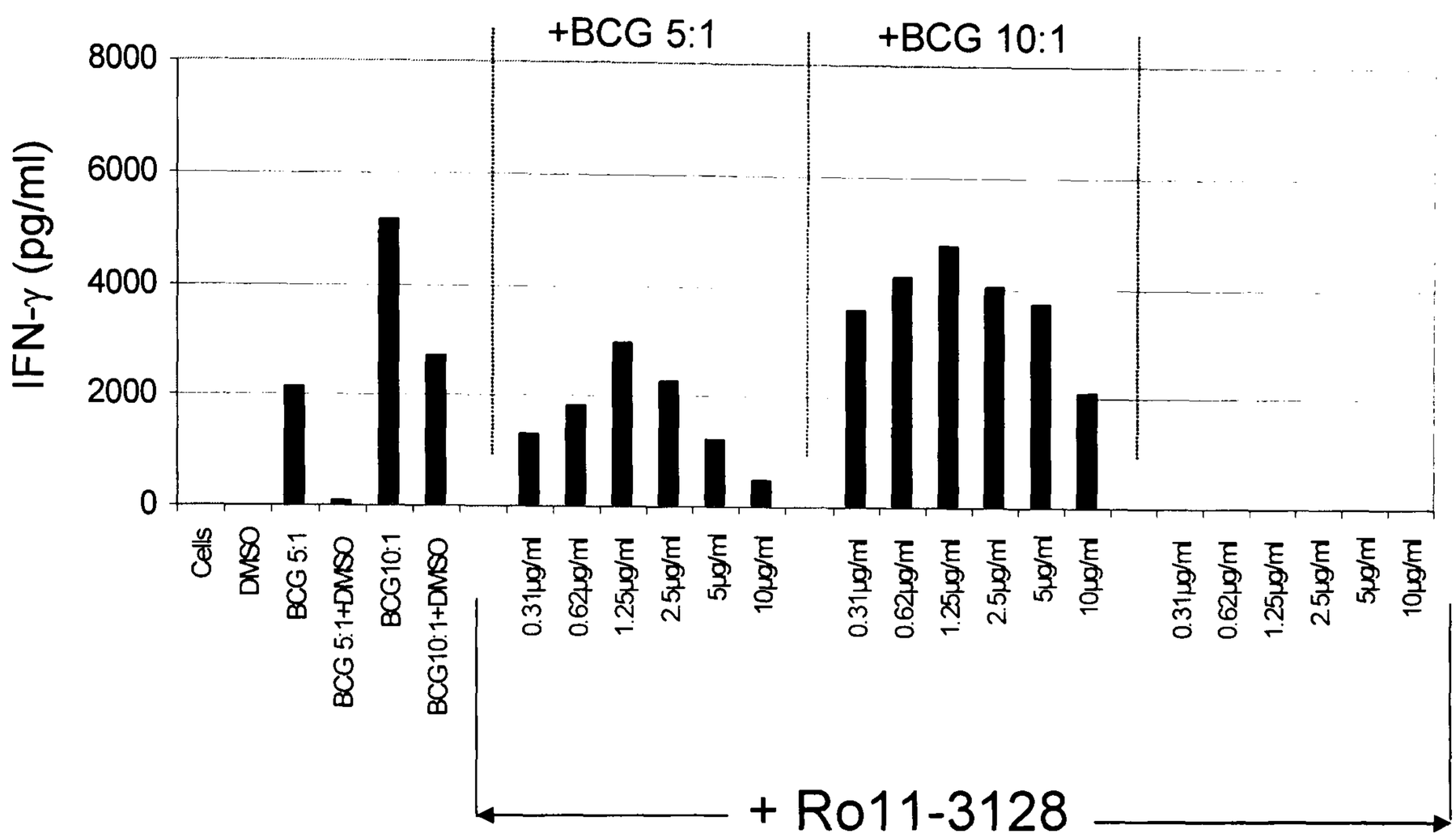


Figure 4.5 Naïve BALB/c spleen cells were cultured with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) in two BCG:cells ratio concentrations, 5:1 and 10:1 ($15x10^5$ BCG:$3x10^5$ cells/well and $30x10^5$ BCG:$3x10^5$ cells/well, respectively). Ro11-3128 was added to cultures ranging from 0.31-10.0µg/ml. The final concentration of DMSO in the cultures was 0.1%. Data were from pooled supernatants of duplicate wells.

In view of this demonstration of an inhibitory effect of 0.1% DMSO this experiment was repeated using higher stock concentrations of Ro11-3128 in DMSO so that the final concentration of DMSO was reduced to 1:16000 (0.00625%) (Figure 4.6). As in the previous experiment BCG alone induced significant IFN-γ production, but in this case the presence of 0.00625% DMSO had an enhancing effect although it did not induce IFN-γ production from cells in the absence of BCG. Concentrations of 0.31-2.5µg/ml Ro11-3128 did not significantly affect IFN-γ production relative to the DMSO alone but concentrations of 5 and 10µg/ml had an inhibitory effect.

Figure 4.6 Observations on the immunomodulatory effect of Ro11-3128 dissolved in DMSO on cells in *in vitro* cultures as judged by IFN-γ production

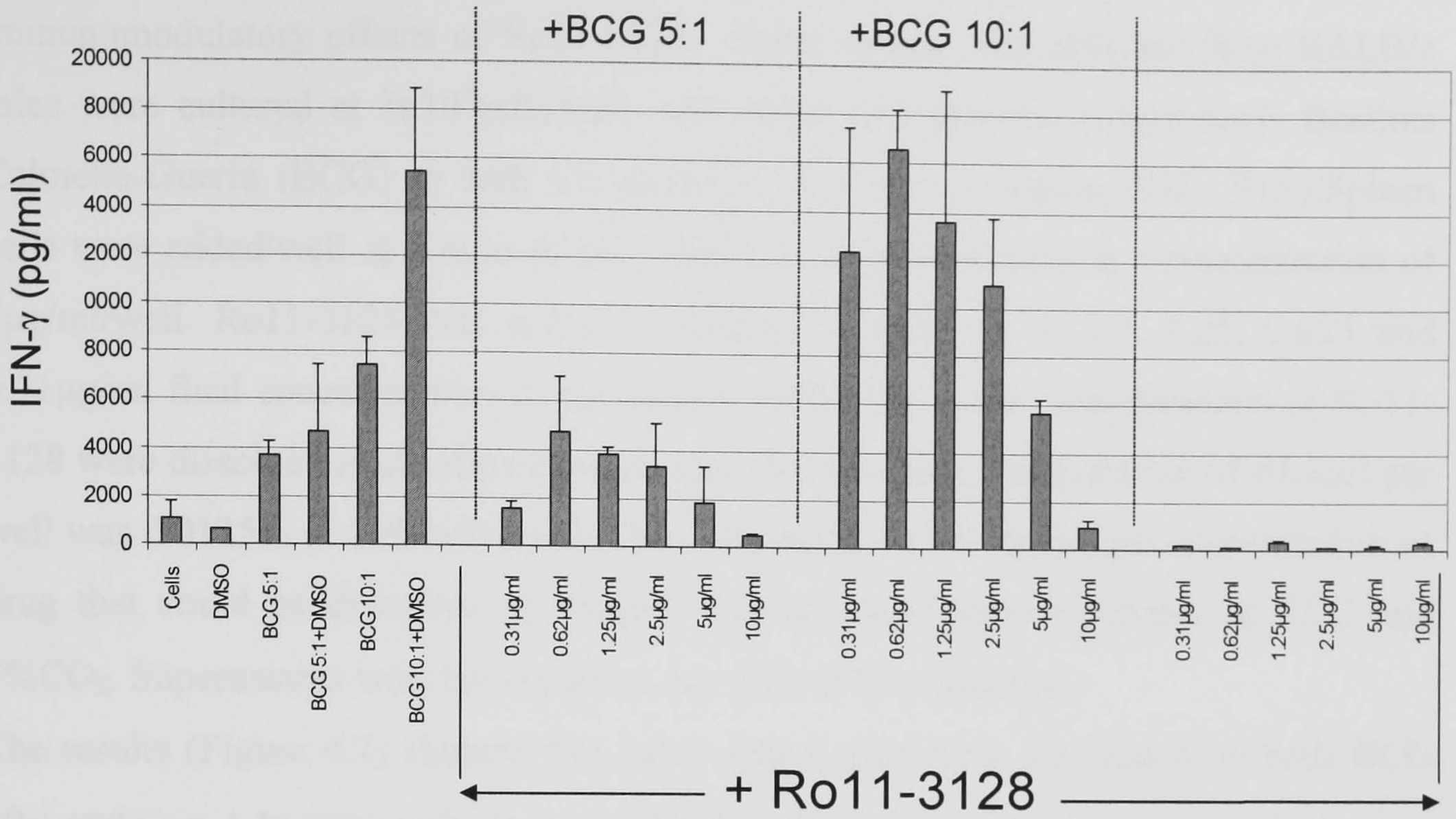


Figure 4.6 Naïve BALB/c spleen cells were cultured with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) in two BCG:cells ratio concentrations, 5:1 and 10:1 ($15x10^5$ BCG:$3x10^5$ cells/well and $30x10^5$ BCG:$3x10^5$ cells/well, respectively). Ro11-3128 was added to cultures ranging from 0.31-10.0µg/ml. The final concentration of DMSO in the cultures was 0.00625%. Data represent the mean+SD for triplicate wells.

From these two experiments it is concluded that there is an interaction between the effects of DMSO and Ro11-3128 on BCG-induced IFN-γ production by spleen cells, which confuses the investigation of any direct immunomodulatory effect of Ro11-3128. At a concentration of 0.1%, DMSO inhibits the IFN-γ production and this can be overcome by increasing concentrations of Ro11-3128 up to 1.25µg/ml but beyond this, Ro11-3128 was itself inhibitory. In contrast DMSO at 0.00625% had a stimulatory effect on IFN-γ production and Ro11-3128 could not enhance this effect.

It is concluded that the BCG/IFN-γ *in vitro* system may be useful to investigate Ro11-3128-induced effects but that alternatives to solubilising Ro11-3128 in DMSO were needed. In the following experiment ethanol was tested.

4.2.3.2 Ro11-3128 dissolved in Ethanol

This was essentially a repeat of the above experiment but the mitogen Concanavalin A alone was used to stimulate some cultures non-specifically as a further control for any immunomodulatory effects of Ro11-3128. Naïve spleen cells obtained from BALB/c mice were cultured at $3x10^5$cells/well with either live *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) or with Concanavilin A (Con A) (Sigma, UK). BCG:Spleen cells were added/well at a ratio of 10:1, and Con A was cultured at a concentration of 5µg/ml/well. Ro11-3128 was added to cultures to give 10, 5, 2.5, 1.25, 0.625 and 0.31µg/ml final concentrations in the culture wells. The stock concentrations of Ro11-3128 were dissolved in absolute ethanol, such that the final concentration of ethanol per well was 0.0125% (1:8000 dilution). This was based on the maximum concentration of drug that could be dissolved in ethanol. Culture plates were incubated at 37°C and 5%CO_2. Supernatants were harvested on day 5 for IFN-γ detection.

The results (Figure 4.7) showed that naïve spleen cells were stimulated by both BCG 10:1 and Con A to produce high levels of IFN-γ. In the absence of either stimulant no

Figure 4.7 Observations on the immunomodulatory effect of Ro11-3128 or Ethanol on cells in *in vitro* cultures as judged by IFN-γ production.

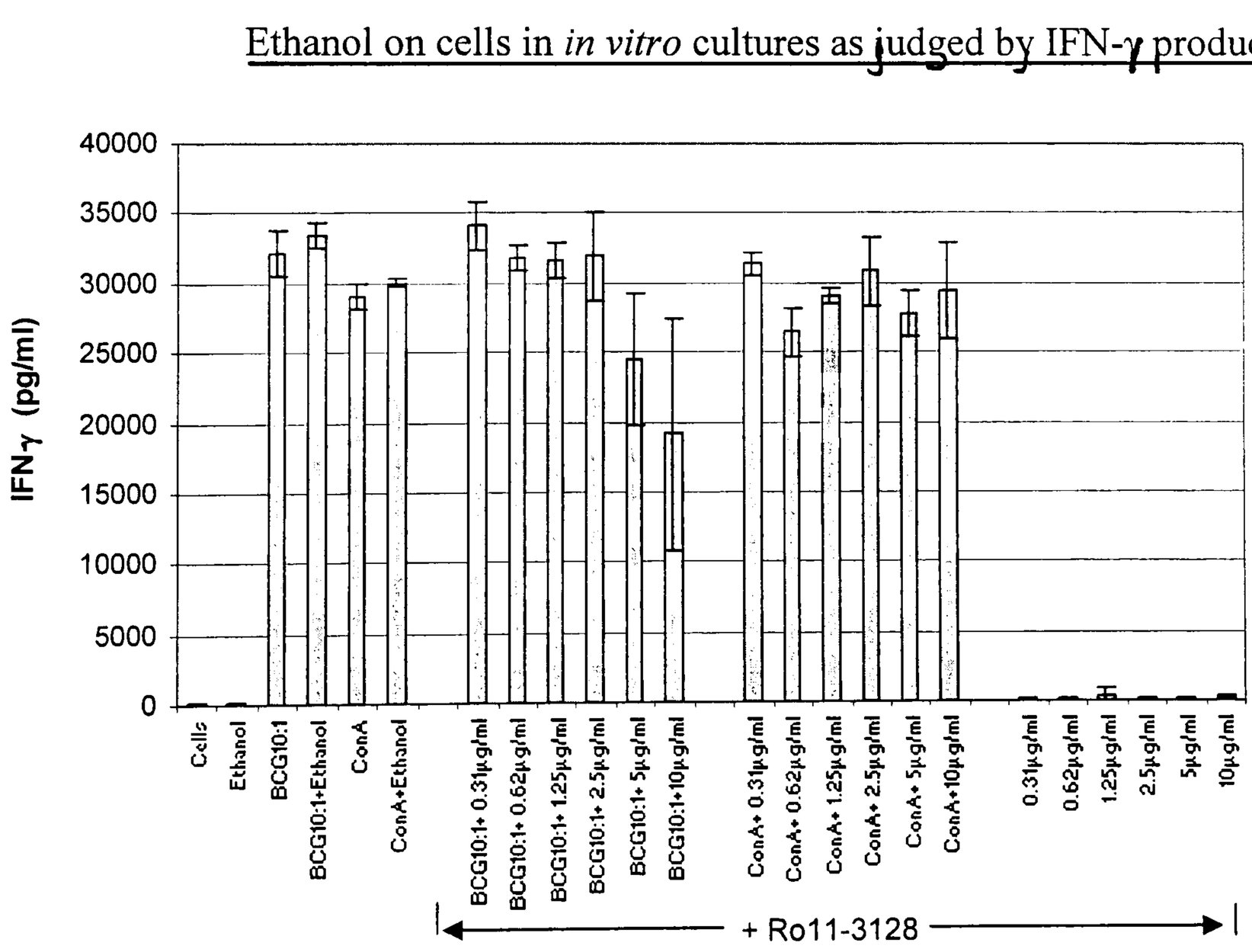


Figure 4.7 Naïve BALB/c spleen cells were cultured for 5 days with either *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) at a BCG:cell ratio of 10:1 ($3x10^6$BCG:$3x10^5$ cells/well) or Con A at a concentration of 5µg/ml. Ro11-3128 was added to cultures ranging from 0.31-10.0µg/ml. The final concentration of Ethanol in the cultures was 0.0125%. Data represents the mean ± SD for triplicate wells.

IFN-γ was detected from cultures containing the different concentrations of Ro11-3128. The presence of ethanol at 0.0125% did not induce IFN-γ production. In BCG-treated spleen cells, IFN-γ was produced at high levels in the presence of low drug concentrations (0.31μg/ml–2.5μg/ml), but this was only comparable to the levels induced by the control cultures containing BCG and BCG+ 0.0125% ethanol. At higher Ro11-3128 concentrations, 5.0μg/ml and 10μg/ml, there was a significant reduction in IFN-γ production. This pattern, however, was not observed with cultures stimulated with Con A in which Ro11-3128 at high or low concentration had no effect, positive or negative, on levels of IFN-γ. As can be seen from Figure 4.8. the presence of ethanol at this low concentration of 0.0125% did not cause the death of the cells *in vitro*. Spleen cell viability was also not affected by concentrations of Ro11-3128 in the range 0.31 – 5μg/ml but there was evidence of reduced viability at 10μg/ml.

Figure 4.8 Viability of spleen cells co-cultured in the presence of varying concentrations of Ro11-3128.

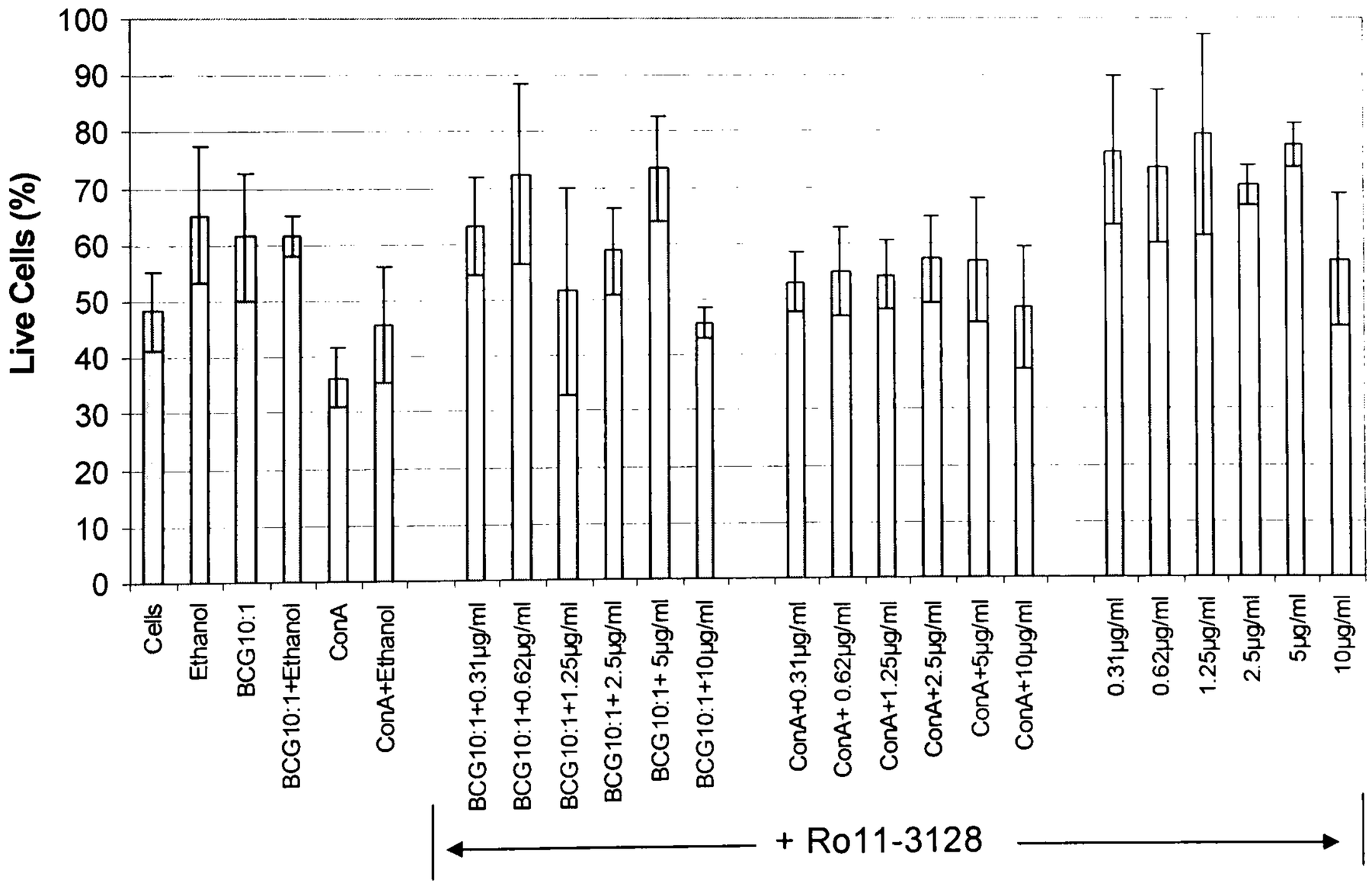


Figure 4.8 See legend to Figure 4.7. Cell viability was assessed by removing cells at day 5 of co-culture and staining for viable cells by Trypan blue exclusion.

It was concluded from these studies that although differing concentrations of DMSO had interesting effects on the BCG/spleen cell IFN-γ production, overall there was no

evidence of an immunostimulatory effect of Ro11-3128 on the spleen cell response to BCG. Specifically there was no evidence for immunostimulatory, inhibitory or cell viability effects of 0.31–2.5μg/ml Ro11-3128 delivered in 0.0125% ethanol but evidence of inhibitory effects at higher Ro11-3128 concentrations. So for subsequent studies on the effects of Ro11-3128 on antigen presentation by schistosomula in culture, Ro11-3128 delivered in 0.0125% ethanol was employed. The experiments shown in Figure 4.1 were repeated using Ro11-3128 dissolved in 0.0125% ethanol and similar levels of blebbing were seen as in Figure 4.1.

4.2.4 Comparison of M169 and DMEM media.

The above experiments on the effect of Ro11-3128 on schistosomula were all carried out using M169 medium (Basch, 1981) which was specially developed for schistosome culture, but in order to study the effects of Ro11-3128 on antigen presentation by treated schistosomula it was planned to co-culture the parasites with sensitized SLN cells and so the culture conditions needed to be suitable for both cells and parasites. Dulbecco's Modified Eagle's medium is one which has been used for culturing schistosomula (Coulson et al., 1998; James et al., 1998) and this is also recommended for mouse cell culture. A preliminary experiment was carried out to compare the viability and blebbing of schistosomula in these two media. The two concentrations of Ro11-3128 that gave high percentage of blebbing for 24 hours (0.625μg/ml and 1.25μg/ml) were once more compared and followed over 48 hr. Levels of blebbing in M169S were similar to previous studies (100% blebbing with 1.25μg/ml, 80% with 0.625μg/ml) but lower in the cDMEM medium (40% with 1.25μg/ml, 10% with 0.625μg/ml). However, in both media viability of drug treated parasites was somewhat better than in the experiments shown in Figure 4.1., with 100% and 50% of the schistosomula treated with 0.625μg/ml and 1.25μg/ml, respectively, still moving at 48hr post treatment. Schistosomula in control cultures without drug looked healthy over the 5 days they were observed with no mortality.

Based on the various above studies, for the purposes of studying the Ro11-3128 induced effects on antigen presentation by Ro11-3128 treated schistosomula *in vitro*. Ro11-3128 was used at a final concentration of 1.25μg/ml (dissolved in 0.0125% ethanol) and co-cultures were set up using cDMEM.

4.2.5 How effective are living (normal or drug-treated) schistosomula in providing antigen for restimulation of sensitized T cells?

These experiments were based on the earlier observation that Ro11-3128 treated schistosomula seemed better than untreated schistosomula at presenting antigen to macrophages as judged by proliferation of lymph node cells from mice injected with crude larval antigen plus Complete Freund's Adjuvant (Smith et al., 1994). For the experiments described here, sensitized cells were derived from the SLN of mice exposed to RoNI. Initial experiments (not shown) had demonstrated that in contrast to soluble larval antigens (see Chapter 3) living drug-treated or normal schistosomula were poorly effective at inducing IFN-γ from total SLN cultures. Although the SLN preparation would have contained antigen presenting cells, it was decided to test the effects of addition of additional myeloid dendritic cells (DCs) produced by *in vitro* culture of bone marrow cells. Dendritic cells have been shown to be stimulated during the skin response to radiation attenuated cercariae (Hogg et al, 2003) and are likely to be involved in the recognition of drug-treated schistomula in the skin. Production of bm-DCs was also required for the studies in Chapter 5.

4.2.5.1 Production and characterisation of *in vitro* generated bone marrow-derived dendritic cells.

Initial experiments were set up to produce bone marrow derived-DCs (bm-DCs) and confirm their phenotype. The method of Lutz et al., (1999), which involved culture of bone-marrow leukocytes for 10 days in 10% GM-CSF (see section 2.9.3), centrifugation steps and changing of medium, was modified following discussion with Dr Jean Langhorne (NIMR, Mill Hill) in some of the initial experiments, where bone marrow leukocytes were cultured in cDMEM containing 1:40 GM-CSF. On day 2, 10 mls of fresh cDMEM containing 1:40 GM-CSF was added to 10 ml cell cultures in their petri-dishes, and on day 4 half of the total volume of medium in the petri-dishes was replaced with fresh cDMEM/1:40 GM-CSF by careful aspiration from the liquid surface as explained in section 2.9.3. The cultures were then left to develop for 6 days after which the non-adherent cells were collected and bm-DCs counted before being added to 96-well culture plates.

In vivo, immature DCs are highly efficient at capturing antigen after which they mature which includes upregulating various surface markers including MHC class II and they become efficient at antigen presentation. For use in antigen presentation experiments, it is important that the *in vitro* generated bm-DCs are immature. Because DCs can be readily activated even by physical manipulation (Gallucci and Matzinger, 2001) it is necessary to check that the majority of the cells used in subsequent experiments were immature bm-DCs. For this a FACScan analysis of day 6 cultures was carried out. Gating was carried out to include cells exhibiting $CD11c^{+}$ and have low to moderate to high surface levels of MHC class II molecules which are characteristic of both immature and mature DC populations in such cultures (Lutz et al., 1999; Shortman & Liu, 2002; Ardavin et al., 2001). Visual inspection of the cultures showed the abundance of cells with typical DC morphology (Figure 4.9).

Figure 4.9 Dendritic cells prepared from mouse bone-marrow cultures

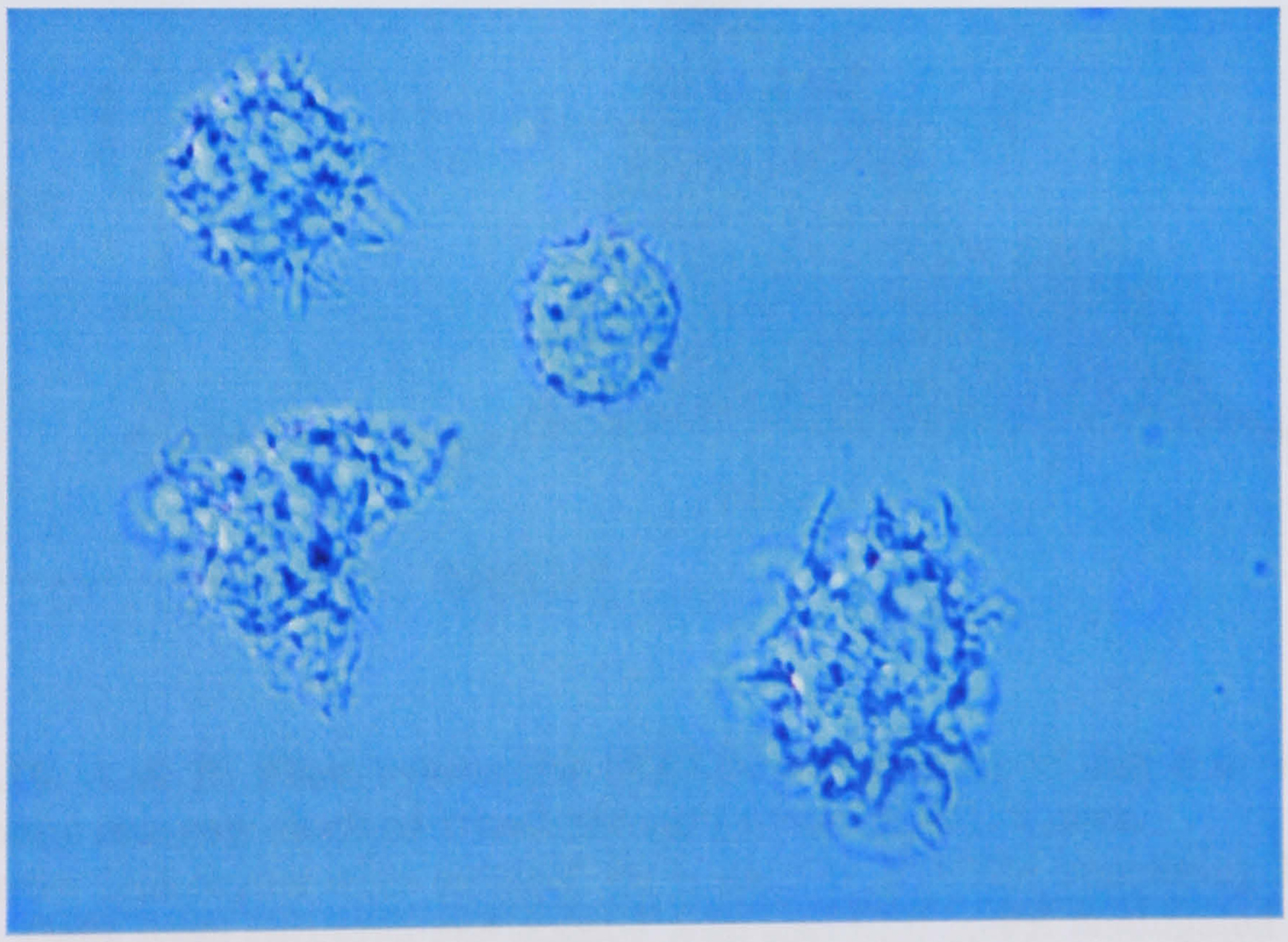


Figure 4.9 Dendritic cells prepared from mouse bone-marrow cultures as described in Figure 4.9. were visualised under light microscopy at x400 magnification. Note the typical irregular dendritic protrusions.

Figure 4.10.A shows that among the day 6-old cells about 45% of cells were CD11c high (regarded as DCs). Of these about 81% were in the $MHCII^{Low\text{-}to\text{-}medium}$ range characteristic of immature DCs (Figure 4.10.B).

Figure 4.10. FACScan analysis of *in vitro* derived dendritic cells

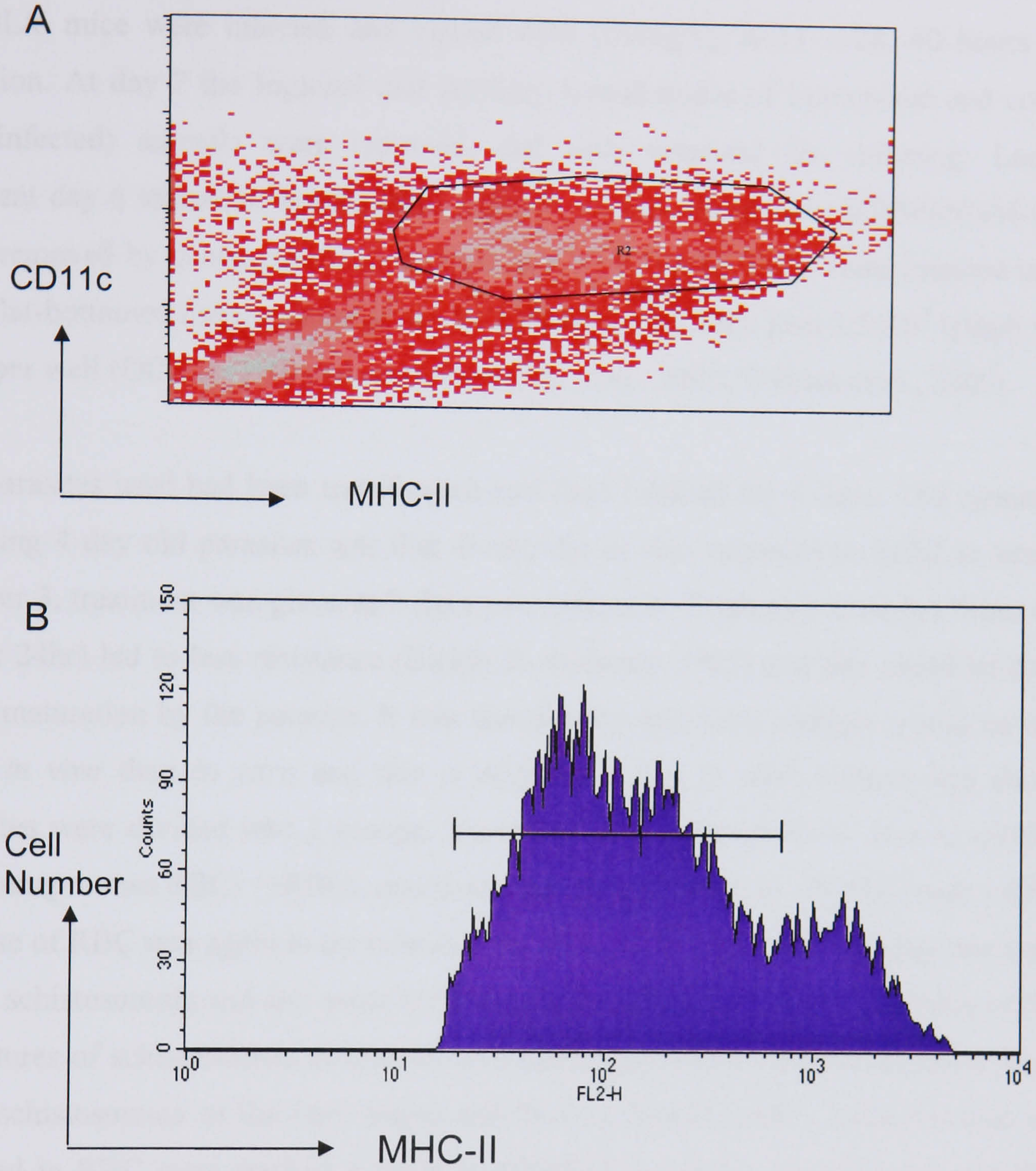


Figure 4.10. A & B: Flow cytometric (FACScan) analysis of day 6 *in vitro* derived bone marrow-derived Dendritic cells from C57BL/6 mice.

Bone marrow leukocytes were cultured for 6 days in cDMEM containing 1:40 GM-CSF. Cells were collected and stained with anti CD11c-PE conjugated and anti –MHCII-FITC conjugated antibodies and analysed by FACS. Dot plot [A] shows the CD11c+ve, MHC class II+ve bm-DC population (shown gated). Histogram plot [B] shows the immature bm-DC population expressing low - medium MHC class II molecules (|——|) and the mature MHC class II high population. About 80% of CD11c+ MHCII+ cells were MHC II $^{low\text{-}medium}$ (Immature DCs) whereas 20% were MHC class II ^{high} (Mature DCs).

4.2.5.2 Cytokine production from sensitized SLN cells during *in vitro* culture with living schistosomula ± Ro11-3128 ± DCs.

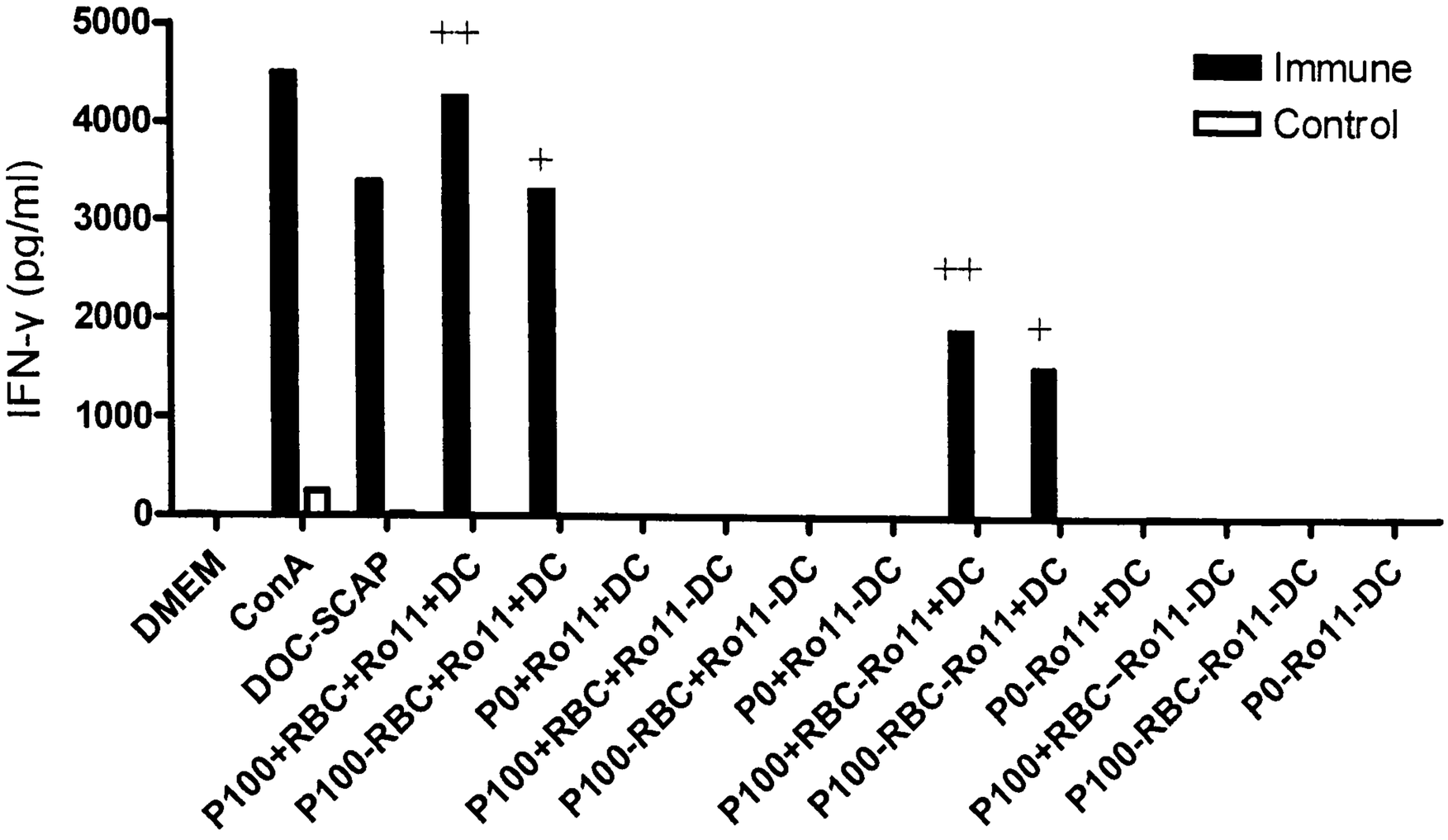
C57BL/6 mice were infected and treated with 200mg/kg Ro11-3128, 40 hours post infection. At day 7 the Inguinal and Axillary lymph nodes of immunised and control (non-infected) animals were removed, and cells prepared for culturing. Loosely adherent day 6 immature bm-dendritic cells (bm-DCs), cultured in cDMEM/GM-CSF, were removed by gentle pipetting, washed once in cDMEM. Cells were cultured in 96-well flat-bottomed tissue culture plates by adding $2.5x10^4$ DCs plus $2.5x10^5$ lymph node cells per well (DC:LN 1:10 cell ratio) (Semnani et al., 2001; Whelan et al., 2000).

The parasites used had been transformed and then cultured for 4 days. The reason for choosing 4 day old parasites was that during the *in vivo* response to RoNI as used in Chapter 3, treatment was given at 2 days post infection. Treatment at earlier times (e.g. 3hr or 24hr) led to less resistance (Bickle & Andrews, 1985) and this could be due to some maturation by the parasite. It was thought that any such changes would be more rapid *in vivo* than *in vitro* and this is why the 4 day *in vitro* culture was chosen. Parasites were divided into 2 groups, those that were cultured for 4 days in cDMEM containing mouse RBCs (+RBC), and those that were cultured in cDMEM only (-RBC). The use of RBC was again to try to mimic the *in vivo* situation by promoting maturation of the schistosomula and any associated important antigenic changes. Addition of RBC to cultures of schistosomula is known to be sufficient to promote development of skin stage schistosomula to the lung stages and beyond (Basch, 1981). Parasites that were cultured in RBC were washed 4 times in cDMEM to remove any adhering red blood cells before finally adding them to the cells in culture. Approximately 100 parasites from either group (P100+RBC, P100-RBC) were added in wells containing either Ro11-3128 (1.25μg/ml) and bm-DCs (+Ro11+DC), or in the absence of one of them (-Ro11+DCs, +Ro11-DC) or in the absence of both (-Ro11-DC). Those cultures without Ro11-3128 contained DMSO instead, at 0.00625%. DOC-SCAP or Con A were added at 10μg/ml and 5μg/ml to control cultures, respectively (bm-DCs were not added to these). Supernatants were collected at 72 hours and IFN-γ and IL-5 levels determined by ELISA to indicate the level of Th1 or Th2 cytokine production.

As seen in Figure 4.11., the immune SLN cells showed a high level of IFN-γ production following stimulation with Con A or DOC-SCAP whereas the control cells showed only a low level response to Con A. In contrast IL-5 production by the immune SLN cells was very low, lower than the naïve cells. In the presence of living schistosomula variable levels of IFN-γ was produced depending on the culture content.

The results clearly showed that addition of bm-DCs to cultures containing schistosomula and sensitised LN cells strongly enhanced IFN-γ production. In fact in this experiment no IFN-γ was detected in cultures without bm-DCs whether there were schistosomula and/or drug present or not. Also there was no production of IFN-γ from control cells showing that this was a recall response and not a primary effect of parasites on naïve SLN cells (e.g. as was shown for the interaction of BCG and spleen cells in section 4.2.3). IFN-γ production was however, dependent on the presence of schistosomula as no IFN-γ was produced in control cultures lacking parasites. The IFN-γ level in cultures containing drug-treated schistosomula, in the presence of bm-DCs, were comparable to the levels induced by Con A or DOC-SCAP. In the absence of Ro11-3128 there was still enhanced cytokine production in the presence of DCs but this was lower than in cultures containing Ro11-3128 (compare values with + or ++). A low level of IL-5 was also seen in cultures with Ro11-3128 treated schistosomula and immune cells relative to control cells (Figure 4.11.). There was evidence of slightly lower IFN-γ production in cultures containing parasites previously cultured in the absence of RBCs than from those previously cultured in the presence of RBCs (Figure 4.11.). This was not seen with IL-5.

Figure 4.11.: Cytokine recall responses to living normal or Ro11-3128 treated schistosomula *in vitro*.



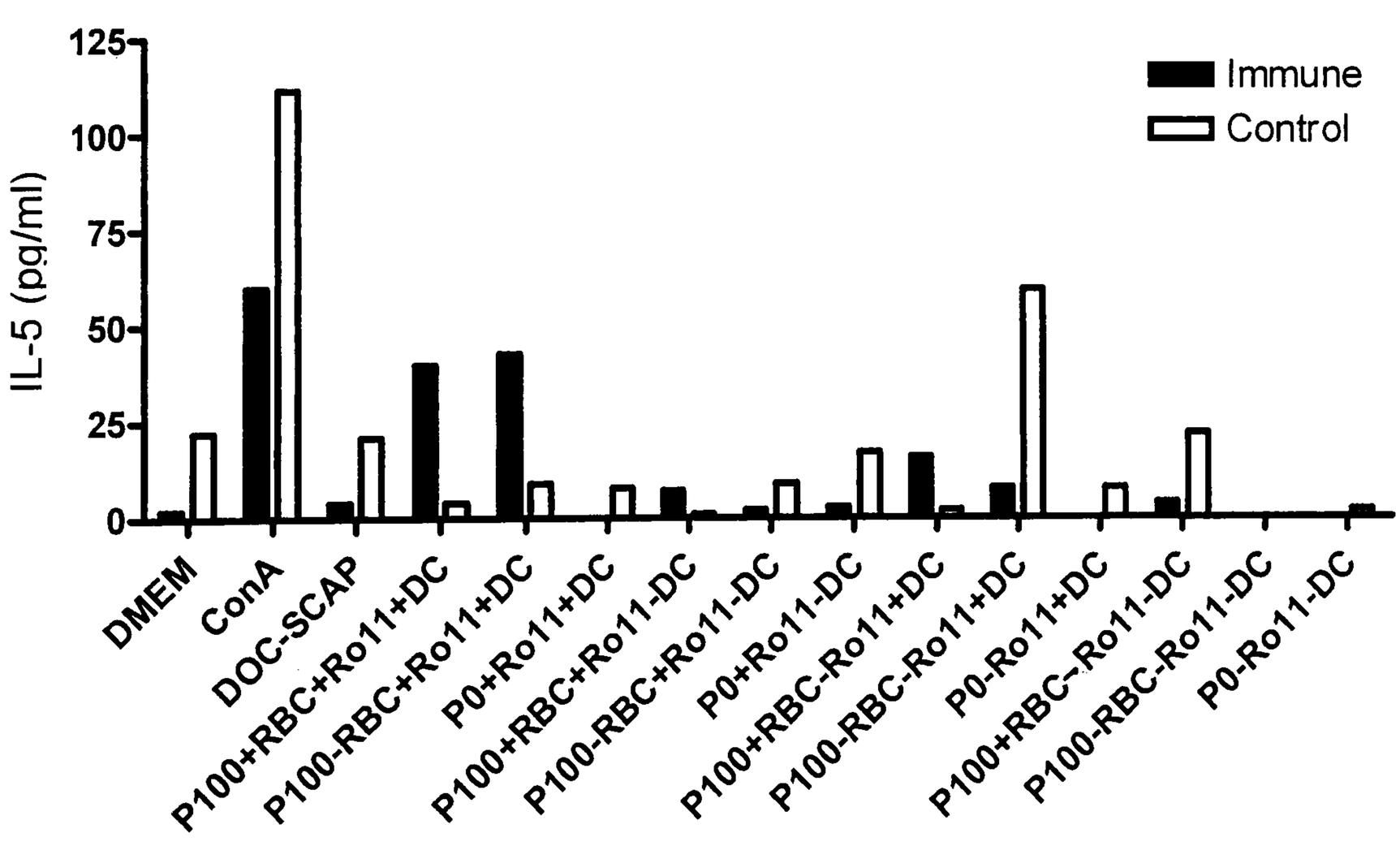


Figure 4.11.: IFN-γ and IL-5 cytokine responses from lymph node cells co-cultured with additional Dendritic cells (DCs) and parasites.
Day 7 inguinal and axillary LNs were pooled from 3 C57BL/6 mice in each group given RoNI or left untreated as controls. **Cells** were added at $2.5x10^5$ cells/well. Bm-DCs were added to some wells (**±DC**) at $2.5x10^4$ DCs per well.
±RBC: Parasites pre-cultured in cDMEM for 4 days with or without mouse red blood cells. **±Ro11**: in the absence or presence of Ro11-3128 (1.25µg/ml).
P100:100 mechanically transformed schistosomula per well ; **P0** : 0 parasites per well.
Control antigens used: **Con A** (5µg/ml), **DOC-SCAP** (10µg/ml). Data for IFN-γ & IL-5 were from pooled supernatants collected at 72 hours of culture.

4.2.5.3 Repeat of experiment 4.2.5.2 including an attempt to define the source of stimulating schistosomula antigen.

The above experiment indicated enhanced antigen presentation by living schistosomula in the presence of Ro11-3128. The next experiment was set up to try to repeat these observations and to gain insight into the source of the antigens whose production was apparently enhanced by Ro11-3128, i.e., (i) soluble released antigen or (ii) particulate antigen including membranous blebs.

To investigate this, sensitized SLN cells were produced as above (7 days post RoNI). Mice were infected with 500 cercariae or left as controls and, were drug treated on day 2 post-infection. Day 6 bm-DCs were cultured with LN cells at $2.5x10^4$: $2.5x10^5$cells/well, respectively. Cells were stimulated with DOC-SCAP (5µg/ml) and Con A (5µg/ml) in the absence or presence of bm-DCs. Parasites were cultured for 4 days in cDMEM and mouse RBCs, washed 3x in cDMEM and added at 100 parasites/well. In this experiment cultures were set up in triplicate in order to allow an estimate of the variation (standard deviation) to be calculated.

In view of the evidence that Ro11-3128 treated parasites gave better recall response than untreated larvae an attempt was made to test separate components of the drug treated parasites. To generate the soluble or particulate fractions, around 3000 of the day 4 schistosomula were placed in a siliconized eppendorf tube containing 1.25µg/ml of Ro11-3128 and left at 37°C and 5% CO_2, for 5 hours. Small particulate material including membraneous blebs were separated from parasite bodies by vigorous pipetting, microscopic examination confirming that most blebs had become detached. The intact Ro11-3128-treated schistosomula (RS) were then sedimented out leaving a supernatant containing soluble released antigen (SA) and shed particulate antigen including blebs (BB). This supernatant was carefully transferred into a separate tube. RS parasites were then resuspended in 1ml of cDMEM and aliquoted in appropriate volume into culture wells (100 parasites/well). The SA and BB medium was centrifuged at 16,000 xg for 30 minutes at room temperature to form a pellet of BB. The SA-containing supernatant was carefully transferred into a third eppendorf tube, leaving behind a pellet of BB. BB pellet was resuspended in 1ml of cDMEM and vortexed. The concentrations of SA and BB were adjusted such that material corresponding to culture

Figure 4.12. IFN-γ and IL-4 production by sensitized lymph node cells co-cultured with living schistosomula or antigens released from them by Ro11-3128 treatment.

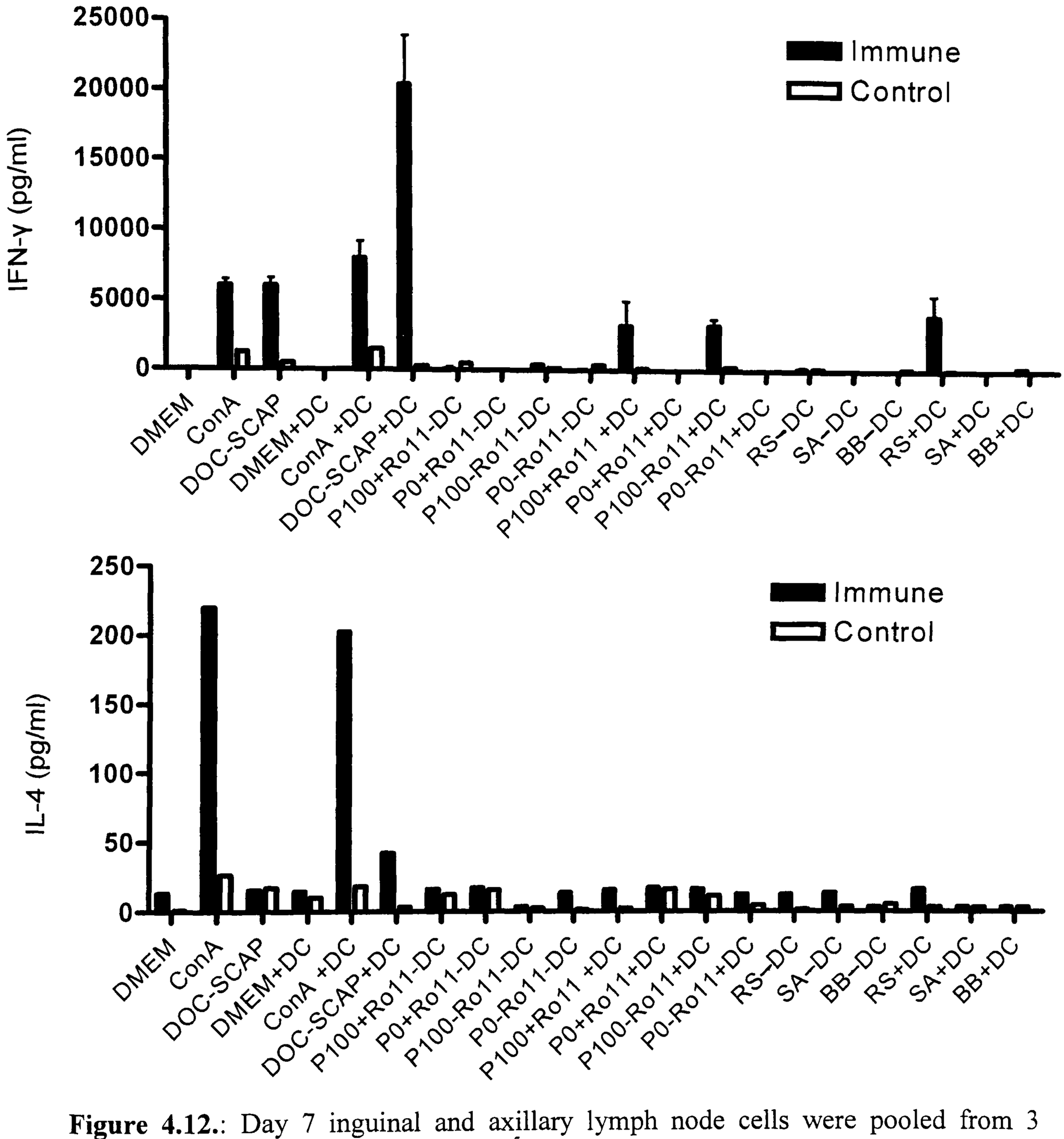


Figure 4.12.: Day 7 inguinal and axillary lymph node cells were pooled from 3 C57BL/6 mice and cultured at $2.5x10^5$ cells per well, in the absence or presence of day 6 **DCs** at $2.5x10^4$ DCs per well. Cells were stimulated with either **Con A** (5μg/ml); **DOC-SCAP** (5μg/ml); 4-day old living schistosomula cultured in cDMEM and mouse RBCs at **100 parasites/well** with or without 1.25μg/ml **Ro11-3128** (in 0.00625% DMSO) in culture; **RS** (residual parasites [after treatment with Ro11-3128 and removal of blebs]) (P100/well), **SA** (soluble released antigens) or **BB** (released blebs). Data for IFN-γ were from supernatants collected at 72 hours of culture. Data were mean+SD of triplicate wells. Data for IL-4 were from pooled supernatants collected at 24 hours of culture. (Immune = Infect+Ro11; Control = Non-infect+Ro11).

of 100 parasites was added to appropriate wells. Day 4 parasites which had not been treated were added to other wells with or without drug. All wells contained the same final volume. Supernatants for IL-4 and IFN-γ were collected at 24 and 72hours, respectively, and the cytokine profile was determined by ELISA.

The results are shown in Figure 4.12. Apart from ConA there was very little IL-4 demonstrable with any of the antigens. In the positive control cultures the immune SLN cells produced high levels of IFN-γ and low levels of IL-4 in response to ConA and this was not significantly affected by addition of bm-DC. High levels of IFN-γ were produced by immune cells in the presence of DOC-SCAP and addition of bm-DCs dramatically raised this to very high levels ($P<0.001$ with and without bm-DC).

Regarding the stimulation by living schistosomula the data in Figure 4.12. is redrawn for clarity in Figure 4.13. This shows that living schistosomula were again shown to induce significant levels of IFN-γ from immune cells in the presence of bm-DCs but not in their absence. However no statistically significant difference in IFN-γ levels was seen between cultures with 100 schistosomula in the presence or absence of Ro11-3128. This differs from the experiment in Figure 4.11. in which a marked difference was seen although in this case no statistical comparison was possible.

Referring back to Figure 4.12., it is also clear that the levels of stimulation of IFN-γ with the living schistosomula (P100 ± Ro11+DC) was comparable to that seen by addition of RS (RS+DC), i.e., parasites that had been exposed to Ro11-3128 and then washed free of any blebbing or soluble released materials. *In vitro* these parasites did not continue to produce blebs, and remained alive up to 24 hours and so this suggests that the particulate material including blebs released by drug treated parasites is not the prime stimulus for the recall response produced by drug treated parasites. However, stimulatory activity was not demonstrated using either soluble antigen (SA) or blebs (BB) removed from the drug treated parasites.

The two experiments (Figures 4.11., 4.12. and 4.13.) both showed the dramatic difference by adding bm-DCs to the cultures, but they were not consistent regarding the

effects of Ro11-3128 on SLN cell re-stimulation. So a third experiment was carried out to try to confirm this point.

Figure 4.13: Selected data from Figure 5.12 redrawn to focus on the stimulation with living schistosomula.

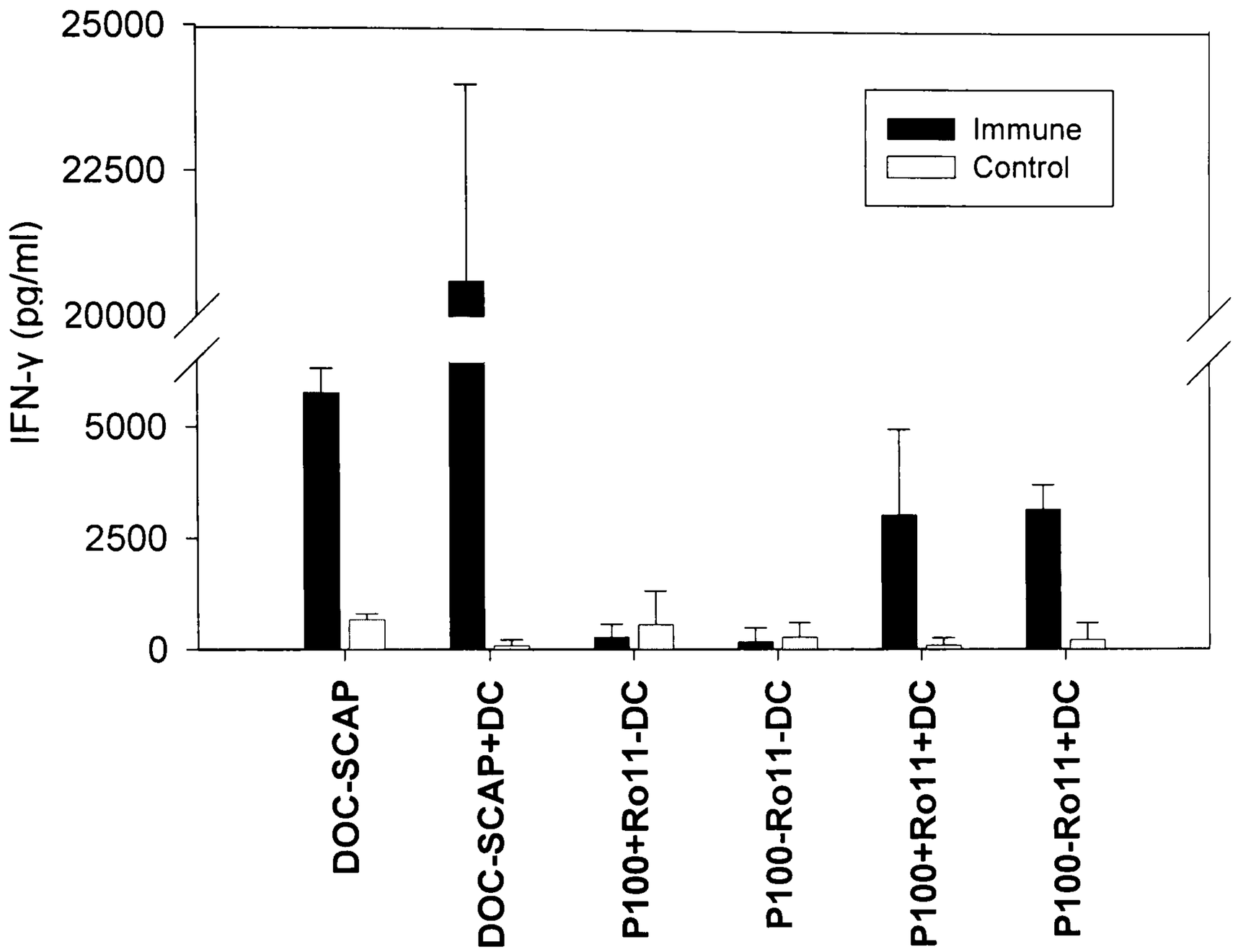


Figure 4.13: Selected redrawn data from Figure 4.12. See legend to Figure 4.12.

4.2.5.4 Further investigation of the effects of re-stimulation using living schistosomula with or without Ro11-3128.

Through experience of trying slightly modified ways of producing DCs a method was settled on which produced a relatively high percentage of immature DCs which would be required for antigen processing. Mature DCs in the co-culture experiments would not be efficient antigen processors but could influence the co-cultures by secreting cytokines. In this experiment bm-DCs were prepared in this way based on the 10 day protocol developed by Lutz et al., (1999), using 10%GM-CSF/cDMEM and using minimal physical disturbance to the cells.

The experiment was set up as for 4.11. and 4.12. except that the schistosomula used were cultured for 24hr in cDMEM in the absence of mouse red blood cells since there was little evidence from experiment 4.11. that the RBC affected the immunogenicity. The other difference was that this experiment used Ro11-3128 in 0.0125% ethanol as the experiment in Figure 4.7 demonstrated no significant effect of this concentration of ethanol on the BCG/splenocyte production of IFN-γ. The results are shown in Figure 4.14.

Figure 4.14.: IFN-γ recall responses to living normal or Ro11-3128 treated schistosomula *in vitro*.

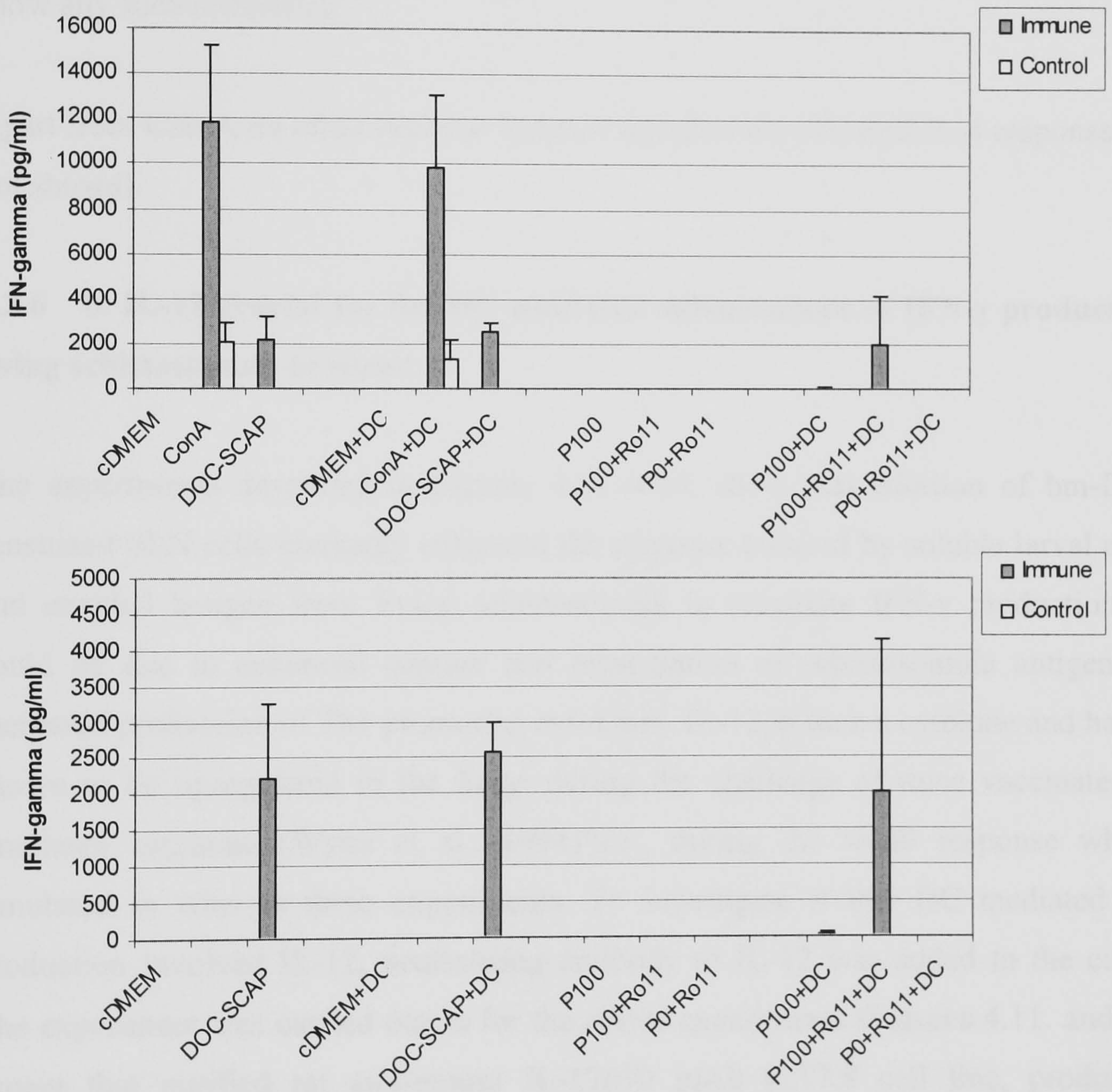


Figure 4.14: Day 7 inguinal and axillary lymph node cells were pooled from 3 C57BL/6 mice and cultured at $2.5x10^5$ cells per well, in the absence or presence of day 10 DCs at $2.5x10^4$ DCs per well. Cells were stimulated with either **Con A** (5μg/ml); **DOC-SCAP** (5μg/ml); living schistosomula previously cultured in cDMEM for 24hr and used at 100 parasites/well with (**P100+Ro11**) or without (**P100**) 1.25μg/ml Ro11-3128 (in 0.0125% ethanol) in culture. Data are from supernatants collected at 72 hours of culture. Data were mean+SD of triplicate wells. (Immune =lymph node cells from Infect+Ro11; Control = Non-infect+Ro11). Note: the bottom graph shows the same data as the top one but with the Con A data removed to highlight the comparison of antigen specific responses.

In agreement with both Figures 4.11. and 4.12., high levels of IFN-γ were induced by Con A and DOC-SCAP was seen. Unlike experiment 4.12. there was no marked and significantly raised response to DOC-SCAP in the presence of DCs. The reason for this is not apparent as the same batch of antigen was used in both experiments. Regarding stimulation by schistosomula, only schistosomula in the presence of DCs and Ro11-3128 treatment stimulated any response. The lack of any response in the absence of DCs was the same as in the other two experiments. The lack of any response with non-drug treated schistosomula is a similar pattern to Figure 4.11. in which the response with Ro11-3128 treatment was higher with Ro11-3128 than without. Figure 4.12. did not show any such difference.

Apart from Con A no other stimulus induced significantly elevated IL-4 responses (data not shown).

4.2.6 Is IL-12 crucial for the DC mediated enhancement of IFN-γ production to living schistosomula *in vitro*?

The experiments described in Figures 4.11.-4.14. show that addition of bm-DCs to sensitized SLN cells markedly enhanced the response induced by soluble larval antigen and enabled antigen from living schistosomula to stimulate IFN-γ production. This could be due to enhanced capture and presentation of schistosomula antigen or to increased production of Th1 promoting cytokines. IL-12 is such a cytokine and has been shown to be upregulated in the lungs during the challenge of mice vaccinated with irradiated cercariae (Wynn et al., 1994) i.e., during the recall response which is simulated *in vitro* in these experiments. To investigate if this DC mediated IFN-γ production involved IL-12, neutralising antibody to IL-12 was added to the cultures. The experiment was carried out as for the above experiments (Figures 4.11. and 4.12.) except that purified rat anti-mouse IL-12p70 mAb (C17.8 cell line, produced as described in section 2.13) was added to some wells (final concentration 10μg/ml). This concentration of anti-IL-12p70 mAb was selected from the results of a preliminary experiment in which production of IFN-γ by spleen cells activated by *in vitro* incubation with *Burkholderia pseudomellii* was abolished (carried out in collaboration with Dr Ganjana Lertmemongkolchai, LSHTM [Data not shown]) (Lertmemongkolchai et al.,

2001). As controls other wells had purified Rat IgG (Sigma, UK) added (at 10μg/ml) or were untreated.

There was no effect of neutralising IL-12 on the very low level of IL-4 production in this experiment (data not shown). Again the DC dependence of the IFN-γ production was clear. The mean level of IFN-γ produced was slightly lower in the wells with rat IgG compared with untreated wells but the difference was not significant (Figure 4.15). With anti-IL-12p70 the mean levels were lower again, less than half the values in the untreated controls and also lower than in the wells with rat IgG but in both cases the differences were not significant. So although this indicated that IL-12 may play a role in the DC mediated effects it was not totally dependent on its presence.

Figure 4.15 Effect of neutralising IL-12 on DC function *in vitro*

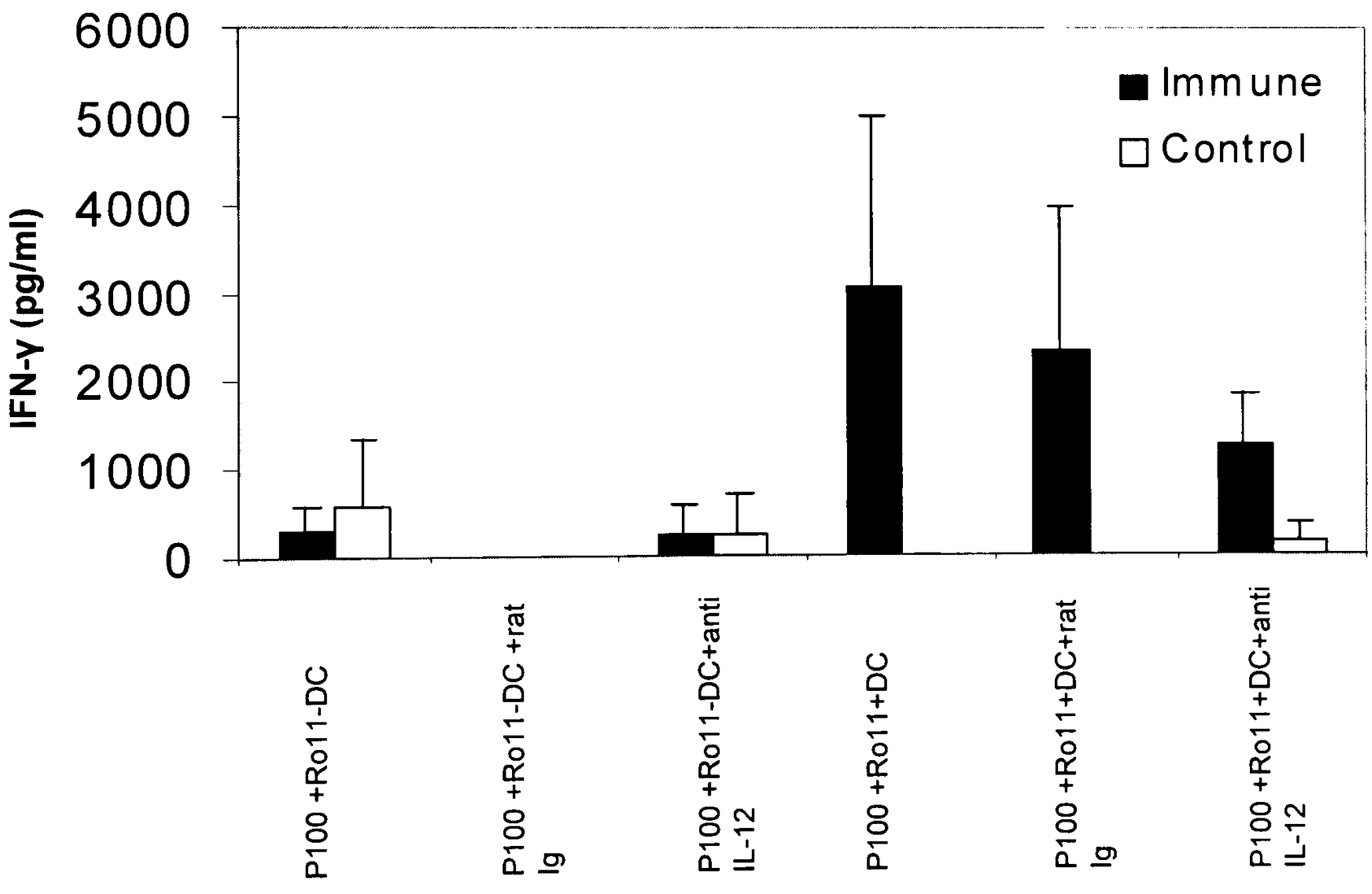


Figure 4.15 IFN-γ production from cultures containing day 7 inguinal and axillary LN cells cultured at $2.5x10^5$ cells/well, 100 parasites/well (P100), Ro11-3128 (at 1.25μg/ml), in the absence or presence of DCs ($2.4x10^4$ cells/well), and either rat monoclonal anti-IL-12 immunoglobulin, rat IgG or no antibody. Supernatants were collected at 72 hours of culture and the data is the mean + SD of triplicate wells. (Immune = Infect+Ro11[RoNI]; Control = Non-infect+Ro11[RoCC]).

4.3 Discussion

Exposure of mice to Ro11-3128 treated infection results in high levels of immunity and is known to arrest the vast majority of the larvae at the skin stage (Bickle & Andrews, 1985; Mastin et al., 1985a; Mountford et al., 1989). The fact that similar treatment with other drugs or exposure to highly irradiated cercariae which also arrest larvae in the skin induce relatively poor levels of protection (Bickle & Andrews, 1985; Bickle et al., 1990) led to investigations to try to explain why Ro11-3128 seems to be so effective at inducing this immunity. Possibilities considered were (i) Production of (protective) membraneous material (blebs) by the drug, (ii) Non-specific adjuvant effects of Ro11-3128, promoting protective Th1 immune responses, (iii) Increased production/release of other protective antigens including soluble antigens, (iv) Sensitization of the lung due to migration of a proportion of the lethally damaged larvae there, and (v) Persistent antigen release due to protracted survival of drug damaged larvae in the skin. The work in this Chapter specifically addresses the first three of these possibilities.

Production of protective membraneous material.

It has been suggested that the efficacy of infections terminated at the skin stage with Ro11-3128 on 1 or 2 days post-infection in inducing immunity may be related to the way in which the drug affects antigen presentation by schistosomula of this age. *In vitro* studies had shown the production of large and multiple membranous vesicles (blebs) at the parasite's surface, and the release of smaller vesicles and particulate matter (Bickle et al., 1990). More recently, by injecting schistosomula into skin pouches (Daly et al., 1999) of drug treated mice and recovering the larvae, Dr Yaobi Zhang (personal communication) has shown that this blebbing also occurs *in vivo*. The antigenic composition of these blebs has been shown to be qualitatively different from that of the rest of the larval surface. For example, the membrane glycoprotein Sm16, a vaccine candidate antigen (Bickle et al., 1986) is present in the blebs (Bickle et al., 1990) but Sm38 is not. Sm16 is associated with the preformed membraneous bodies (Bickle & Oldridge, 1999) which give rise to the schistosomular membrane (Hockley & McLaren, 1973) suggesting that Ro11-3128-induced blebs may originate from this membrane reservoir. The production of membraneous material may explain why Ro11-3128-abbreviated infections stimulate strong antibody responses to larval surface antigens (Bickle, Sacko & Vignali, 1990).

Smith et al. (1994) found that *in vitro* Ro11-3128 treatment of schistosomula co-cultured with macrophages led to increased cell proliferation when these macrophages were subsequently cultured with cells sensitized by injection of larval antigen with Complete Freund's adjuvant. It was further shown that this enhanced response was associated with the particulate matter released from the drug-treated parasites. So it was suggested that the membraneous blebs might be responsible. However, drug-treated irradiated infections (RoGI) are poorly protective (Bickle & Andrews, 1985; Mountford et al., 1989; Chapter 3 section 3.2.1) and so if the simple production of blebs was responsible alone for the optimal immunity induced by RoNI, it is necessary to suggest that either fewer blebs are produced by the irradiated parasites and/or that they are antigenically different. To test the first of these possibilities, irradiated and normal schistosomula were cultured with increasing concentrations of native drug. Another purpose of these studies was to establish the dose of native drug that induced optimal blebbing. Native drug was tested rather than use of 50% heat inactivated serum from mice treated with curative doses of Ro11-3128 (serum-derived drug, SDD, as used by Smith et al., 1994) because SDD was inconvenient to produce in bulk and not suitable for the planned co-culture of sensitised mouse lymphocytes with schistosomula for assessment of released cytokines.

There was a clear bell-shaped dose response with doses of 0.625-10μg/ml inducing blebbing in a high proportion of parasites but both lower and higher doses producing little blebbing probably because of rapid killing of the larvae at the higher concentrations (50μg/ml). 1.25μg/ml was selected as the best dose for use in treating schistosomula *in vitro* for co-culture experiments because it produced the maximum level of blebbing, similar to that reported for 50% SDD (Smith et al., 1994; Bickle et al., 1990) but more protracted survival than higher doses. No differences were observed between normal and irradiated schistosomula regarding the survival or percentage of parasites with drug-induced morphological changes, notably production of blebs. So it appears that, assuming the drug interactions seen *in vitro* reflect what happens *in vivo*, simply inducing blebs is not adequate to induce optimal levels of immunity. This did not necessarily rule out the possible involvement of the membraneous blebs in RoNI immunity, as it was possible that there are subtle differences in the antigenic

composition of the particulate material produced/released in irradiated and normal infections and/or differences in the release of soluble antigens. It nevertheless remains a possibility that the unique Ro11-3128-induced production of membraneous material and/or associated release of soluble antigens accounts for the high levels of antigen specific IFN-γ production by SLN cells at day 21 post infection (Figure 3.3 and 3.4, Chapter 3). Further work on this possibility could compare such skin and SLN responses following Ro11-3128 terminated infections and those terminated with Ro15-5458 which seems to be ineffective in inducing resistance (Bickle et al., 1990).

Further comparisons of antigen production between the normal and irradiated drug-treated schistosomula, e.g., by Western blotting or metabolic labelling and co-precipitation were not carried out here but the possibility that Ro11-3128 promoted production/release of protective antigen from normal schistosomula was directly tested by a simple immunization experiment. This involved the intradermal injection of the equivalent of 500 schistosomula plus attached blebs and any soluble released antigens, and this compared with the RoNI protocol which uses 500 percutaneously applied larvae drug treated *in vivo*. It is clear that the RoNI induced marked IFN-γ production by the spleen cells whereas the killed vaccine was largely ineffective at inducing IFN-γ although there was some production of antibody albeit much less than with RoNI. At challenge there was little evidence of protection. Low worm counts in the three mice, given the killed vaccine did not correlate with higher antibody or IFN-γ levels and the reason for the low counts is unknown. In spite of this, it is clear that any short term induction of increased antigen release/production induced by Ro11-3128 such as occurs *in vitro* is not sufficient to induce optimal protection, confirming the idea that the RoNI requires the protracted survival of the drug-treated schistosomula.

There has been limited success in immunizing with crude dead antigens in schistosomiasis (Dean, 1983). The most successful series of experiments were also based on attempts to induce IFN-γ responses following studies of the irradiated vaccine (James et al., 1984) discovered that significant protection (35-70% protection) could be induced by freeze/thawed schistosomula injected i.d. together with BCG. In addition to the requirement for this vaccine to be given with BCG it also involved injection of 10,000 schistosomula. Any enhanced production/release of protective antigens by the *in*

vitro Ro11-3128-treated 500 schistosomula which was the number used per mouse in the present study was obviously not sufficient to compensate for the vastly greater numbers of parasites or the adjuvant activity of the BCG used in the James et al. (1984) studies. However, one possibility which was considered was that, *in vivo*, Ro11-3128 itself may be able to act as an adjuvant for Th1 mediated immune responses.

Are there non-specific adjuvant effects of Ro11-3128 which promote protective Th1 immune responses?

A number of drugs against infectious agents are known to have immunostimulatory effects and for some of these, notably the intracellular organisms like *Leishmania* the therapeutic action is based on this immune activation (Croft and Coombes, 2003). The possibility that Ro11-3128 might have immunostimulatory effects was investigated *in vitro* by its effects on the interaction of macrophages with BCG. This is a system that is known to induce a strong Th1 response (Bancroft, 1993). Naïve mouse spleen cells cultured with live *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) results in induction of IFN-γ by NK cells stimulated, in culture, by the pro-inflammatory cytokines (IL-12, IL-18 and TNF-α) produced from infected macrophages, early in the response. By 5 days the action of this IFN-γ promotes IFN-γ production from antigen-sensitised Th1 cells. So this is an assay for both the innate IFN-γ response and the primary antigen-specific response.

This *in vitro* system also provided an ideal way to test if Ro11-3128 and/or solvents used in the preparation of the Ro11-3128 stock solution, DMSO or ethanol, would have a damaging effect on lymphocytes in culture. This was important to determine as experiments had been planned to extend the work of Smith et al. (1994) by co-culture of living drug-treated parasites directly with sensitized cells which it was hoped would provide a more sensitive way of looking at Ro11-3128 effects on antigen presentation.

Three experiments were conducted testing the effects of a range of concentrations of Ro11-3128. The stock drug had initially been dissolved in DMSO, and in the first experiment the final concentration of DMSO was 1:1000 but in the second experiment the concentration was lowered to 1:16000. In both experiments the dominant cytokine produced from the BCG-stimulated cells was IFN-γ. No IL-4 was detected in any of the

cultures. In both experiments, cell cultures containing BCG showed the expected increase in IFN-γ production. The presence of 1:1000 DMSO markedly reduced the IFN-γ production, but the presence of Ro11-3128 appeared to overcome this DMSO effect in a dose-dependent manner up to 1.25μg/ml at which concentration the IFN-γ production was comparable to the non-DMSO treated cultures. The negative effect of 1:1000 DMSO was overcome by reducing the concentration to 1:16000 in a subsequent experiment. In fact this concentration had an enhancing effect on IFN-γ production with higher levels of IFN-γ production in cultures containing BCG/DMSO/cells than in cultures containing BCG/cells only. At this DMSO concentration, Ro11-3128 had no significant enhancing or inhibitory effect at 0.31 -2.5 μg/ml but at 5-10μg/ml the IFN-γ production was significantly reduced.

From these results, two conclusions were reached: (i) within the range of concentrations of Ro11-3128 from 0.31-10μg/ml there was no evidence of a stimulatory effect of Ro11-3128 but at 5-10μg/ml there was an inhibitory effect. (ii) for the purposes of using drug-treated schistosomula as antigen sources, the use of Ro11-3128 at 1.25μg/ml and final concentrations of DMSO of 0.00625% was the optimum, producing good production of blebs, reasonable parasite viability but not inhibiting the splenocyte production of IFN-γ by BCG. Because DMSO itself had either inhibitory or stimulatory effects on the BCG/splenocyte system depending on the concentration, ethanol was also used as the solvent for Ro11-3128. A concentration of 0.0125% had no effect on IFN-γ production in the BCG/splenocyte cultures and using this it was also shown that concentrations of Ro11-3128 from 0.31-2.5μg/ml there was no evidence of a stimulatory effect of Ro11-3128 but at 5-10μg/ml there was an inhibitory effect. Interestingly non-specific stimulation with Con A was not affected by any of the drug concentrations, suggesting that high concentrations of Ro11-3128 were having an effect at a different point in the pathway to induction of IFN-γ rather than simply being cytotoxic to lymphocytes.

In conclusion to these BCG/macrophage studies there was no evidence that Ro11-3128 either caused spontaneous production of IFN-γ in culture or promoted the generation of the Th1 response following prolonged BCG co-culture with spleen cells. So there was no evidence that the drug has an adjuvant effect at least using this *in vitro* system. It

would be possible to test if the drug affected *in vivo* immune responses by looking at the response to a defined antigen or to a live antigen such as BCG but this was not followed up in the present studies.

Does Ro11-3128 promote antigen presentation to sensitized cells?

To assess if Ro11-3128 induces enhanced production/release of antigenic material (possibly including protective antigens) which is relevant to the *in vivo* RoNI immunization, a system was set up to culture living schistosomula with SLN cells from RoNI immunized mice and to look at the effects of addition of Ro11-3128 on cytokine production. This was based on the work of Smith et al., (1994) who cultured schistosomula with macrophages and then used these as antigen presenting cells (APCs) with lymph node cells from mice given crude antigen plus Complete Freunds adjuvant. They reported that Ro11-3128 enhanced proliferative responses in this system. The system used here was considered an improvement since RoNI sensitised LN cells were used directly in cultures with living schistosomula (± Ro11-3128) together with bm-DCs (as APCs) and the effect of drug-treatment was assessed by production of IFN-γ which has been implicated in protection in Chapter 3.

Quite a lot of effort went into developing the conditions for preparation and culture of the schistosomula. Initially use was made of M169 (Basch, 1981) a complex medium capable of supporting the *in vitro* development of a high proportion of larvae into adult worms but not suitable for culture of murine cells. For co-culture of parasites with mouse SLN cells, however, use of DMEM throughout proved to be the best compromise; the schistosomula proved to be less healthy if they were initially set up in M169 but then transferred to DMEM when the SLN cell co-cultures were set up. In terms of parasite viability DMEM was superior to RPMI-1640 although both fine for mouse cells. Various methods were tested for production and purification of mechanically transformed schistosomula by vortex shearing (Ramalho-Pinto et al., 1974) or needle passage (James & Taylor, 1976) of cercariae for breaking the cercarial heads from their tails; repeated orbital shaking in Petri-dishes (Basch, 1981) or sedimentation, or density centrifugation on Percoll gradients (Lazdins et al., 1982) for separation of larval heads and tails. Needle passage and gradient centrifugation proved the best methods in terms of purity and viability of the larvae and production of sterile

preparations. Schistosomula produced in this way, followed by 5 washes in E/LAC containing 300U/ml Penicillin, 300µg/ml Streptomycin and 160µg/ml gentamicin (see section 2.4) did not get contaminated with bacteria or fungi. In the co-culture experiments described, aliquots of the schistosomula were cultured alone for 1-2 weeks to ensure that there was no bacterial or other growth among the parasites, which might have been hidden in the co-cultures due to phagocytosis/ attachment by macrophages or bm-DCs.

Use of such living schistosomula as sources of antigen in *in vitro* recall experiments has not been reported previously. These co-culture experiments are aimed at mimicking the interactions that might occur between drug treated schistosomula and DCs as antigen presenting cells in skin/SLN *in vivo*. In the Ro11-3128 model it is supposed that the skin Langerhans cells (LC) would be required for antigen transfer from the skin to the draining lymph nodes and for the priming of the primary T-cell response at that site. Any Ro11-3128 attenuated schistosomula reaching the SLNs would be expected to make intimate contact with SLN DCs and would boost the response primed by LC carrying antigen from the skin.

Released antigen and the dying/blebbing parasites themselves would be expected to make contact with epidermal LC (Wang et al., 1999; Jakob et al., 2001). LCs express a range of myeloid markers, including C11b, stains low for CD8 and has high surface levels of DEC-205 (Shortman & Liu, 2002). After contact with antigen in the skin the LCs move to the SLNs where they express high levels of langerin a characteristic marker of epidermal LCs, and these are believed to be the mature form of the LC (Henri et al., 2001). One cell surface molecule that is decreased on LN LC compared to epidermal LC is E-cadherin (Borkowski et al., 1994) ; Schwarzenberger & Udey, 1995) which has led to the speculation that decreased E-cadherin expression may be one mechanism permitting LC to depart the epidermis. Upon reaching the LNs , LC have typically differentiated into cells that express higher levels of MHC II and costimulatory molecules like CD80, CD86 (Larsen et al., 1994; Kawamura & Furue, 1995), rendering them more effective at stimulating the proliferation of naive Ag-reactive T cells they encounter in the LN.

Studies on myeloid DCs are difficult because they are rare in the body tissues and therefore isolation procedures are time consuming and cell yields are low (Williams et al., 1994). In mice about $6x10^4$ LCs can be isolated from the epidermis of one ear (Ortner et al., 1996) and about $1\text{-}10x10^5$ DC from one spleen or thymus (Vremec et al., 1992; Inaba et al., 1998). However, $1\text{-}3x10^8$ bm-DCs per mouse can be produced after 10-12 days using mouse bone marrow cells and GM-CSF (Lutz et al., 1999). Such DC are now commonly used and were the ones used in these studies.

In the co-culture experiments (Figs 4.11-4.14) the first point of interest is that addition of 500 parasites/ml (±Ro11-3128) to naïve SLNs ± bm-DC did not stimulate detectable levels of IFN-γ at least within the 3 days of culture. In similar kinds of experimental set up certain other organisms notably the intracellular organisms such as *Trypanosoma cruzi* (Aliberti et al., 1996) or *Burkholderia pseudomallei* (Lertmemongkolchai et al., 2001) can be shown to induce IL-12 and/or IL-18 from macrophages which then induces IFN-γ production by naïve spleen cells. Thus although irradiated or drug-treated schistosomula clearly induce a biased Th1 response characterised in the irradiated infection by IFN-γ production within days of presentation *in vivo* (Hogg et al, 2003; Hogg et al., 2003b) it was not possible to demonstrate interactions with naive cells of the innate immune system leading to IFN-γ production in the *in vitro* system studied. The work in Chapter 5 looks in more detail at activation markers in DC/schistosomula interactions.

In the presence of immune cells, added as a sensitive readout of altered antigen presentation, soluble larval antigen (DOC-SCAP) could induce IFN-γ production by the sensitised SLN cells alone (as in Chapter 3) although this was enhanced by the addition of bm-DCs. In contrast, the living schistosomula were unable to induce IFN-γ production by SLN cells alone but the addition of bm-DCs did induce IFN-γ production in some circumstances in the three experiments (Figures 4.11.-4.14.). Comparing normal and Ro11-3128-treated schistosomula, non-drug-treated schistosomula induced IFN-γ production in the presence of bm-DCs in the first experiment but this was somewhat less than in the presence of the drug. In the second experiment both normal and drug treated schistosomula worked equally well whereas in a third experiment the drug treated parasites induced significant IFN-γ production whereas the normal

schistosomula did not. So in two of the three experiments there was evidence that Ro11-3128 treated schistosomula are better able to present antigen to DCs than non-treated schistosomula.

In the experiments of Smith et al. (1994) using fixed macrophages as APCs, T cell activation (measured by the production of IL-3/GM-CSF i.e., stimulatory activity of T cells) was demonstrated using material recovered from drug-treated schistosomula and this effect was removed by filtration suggesting that it was the particulate material that was the stimulus. In the present experiments no effect on IFN-γ production from RoNI sensitised cells using material recovered from schistosomula treated with native drug was observed. The most obvious difference between these experiments was the use of fixed macrophages in one situation and bm-DCs in the other. Banchereau et al., (2000) stated that DCs have the unique ability of presenting antigens to naïve T cells, whereas macrophages and B cells can only activate primed T cells. In this regard it could be argued that macrophages are a more suitable cell for use in recall responses, but the experiments here were aimed at looking at altered antigen release by Ro11-3128 which might explain enhanced priming of the immune response during RoNI, and the use of sensitized cells was as a read out of any altered antigen release. Other differences were the use of SDD by Smith et al., (1994) which may have altered the antigen production and the use of crude larval antigen in CFA to produce sensitized cells which is likely to have primed responses against many different antigens from RoNI.

It is probable that the enhanced IFN-γ production in the presence of bm-DCs is due to increased antigen presentation by the bm-DCs to supplement the resident APCs from the SLN preparations, but in theory they could also be providing stimulatory cytokines to presentation by other cells in the culture. IL-12 positive DCs have been demonstrated in the skin of mice vaccinated with GI (Hogg et al., 2003). In a preliminary experiment addition of anti-IL-12p70 mAb to the cultures reduced the level of IFN-γ produced but not to a significant extent.

In vivo the schistosomula in the skin are presented with a much more complex set of cell types and immunological components. Apart from direct effects of the drug on the nature and amount of antigen released from the parasites and presented to antigen

specific T cells, it is possible that drug treatment could alter interactions between the parasite and cells of the innate immune system. Previous studies showed that the infective cercarial stages of *S. mansoni* had the capacity to produce a wide range of eicosanoids, including the arachidonic metabolite PGE2 (Fusco et al., 1985; Salafsky & Fusco, 1987). In addition, Ramaswamy et al., (2000) reported that cercariae produced significant quantities of PGE2 following incubation with linoleic, a free fatty acid found on the surface of the skin. The parasites also induced PGE2 and IL-10 from human and mouse keratinocytes. The skin stage schistosomula was also reported to have same effect on keratinocytes, as was observed in the analysis of mouse skin. In the skin of IL-10-deficient mice a prominent cellular reaction occurred around the parasite, and there was considerable delay of the parasitic migration through the skin (Hogg et al., 2003b). Irradiation inhibits this induction resulting in lower IL-10 production. It is possible that drug treatment inhibits the anti-inflammatory strategies developed by the schistosomula. This might explain the strong induction of IFN-γ responses in the SLN of mice exposed to GI and particularly to Ro11-3128 abbreviated infections, but would not explain the fact that comparable responses that were induced in the SLN of both RoNI and RoGI do not result in comparable levels of protection (Chapter 3).

The nature of the antigens released by the living schistosomula which stimulate the IFN-γ production seen *in vitro* is not known. A number of antigens are known to be released from normal schistosomula both *in vitro* and *in vivo*. Membrane proteins labelled with iodine have been shown to be lost *in vivo* much more rapidly than during *in vitro* culture (Pearce et al., 1986). Antigens are also lost from the larval surface during *in vitro* culture. Some surface antigens have been shown to be glycosylphosphatidylinositol (GPI)-anchored to the membrane (Sauma et al., 1991). Molecules released from schistosomula *in vitro* have been shown to include antigens recognized by IgE from immune rats (Auriault et al., 1984), including a 26,000 MW antigen. In addition to the release from the schistosomula surface of membrane-attached antigens, certain enzymes with a normally cytosolic location have been reported to occur at the larval surface and have been implicated in protection e.g. Glutatione-S-transferase (GST) (Taylor et al., 1988), triose phosphate isomerase (TPI) (Harn et al., 1992) and glyceraldehyde-3-phosphate dehydrogenase (Goudot-Crozel et al., 1989). Their apparent lack of membrane-attachment structures and, in the case of GST and

TPI, their transient appearance at the larval surface, suggests that such antigens would be released during *in vitro* culture. It is likely that a large number of antigens released by living schistosomula are able to stimulate IFN-γ production by RoNI SLN cells, but as candidate vaccine larval antigens are identified by proteomic or genomic approaches and become available by recombinant DNA techniques their ability to induce strong IFN-γ production by such cells would give strong support to their possible protective potential and use in a vaccine to mimic the attenuated larval vaccines (James et al., 1986 Coulson, 1997; Chapter 3).

In conclusion, the results in this Chapter show that the normal and 20krad schistosomula have comparable levels of production of membraneous blebs in response to Ro11-3128 *in vitro* indicating that this altered antigen presentation itself is not solely responsible for inducing high levels of immunity. This was further demonstrated by the failure to induce protection by i.d. injection of drug treated freeze/thawed parasites. The conclusion reached in Chapter 3 was that the particular efficacy of RoNI compared with RoGI relates to greater persistence of the antigenic stimulus possibly due to greater longevity of the non-irradiated parasites. It would be very interesting if this could be confirmed and also the morphological state of the parasites e.g. do the drug treated schistosomula reported in the SLN show persistent production of membraneous blebs? Addition of Ro11-3128 to an *in vitro* culture system in which BCG activates spleen cell cultures and which involves macrophage, NK cell and Th1 cell activation leading to IFN-γ production failed to show enhancement of these effects which did not support the idea that adjuvant activity accounts for the high levels of Th1 cell stimulation in the SLN of both RoNI and RoGI. However, there are many other points in generation of an immune response that were not represented in this culture system and direct testing *in vivo*, for example the response to BCG could be of interest. The development of the co-culture protocol for use with immune cells, DC and live schistosomula showed the enhancing effects of adding bm-DCs to the culture and this prompted studies into effects of schistosomula on bm-DC maturation (Chapter 5). Schistosomula, with or without Ro11-3128 did not induce production of IFN-γ from cultures of naive SLN cells with or without bm-DC, unlike certain other organisms which induce Th1 responses, which fails to demonstrate a Th1 priming signal from the living larvae. However, using recall responses by RoNI-sensitized SLN cells there was evidence that the drug

treatment can enhance antigen presentation and this could contribute to the high levels of Th1 responsiveness seen in the SLNs of both RoNI and RoGI mice. To further investigate this possibility further comparisons could be made of the SLN responses to infections terminated with Ro11-3128 or Ro15-5458 which kills larvae in the skin but does not induce the obvious morphological changes to the membrane and does not induce immunity.

CHAPTER 5

IN VITRO INTERACTIONS OF *S. MANSONI* SCHISTOSOMULA WITH BONE-MARROW DERIVED DENDRITIC CELLS

5.1. Introduction:

The studies in Chapter 3 had shown that a Th1 biased response (IFN-γ >> IL-4) was induced in mice following exposure to large numbers of larvae whether or not they were attenuated (γ-irradiation or the drug, Ro11-3128) although the attenuated infections induced markedly higher IFN-γ responses in the SLN and spleen. This is consistent with the earlier demonstration of a Th1 cytokine response during the prepatent period of *S. mansoni* infection even with low numbers of larvae (Pearce et al., 1991) and the demonstration of a skewed Th1 response in the SLNs 18-23 days following irradiated infections (Pemberton et al, 1991).

In Chaper 4 the idea that enhanced antigen production/release caused by the effects of Ro11-3128 on the larvae might underlie the high efficacy of the drug in inducing immunity led to *in vitro* studies in which normal or drug-treated schistosomula were used to re-stimulate cytokine responses from sensitized T cells. These studies showed that when SLN cells from mice exposed to RoNI were co-cultured with schistosomula, IFN-γ production could only be detected when bm-DC were added to the cultures. It is believed that the antigen release caused by Ro11-3128 may facilitate antigen loading of DC, and/or stimulate their functional differentiation in a manner which facilitates protective immunity. No previous experiments have been reported on the effects of schistosomula on DC function but, using *in vivo*-activated peritoneal adherent cells as APC Smith et al. (1994) previously showed that treatment of schistosomula *in vitro* with Ro11-3128 results in enhanced antigen presentation during co-culture of the fixed cells with T cells from *S. mansoni* antigen sensitized mice. Furthermore, in these experiments the blebs and other antigens released by Ro11-3128-treatment could be separated from the otherwise intact schistosomula and also caused enhanced APC induced IL-3/GM-CSF production from sensitized T cells. Most of this APC activity was induced by the particulate fraction as shown by its removal by filtration. In this paper nothing was reported on studies about if and how the macrophages were activated.

The studies in this Chapter were initiated to extend these observations using DC, which are now known to play the central role in antigen presentation (Kelsall et al., 2002). Encounter of immature myeloid-derived DC with microbial antigens in peripheral tissues is believed to have two main consequences. The engagement of 'pattern recognition' receptors (PPR) on DCs initiates the process of maturation which include alteration in cell surface phenotype (e.g., CD80, CD86, CD40 expression) and of sub-cellular MHC class II trafficking. The latter facilitates interaction with microbial antigens, and stabilises newly formed MHC-peptide complexes at the plasma-membrane for subsequent presentation (Pierre et al., 1997; Cella et al., 1997). In addition, maturation is accompanied (and may be facilitated) by DC migration to the T-cell zones of secondary lymphoid organs (Pierre et al., 1997; Randolph et al., 1998). Here they encounter naïve T cells and are responsible for primary T cell activation (Ingulli et al., 1997). DCs are also responsible for directing Th cell development along the Th1 and Th2 pathways. There has been increasing evidence that the local conditions of DC maturation, such as prostaglandin E2 may downregulate the capacity to produce IL-12, so biasing towards Th2 development (De Smedt et al., 1997; Vieira et al., 1998). Identification of microbial characteristics which may modulate DC into cells capable of biasing such differentiation of T cells is critical to understanding the host's immunity.

Two major DC subsets that differ in both CD8 expression and localization in different regions of the lymphoid organs have been reported to have different functions in the induction of T cell responses (Vremec et al., 1992; Pulendran et al., 1997). $CD8\alpha^+$ DC have been suggested to be specialized for promoting Th1 responses and $CD8\alpha^-$ for Th2 responses (Maldonado-Lopez et al., 1999; Pulendran et al., 1999), however, subsequent studies have shown that distinct DC subsets are able to promote either type of response depending upon pathogen-derived signals and host-derived cytokines present in the microenvironment (Vieria et al., 2000; De Smedt et al., 2001; MacDonald et al., 2001: Maldonado-Lopez et al., 2001).

In vivo studies of the GI model have indicated that IL-12 is an important cytokine in induction of the IFN-γ mediated immunity (Wynn et al., 1996; Anderson et al., 1998). Hogg et al. (2003) have reported finding CD11c+ve DC within the inflammatory site induced in the skin post infection and shown persistent IL-12 production in the skin

following GI as compared with unattenuated infections. They suggest that the majority of the IL-12+ve cells trafficking from cultured isolated skin sections following GI are myeloid DCs perhaps migrating Langerhans cells although macrophages were also represented. In the RoNI model, immuno-histochemical analysis, in collaboration with Dr Chris Engwerda, showed that IL-12+ve cells with DC morphology can be found in the SLNs 6 days following infection. In Figure 5.1. such cells are seen in close juxtaposition to a drug-treated larva within the draining lymph node.

Figure 5.1

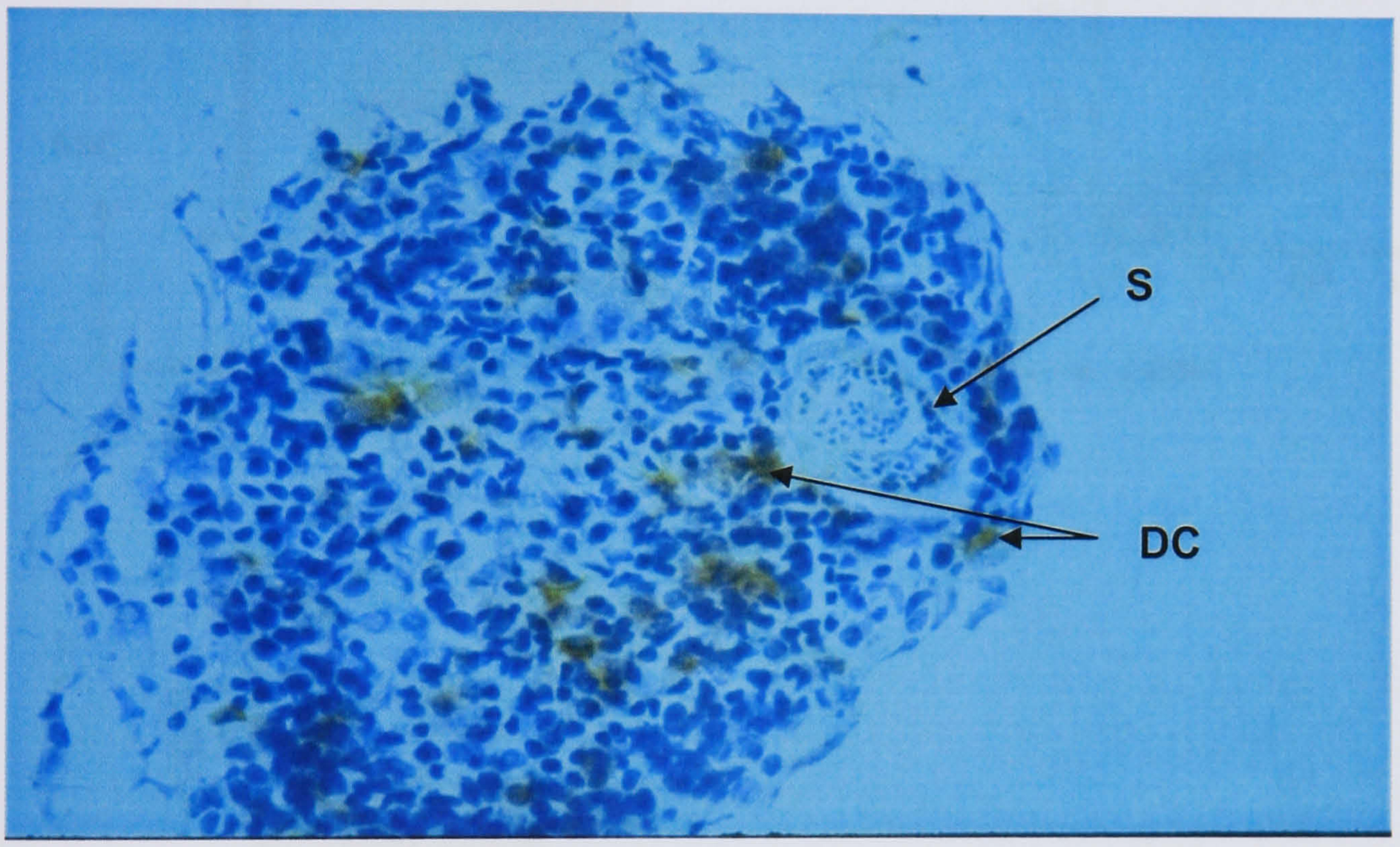


Figure 5.1 Photomicrograph showing IL-12p40+ve, putative dendritic cells (DCs) adjacent to a schistosomulum (S) in the draining popliteal lymph node 6 days following RoNI (Courtesy of Chris Engwerda and Quentin Bickle).

The studies in this Chapter were initiated to investigate the interaction of bm-DCs, which are of myeloid origin, with living normal, drug-treated and/or irradiated schistosomula *in vitro* to see if such DCs can be activated towards a typical Th1-promoting phenotype.

5.2. Results:

Activation of DCs is associated with upregulation of a number of surface markers including MHCII and the costimulatory molecules CD86 and CD40 (Banchereau et al.,

2000) and these were used to assess activation in these studies. Figure 5.2 shows the population of cultured bone marrow cells gated for the FACS staining. The CD11c+ve cells in this population were deemed bm-DCs and gated on for analysis of MHCII and CD86/40 expression. The negative staining using the isotype controls is also shown. Examples of positive staining FACS for MHCII and CD86 are shown later in Figure 5.12 .

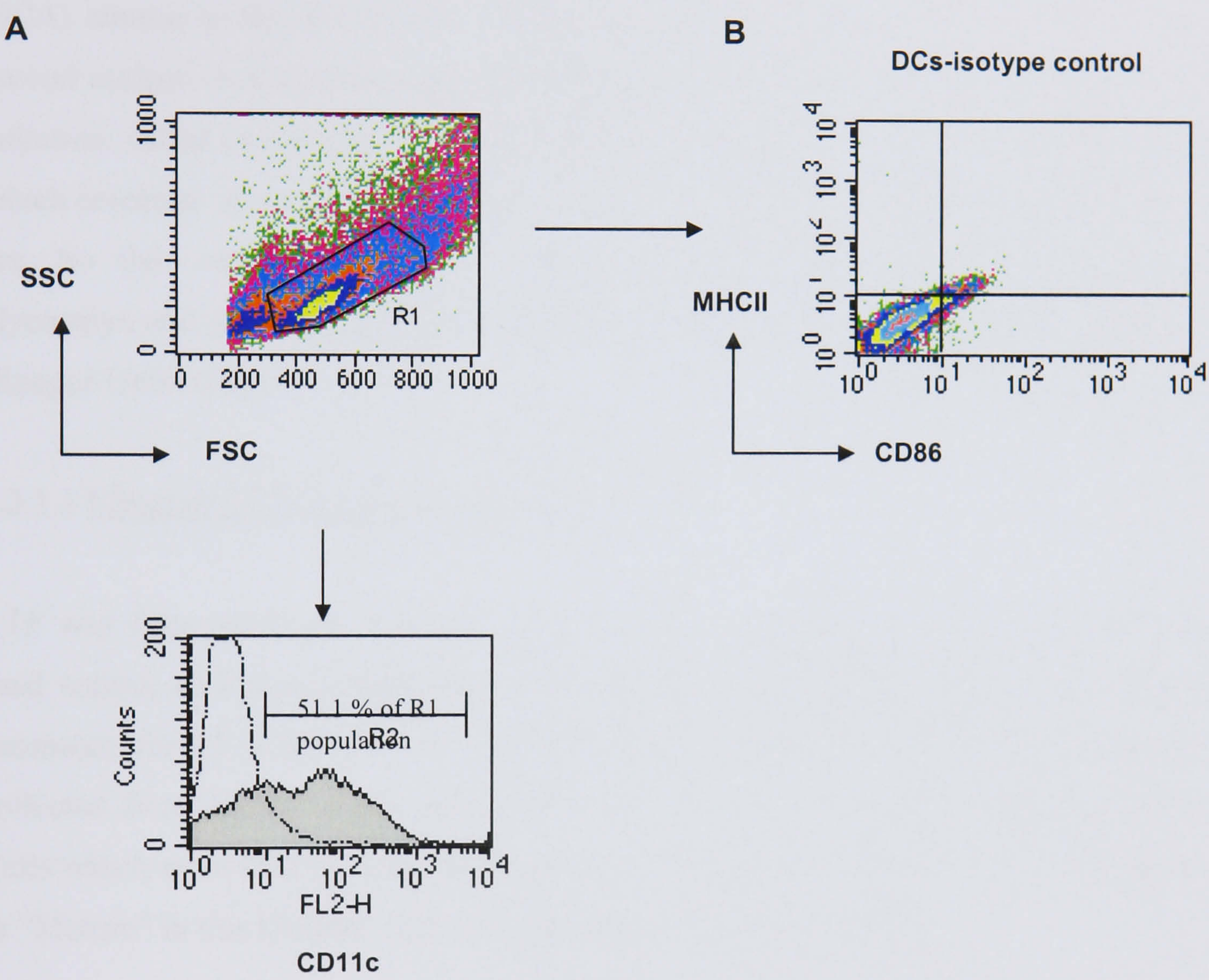


Figure 5.2: Flow cytometry settings gated on the population of bm-DCs. The above settings were used to gate on day 10 bone-marrow derived DCs. **A** shows the R1 population which according to SSC (size of the cells) vs FSC (granularity of the cells) indicates a population where the majority of the cells are bm-DCs. **B** shows a dot plot of the isotype controls for MHCII and CD86. **C** shows that 51% of the R1 gated population in **A** were CD11c+ i.e., bm-DCs (gated as R2). Combining the gates R1+R2 represents the cells that according to size, granularity and CD11c expression are considered bm-DCs.

5.2.1 Effect of soluble antigens released from the skin stage larvae on the maturation of bone-marrow derived DCs:

The work in Chapter 4 had shown that bm-DC could enhance stimulation of sensitized cells by the soluble crude antigens SCAP and DOC-SCAP. So initial experiments in this Chapter were set up to see if soluble larval preparations would induce maturation in bm-DCs. The first type of antigen used was a soluble preparation of whole cercariae (SCA) similar to the SCAP used in other parts of this thesis (see section 2.5). The second antigen was a preparation of molecules released by the parasite at the onset of infection, called cercarial transformation fluid (CTF) which consists of the material in which cercariae are mechanically transformed to schistosomula and then cultured for 3 hrs. So this contains penetration cercarial enzymes, remnants of the cercarial glycocalyx and other antigens. Both of these were kind gifts from Prof. Mike Doenhoff (Bangor University).

5.2.1.1 Effect of CTF antigens on bm-DCs.

CTF was filter sterilized and added at 1, 5, 25 and 125µg/ml (stock = 1mg/ml) to a final volume of 1ml/well containing $5x10^5$ DCs, in 24-well culture plates. These were incubated for 22 hours at 37°C and 5% CO_2, after which bm-DCs were carefully collected from the wells and stained for CD11c, MHCII and CD86 surface markers. Cells which were CD11c +ve, CD86 and Class II high (see Figure 5.3.) are referred to as "Mature" in this Chapter. LPS was included as a positive control.

The naïve bm-DCs showed 36% mature DCs and this was unaffected by the addition of 1µg/ml CTF but with higher concentrations there was a dose-dependent increase in mature cells with 73% becoming activated by the highest concentration of CTF (125µg/ml). LPS (1µg/ml) induced the highest % maturation among DCs of 91%.

Figure 5.3

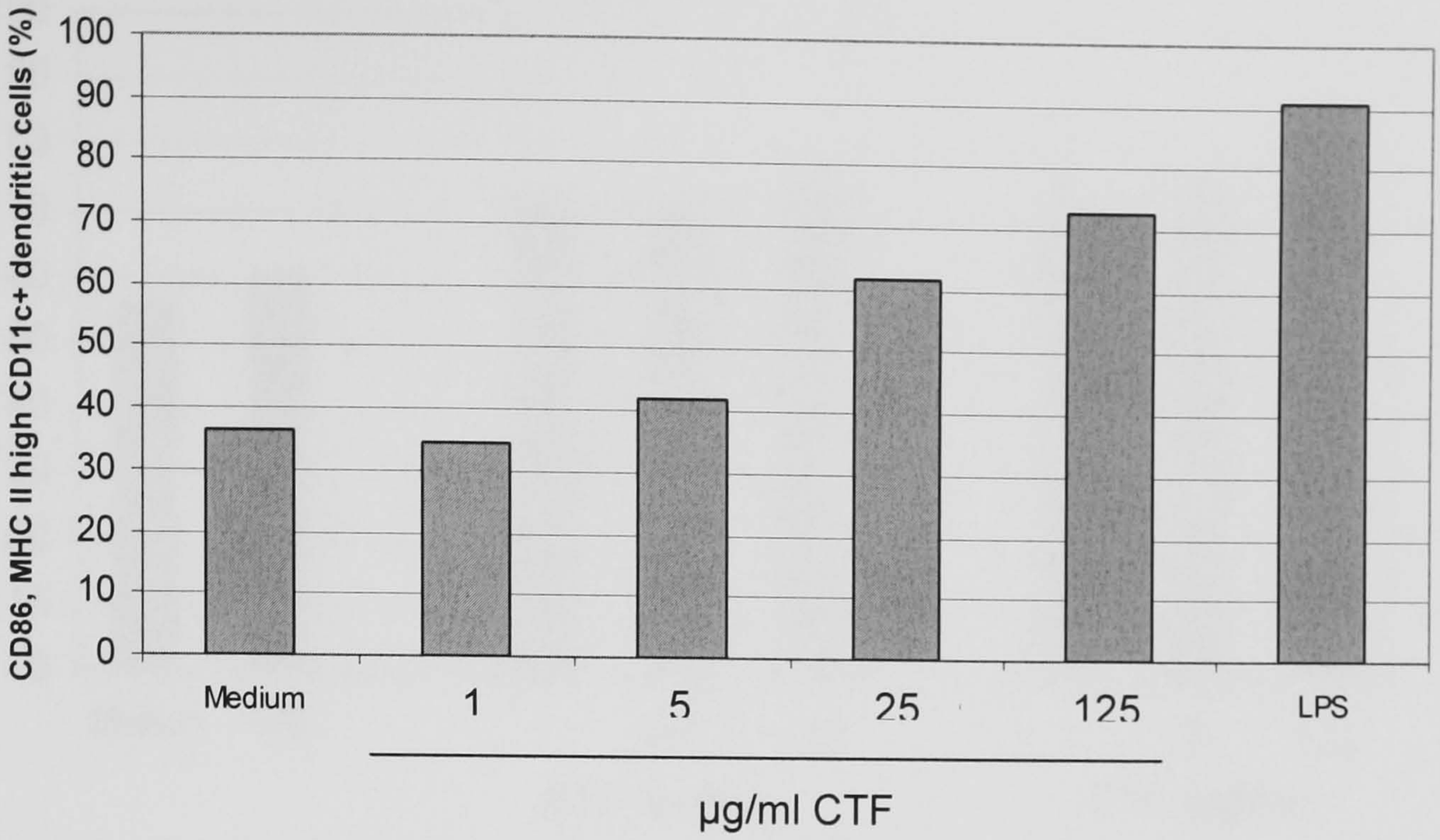


Figure 5.3 Cercarial Transformation fluid (CTF) was added at 1, 5, 25, and 125µg/ml to $5x10^5$ immature day 10 bm-DCs. For positive control, bm-DC cultures were stimulated with 1µg/ml LPS. Cultures were left incubating for 22 hours. Cells were stained for CD11c, MHCII and CD86. Data represents the percentage of CD11c+ cells that were CD86, MHCII high. Data were from single well observations.

5.2.1.2 Comparison of the activation of bm-DCs by whole cercarial antigen (SCA) and secreted antigens (CTF).

The effect of whole cercarial antigen, SCA, was then tested and compared to CTF. SCA was added at the same protein concentrations as the CTF in the first experiment i.e. 1, 25, 125µg/ml (stock =4.9mg/ml). These cultures were incubated for 20 hours at 37°C and 5% CO_2, and the cells were then carefully removed and stained as above. The results are shown in Figure 5.4. A high percentage (56%) of the immature bm-DCs were activated in the medium control and the LPS made little difference to this but the highest percentage maturation was among bm-DCs stimulated with both SCA and CTF at 125µg/ml (76% and 79%, respectively).

Figure 5.4

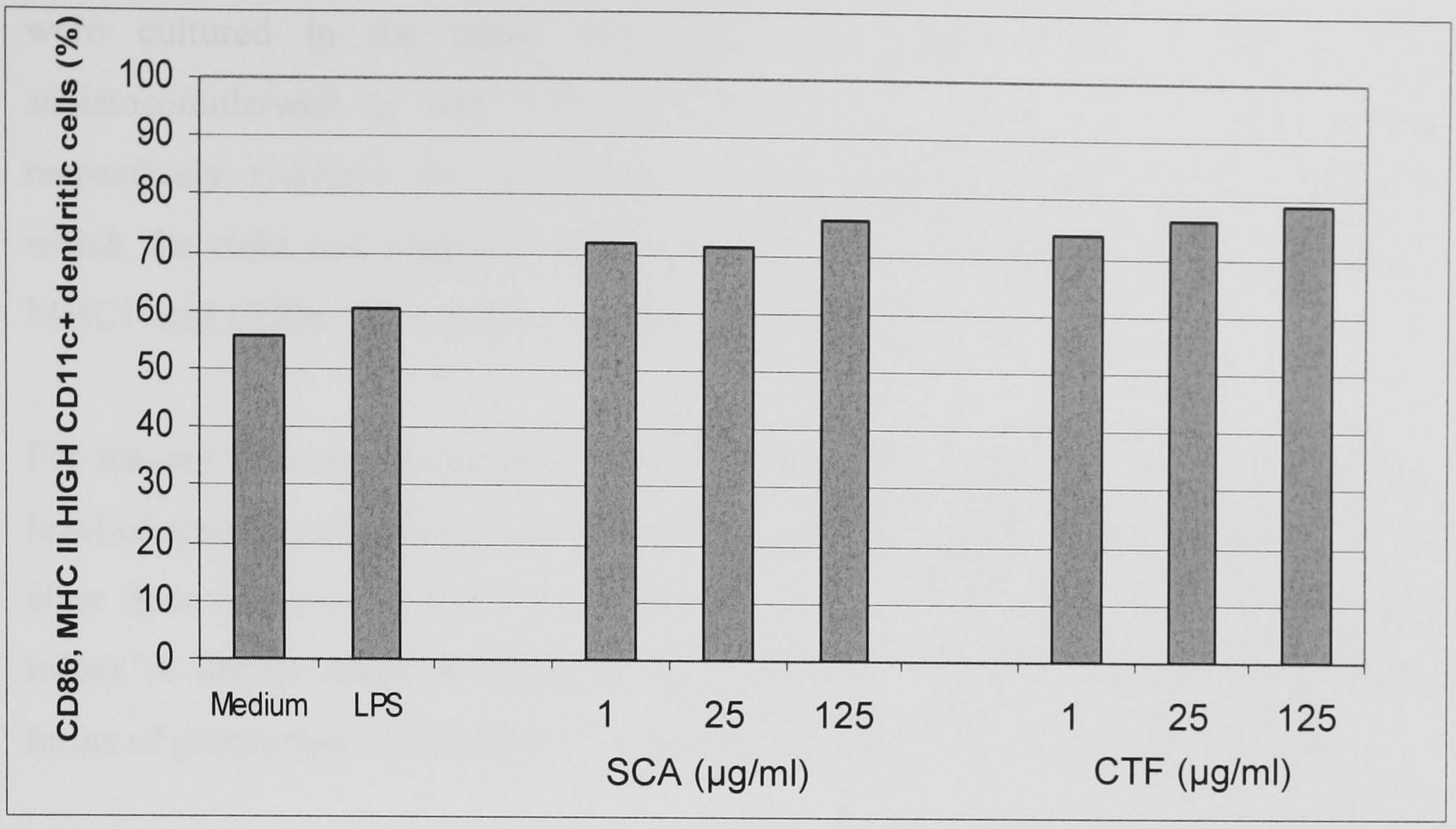


Figure 5.4 Soluble cercarial antigen (SCA) and Cercarial Transformation Fluid (CTF) were added at 1, 25 and 125µg/ml to $5x10^5$ immature day 10 bm-DCs. For positive control cultures bm-DCs were stimulated with LPS (1µg/ml). Cultures were left incubating for 20 hours after which the cells were carefully removed and stained for CD11c, MHCII and CD86. Data represents the percentage of CD11c+ cells that were CD86, MHCII high. Data were from single well observations.

This suggested that the cercariae contain/release antigens that are taken up by bm-DCs and induce their maturation, but the possibility that the preparations might be contaminated with endotoxin derived from contamination of the preparations with bacteria from the snail water into which the cercariae were shed was considered and an experiment was set up to test this.

5.2.1.3 Do parasites and parasite-specific antigens (CTF, SCA) stimulate DCs in the absence of TLR-4 signalling?

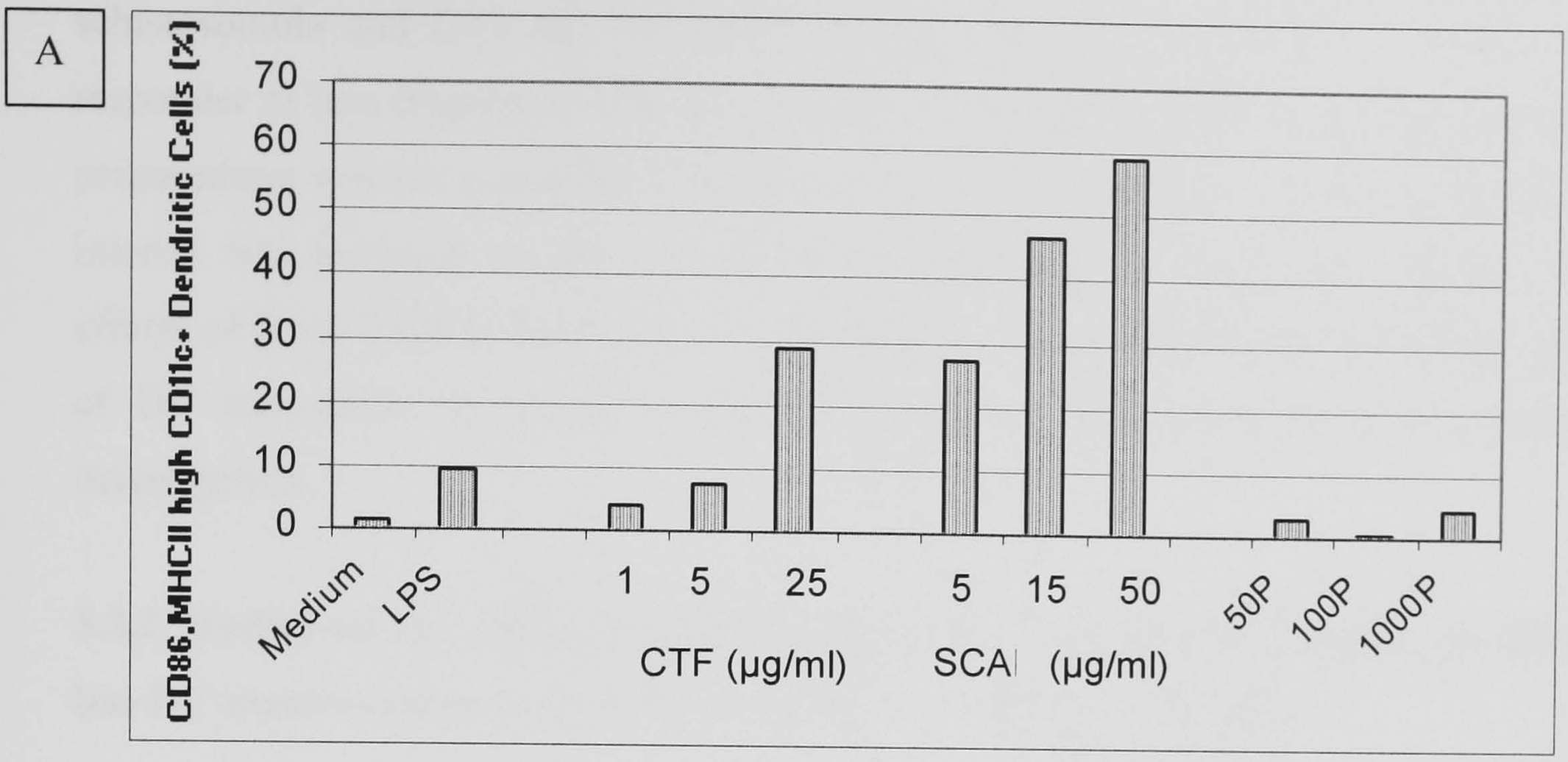
C3H/OuJ mice possess the gene for LPS responsiveness whereas C3H/HeJ mice have a null mutation in the gene encoding for the Toll-like receptor 4 (TLR-4), which is involved in recognition of, and signal transduction from, the LPS receptor complex. This means these mice do not develop rapid inflammatory responses to LPS (Hopkins et al., 1996).

In this experiment, bone marrow leukocytes from female C3H/HeJ and C3H/OuJ mice were cultured in the usual way and then cultured with 50, 100 or 1000 schistosomula/well or with CTF and SCA at 1, 5, 25 and 5, 15 and 50 μg/ml respectively. Cultures were incubated for about 20 hours at 37°C and 5% CO_2, after which the cells and parasites were separated and all cells were stained for CD11c, MHCII and CD86. The results are shown in Figure 5.5.

For reasons that are not apparent the naïve cells in this experiment showed a very low level of spontaneous maturation. However, compared to medium alone SCA induced a clear dose response in bm-DC maturation of normal LPS responder cells (C3H/OuJ mice). A similar response was seen with C3H/HeJ cells but this was much lower in terms of percentage maturation (14% compared with 58% for the responder cells).

CTF antigen, on the other hand, induced some maturation of the C3H/OuJ (Normal LPS-responder) cells but no maturation of the non-responder cells. As for schistosomula, these did not induce any significant bm-DC maturation of either –ve or +ve LPS responder cells. These results suggested that there was endotoxin contamination in the CTF and SCA preparations. Independent testing of these (European endotoxin testing service, Biowhittaker, Inc) showed that the CTF and the SCA had respectively 11.4 and 5.3 EU/ml of endotoxin (IEU=100pg) and it is probable that this contributed significantly to the activation seen. (NB. This experiment was carried out using a different preparation of SCAP from the one used in the rest of the thesis which was prepared at LSHTM. This was also tested and found to have 0.05 EU/ml a value considered acceptable for the restimulation studies).

Figure 5.5



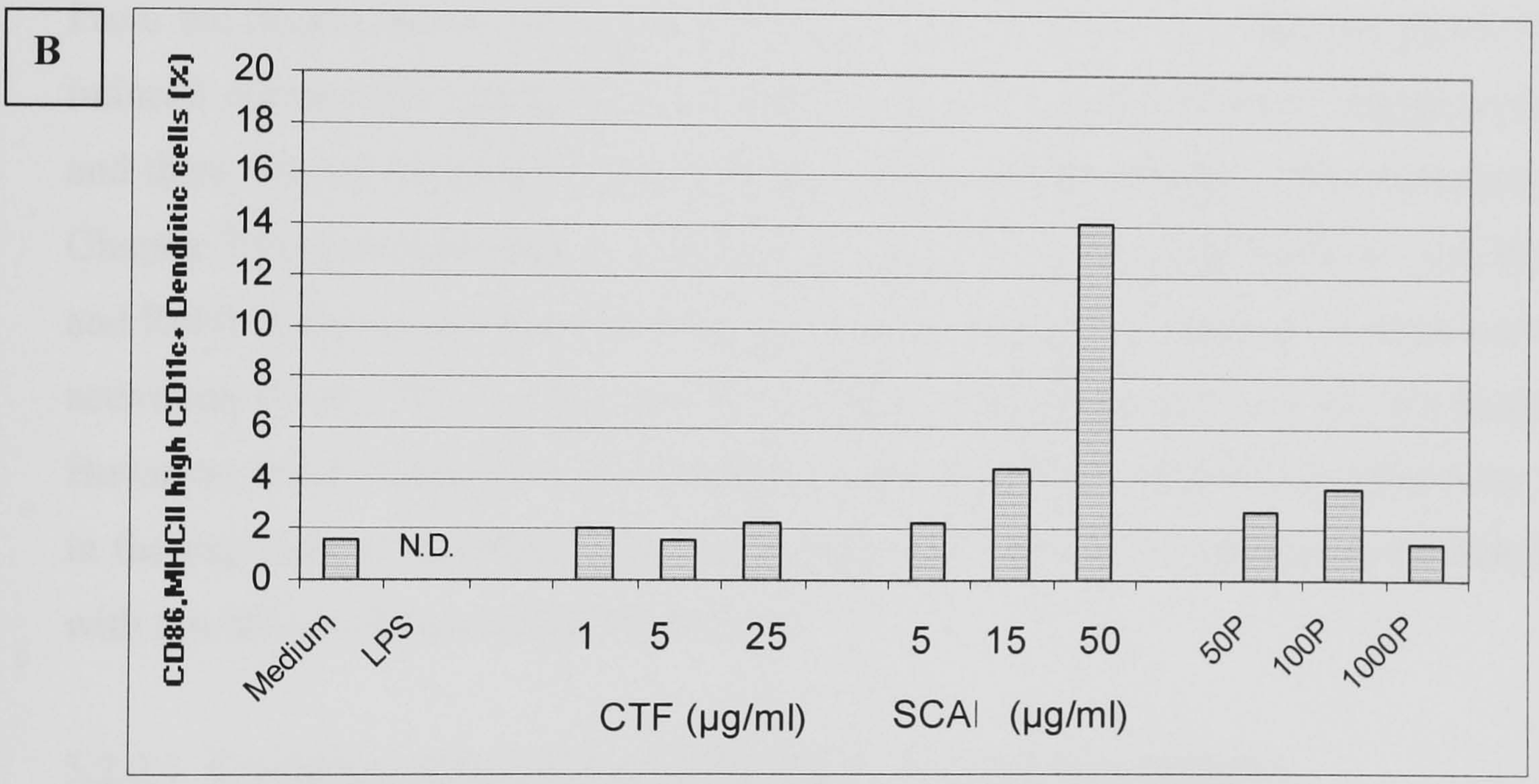


Figure 5.5
(A) Stimulation of C3H/OuJ (Normal LPS-responders) bm-DCs.
(B) Stimulation of C3H/HeJ (LPS-non-responders) bm-DCs.
Live and transformed schistosomula (50,100,1000P/ml) and the antigens CTF and SCA were added to $5x10^5$ immature day 10 bm-DCs/well. For positive control, bm-DC cultures were stimulated with 1µg/ml LPS. Cultures were left incubating for 20 hours, after which cells were collected and stained for CD11c, MHCII and CD86. Data represents the percentage of CD11c+ cells that were CD86, MHCII high. Observations were from single wells. [N.D.=Not Done].

The main value of these studies on CTF and SCA was that they allowed experience with the FACS techniques but also this experiment was also the first one with living schistosomula and DCs and the failure to demonstrate DC activation in either the responder or non-responder cells showed that LPS contamination in these live parasite preparations was not a problem. Further work on CTF and SCA was not continued as interest was focussed on the use of living schistosomula because this allowed the effects of Ro11-3128 to be assessed. Although this first experiment showed little signs of DC maturation by living schistosomula this needed confirmation and further investigation.

5.2.2 Studies on the ability of Drug-treated and/or Irradiated parasites to affect bm-DC maturation as judged by upregulation of surface markers.

From the results shown in Chapter 4 (Figures 4.1 A&B), it was clear that Ro11-1328 induced comparable blebbing from both irradiated and non-irradiated schistosomula, and there was no difference in the overall viability of the parasites. Also the results in Chapter 3 indicate that there is a strong induction of Th1 responsiveness in both RoGI and RoNI at least in the short term post-vaccination indicating that early events such as activation of skin DCs are unlikely to explain the difference between the two models. However, these results were not available at the start of the studies described here. So in the experiment described below both normal and irradiated parasites were cultured with bm-DCs with or without Ro11-3128.

5.2.2.1 Co-culture of bm-DCs with 50,100 or 1000 schistosomula/ml.

Cercariae were irradiated, as described in section 2.3, at an optimum dose of 20krad. Then both irradiated and non-irradiated cercariae were transformed and schistosomula were added at 50, 100 and 1000P/ml/well. Bm-DCs were cultured at $5x10^5$cells/well together with either non-irradiated parasites ± Ro11-3128, or irradiated parasites± Ro11-3128. Control cultures received either no stimulation ± Ro11-3128 or were stimulated with LPS at 1μg/ml. After 22 hours bm-DCs were separated from parasites and stained for CD11c, MHCII and CD86. Photomicrographs showing the appearance of the types of cultures used throughout this Chapter are shown in Figure 5.6. The experiment shown is one in which 10,000 parasites/well were used.

Figure 5.6.

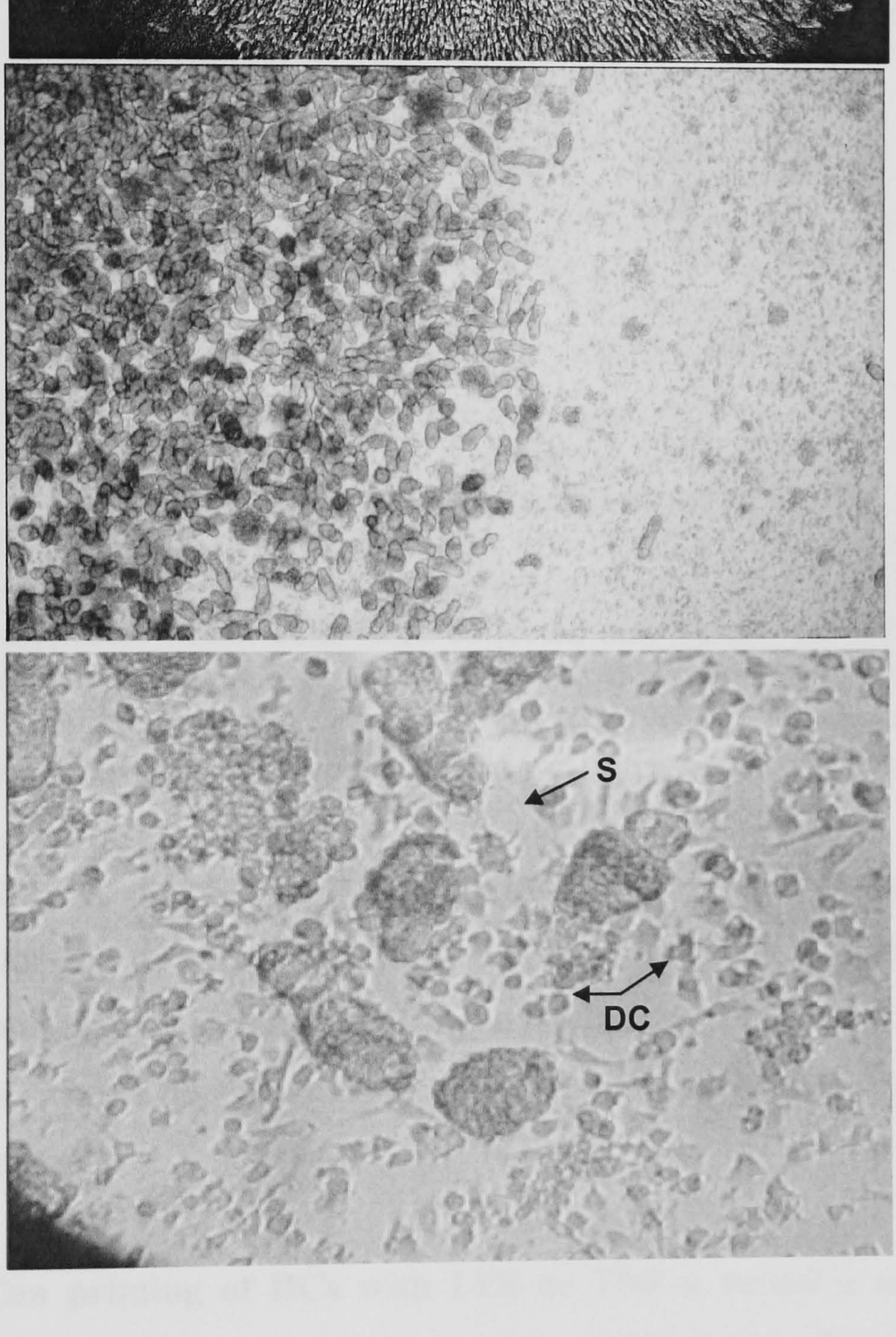


Figure 5.6. Photomicrographs of culture of MS and bmDC. The well shown is of a culture containing 10,000 MS treated with Ro11-3128. The top picture shows the clustering of the parasites at the centre of the flat bottomed well of the 24 well plate. Blebbing parasites (S) and DCs can be seen in the bottom picture.

The results of the DC surface marker expression are shown in Figure 5.7.

Figure 5.7

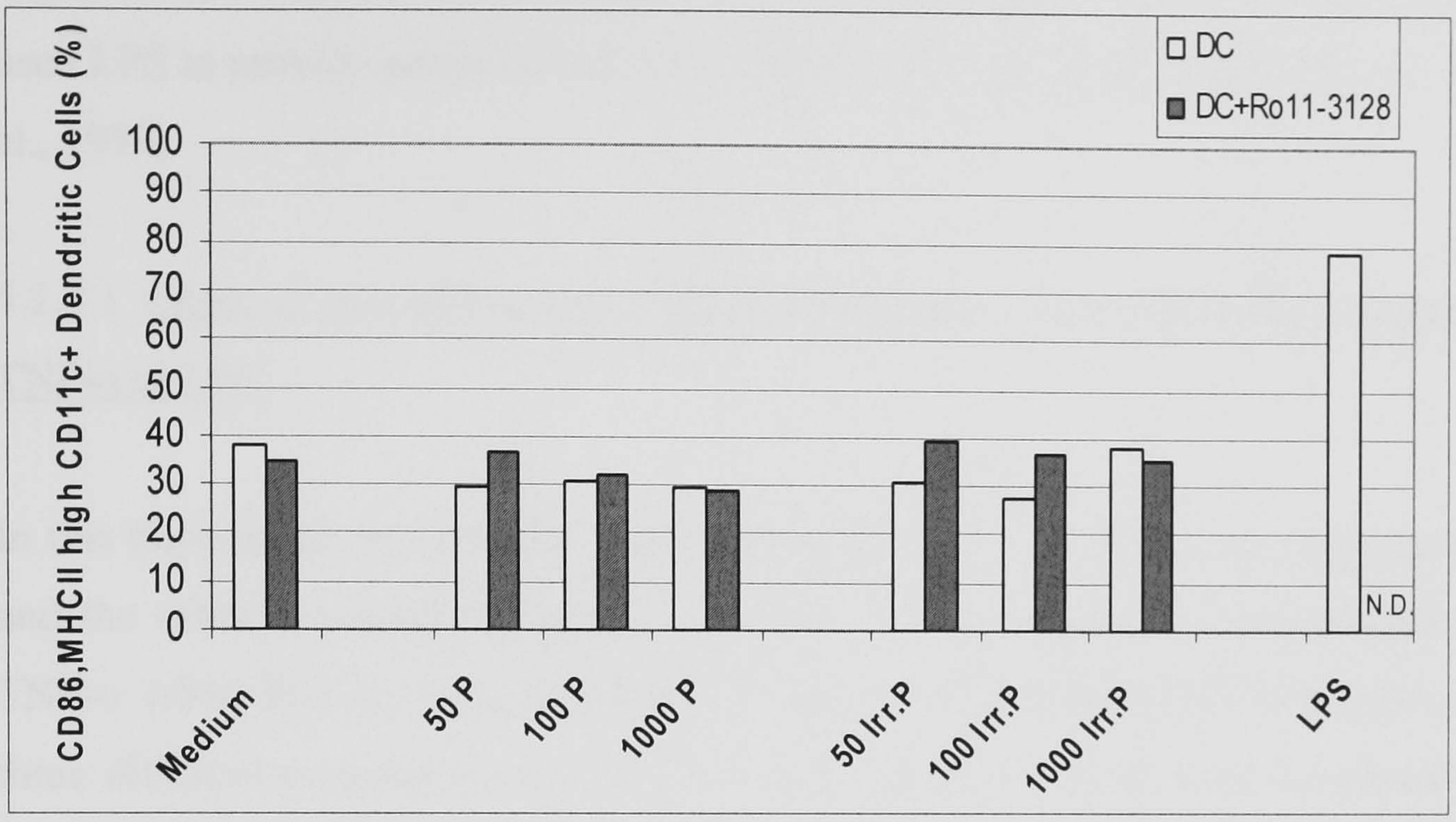


Figure. 5.7 Irradiated and non-irradiated schistosomula (50,100,1000P/ml) were added to $5x10^5$/well immature day 10 bm-DCs from C57BL/6 mice. Ro11-3128, dissolved in 0.0125% ethanol, was added to the appropriate wells at 1.25µg/ml/well. Cultures were left for 22 hours, after which cells were collected and stained for CD11c, MHCII and CD86. Data represents the percentage of CD11c+ cells that were CD86, MHCII high. Observations were from duplicate wells. (Total events [i.e. No. of cells counted by FACScalibur =25,000]).P=non-irradiated schistosomula, Irr.P=Irradiated schistosomula.[N.D.=Not Done].

The naïve DC population showed around 35-38% activation but increasing numbers (50-1000P/ml) of normal or irradiated parasites did not induce noticeably enhanced DC maturation as assessed by upregulation of MHCII and CD86 (compared to controls with LPS, maturation was 78%). Neither did drug treatment lead to upregulation.

5.2.3 Can priming of DCs with LPS or TNF-α reveal a secondary maturation signal from co-cultured MS as judged by upregulation of surface markers?

It was observed from the above results that up to 1000 parasites/ml (whether normal, irradiated or drug-treated) had no effect on activating bm-DCs. Possibilities considered were: that the schistosomula might simply not have the ability to directly activate DCs; that they secrete products (e.g., enzymes, etc.) that inhibit DC maturation; or that a

secondary signal may be required for activation e.g., host derived cytokines produced by other cells of the innate immune system. The possibility that schistosomula may be able to further activate DCs which are already primed by an additional signal was tested below. Various studies on the activation potential of infectious organisms have used LPS to provide partial activation of DCs (e.g., Van Overtvelt et al, 1999; Urban et al., 1999).

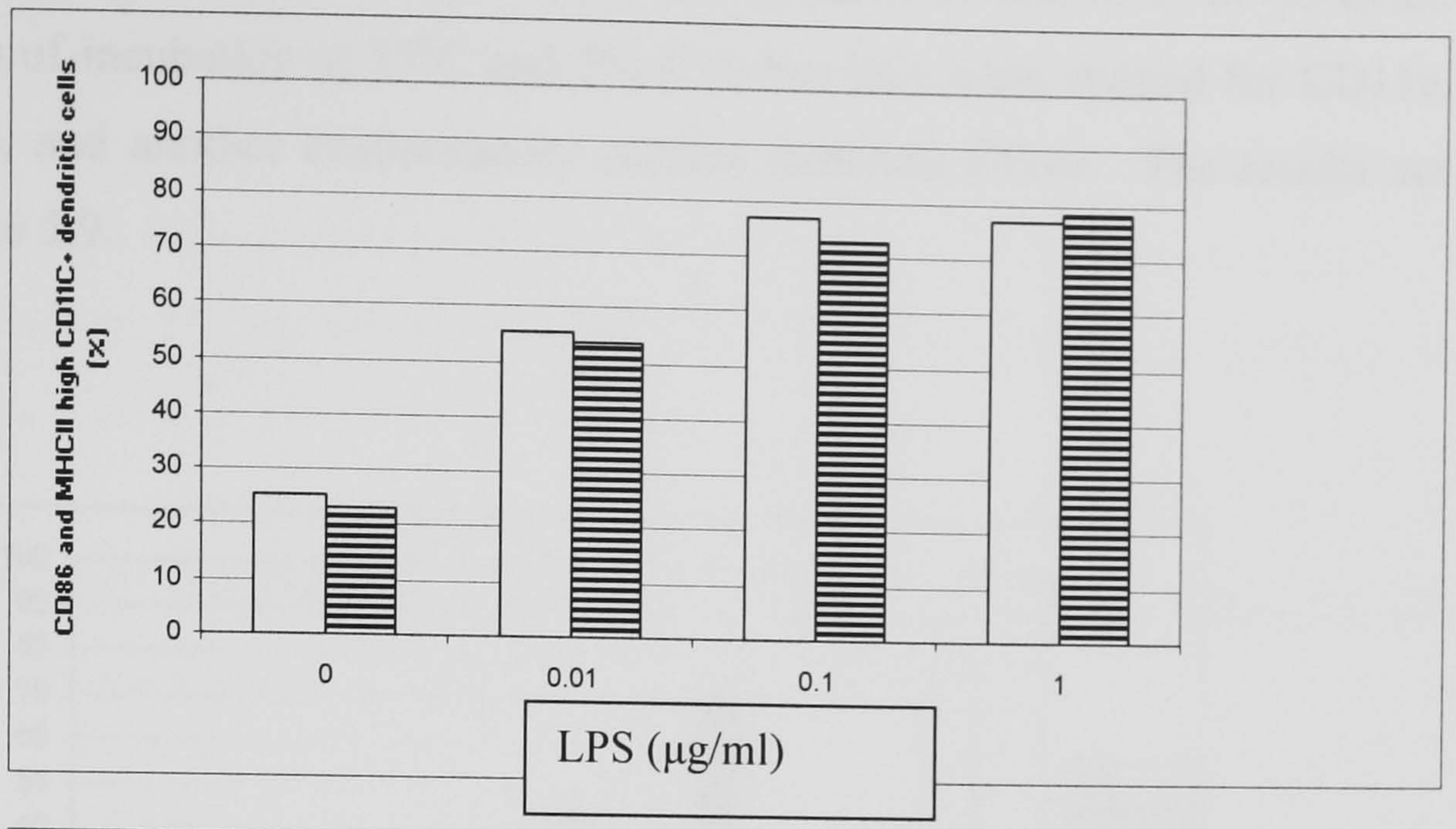
5.2.3.1 Effect of co-culture with 400 schistosomula on bm-DC also activated with TNF-α or LPS.

In this experiment two priming signals were used: one, an activating cytokine TNF- α and the other, a microbial ligand, LPS. Four concentrations of recombinant murine TNF-α (rMu-TNF-α) were tested, 0.5, 5, 50 and 500U/ml, and LPS was also added at three different concentrations, 0.01, 0.1 and 1μg/ml. Cultures were incubated for 16 hours at 37°C and 5% CO_2, after which bm-DCs were collected and stained for CD11c/MHCII/CD86. The results are shown in Figure 5.8.

Bm-DCs showed increasing percentage maturation with increasing concentration of TNF-α (0.5, 5, 50, 500U/ml). Adding parasites to such cultures neither reduced nor enhanced the levels of DC activation. The same pattern was observed when parasites were cultured with increasing concentrations of LPS (0.01, 0.1 and 1μg/ml,). So there was no evidence that 400 schistosomula/ml could (i) provide an activation signal to primed DCs or (ii) inhibit LPS or TNF-α induced DC maturation.

Figure 5.8.

A



B

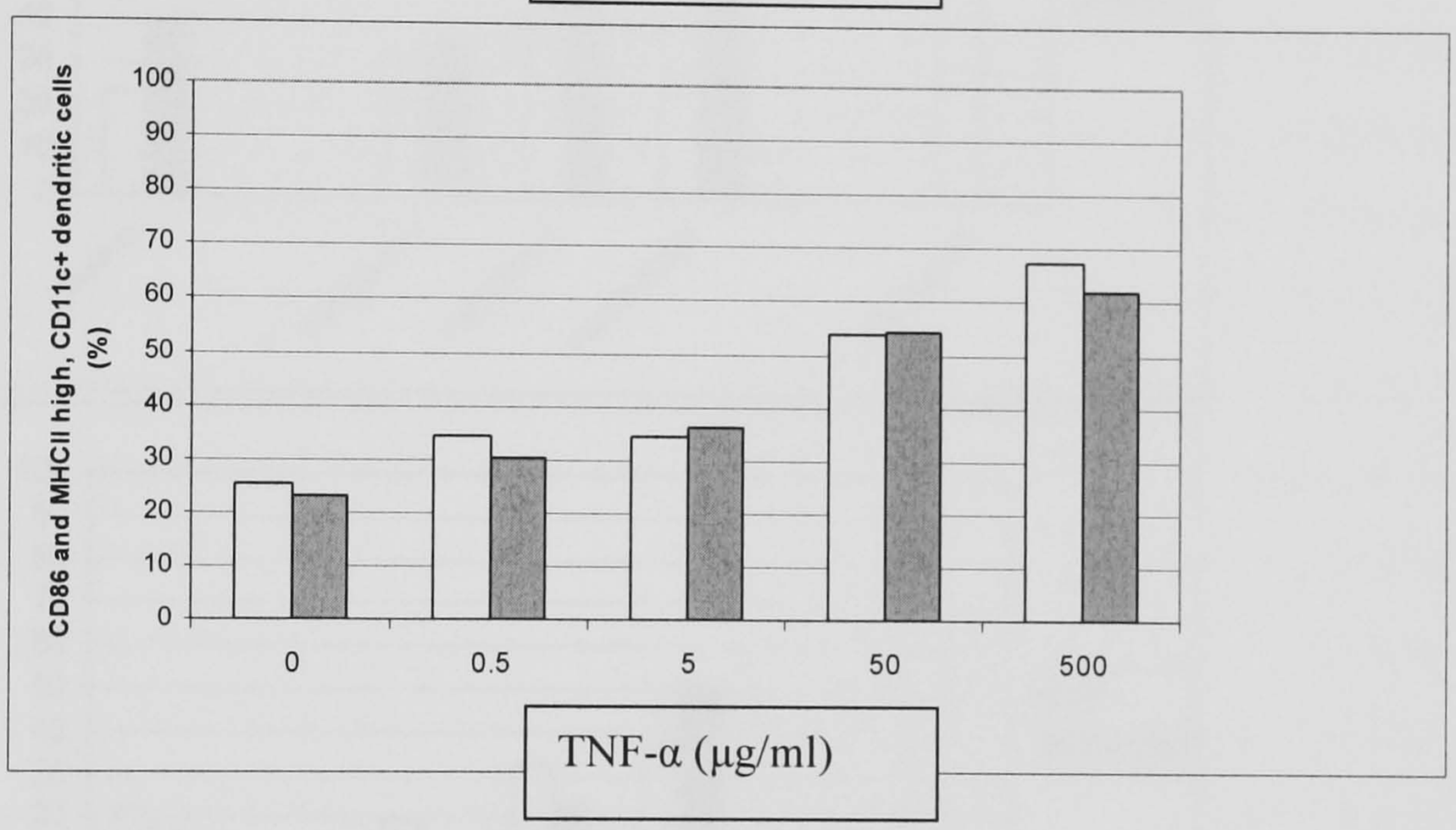


Figure 5.8. (A & B). Live schistosomula (400/ml) were added to $5x10^5$ cells/well of day 10 immature bm-DCs from C57BL/6 mice. **(A)** LPS was added to these cultures at 0.01, 0.1 and 1µg/ml and **(B)** TNF-α added at 0.5, 5, 50 and 500U/ml. Cultures were left incubating for 16 hours, after which they were collected and stained for CD11c MHCII and CD86. Open bars are DC alone and shaded bars DC plus 400 MS/ml. Observations were from single wells.

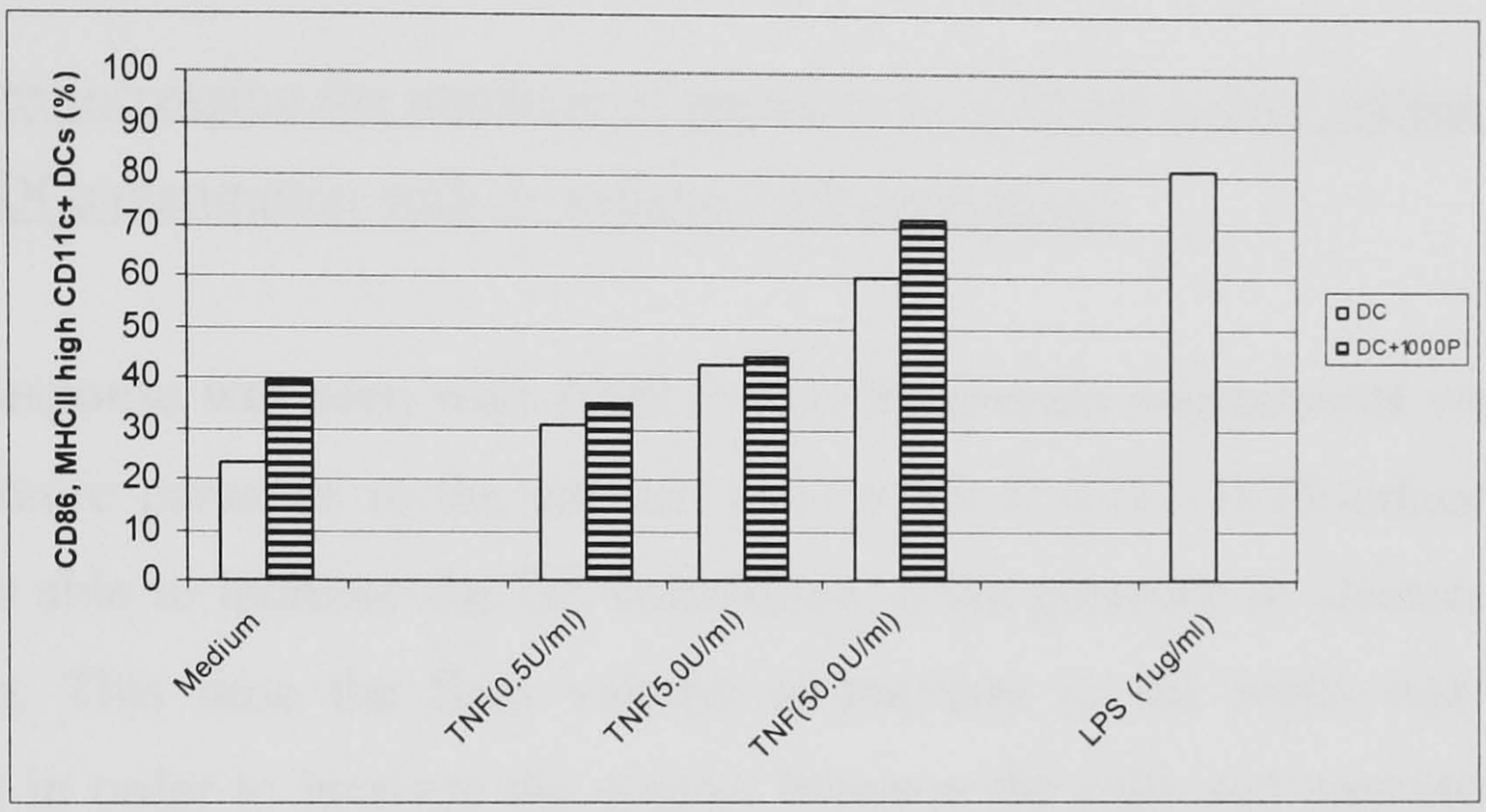
5.2.3.2 Effect of increasing parasite numbers to 1000/ml on the maturation of TNF-α-stimulated bm-DCs as judged by MHCII/CD86/CD40 upregulation.

In the last experiment 400 schistosomula/ml did not affect TNF-α- nor LPS-stimulated bm-DC maturation. Therefore, in this experiment parasite numbers were increased to

1000/ml, to try and see if this would increase bm-DC maturation in the presence of TNF-α stimulant. TNF-α was added to cells and parasites at 0.5, or 5, or 50 U/ml. After 20 hours of incubation at 37°C and 5% CO_2 bm-DCs were stained for CD11c, MHCII, CD86, and another costimulatory surface molecule CD40. The results are shown in Figure 5.9.

Figure 5.9.

A



B

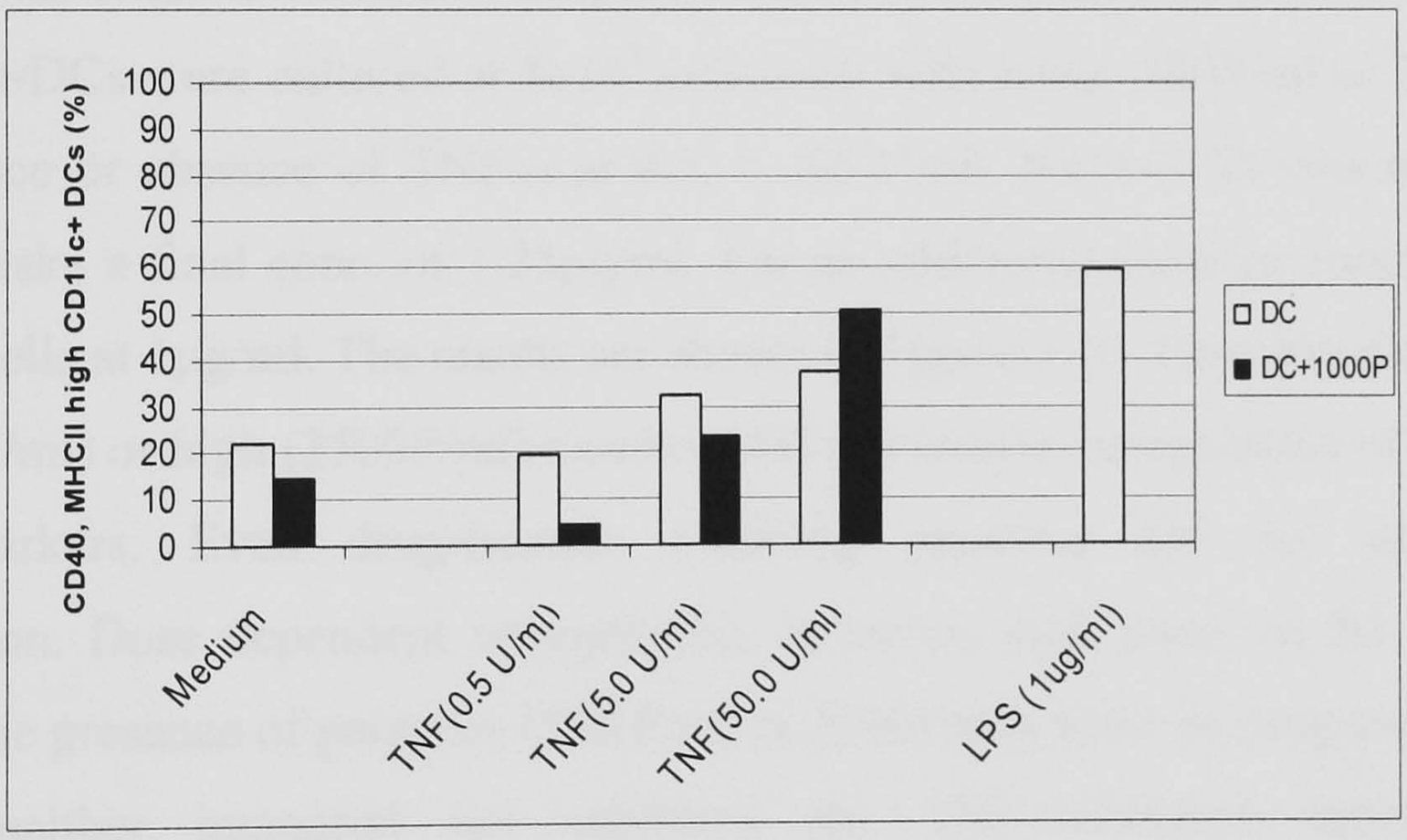


Figure 5.9. Schistosomula (1000 P/ml) were added to $5x10^5$cells/well of day 10 immature bm-DCs from C57BL/6 mice. TNF-α was added at 0.5, 5, 50 U/ml. Cultures were left incubating for 20 hours, after which bm-DCs were collected and stained for **(A)** CD11c, MHCII and CD86, or **(B)** CD11c, MHCII and CD40. Observations were from single wells. Final volume/well was 1ml.

As judged by CD86/MHCII upregulation the pattern was like that seen before (Figure 5.8.B) i.e., there was a dose response in activation by TNF-α but addition of the

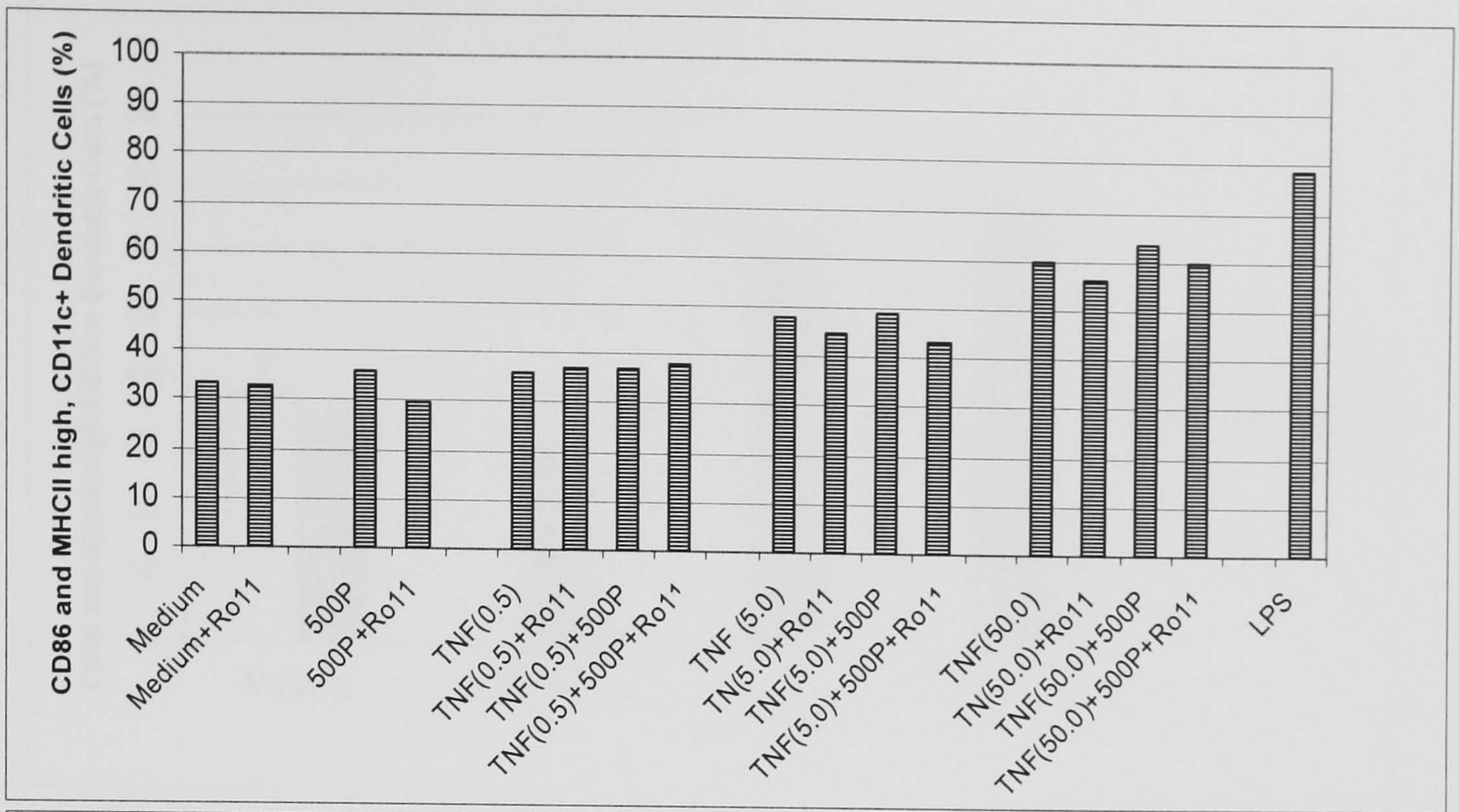
parasites did not increase this although there was some indication of enhanced expression with schistosomula in the medium alone. However there was no evidence of activation by the parasites alone as judged by CD40 expression (Figure 5.9.B). As with CD86, expression of CD40 showed a dose response with increasing TNF-α. Addition of parasites did not enhance this expression and there was some indication at lower TNF-α concentrations that the parasites inhibited the expression, although at high TNF-α concentrations there was no evidence of inhibition.

5.2.3.3 Does increasing the numbers of parasites to 2500/ml and/or adding Ro11-3128 affect bm-DCs maturation with or without TNF-α priming?

Since no response was seen with 1000 live schistosomula experiments were set up to add even more parasites to the cultures and to see if Ro11-3128-induced blebbing/ killing was able to increase the DC maturation in the presence or absence of TNF-α-stimulation. This time the final volume of medium in the wells was reduced to 400µl/well in order to increase the contact between the cells and parasites. Immature day 10 bm-DCs were cultured at $5x10^5$ cells/well with either 500P/ml or 2500P/ml in the presence or absence of TNF-α at 0.5, 5, 50 U/ml. Ro11-3128 was added to the wells to make a final conc. of 1.25µg/ml. For an additional positive control LPS was added to cells at 1µg/ml. The results are shown in Figure 5.10. Parasites alone at either low (500P/ml) or high (2500P/ml) numbers did not induce upregulation of MHCII and CD86 markers. Even drug-treated, blebbing parasites did not enhance this upregulation. Dose dependent upregulation, however, took place in the presence of TNF-α. The presence of parasites (500 P/ml or 2500P/ml) alone or drug-treated in such cultures neither increased nor inhibited the TNF-α-induced upregulation of CD86/MHCII markers. It was considered that this large number of schistosomula would have been enough to show any physiologically relevant effects on DC surface maturation but in final experiments on this theme the concentration was increased to 10,000/ml/well.

Figure 5.10.

A



B

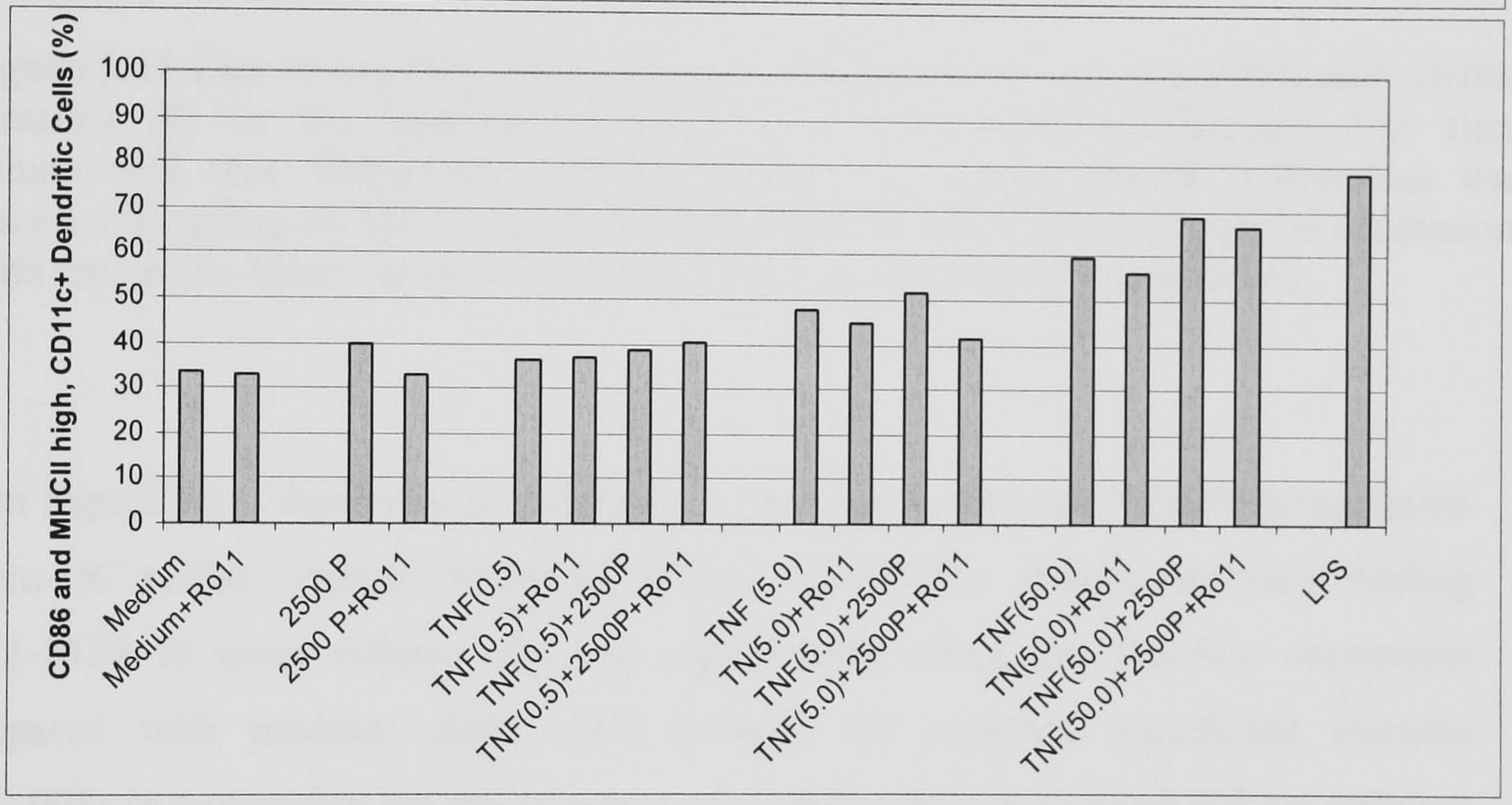


Figure 5.10. Schistosomula were added at **(A)** 500P/ml or **(B)** 2500P/ml to $5x10^5$ cells/well of day 10 immature bm-DCs from C57BL/6 mice. TNF-α was added at 0.5, 5, 50 U/ml. Ro11-3128 was added to the appropriate cultures at 1.25μg/ml. For positive control LPS was added to cells at 1μg/ml. Cultures were left incubating for 22 hours, after which bm-DCs were collected and stained for CD11c, MHCII and CD86. Final volume in wells was 400μl/well (Total events = 45,000-50,000).

5.2.3.4 Can bm-DC maturation with or without LPS stimulation be affected by adding very high numbers of schistosomula (10,000/ml) with or without Ro11-3128?

The experiment was set up as above but instead of TNF-α, LPS was used as a priming signal. The results are shown in Figure 5.11.

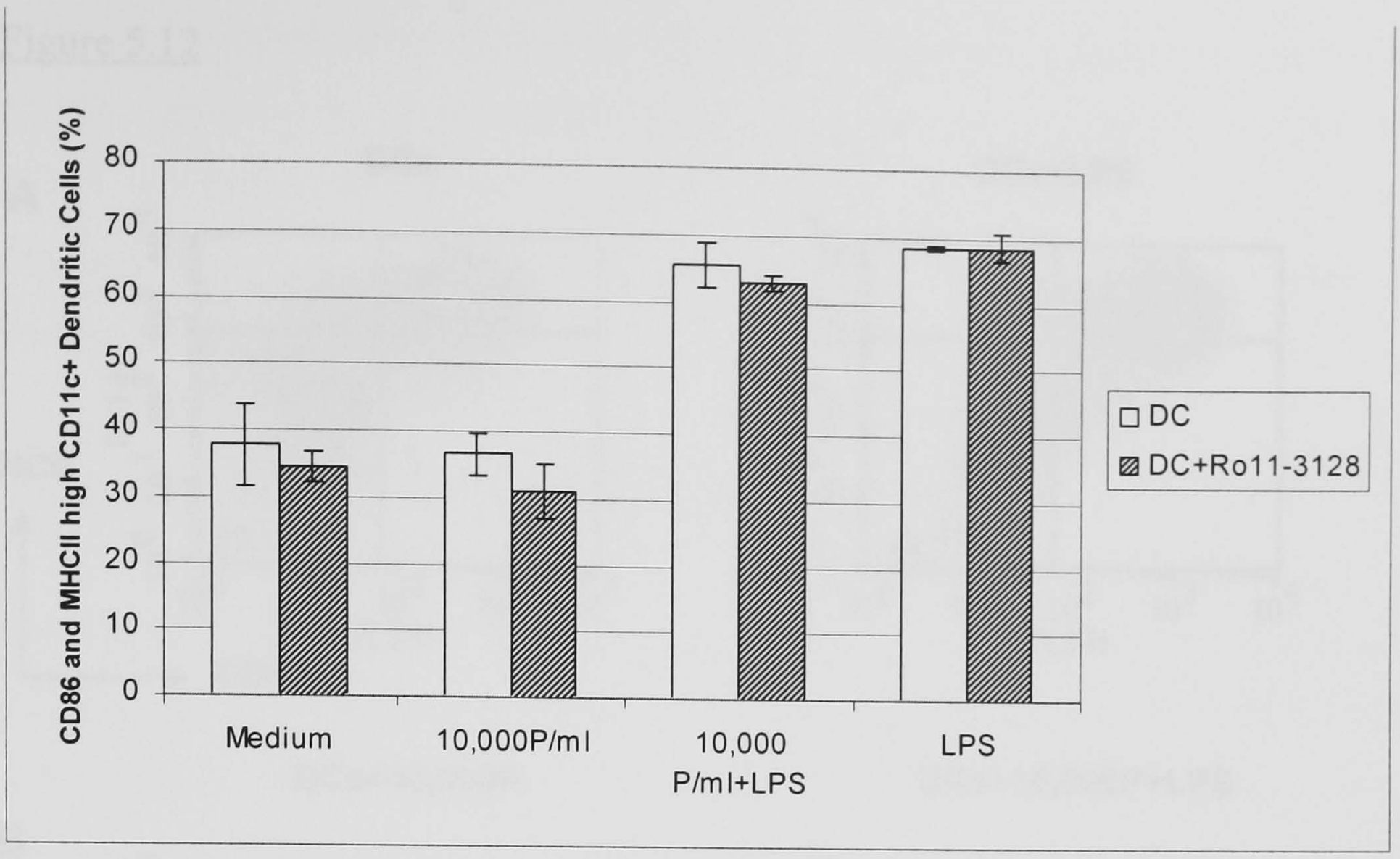


Figure 5.11 Day 10 bm-DCs, were cultured at $5.0x10^5$/well and stimulated with 10,000 parasites (P) in the absence or presence of Ro11-3128 (1.25µg/ml). The final volume/well was 500µl/well. LPS was added at 1µg/ml. Bm-DC maturation was assessed by gating on MHCII and CD86 high CD11c+ DCs after 19 hours of incubation. Data represents Mean values ± Standard Deviation of triplicate culture wells.

From Figure 5.11. there was no evidence of increased CD86 and MHCII upregulation of bm-DC surface markers following culture with 10,000 schistosomula/ml. Adding Ro11-3128 to these cultures did not significantly affect this baseline expression compared with medium alone. LPS induced the expected significant increase ($P<0.001$) in expression but the presence of 10,000 schistosomula/ml did not enhance this expression in fact it was slightly reduced but not significantly ($P= 0.32$, as compared to LPS+DC). Addition of Ro11-3128 to the schistosomula and LPS cultures did not enhance surface marker expression.

This experiment was essentially repeated and the data shown as FACS plots is shown in Figure 5.12. The three left hand plots show the three control cultures to demonstrate the reproducibility of the system (i.e. 39.4, 40.7 and 40.0% activated DCs). DC+LPS alone showed 70.9% activation, with 10,000 schistosomula added this was 70.7 and with parasites plus Ro11-3128, 63.7%.

Figure 5.12

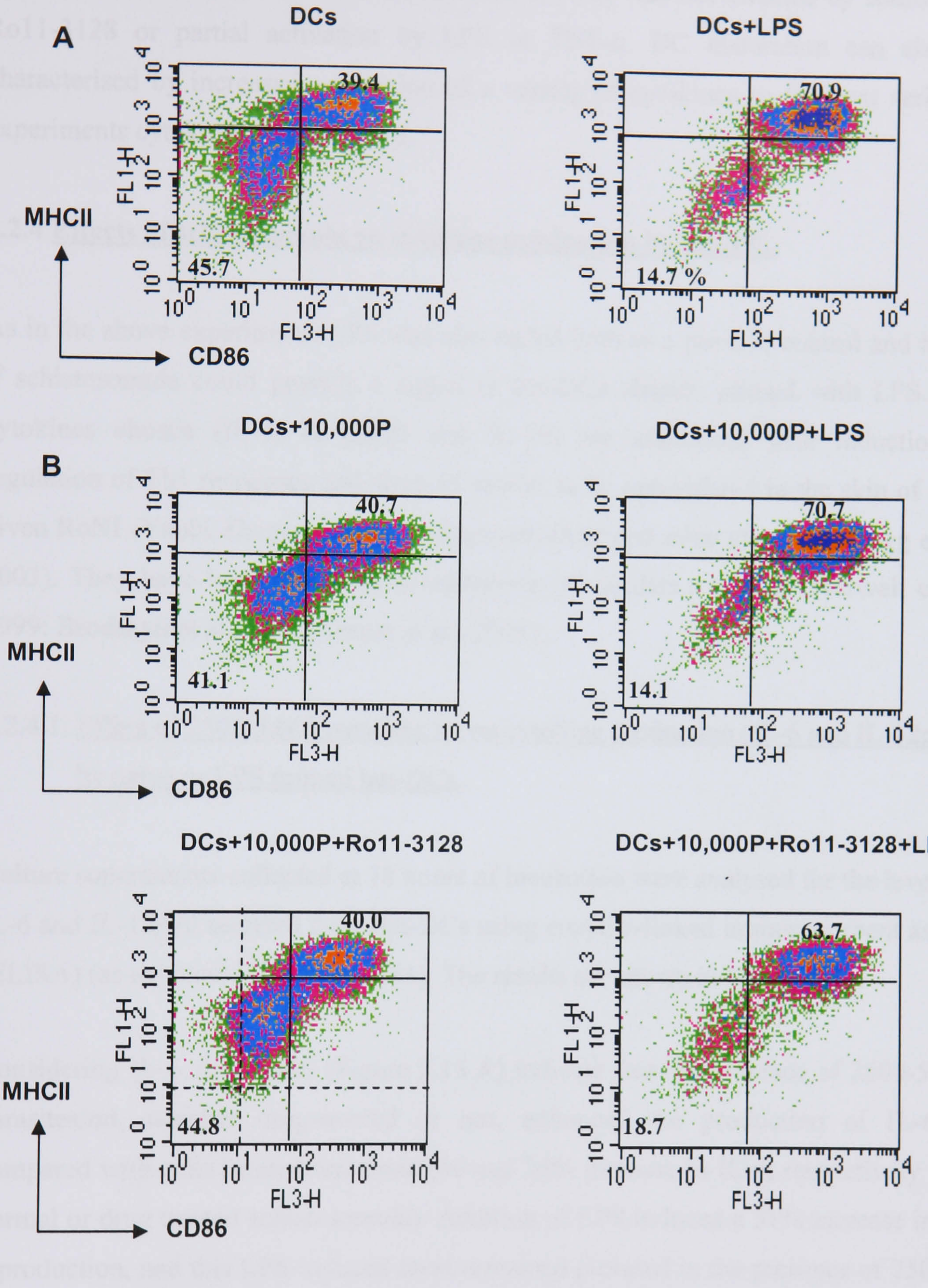


Figure 5.12. Upregulation of MHCII and CD86 in the presence or absence of 10,000 *S. mansoni* schistosomula. $5x10^5$ Bm-DCs were cultured for 19 hours ± 10,000parasites ± LPS (1μg/ml) ± Ro11-3128 (1.25μg/ml). The cells were then stained with CD11c-PE, MHCII-FITC and CD86-QR, and analysed by three colour flow cytometry. Results were gated on 50,000 CD11c+ cells. Numbers within quadrants represent % of cells expressing either immature bm-DCs $MHCII^{low}$ and $CD86^{low}$ (bottom left quadrants) or activated bm-DCs $MHCII^{high}$ $CD86^{high}$ markers (top right quadrants). Dotted lines represent isotype controls for MHCII and CD86 (see Fig. 5.2.B).

The above experiments failed to show upregulation of surface activation markers by DC in the presence of 50-10,000 schistosomula. This was not affected by addition of Ro11-3128 or partial activation by LPS or TNF-α. DC maturation can also be characterised by increased expression of a variety of cytokines so the next series of experiments cytokines were assayed.

5.2.4 Effects of schistosomula on cytokine production by bm-DC.

As in the above experiments LPS was also added both as a positive control and to see if schistosomula could provide a signal to bm-DCs already primed with LPS. The cytokines chosen (IL-6, IL-12p70 and IL-10) are associated with induction or regulation of Th1 responses and were all shown to be upregulated in the skin of mice given RoNI (Yaobi Zhang- personal communication) and mice given GI (Hogg et al., 2003). They have been measured in numerous DC studies e.g. Van Overtvelt et al., 1999: Brodskyn et al., 2002; Seixas et al., 2001). .

5.2.4.1. Effect of 2500-5,000 parasites/ml on cytokine production (IL-6 and IL-12p70) by naïve or LPS primed bm-DCs.

Culture supernatants collected at 18 hours of incubation were analysed for the levels of IL-6 and IL-12p70 secreted from bm-DCs using enzyme-linked immunosorbent assays (ELISA) (as explained in section 2.11). The results are shown in Figure 5.13.

Considering IL-6, the results (Figure 5.13.A) indicate that the presence of 2500-5,000 parasites/ml, whether drug-treated or not, enhanced the production of IL-6 as compared with cells in medium alone (36 and 38% increase in IL-6, respectively with normal or drug treated schistosomula). Addition of LPS induced a 51% increase in IL-6 production, and this LPS-induced level remained elevated in the presence of 2500 or 5000P/ml but was not increased whether the added parasites were normal or drug treated. So this result indicated that living schistosomula may increase the level of IL-6 production compared with medium alone but that they were not able to enhance the level of activation induced by LPS.

From Figure 5.13.B, neither the presence of 2500 nor 5000 P/ml induced the production of IL-12p70 from bm-DCs. However, IL-12p70 was produced at an

Figure 5.13.

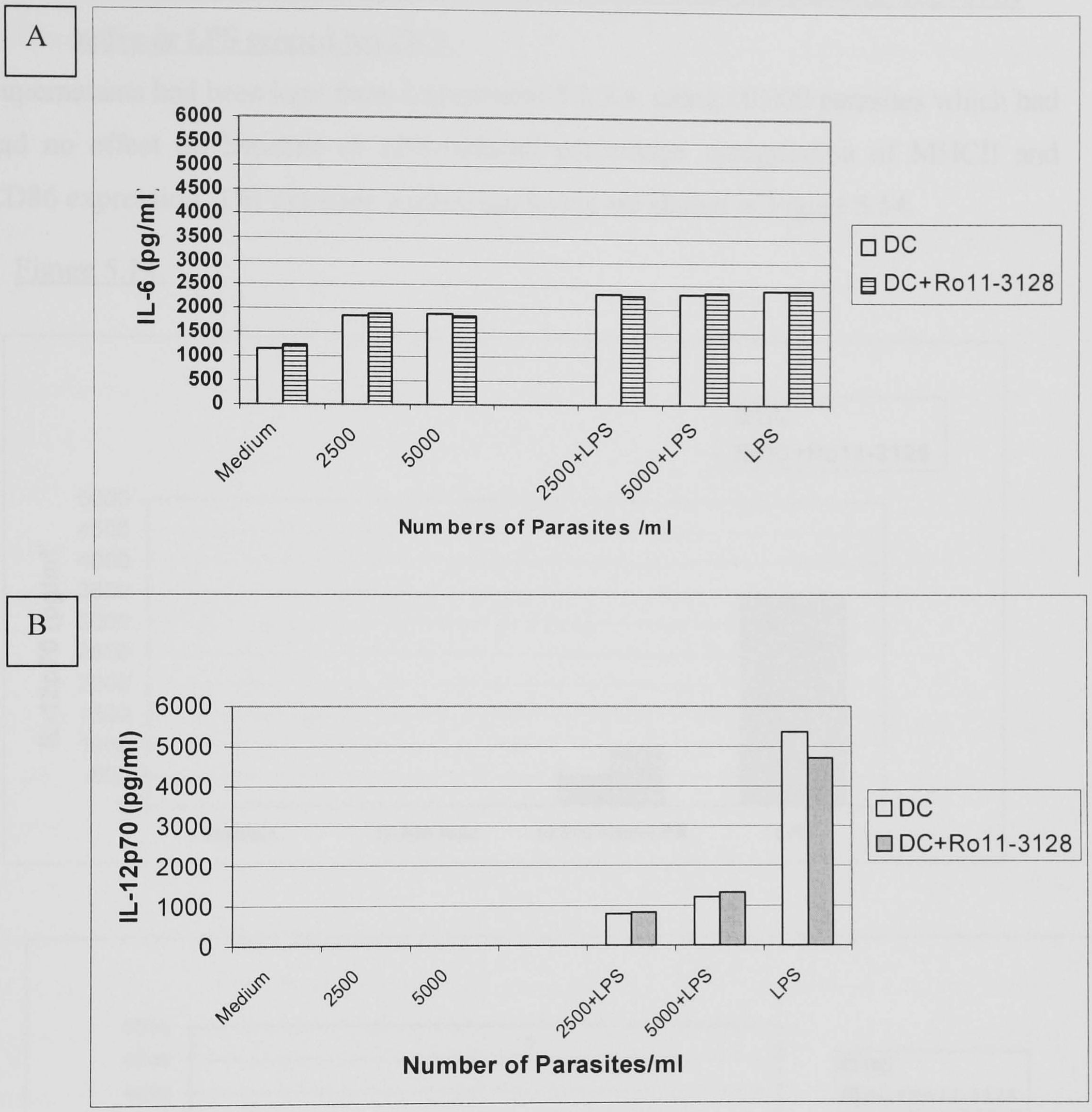


Figure 5.13. Day 10 bm-DCs, were cultured at $5.0x10^5$/well and stimulated with 2500 or 5000 schistosomula/ml in the absence or presence of Ro11-3128 (1.25μg/ml) and the presence or absence of LPS at 1μg/ml. Supernatants were recovered 18 hr later and IL-6 (Top graph) or IL-12p70 (bottom graph) assayed by ELISA. Data represents single well observations.

elevated level from LPS-stimulated bm-DCs and interestingly, this IL-12p70 level was much lower from LPS-stimulated bm-DCs in the presence of either 2500 or 5000 parasites/ml (reduced 85% and 77% respectively, as compared to LPS+DC cultures).

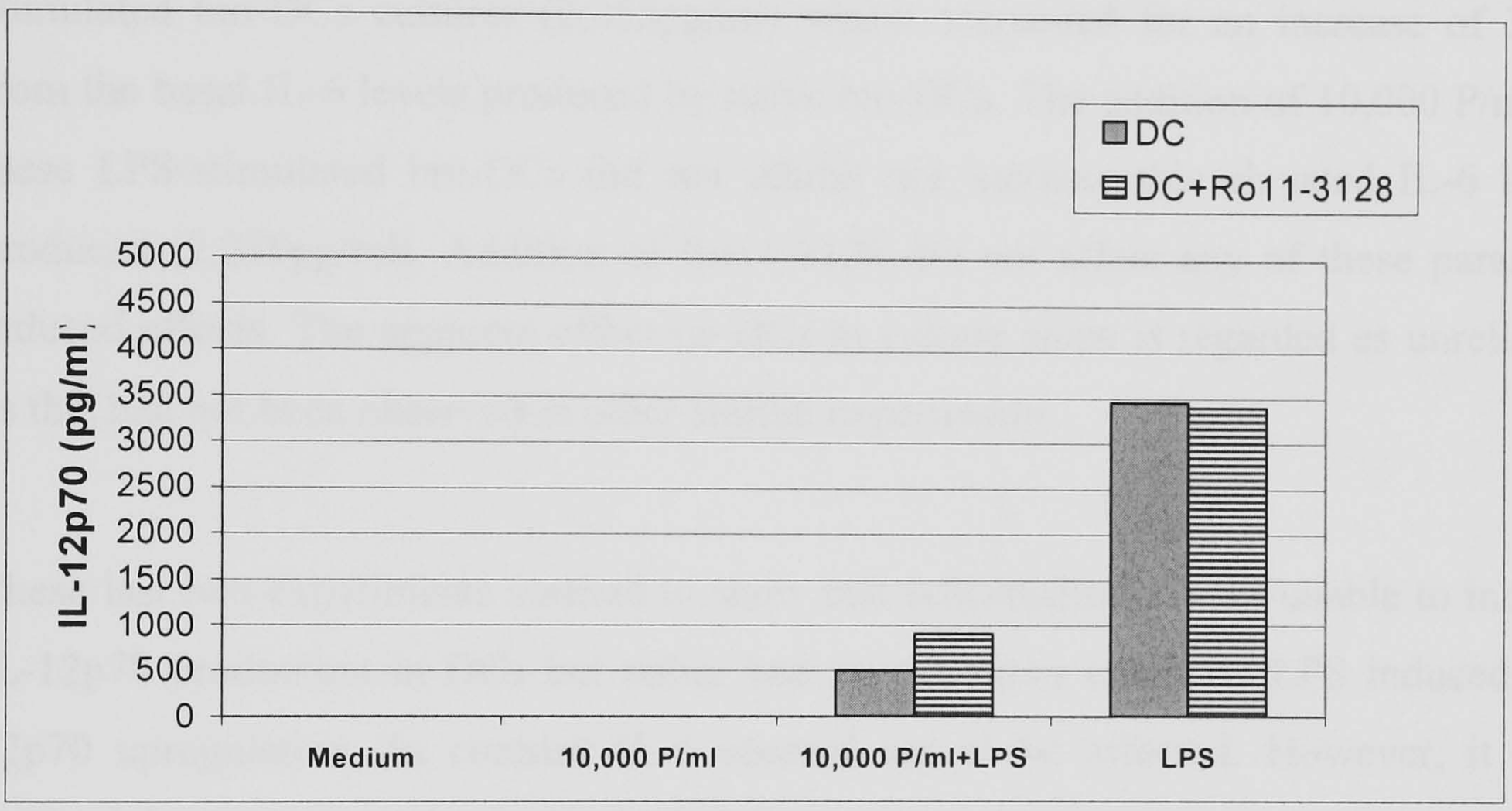
This effect was independent of the presence of Ro11-3128. Further investigations of this negative effect on IL-12p70 production were carried out.

5.2.4.2 Effect of 10,000 parasites/ml on cytokine production (IL-6 and IL-12p70) by naïve or LPS primed bm-DCs.

Supernatants had been kept from Experiment 5.2.3.4. using 10,000 parasites which had had no effect on baseline or LPS-induced percentage upregulation of MHCII and CD86 expression. The cytokine expression levels are shown in Figure 5.14.

Figure 5.14.

A



B

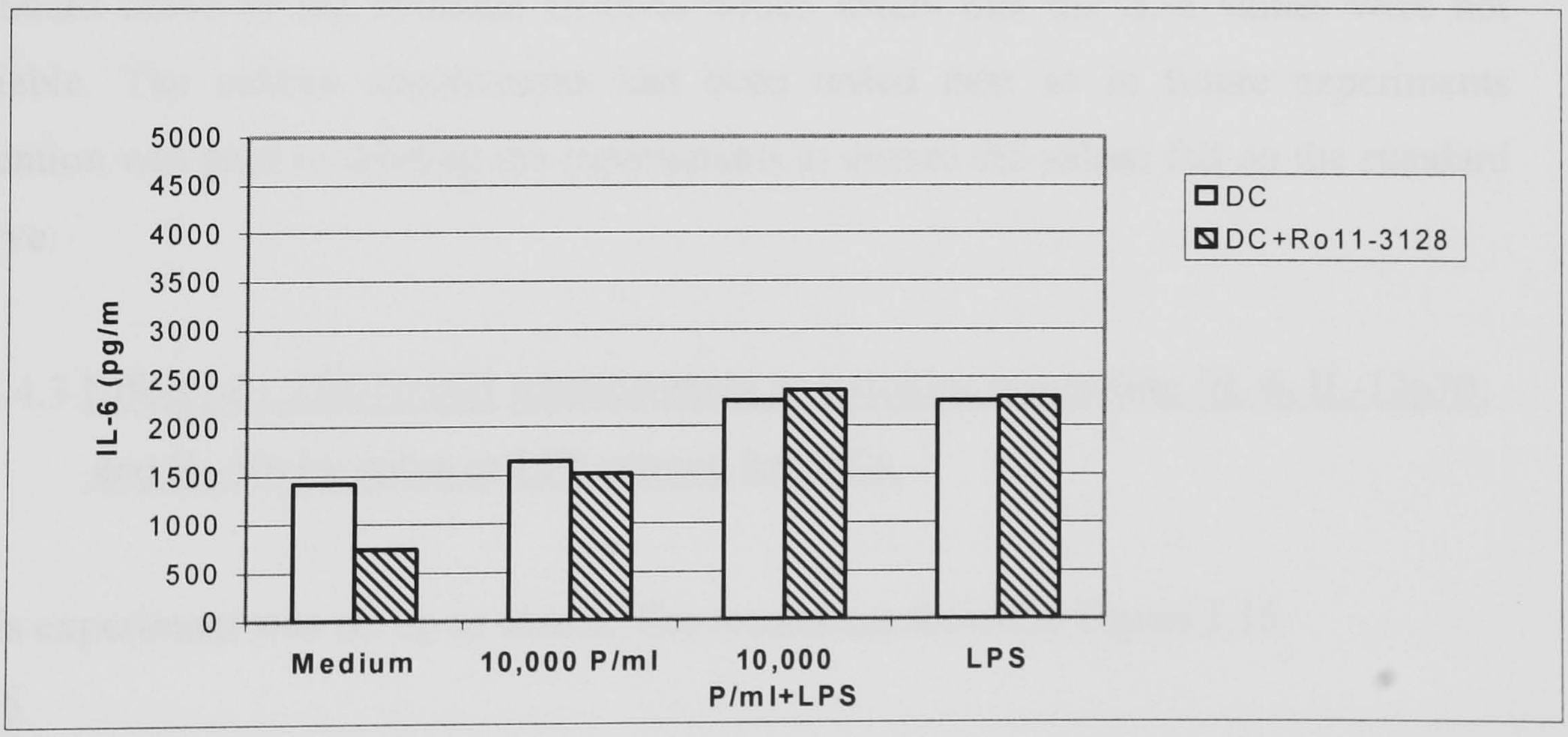


Figure 5.14. Day 10 bm-DCs, were cultured at $5.0x10^5$/well and stimulated with 10,000 parasites (P) in the absence or presence of Ro11-3128 (1.25µg/ml). The final volume/well was 500µl/well. LPS was added at 1µg/ml. Supernatants were recovered after 19 hours of incubation and assayed for cytokines by ELISA. Data represents Mean values from pooled supernatants.

Figure 5.14.A shows that adding 10,000P/ml produced the same IL-12p70 pattern seen in Fig.5.13.B. There was no evidence of production of IL-12p70 from cultures containing the parasites only, either in the presence or absence of Ro11-3128. However, such large numbers of parasites had markedly reduced the levels of LPS-induced IL-12p70 (501pg/ml) production by 85% as compared to the positive control cultures with LPS alone (3,453pg/ml).

From Figure 5.14.B, 10,000P/ml in culture induced a 14% increase in IL-6 production from bm-DCs as compared to bm-DCs only. IL-6 levels were highest in LPS-stimulated bm-DCs cultures (2,253pg/ml) which accounted for an increase of 37% from the basal IL-6 levels produced by naïve bm-DCs. The addition of 10,000 P/ml to these LPS-stimulated bm-DCs did not inhibit nor increase this elevated IL-6 level produced (2,259pg/ml). Addition of Ro11-3128 did not affect any of these parasite-induced effects. The apparent effect on DCs in culture alone is regarded as unreliable as this had not been observed in other similar experiments.

These last two experiments seemed to show that schistosomula were unable to induce IL-12p70 production in DCs but rather had an inhibitory effect on LPS induced IL-12p70 upregulation. In contrast IL-6 seemed not to be affected. However, it was realised that the values for the IL-6 ELISA from the culture wells were at the top of the standard curve of the cytokine ELISAs which meant that the IL-6 values were not reliable. The culture supernatants had been tested neat so in future experiments attention was paid to diluting the supernatants to ensure the values fell on the standard curve.

5.2.4.3 Effect of 1,250-10,000 schistosomula on cytokine production (IL-6, IL-12p70, and IL-10) by naïve or LPS primed bm-DCs.

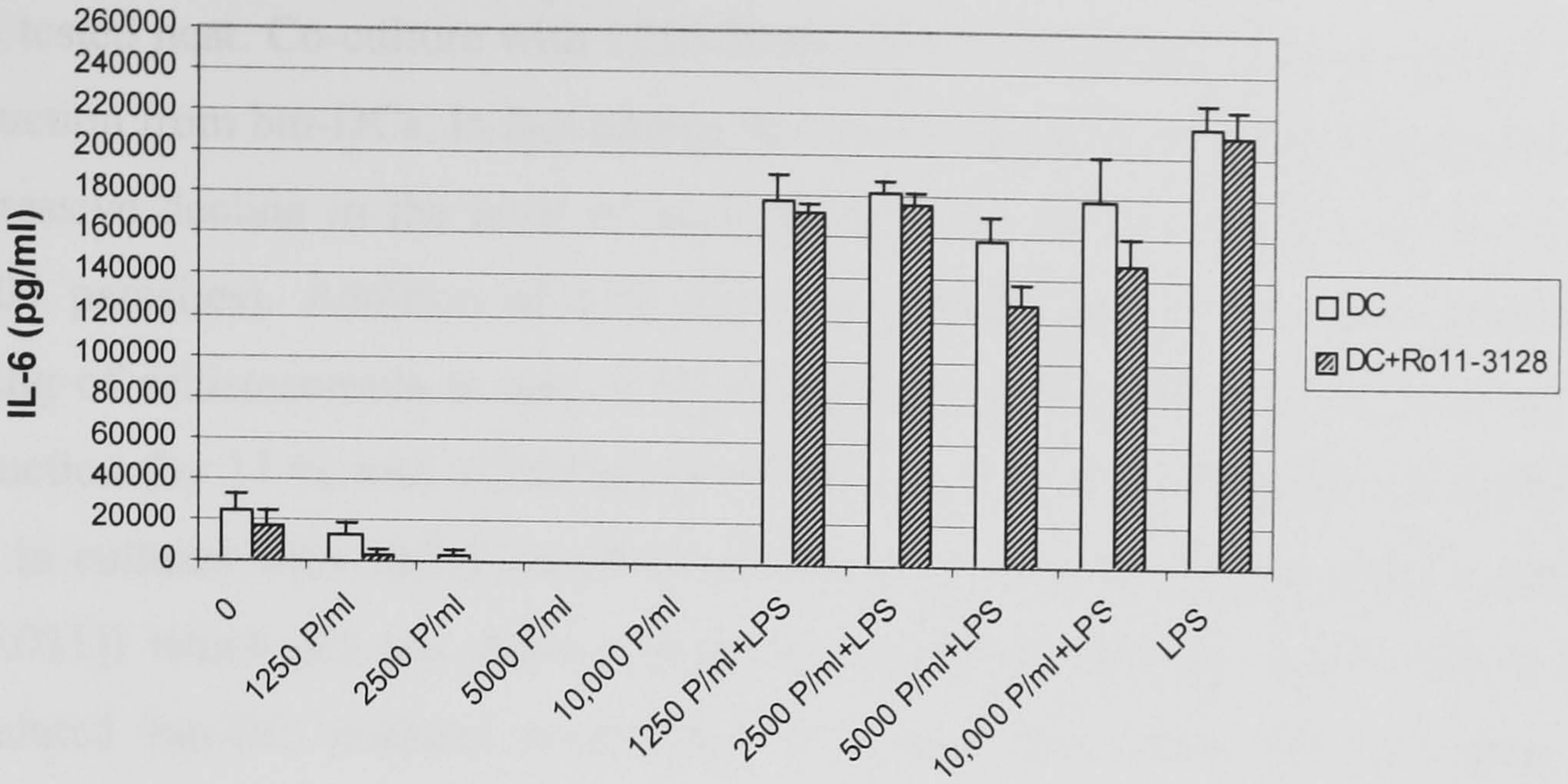
This experiment was set up as above. The results are shown in Figure 5.15.

IL-6

Following preliminary testing the supernatants were eventually tested at 1:500 dilution. As seen from Figure 5.15.A, when this was done the levels of IL-6 detected were vastly increased compared with the above experiments in which the supernatants

Figure 5.15.

A



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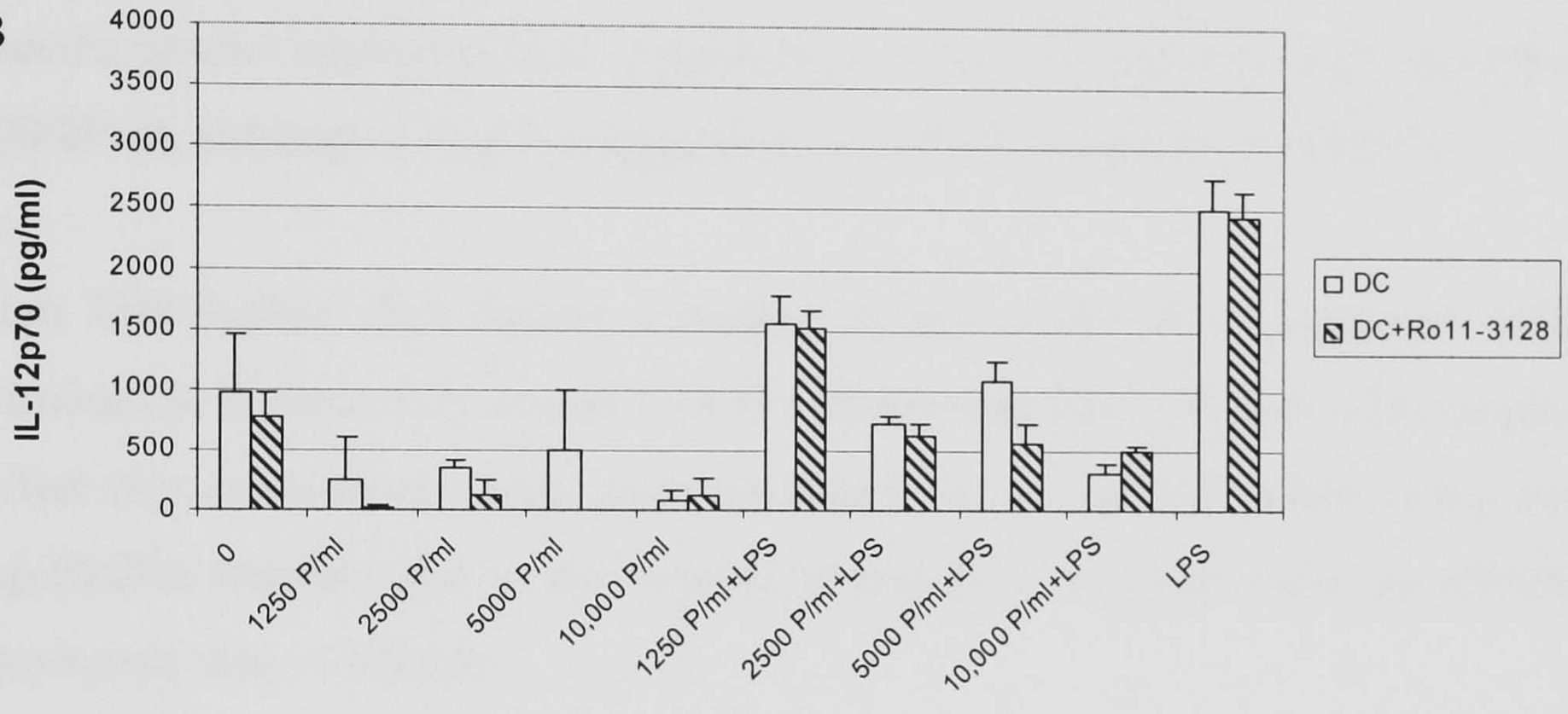


C

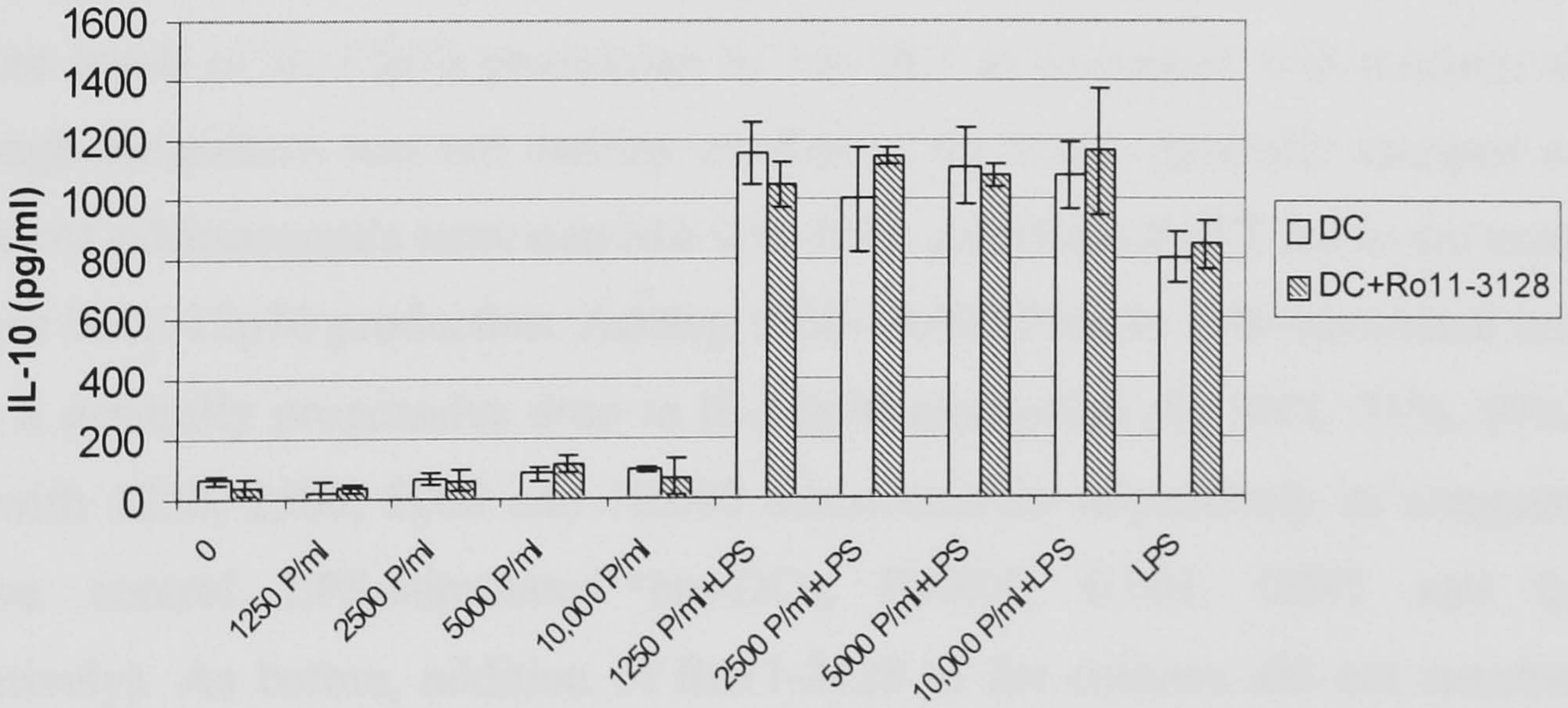


Figure 5.15. Day 10 bm-DCs, were cultured at $5.0x10^5$/well and stimulated with 1250-10,000 parasites (P) in the absence or presence of Ro11-3128 (1.25μg/ml) and/or LPS at 1μg/ml. The final volume/well was 500μl/well. Supernatants were recovered after 19 hours of incubation and assayed for cytokines by ELISA. Data represents Mean values ± Standard Deviation of triplicate culture wells.

were tested neat. Co-culture with 1250-10,000 schistosomula/ml did not increase IL-6 production from bm-DCs. In fact adding increasing numbers of schistosomula led to a progressive decline in the level of background IL-6 production ($P< 0.01$ for 2,500-10,000 parasites). Addition of LPS led to a ten fold increase in IL-6 production. Adding of schistosomula to such LPS-stimulated bm-DCs reduced the level of IL-6 production (by 17 % with 1250P/ml [P=0.009], by 15% with 2500P/ml [P=0.006], by 26% in cultures with 5000P/ml [P=0.0011] and by 16% in cultures with 10,000P/ml [P=0.031]) which did not show a clear dose response. Adding Ro11-3128 to LPS-stimulated bm-DC cultures containing 1250 and 2500 P/ml did not show any significant difference in IL-6 levels from their non-drug-treated equivalents (P=0.47 and 0.21, respectively). However, with larger number of parasites, drug-treatment produced a greater inhibition of IL-6 production. This was significantly lower ($P<0.02$) for 5000P/ml, although it was not significant for 10,000 parasites (P=0.085).

The ten fold higher IL-6 values recorded in this experiment compared with the experiments in Figures 5.13.A and 5.14.B indicate that the cytokine concentrations in these last two experiments were underestimated due to the OD values being beyond the top ELISA standard and so the conclusion that the IL-6 levels were not affected by schistosomula was unreliable.

IL-12p70

As was also observed in Figure 5.13.B, 1250–10,000 schistosomula alone reduced the baseline levels of IL-12p70 production by bm-DCs as compared with medium alone. Although the pattern was not entirely consistent, the levels generally dropped as the number of schistosomula increased like with IL-6. Addition of LPS led to the expected increase in IL-12p70 production. Adding 1250-10,000 P/ml to LPS-stimulated bm-DC led to a generally progressive drop in IL-12p70 production (by 38%, 71%, 57% and 88% with 1250, 2500, 5000 and 10,000 schistosomula respectively as compared to positive control LPS-stimulated bm-DCs, $P<0.01$, 0.001, 0.001 and 0.001, respectively). As before, addition of Ro11-3128 to the cultures did not consistently affect the suppression caused.

IL-10

Low baseline levels of IL-10 were produced in medium alone and the presence of 1250-10,000 schistosomula/ml bm-DCs led to progressive but slight increases (Figure 5.15.C) although these were not statistically significant. LPS induced increased secretion of IL-10 by around 15 fold. Adding 1250, 2500, 5000, and 10,000 schistosomula/ml to LPS-stimulated bm-DCs led to increases of IL-10 production (45%, 25%, 39% and 35% [$P<0.01$, $P=0.095$, 0.011, 0.0126, respectively]). Adding Ro11-3128 to these cultures with parasites did not change IL-10 production from that seen in equivalent non-drug treated cultures.

This experiment showed that co-culture of bm-DCs with schistosomula reduced both baseline and LPS-induced levels of IL-6 and IL-12p70 but increased corresponding IL-10 levels. In view of the apparent different effects of schistosomula on the LPS induction of different cytokines this experiment was repeated and measurement of TNF-α included. Culture with 625-2500 P/ml was used to try to determine the lower range of parasites for inducing these effects.

5.2.4.4 Effect of 625-2500 schistosomula on cytokine production (IL-6, IL-12p70, IL-10 and TNF-α) by LPS primed bm-DCs.

The experiment was set up like the previous ones. The results are shown in Figure 5.16.

IL-6

LPS induced approximately 15 fold increase in IL-6 production (Figure 5.16.A, $P<0.00001$). This LPS-induced high level of IL-6 was reduced in the presence of parasites by 24%, 27%, and 35% in the presence of 625, 1250 and 2500 schistosomula respectively ($P=0.011$, 0.0045, 0.0019, respectively). Cultures containing drug-treated parasites produced similar amounts of IL-6 as their equivalents with non-drug treated parasites.

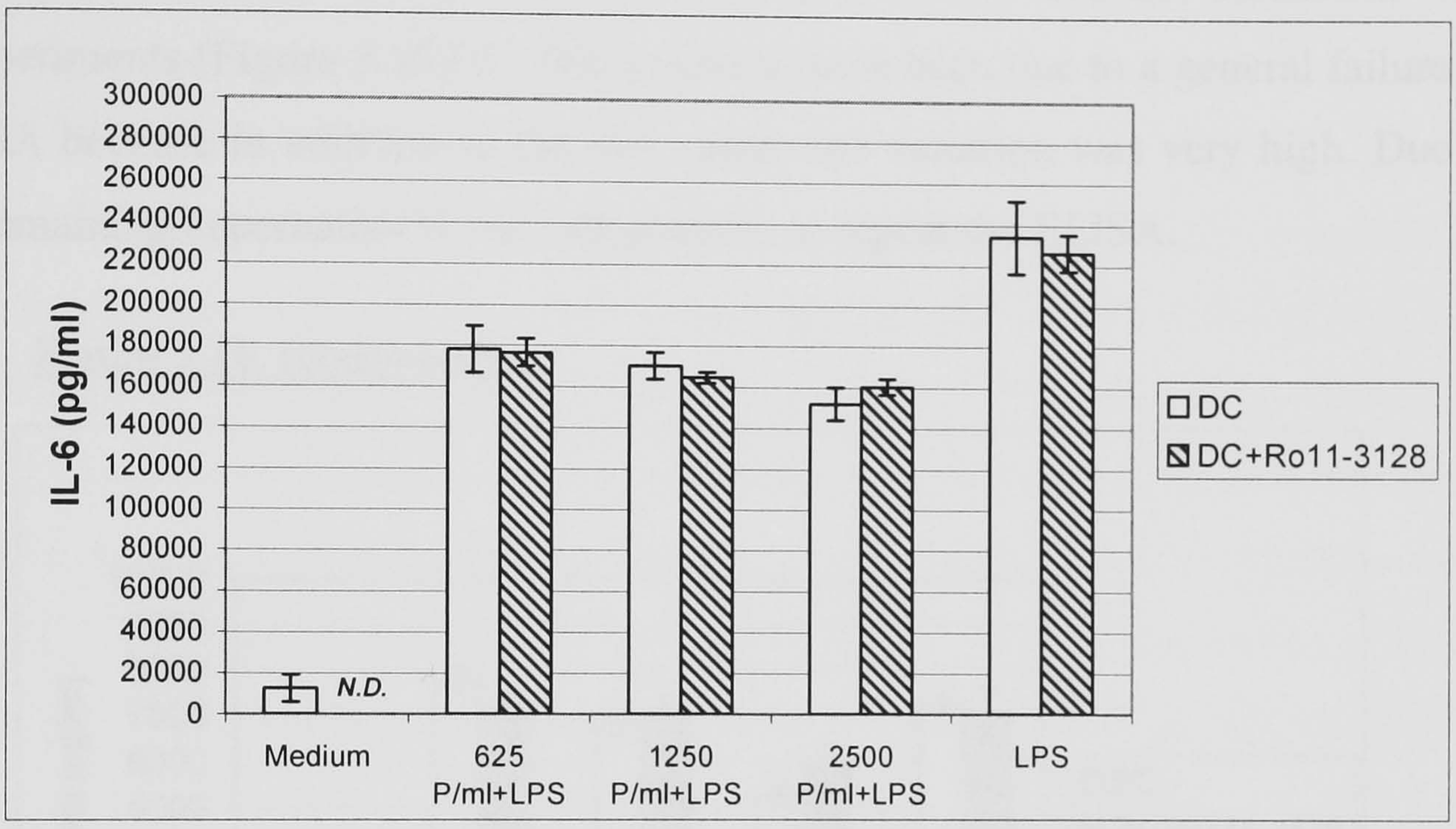
IL-12p70

Stimulation of bm-DCs with LPS induced approximately 40 fold increase in IL-12p70 production (Figure 5.16.B). On addition of increasing numbers of parasites, there was a parasite dose-dependent suppression of IL-12p70 production from LPS-activated

bm-DCs (625, 1250, 2500 schistosomula respectively resulting in 18%, 23% and 72% [P=0.30, 0.14 & 0.00025, respectively]) reduction in IL-12p70 produced as compared to control LPS-stimulated bm-DCs). Drug-treated 625P/ml induced a reduction of only 8% in IL-12p70 production from LPS-stimulated bm-DCs, but 1250 and 2500 drug treated parasites induced higher reductions than equivalent non-drug treated cultures (64% and 86% respectively for 1250 and 2500 P/ml as compared to DC+LPS) although the difference was only statistically significant for the 2500 parasites (P< 0.001).

Figure 5.16.

A



B

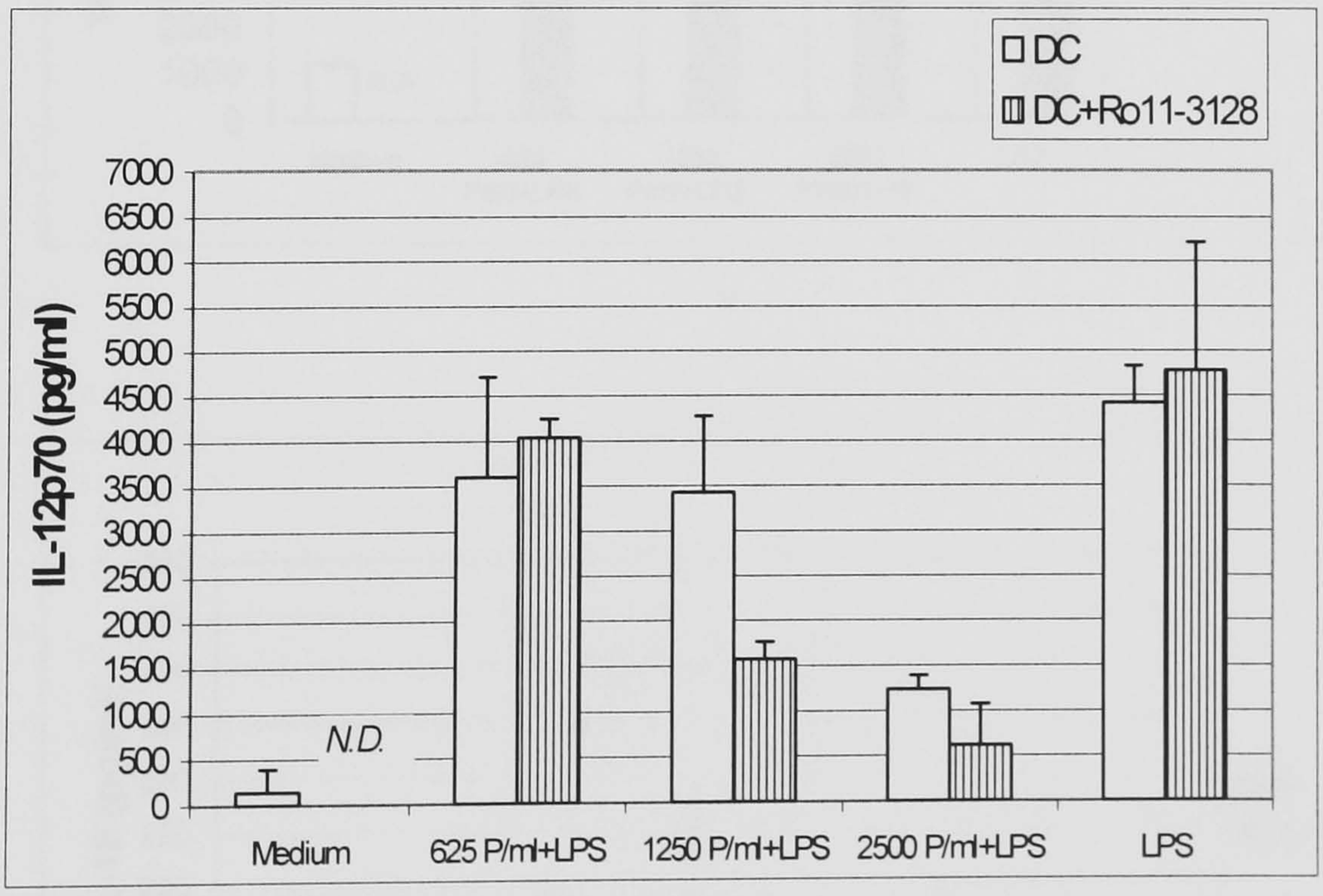


Figure 5.16. A and B above, C and D below. Day 10 bm-DCs, were cultured at $5.0x10^5$/well and stimulated with 625-2,500 parasites (P) in the absence or presence of Ro11-3128 (1.25µg/ml) and/or LPS at 1µg/ml. The final volume/well was 500µl/well. Supernatants were recovered after 19 hours of incubation and assayed for cytokines by ELISA. Data represents Mean values ± Standard Deviation of triplicate culture wells. [N.D. = Not Done].

TNF-α

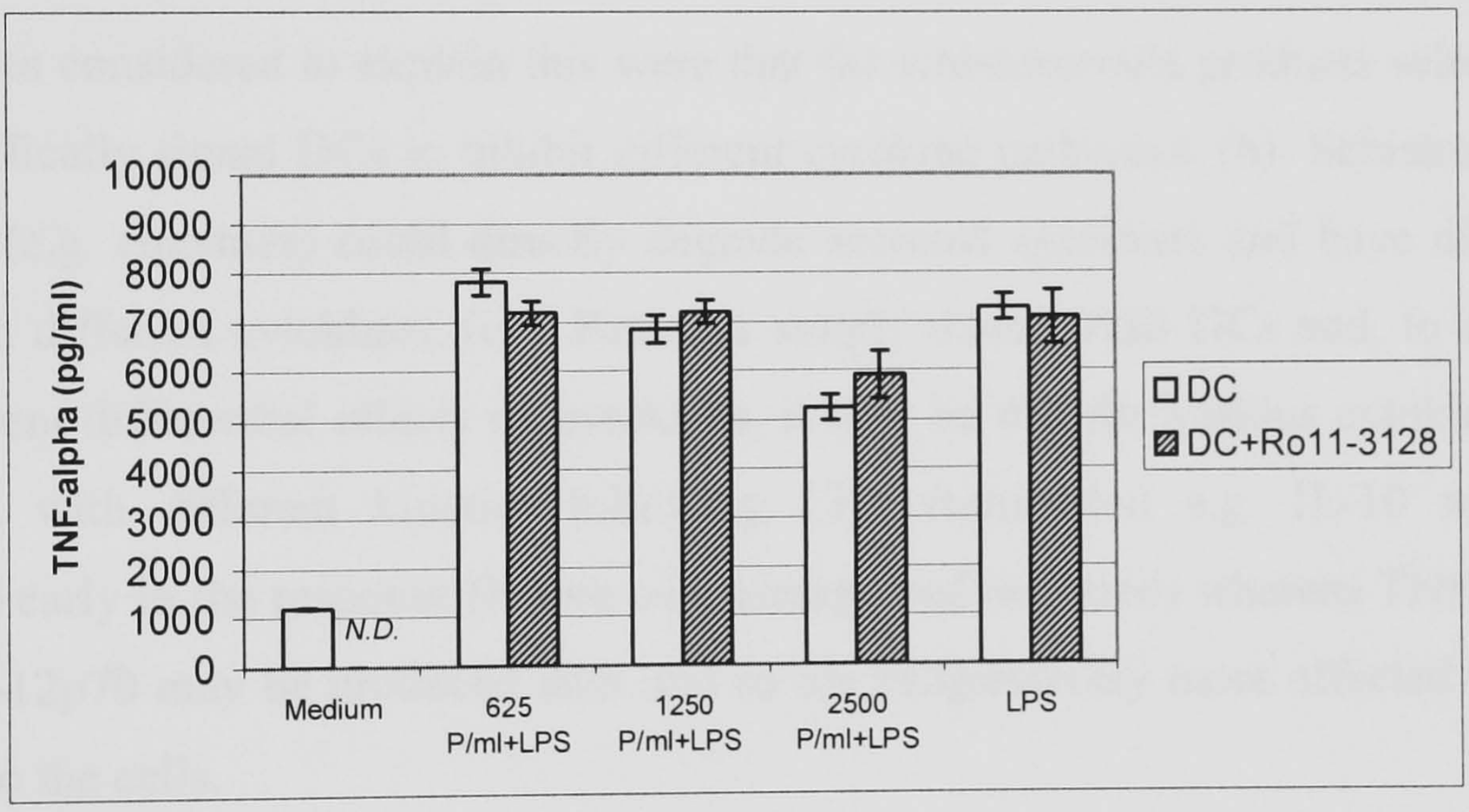
As seen in Figure 5.16.C LPS induced approximately 7 fold increase in TNF-α production from bm-DCs. The lower parasite numbers, 625 and 1250 P/ml did not suppress this level of LPS-induced TNF-α production (P=0.069 and P=0.122, respectively). However, 2500P/ml did reduce TNF-α production by 28% as compared to DC+LPS (P<0.001). Addition of Ro11-3128 had no significant effect on these values.

IL-10

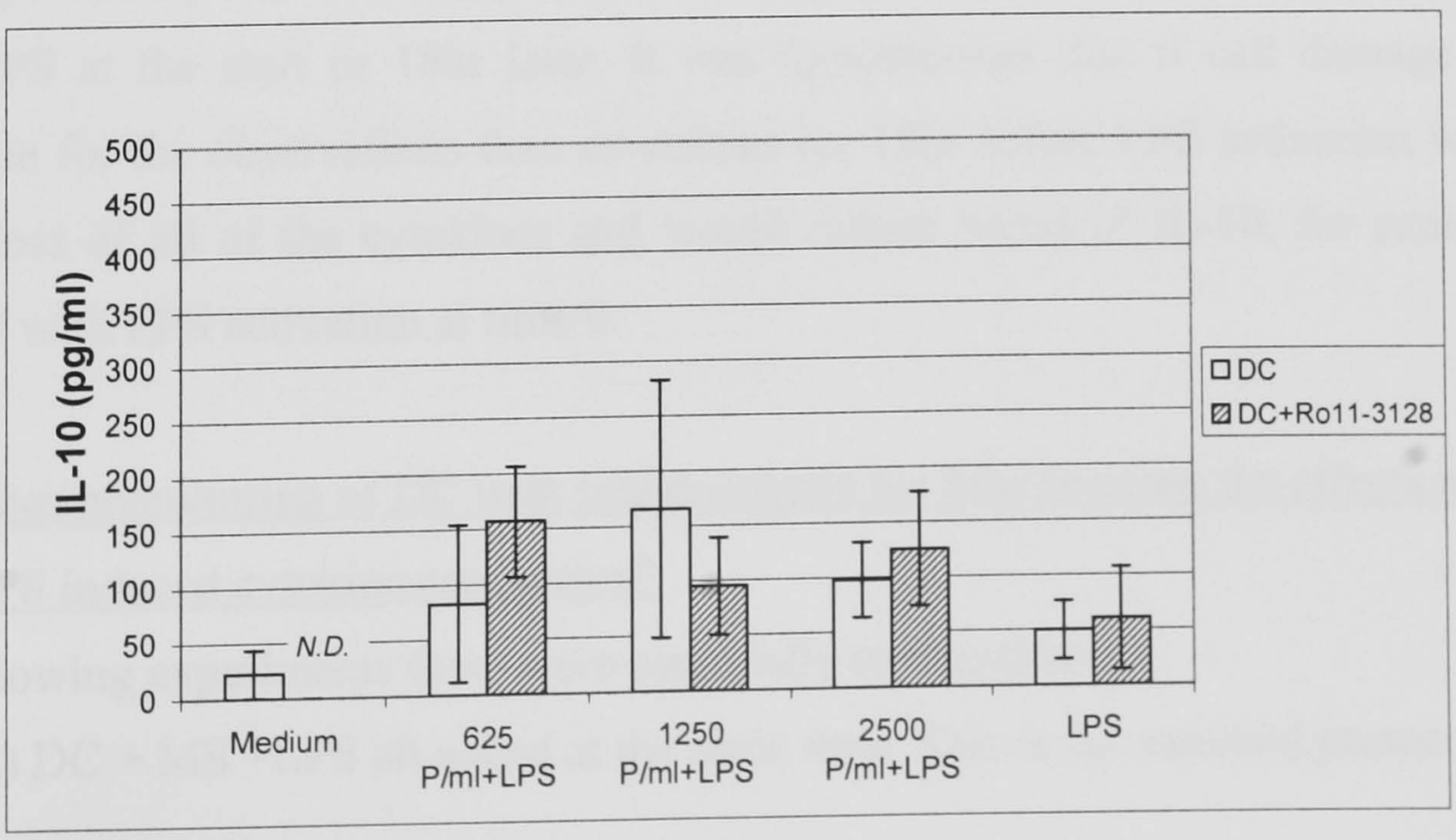
Unfortunately the values obtained for IL-10 were very low and not consistent with other experiments (Figure 5.16.D). This seems to have been due to a general failure of the ELISA because in addition to the low values the variation was very high. Due to lack of remaining supernatant it was not possible to repeat the ELISA.

Figure 5.16. (continued)

C



D



Because of the very high variation little can be learned from this data although the general pattern of the mean values was like that in 5.15.C. i.e., LPS values were higher than medium alone, in the cultures with parasites the values were higher than LPS alone and presence of drug did not induce marked differences.

IL-4

IL-4 was assayed also but none was found (data not shown).

5.2.5 Are the differential effects on cytokines detected in culture due to parasite-induced cell damage combined with different kinetics of cyokine production?

The above experminents show that there were differential effects of co-culture of mechanically-transformed schistosomula (MS) and DCs on the LPS-induced secretion of the different cytokines (IL-12p70>IL-6>TNF-α) and IL-10 seemed to be unaffected. Possiblities considered to explain this were that (a) schistosomula products selectively and specifically signal DCs to inhibit different cytokine pathways. (b) Schistosomula products (e.g. enzymes) could directly degrade secreted cytokines and have different effects on different cytokines. (c) Parasites simply damage/kill DCs and, to explain the apparent differential effects on cytokines, it may be that the various cytokines are produced with different kinetics following LPS stimulation e.g. IL-10 may be produced early in the response (before cell damage had occurred) whereas TNF-α, IL-6 and IL-12p70 may be produced later and so are progressively more affected by any damage to the cells.

This last possibility was investigated by putting parasites and cells together and either adding LPS at the start or 18hr later. It was hypothesised that if cell damage was responsible for the observations then co-culture for 18hr before LPS activation would produce less of all of the cytokines and would reduce levels of IL-10, for example, compared with LPS activation at time 0.

5.2.5.1 Does preculturing of DC with schistosomula for 24hr increase the effects on LPS induced cytokine production?

In the following experiments there were essentially two treatments:

Group (A) DC + MS +LPS all added at the same time. This is the standard protocol

Group (B) DC + MS added as in (A) but LPS added 24hr later. The schistosomula used were 3hr old. Supernatants were sampled at both +6hr and +24hr post LPS-treatment in order to test if the different cytokines were produced with different kinetics (e.g. IL-10 predominantly early and IL-12p70, IL-6 and TNF-alpha predominantly later). The results are shown in Figures 5.17.A to D. Control values in medium alone are not shown but were low, in the usual range.

IL-6

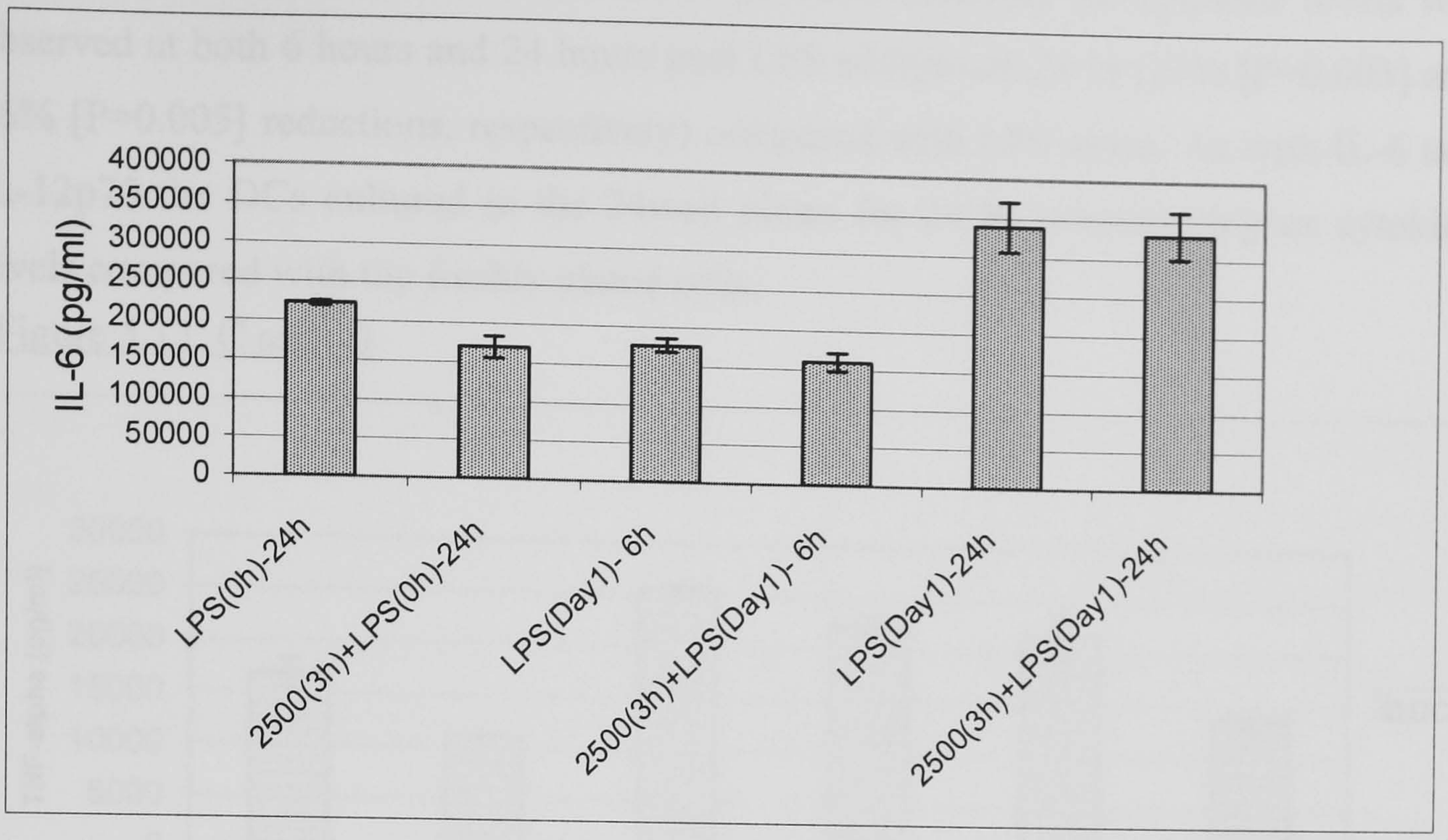
It can be seen from Figure 5.17.A that in the standard protocol (treatment group A) LPS induced about 220 ng/ml IL-6 by 24hr, exactly comparable to Figure 5.16.A. Addition of the parasites to the cultures (2500(3h)+LPS(0h)-24h) caused a mean level of IL-6 at 24hr which was 24% lower than with LPS in the absence of parasites [P=0.003] (i.e., similar to the reduction seen in Figure 5.16.A). However, the levels of IL-6 produced by bm-DCs cultured overnight with MS (treatment group B), was exactly comparable to that induced in the absence of MS at both 6hr and 24hr post LPS treatment. This indicated that the DCs were perfectly able to respond to LPS by producing IL-6 after 24hr with the parasites and so there was no evidence that the cells were killed or damaged by the O/N culture with parasites. It is interesting to note that the DCs which has been cultured overnight in the 24well plates produced similar levels of IL-6 by just 6 hrs post LPS (176 ± 8ng/ml) as compared with the 24hr cultures set up at the start of the experiment (222.479 ± 3.234 ng/ml). But by 24hr these cells produced much higher levels of IL-6 (338± 3ng/ml, P=0.003) indicating that the harvesting and plating of the DCs primed them for LPS activation. A similar apparent priming of cytokine production was also seen for IL-12p70 (see below).

IL-12p70

As in previous studies addition of parasites and LPS to cultures at time 0h (treatment group A) induced 63% reduction in IL-12p70 measured in the cultures (P=0.0002). When DCs and parasites were co-cultured for 24hr and then LPS added the level of parasite induced reduction in IL-12p70 was 31% at 6hr (P=0.015) and only 38% by 24hr(P=0.0365).

Figure 5.17. A and B

A



B

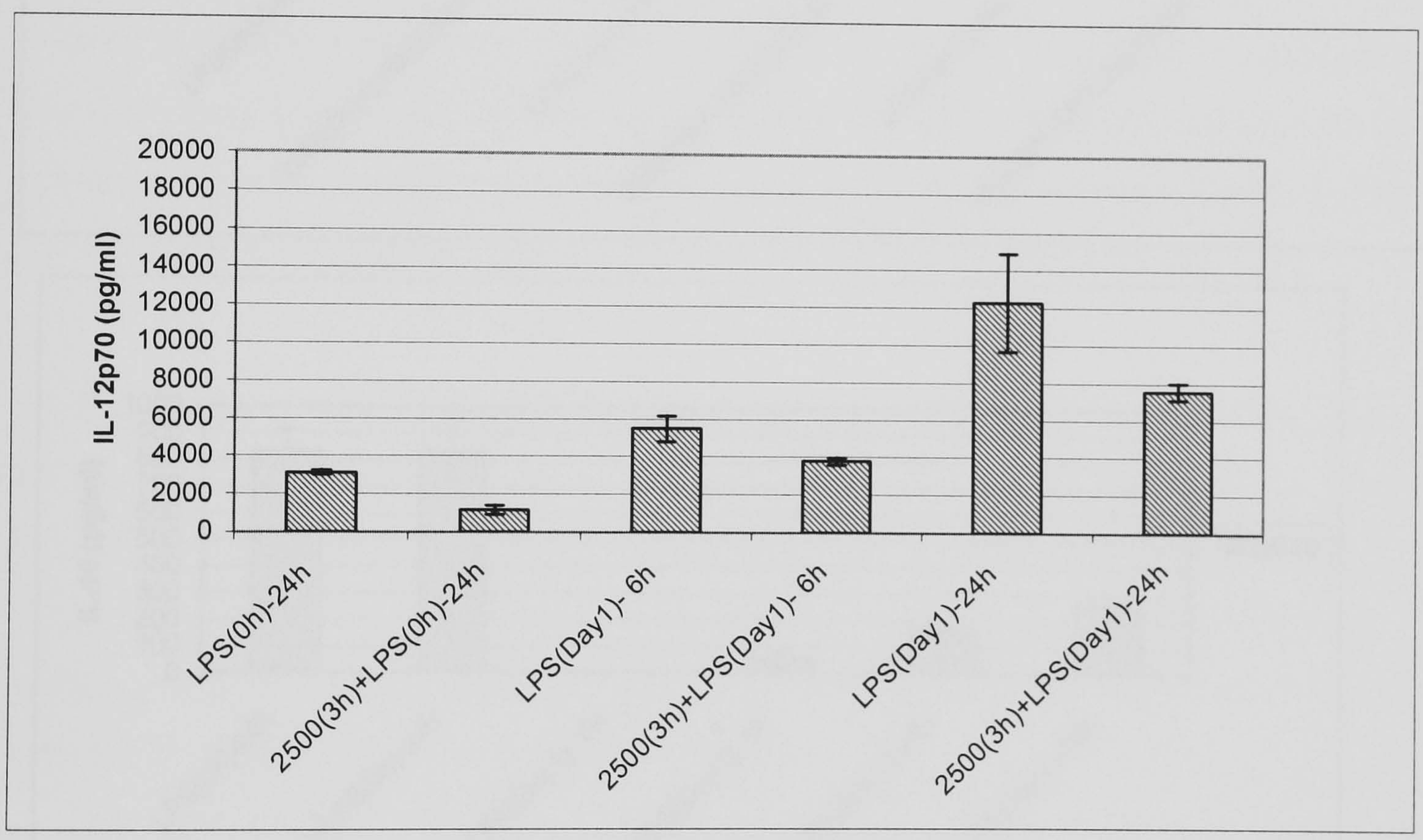


Figure 5.17. A and B. Day 10 bm-DCs, were cultured at $5.0x10^5$/well with or without 2,500 3hr old parasites (2500 [3h]) P)/ml and LPS at 1μg/ml. LPS was either added at time 0 [LPS (0h)]or at 24h [LPS (Day 1)]. Supernatants were recovered after 6h or 24h after LPS stimulation and assayed for cytokines by ELISA. Data represents Mean values ± Standard Deviation of triplicate culture wells.

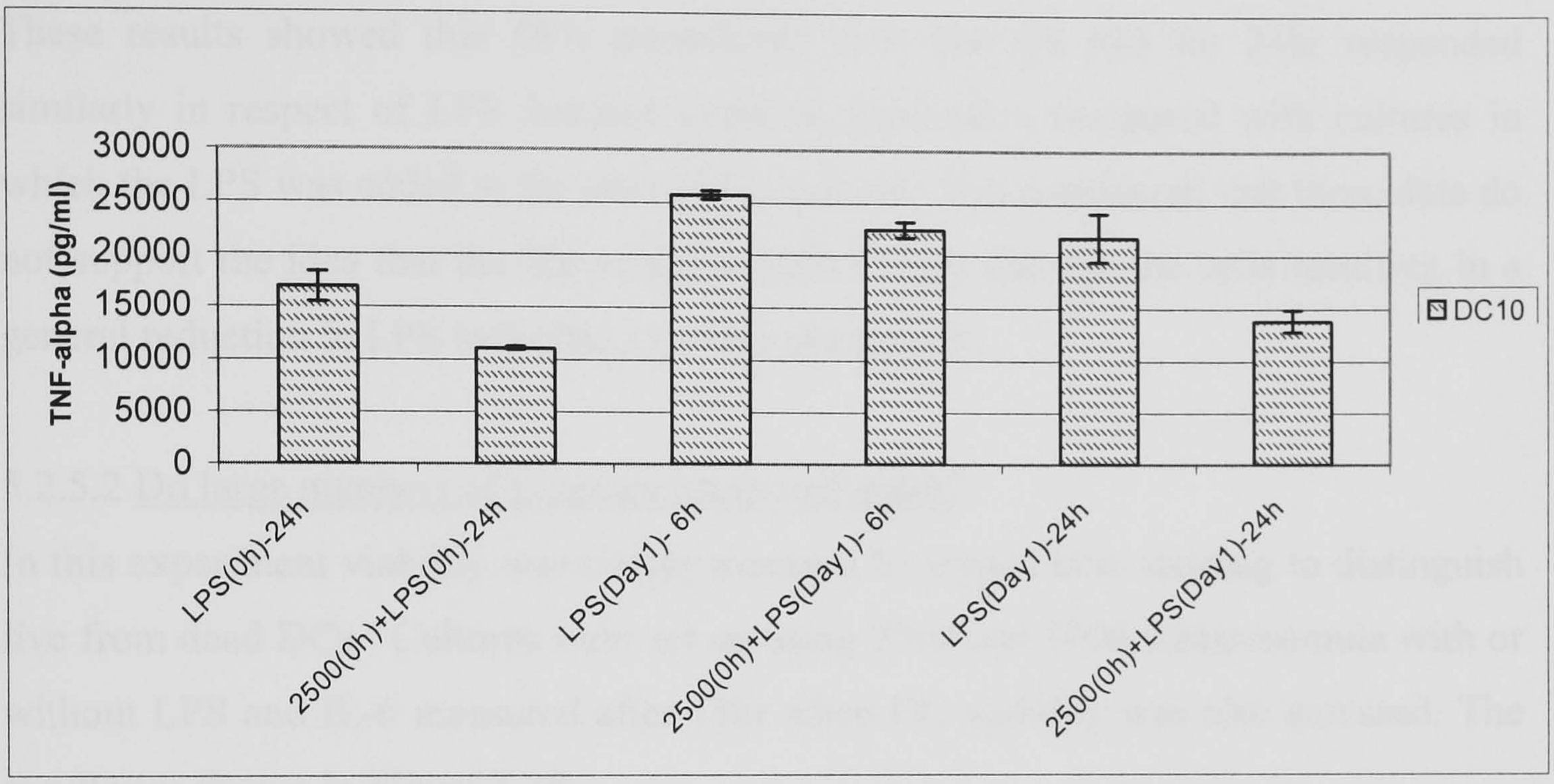
TNF-α

The pattern of TNF-α production (Figure 5.17.C) was similar to IL-12p70 (Figure 5.17. B) but the parasite inhibition was less pronounced. Addition of parasites and LPS to cultures at time 0 resulted in 34% reduction in TNF-α levels compared without

parasites (P=0.002). A similar pattern of parasites reducing the cytokine levels was observed at both 6 hours and 24 hours post LPS addition at 24 hr (13% [P=0.003] and 36% [P=0.005] reductions, respectively) compared with LPS alone. As with IL-6 and IL-12p70 the DCs cultured in the 24well plates for 24 hr produced higher cytokine levels compared with the freshly plated cells.

Figure 5.17. C and D

C



D

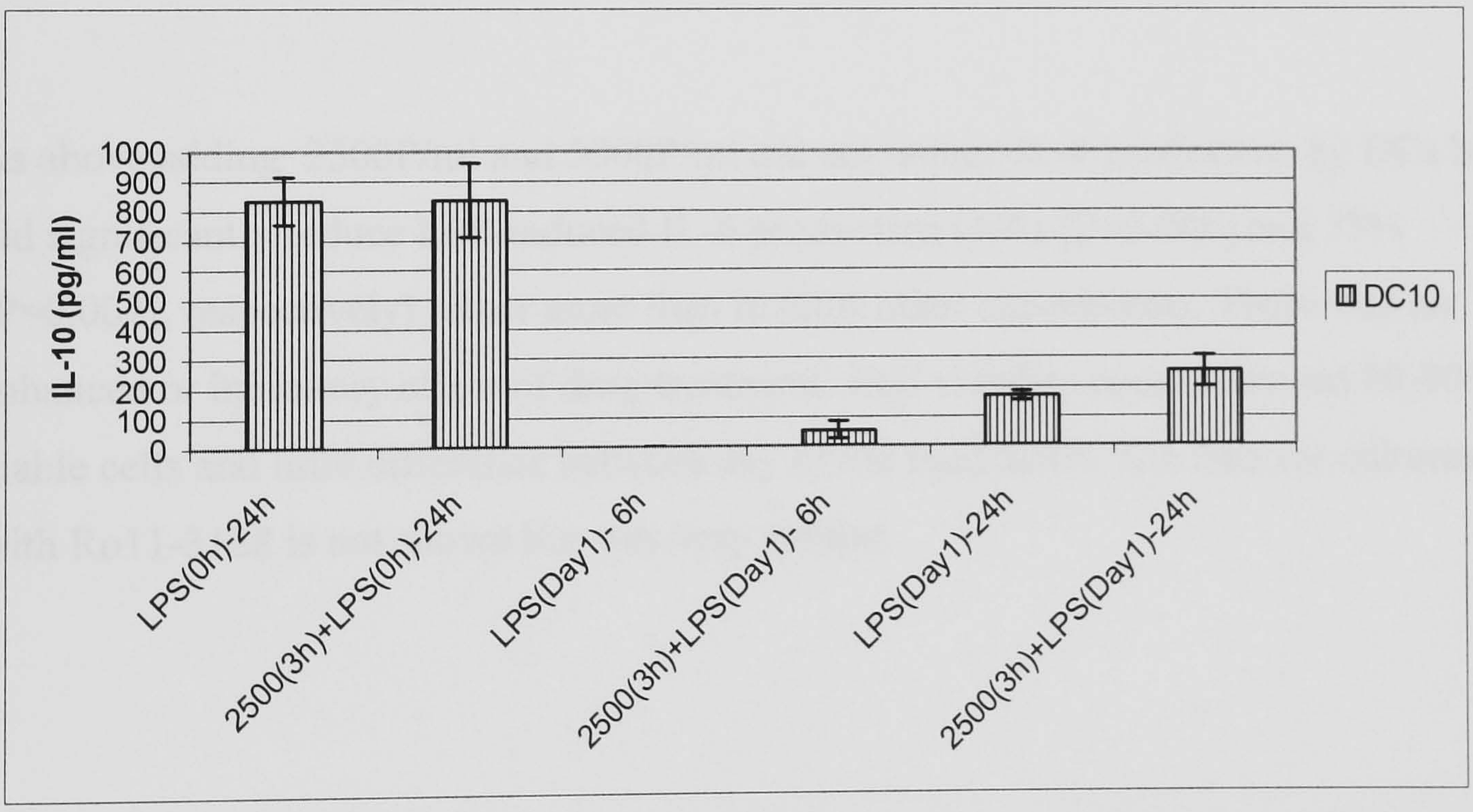


Figure 5.17. C and D (See Figure 5.17.A and B).

IL-10

Levels of IL-10 (Figure 5.17.D) showed a quite different pattern. As in the previous studies, co-culture of freshly plated DCs with schistosomula [2500(3h)+LPS (0h)-24h] did not affect LPS induced IL-10 production at 24hr relative to LPS-activated DCs alone [LPS (0h)-24h]. In marked contrast to IL-6, IL-12p70, and TNF-α, DCs cultured

overnight in 24 well plates (in the absence of parasites) produced much less IL-10 following LPS activation compared with activation of freshly plated cells. Even so the lowish level of IL-10 production following adding LPS at 24hr was increased rather than decreased in the cultures containing parasites (252±53 pg/ml as compared to 169±16 pg/ml, a 33% increase at 24 hr after LPS activation [P=0.062]).

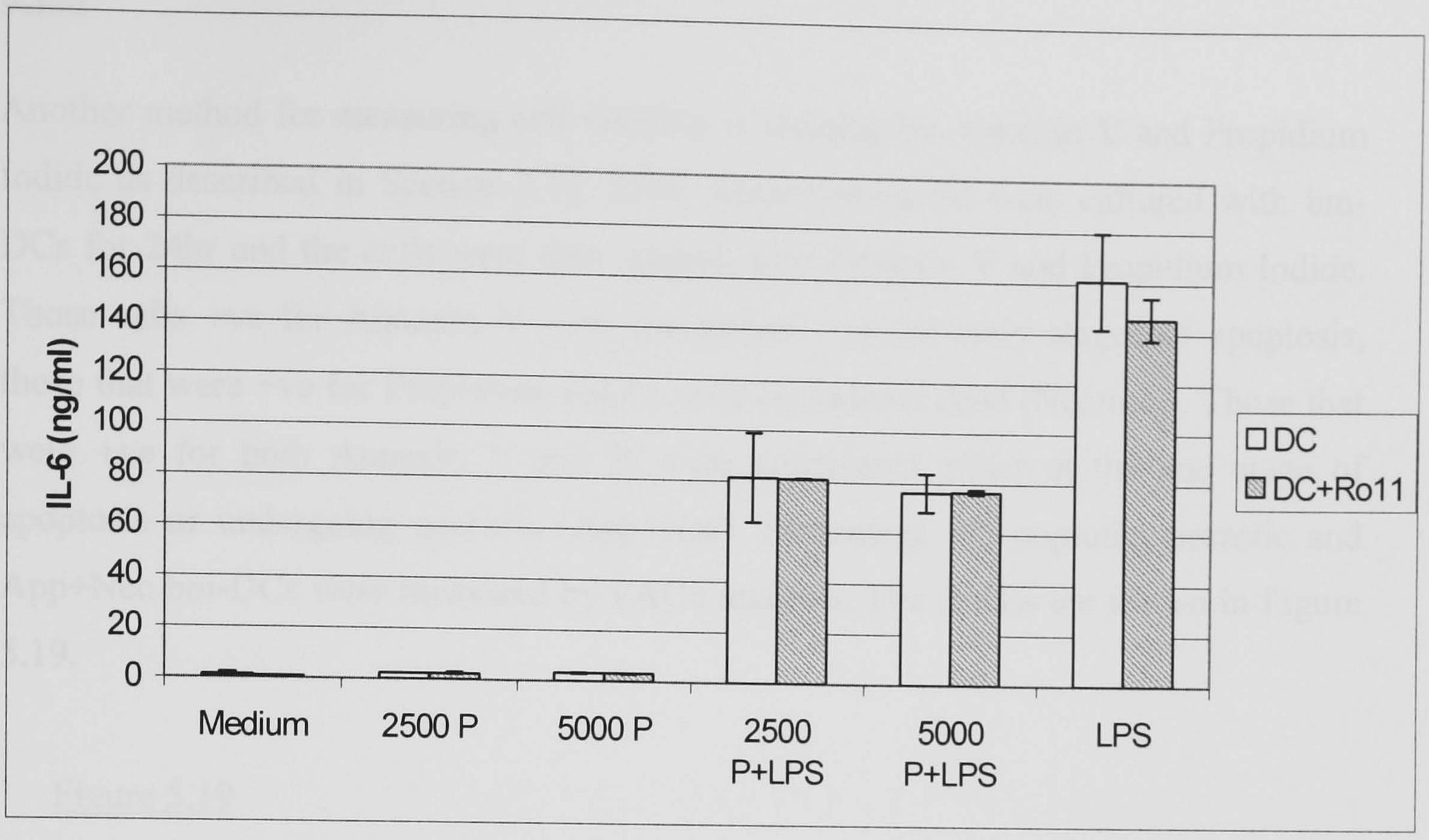
These results showed that DCs precultured with 3hr old MS for 24hr responded similarly in respect of LPS induced cytokine production compared with cultures in which the LPS was added at the start of the cultures. It is considered that these data do not support the idea that the 3hr schistosomula simply damage the cells resulting in a general reduction in LPS inducible cytokine production.

5.2.5.2 Do large numbers of parasites cause cell death?

In this experiment viability was simply assessed by trypan blue staining to distinguish live from dead DCs. Cultures were set up using 2500 and 5000 schistosomula with or without LPS and IL-6 measured after 18hr when DC viability was also assessed. The results are shown in Figure 5.18.

As above adding 2500P/ml and 5000P/ml did not induce IL-6 production by DCs but did significantly reduce LPS-induced IL-6 production (49% [P=0.006] and 53% [P=0.002], respectively) rather more than in most other experiments. There was no enhanced or inhibitory effect of drug-treatment. Cell viability counts showed 80-90% viable cells and little difference between any of the treatments. The data for cultures with Ro11-3128 is not shown but was very similar.

Figure 5.18



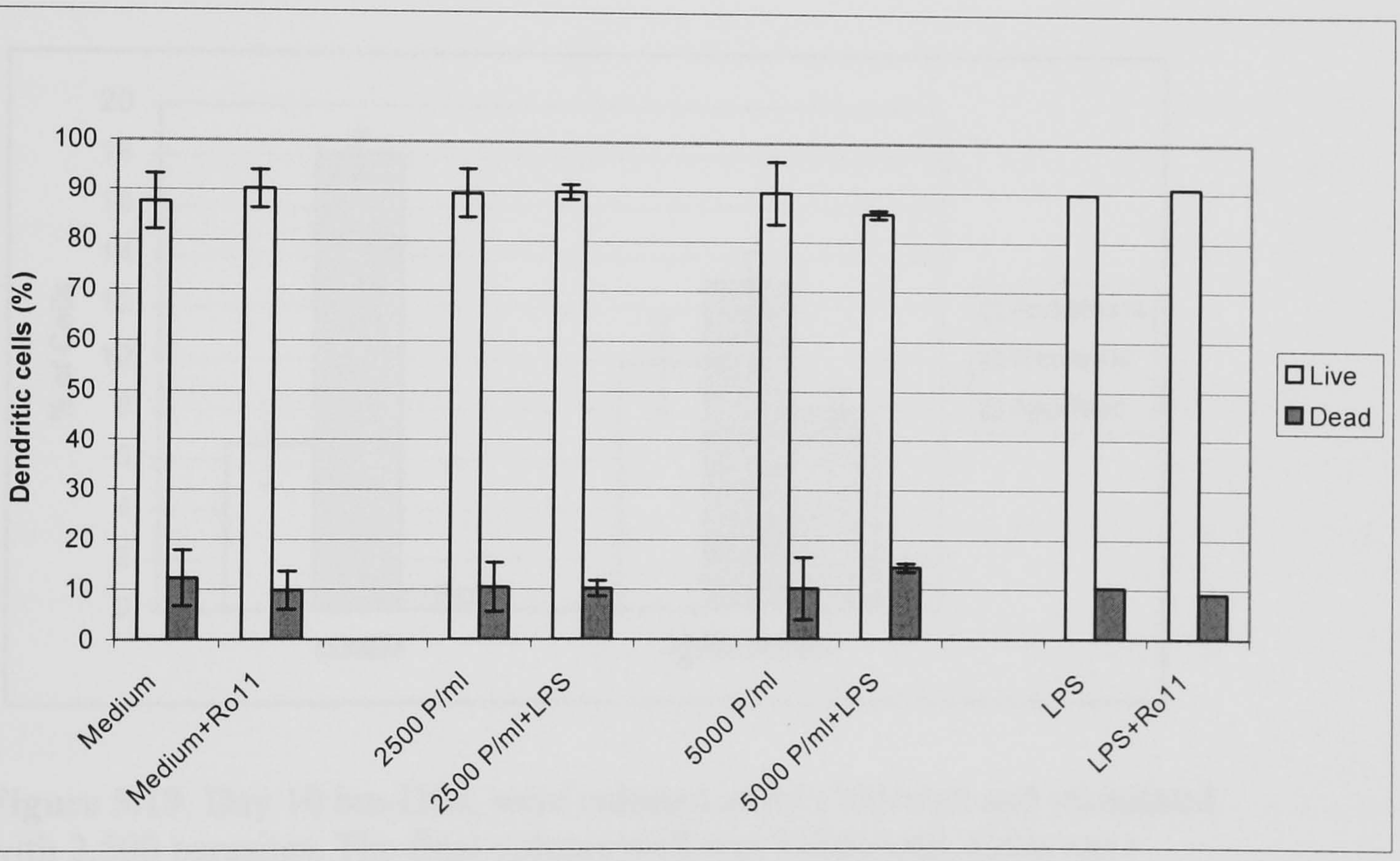


Figure 5.18. Day 10 bm-DCs, were cultured at $5.0x10^5$/well and stimulated with 2,500 - 5,000 parasites (P) in the absence or presence of Ro11-3128 (1.25μg/ml) and/or LPS at 1μg/ml. The final volume/well was 500μl/well. Supernatants were recovered after 18 hours of incubation and assayed for cytokines by ELISA. DC viability was assessed by Trypan blue staining, after 18 hours of incubation. Cells not stained blue with the dye were classified as viable, whereas those stained blue were classified as dead. Data represents Mean values ± Standard Deviation of triplicate culture wells.

5.2.5.3 Annexin V and propidium iodide staining method for apoptotic cells and cell death

Another method for measuring cell viability is staining by Annexin V and Propidium Iodide as described in Section 2.12. 2500 schistosomula/ml were cultured with bm-DCs for 24hr and the cells were then stained with Annexin V and Propidium Iodide. Those cells +ve for Annexin V were considered +ve for early stages of apoptosis, those that were +ve for Propidium Iodide were considered dead (Necrotic). Those that were +ve for both Annexin V and PI were considered either at the end stage of apoptosis or undergoing necrosis (App+Nec). Percentage of apoptotic, necrotic and App+Nec bm-DCs were measured by FACS analysis. The results are shown in Figure 5.19.

Figure 5.19

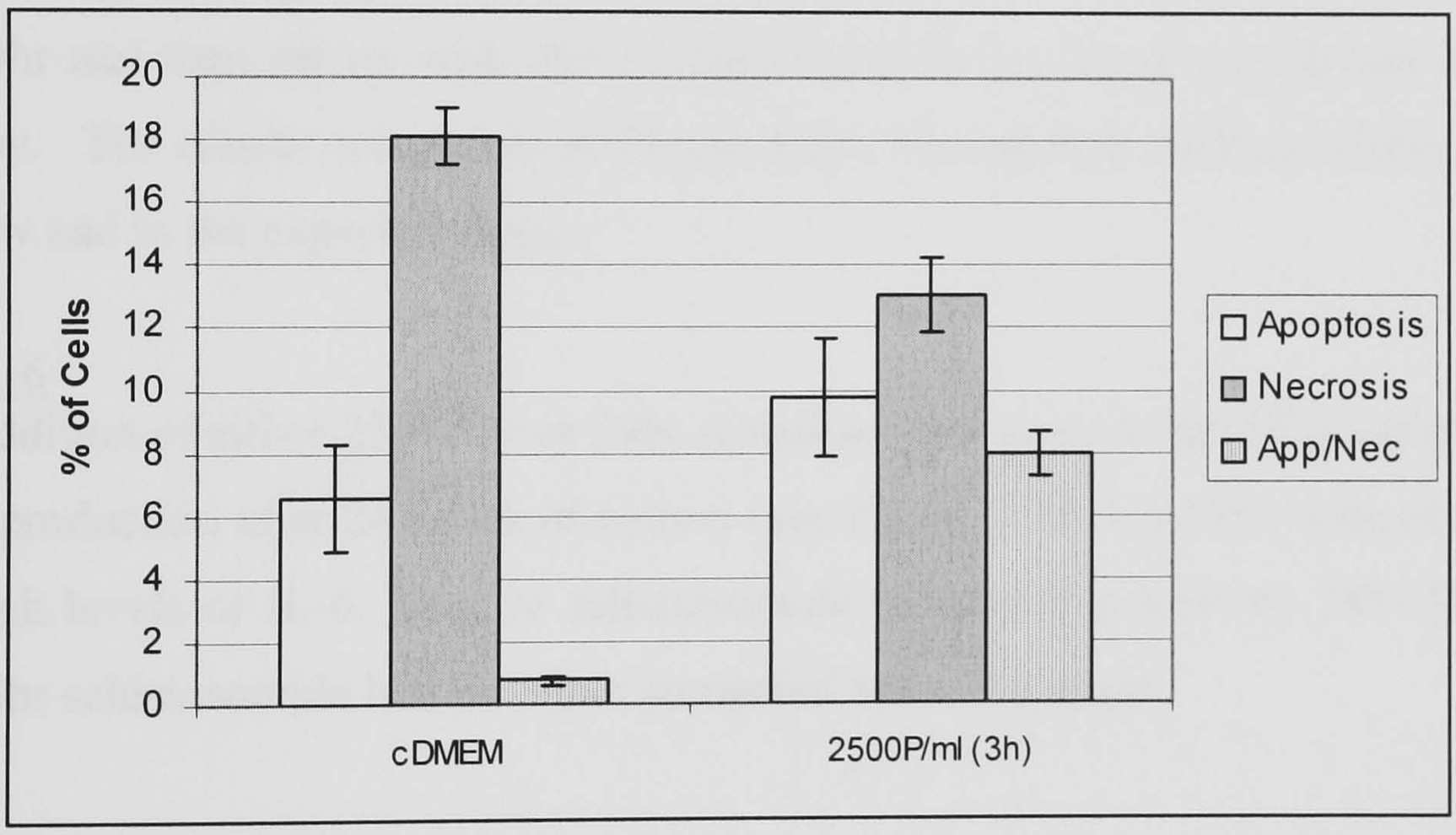


Figure 5.19. Day 10 bm-DCs, were cultured at 5.0x105/well and stimulated with 2,500 parasites. The final volume/well was 500µl/well. Cells were recovered at 18hr, stained using Annexin V and propidium iodide as described in section 2.12.

Although there was some difference in the different categories, the results show a comparable level of necrotic and apoptotic cells with or without the schistosomula (25% and 30% respectively for apoptotic and necrotic cells together) which indicates that 2500 schistsomula did not induce singificantly greater cell damage compared to

medium alone. A similar result was obtained using 10,000 schistosomula (data not shown).

The above studies had used schistosomula just 3hr after mechanical transformation. Because drug treatment in the RoNI is not given until day 2 post infection it was of interest to see if older schistosomula would also induce these effects on DCs. To investigate this, experiments were carried out with schistosomula cultured for 24hr.

5.2.6 Comparison of the effects of 3hr and 24hr schistosomula on LPS induced cytokine production.

5.2.6.1 Experiment 1

Cultures were set up as in earlier experiments. In this experiment 3hr schistosomula were set up with day 10 bm DCs on one day and the remaining schistosomula kept for 24hr and then set up with day 11 bm-DCs from the same preparation the following day. The results are shown in Figure 5.20. The control, medium alone, values were low and in the expected range.

IL-6

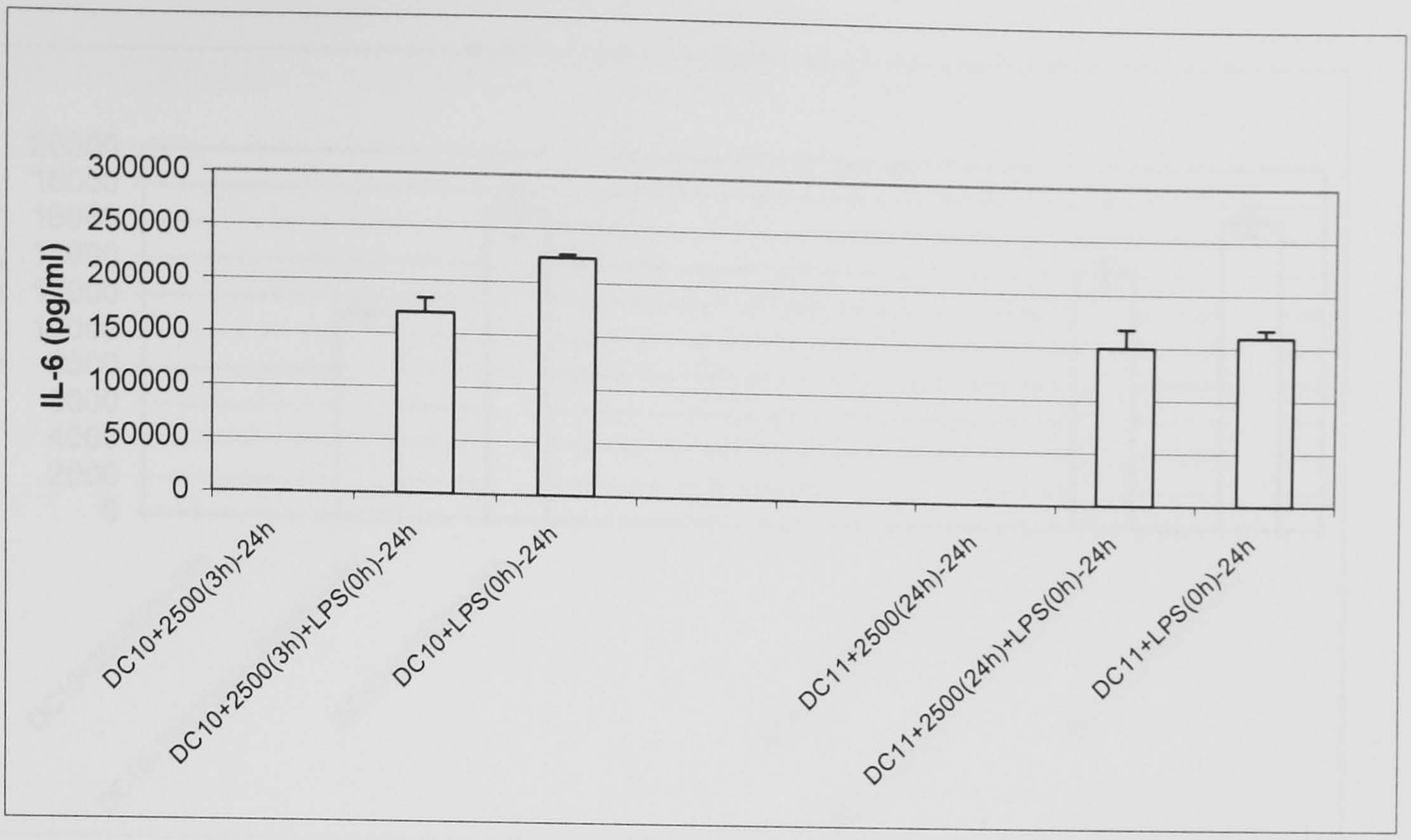
Addition of either 2500 3hr or 24hr schistosomula alone to bm-DCs did not induce IL-6 production after 24 hours of culture (see Figure 5.20.A.). LPS induced the expected high levels of IL-6. The 3hr schistosomula reduced this level by 24% [P=0.003] but 24hr schistosomula had no effect compared with LPS alone.

IL-12p70

As seen in Figure 5.20.B adding LPS (1μg/ml) stimulated day 10 bm-DCs to produce 3,109±134 pg/ml of IL-12p70 by LPS, a level that was significantly reduced by 63% in the presence of the 3hr schistosomula [P=0.00021]. The LPS-stimulated day 11 bm-DCs produced lower IL-12p70 (1,907± 150 pg/ml) than the day 10 bm-DCs but interestingly, 24hr schistosomula did not significantly suppress this.

Figure 5.20.

A



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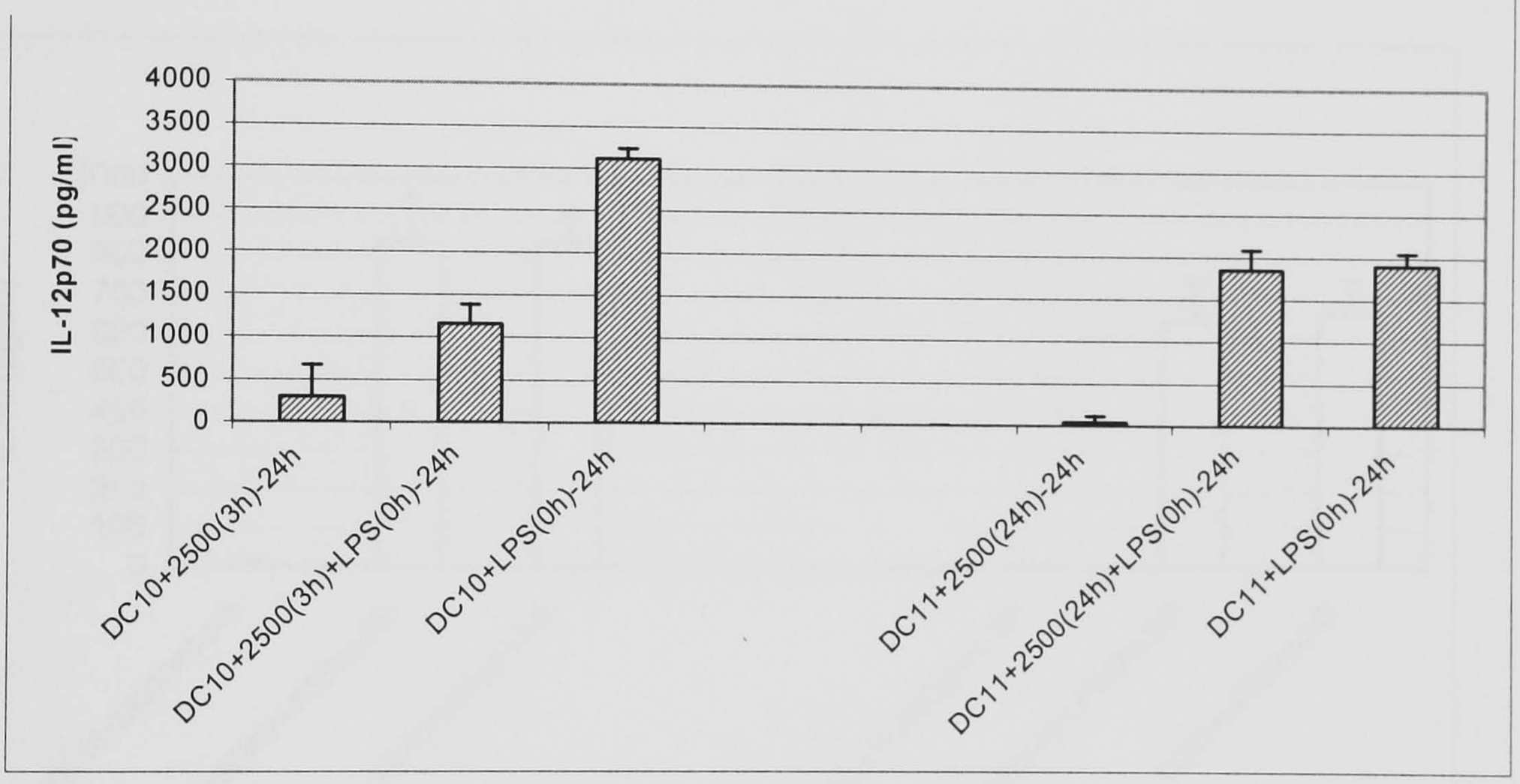


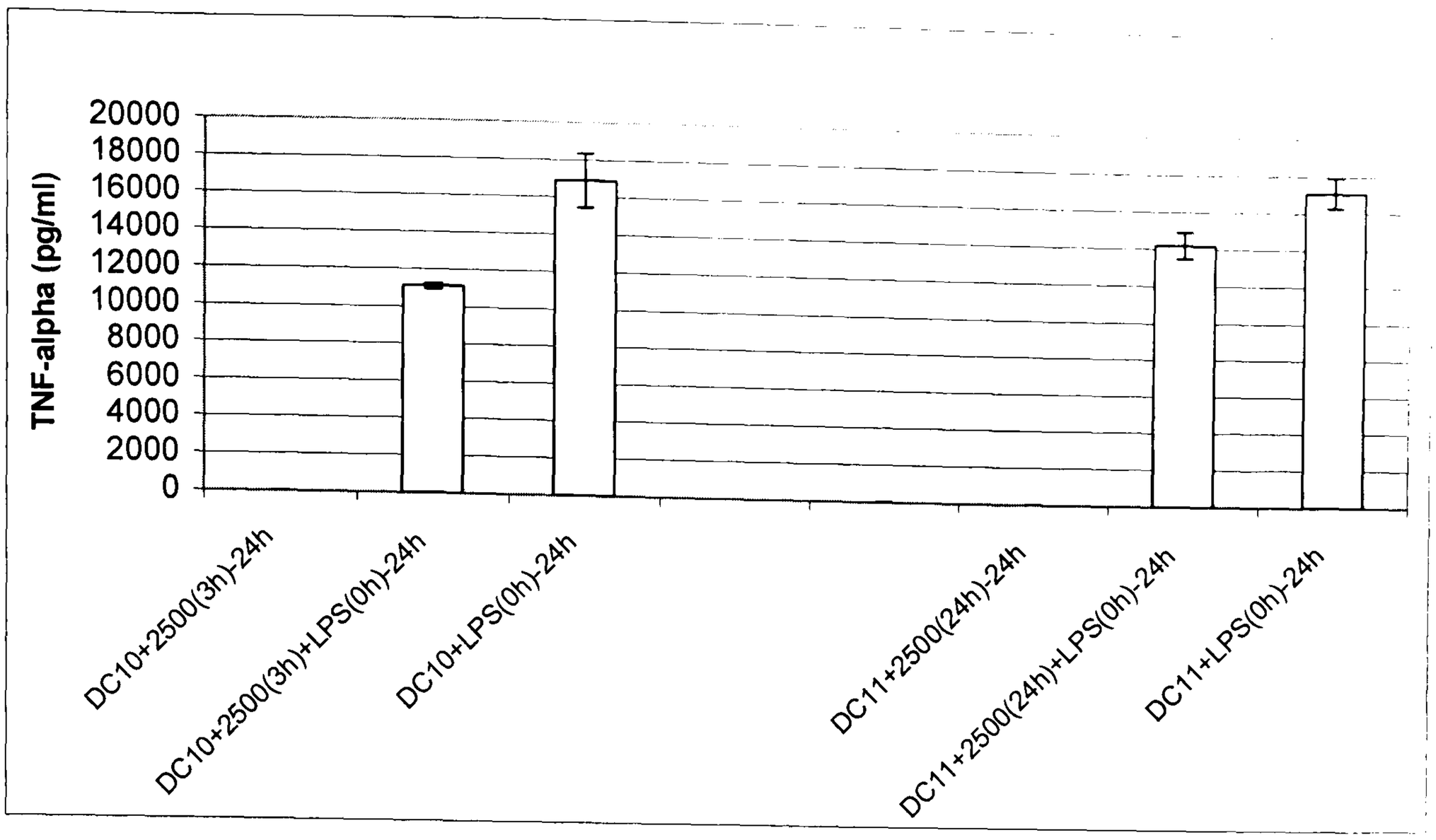
Figure 5.20. A and B. Day 10 bm-DCs, were cultured at $5.0x10^5$/well with or without 2,500 3hr old parasites (2500 [3h])/ml. The following day 24hr old parasites (2500 [24h])/ml were added to day 11 bm-DCs. LPS was added at 1μg/ml to some of the cultures. Supernatants were recovered 24h after LPS stimulation and assayed for cytokines by ELISA. Data represents Mean values ± Standard Deviation of triplicate culture wells.

TNF-α

Neither 3hr nor 24hr schistosomula stimulated any TNF-α production from bm-DCs (Figure 5.20.C). The LPS activation produced comparable TNF-α production from the day 10 and day 11 cultures. Adding 3hr parasites to LPS-stimulated day 10 bm-DCs suppressed the level of TNF-α by 34% compared to LPS control cultures [P=0.002] and adding the 24hr parasites also suppressed production (by 17%, P=0.01).

Figure 5.20. (contd.)

C



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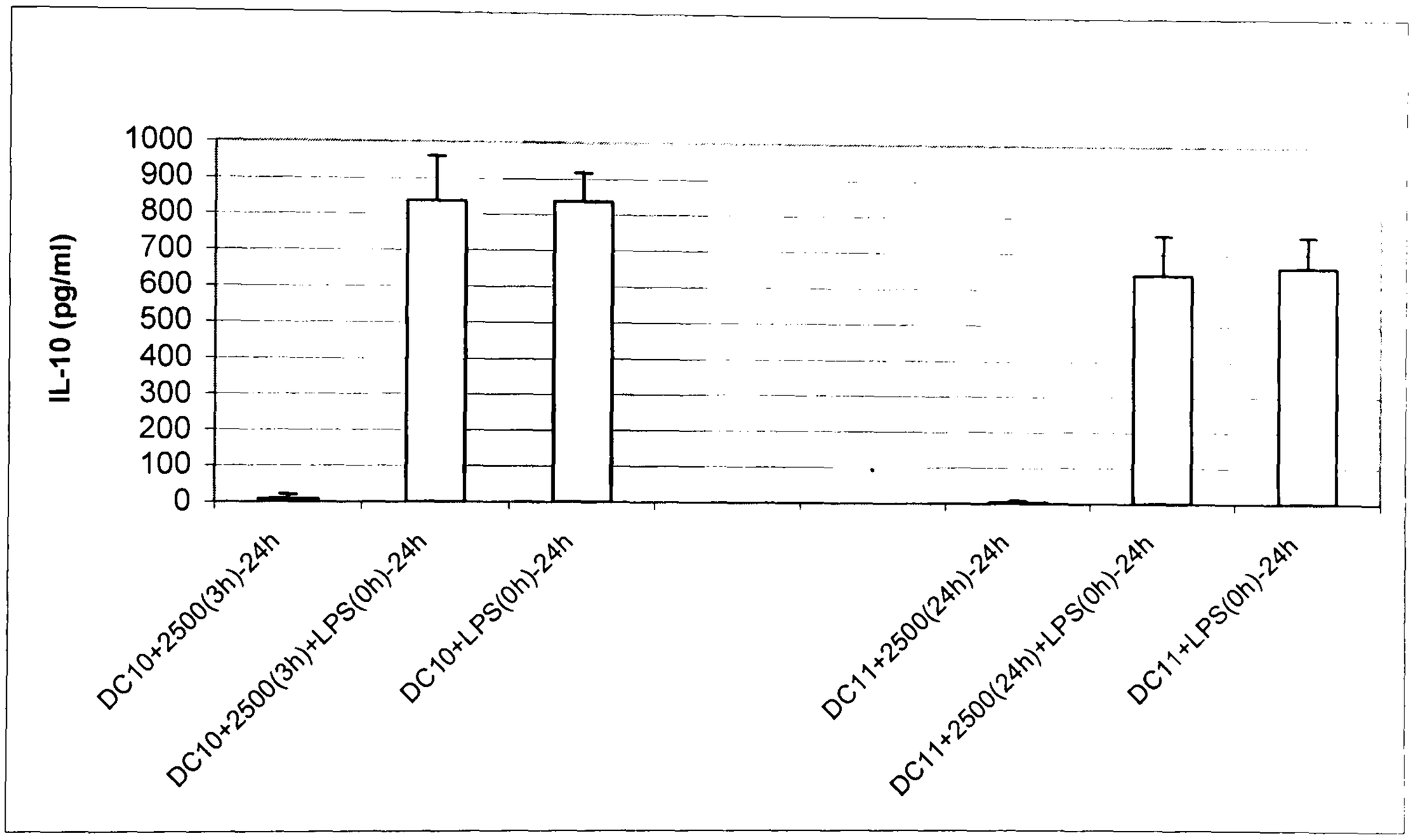


Figure 5.20.C and D. See legend to Figure 5.20.A and B.

IL-10 production

The 3hr parasites alone did not stimulate IL-10 (Figure 5.20.D). The 24hr parasites did seem to have stimulated IL-10 production at 6hr following co-culture (data not shown) but none was detected at 24hr. Adding LPS to cultures containing freshly prepared parasites induced the production of high levels of IL-10, as with IL-6. Neither 3hr nor 24hr schistosomula significantly reduced or enhanced this level.

These results confirm earlier ones showing that 3hr schistosomula can reduce LPS induced cytokine levels in bm–DC culture (IL-12>TNF-α & IL-6) but IL-10 is

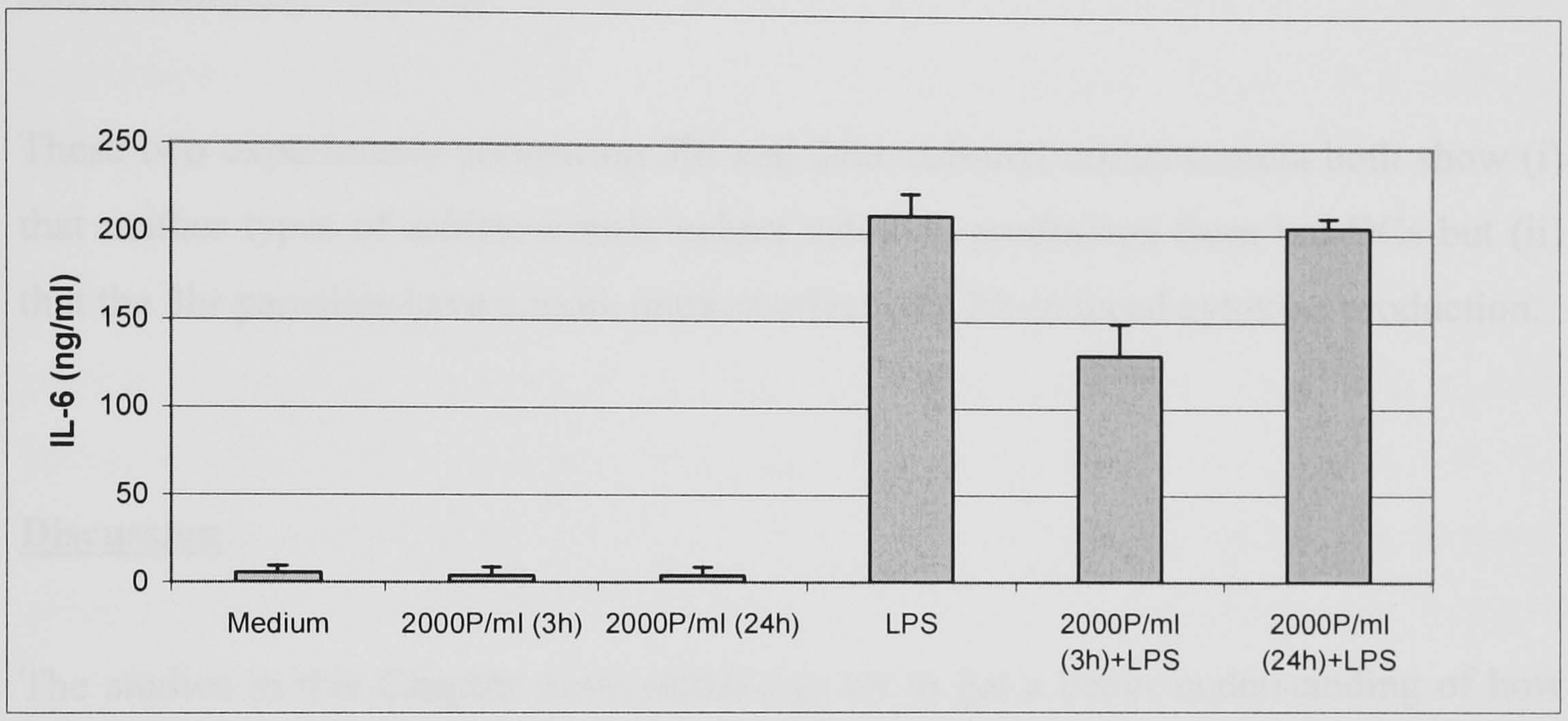
unaffected. The 24hr schistosomula, however, did not significantly affect the levels of any of these cytokines.

5.2.6.2 Repeat comparison of 3hr and 24hr parasites.

This was similar to the previous experiment but this time the 24hr schistosomula were prepared on one day and the 3hr schistosomula the following day. These were then added to the same batch and age of bm-DCs. The results are shown in Figure 5.21.

Figure 5.21.

A



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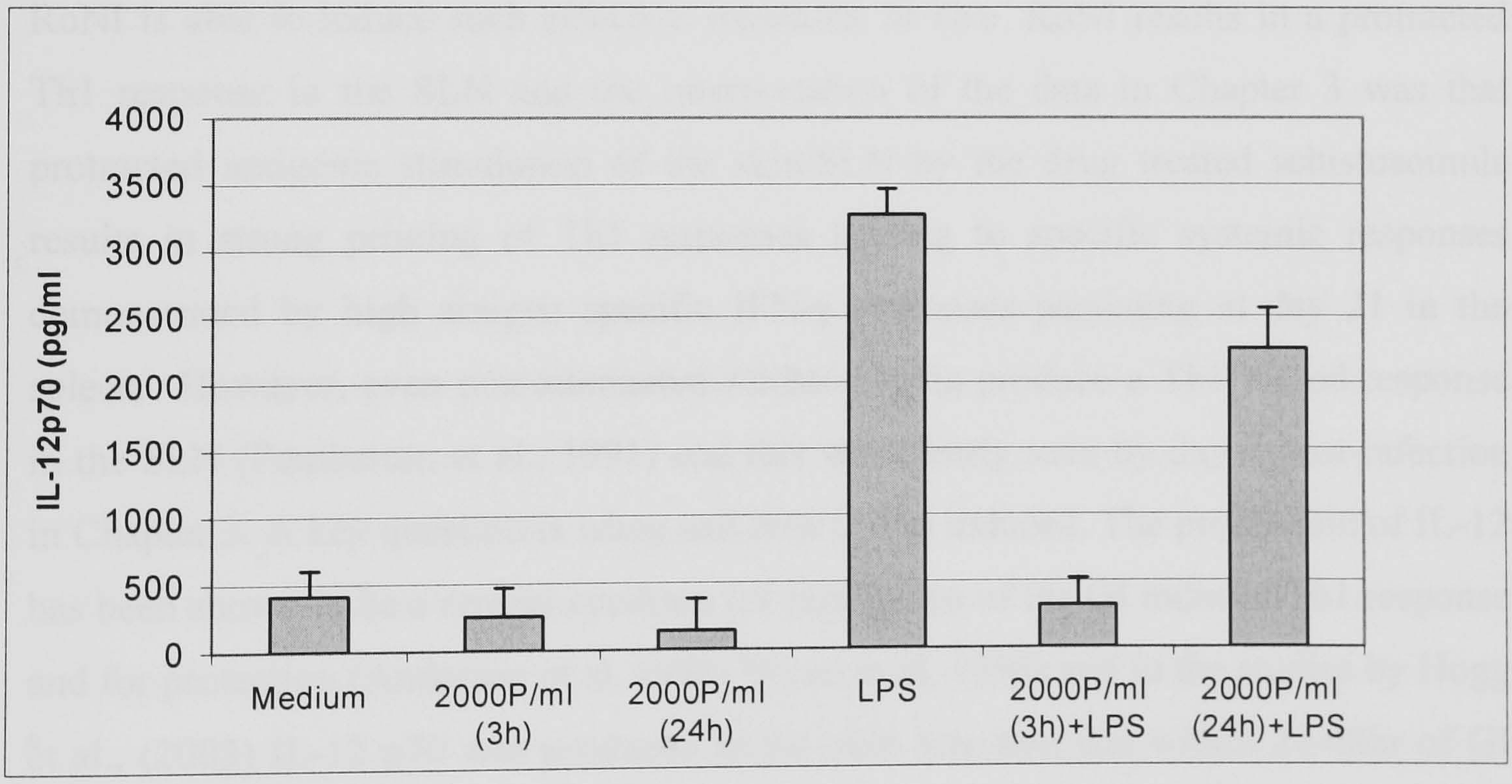

Figure 5.21. A and B. Day 10 bm-DCs, were cultured at $5.0x10^5$/well with or without 2,500 3hr old or 24hr old parasites [2500 (3h)/ml] or [2500 (24h)/ml]. LPS was added at 1μg/ml to some of the cultures. Supernatants were recovered 24h after LPS stimulation and assayed for cytokines by ELISA. Data represents Mean values + Standard Deviation of triplicate culture wells.

As was observed in previous experiments neither 3hr or 24hr schistosomula induced elevated levels of IL-6 or IL-12p70 production by DCs on their own. Also as in earlier experiments 3hr schistosomula reduced the level of IL-6 (38%, P=0.00012) and IL-12p70 production (90%, P<0.0001). But 24 hr cultured schistosomula had no effect on the IL-6 production by DC and caused a slight reduction in IL-12p70 (31%, P=0.00048) although the levels remained significantly higher than with 3hr schistosomula (P=0.0008).

These two experiments comparing 3hr and 24hr cultured schistosomula both show (i) that neither types of schistosomula induce cytokine production from bm-DCs but (ii) that the 3hr parasites have a more marked effect on LPS-induced cytokine production.

Discussion

The studies in this Chapter were initiated to try to get a better understanding of how RoNI is able to induce such effective immunity *in vivo*. RoNI results in a protracted Th1 response in the SLN and the interpretation of the data in Chapter 3 was that protracted antigenic stimulation of the skin/SLN by the drug treated schistosomula results in strong priming of Th1 responses leading to specific systemic responses demonstrated by high antigen specific IFN-γ responses persisting at day 21 in the spleen. However, even non-attenuated schistosomula produce a Th1 biased response in the SLN (Pemberton et al., 1991) and this was clearly seen by day 7 post-infection in Chapter 3. A key question is when and how this is induced. The production of IL-12 has been shown to be a crucial cytokine for production of the GI induced Th1 response and for protection (Anderson et al, 1998; Wynn et al. 1996) and in the studies by Hogg et al., (2003) IL-12 p70 was produced in the skin infection site within 24-48hr of GI and NI. Also the majority of MHCII, IL-12p40 positive cells emerging from skin explants taken at day 4 post vaccination were myeloid DCs or macrophages. Other studies have shown that certain Th1 promoting infectious organisms can activate DCs directly *in vitro* (Gorak et al, 1998; Marriott et al., 1999; Seixas et al., 2001) and so the experiments in this chapter focussed on finding out if schistosomula could similarly

activate DCs and whether this could be enhanced by Ro11-3128 treatment. No previously published data on this was available. It was hoped that by studying the interaction of DCs, which are so important in influencing the nature of the response to a new antigenic stimulus, with normal or drug treated schistosomula *in vitro* some clue would be found about how this Th1 biased response is generated.

Technically these experiments worked well. The 'naïve' bm-DC preparations prepared on different occasions showed varying proportions with the mature phenotype which was defined as MHCII and CD86 high. This was especially so in the early experiments. Bm-DC activation was low among immature DCs (~33%) and the separation of the DCs from the schistosomula by filtration, which was very effective, did not further activate the cells. LPS or TNF-α stimulation normally increased this surface expression more than two fold. The schistosomula preparations using the percoll separation technique produced pure preparations of schistosomula uncontaminated with cercarial tails. The percoll separation combined with the extensive washing in antibiotics produced preparations that did not develop bacterial contamination. Samples of each preparation were kept for at least 10 days and bacterial or fungal contamination was monitored and was a very rare event. Experiments in which this was seen in these controls even if not seen in the parasite DC cultures were abandoned. As it turned out the lack of bacterial/endotoxin contamination in the parasite preparations was clear from the uniform lack of DC activation seen.

Experiments focused on use of living schistosomula but initial studies showed that soluble antigens derived from the penetrating parasites such as CTF (cercarial transformation fluid) and SCA (soluble cercarial antigen) upregulated the expression of MHCII and CD86 in a dose-dependent manner. However, it was found that both CTF and the cercarial antigen preparation used in this experiment had measurable levels of endotoxin and when the experiment was repeated in C3H/HeJ mice which are LPS non-responders (Hopkins et al., 1996) there was no upregulation in the case of CTF and a markedly lower response for SCA. It was concluded that there was no reliable evidence of parasite induced DC activation by these soluble antigens. Since a focus of the work was to look at the effects of Ro11-3128 treatment of schistosomula on DC activation this work on soluble products was not continued.

In numerous experiments, of which 6 separate experiments using 50-10,000 schistosomula/ml are presented in this Chapter there was no evidence of upregulation of surface markers (MHCII, CD86, CD40). In these cultures the parasites showed good viability throughout the duration of the experiment. Addition of Ro11-3128 to the cultures caused the expected effects on the schistosomula surface but had no significant effects on the surface marker expression and neither did exposure to irradiation. In an attempt to prime the DCs by partial activation with LPS or TNF-α no extra surface marker expression was seen. Overall therefore the data indicates that bm-DC do not respond to molecules released by living schistosomula at least in the time frame of the experiments carried out here, 24hr culture. Seixas et al. (2001), using the same type of bm-DC with *Plasmodium c. chabaudi* schizonts have found upregulation of these markers at 20hr of co-culture.

In five separate experiments schistosomula (650-10,000/ml) also failed to induce significant effects on DCs as judged by levels of cytokine production (IL-12p70, TNF-α, IL-10 or IL-6). These effects were also independent of the presence of Ro11-3128.

However, co-culture of DC with schistosomula reduced the levels of LPS-induced cytokine production by bm-DCs in a dose-dependent manner and had differential effects on production of the different cytokines. A marked reduction occurred with high numbers of larvae (2500-10,000/ml), while this inhibition was less with lower numbers 625-1250/ml. IL-12p70 production was most affected (mean reduction of 80%) followed by IL-6 (mean reduction of 34%) and TNF-α (mean reduction of 32%). IL-10 was relatively unaffected, and IL-4 levels were undetectable. As with the CD86 and MHCII expression, the negative effects of schistosomula on cytokine expression was unaffected by the addition of Ro11-3128 to the cultures.

To explain the lack of upregulation of surface markers and of cytokine production during schistosomula/DC co-culture and the differential effects on LPS-induced secretion of the different cytokines various possibilities were considered: (a) schistosomula products (pathogen-associated molecular patterns, PAMPs) fail to signal activation of DCs but rather selectively and specifically signal DCs to inhibit different cytokine pathways (b) schistosomula simply damage/kill DCs and, to explain the apparent differential effects on cytokines, it may be that the various cytokines are

produced with different kinetics following LPS stimulation e.g. IL-10 may be produced early in the response (before cell damage had occurred) whereas TNF-α, IL-6 and IL-12p70 may be produced later and so are progressively more affected by any damage to the cells. Differential rates of production during co-culture of bm-DCs with *P.c. chabaudi* have been reported (Seixas et al., 2001) although in this study TNF-α peaked at 1hr whereas both IL-6 and IL-12p70 increased to peak at 20hr, (c) schistosomula products may promote cytokine production but other larval products (e.g. enzymes) could selectively degrade secreted cytokines (IL-12p70>>IL-10).

The explanation that progressive loss of cell viability combined with different rates of cytokine production was investigated by setting up the co-cultures and adding LPS either at the start or after 24hr. The same patterns of effect on cytokine production with the schistosomula were seen for each cytokine for LPS addition at time 0 or 24hr and in fact were less pronounced at the 24hr time point compared with time 0 (36% reduction at 24h compared with 34% reduction at 0h in TNF-α levels, and 38% reduction at 24h compared with 63% at 0h in IL-12p70 levels). For IL-10 there was an enhancement of 33% in the presence of the schistosomula compared with no effect at time zero. This supports the idea that the schistosomula have specific and differential effects on the production of the different cytokines rather than having cytotoxic effects and this is also indicated by the lack of evidence for general loss of cell viability or for apoptosis by methylene blue staining and the Annexin V and propidium iodide staining. However, further experiments would be needed to extend and confirm this. The possibility that parasite products may degrade cytokines can be investigated by adding known cytokine concentrations to the cultures and then assessing their levels at intervals during culture. Since the 24hr cultured parasites did not have the same effect as the 3hr cultured parasites it would be useful to compare these in such an experiment. It would also be important to establish if the effects on cytokine production can be seen at the mRNA level by RNAse protection or RT-PCR methods. Inclusion of house-keeping genes in the assays would control for the general effects on cell viability. If differential effects could be seen at the mRNA level it would indicate some specific signalling by parasite factors. In addition other DC functions could be measured such as the ability to process and present other antigens and for this the use of ovalbumin and the ovalbumin specific transgenic T cells would be suitable (Hsieh et al., 1993).

Angeli et al. (2001) looked at activation of skin Langerhans cells (LC) *in situ* following infection of mice with *S. mansoni* cercariae. They reported that LC showed upregulation of MHCII and CD86 from 1-120hr post infection as judged by immunohistochemistry and were also positive for the DC activation marker DEC-205. It was also reported that the most intensely stained LC were surrounding the parasites. In collaboration with Dr Stephen Jolles (National Institute for Medical Research, Mill Hill) experiments were carried out co-culturing schistosomula with cells extracted from mouse epidermal sheets using trypsin (Tokura et al., 1994) and staining for MHCII and CD86. Unfortunately, the process of extraction and manipulation of these cells, which contain around 5% LC amongst keratinocytes, resulted in 100% of the cells upregulating these markers even in control cultures and the mean fluorescence intensities were also comparable with or without parasites. The work of Angeli et al. (2001) demonstrates a rapid activation of LC after infection and a close association between the parasite and the activated cell but it does not show that parasite products induce the activation directly. Keratinocytes, the most abundant cell type in the skin are known to be activated by microbial stimuli to produce a range of cytokines (Tokura et al 1994) and it is possible that the activation of LC seen by Angeli et al. (2001) is secondary to these effects and not caused directly by LC/schistosomula molecular interactions.

The observed upregulation of MHCII and CD86 (Angli et al, 2001) is typical of the response of DCs to Th1 activating signals such as heat-killed *Propionebacterium acnes* (MacDonald et al., 2001), and indicates a Th1 priming signal in the skin soon after infection. In contrast, the Th2 priming signal, *S. mansoni* egg antigen, did not up-regulate the expression of DC costimulatory molecules *in vitro*: CD40, CD54, CD80, CD86 or OX40L and produced no detectable IL-4, IL-10, or IL-12, displaying only a minor increase in MHC class II expression, yet were able to prime a strong Th2 response when injected into naïve recipient animals (MacDonald et al., (2002b). A Th1 response can be demonstrated in cultured skin from isolated pinnae soon after infection of mice with either normal (NI) or irradiated (GI) cercariae but is more pronounced in the GI (Hogg et al., 2003). These authors did not look before 24hr post infection but at that time the Th1 promoting cytokines IL-12p70 and IL-18 were elevated in both NI and GI at 24hr, and the majority of MHCII, IL-12p40 positive cells emerging from skin explants taken at day 4 post GI vaccination were myeloid DCs or macrophages.

However, the response at this early time is not exclusively Th1 dominated, both antigen specific IL-4 and IFN-γ were demonstrated to be produced in SLN at day 4 post infection (Hogg et al., 2003b). It was only subsequent to this that production of both of these cytokines declined in the NI but IFN-γ persisted until day 14. So on balance it is uncertain precisely what feature of the attenuated infections favours the Th1 dominance and where and when it is induced but the results of the studies in this chapter suggest that schistosomula do not release factors that can activate DCs but rather that they are inhibited.

The balance of effects observed, reduced IL-12p70 and TNF-α but no effect on IL-10 are likely to be anti-inflammatory as IL-12 is a proinflammatory cytokine governing the development of IFN-γ producing T cells (Trinchieri, 1998) and IL-10 is a strong inhibitor of IL-12 transcription (Aste-Amezaga et al., 1998) and inhibits IL-12 production by DC (De Smedt et al. 1997). Various *in vitro* studies have shown that IL-12 is produced by dendritic cells following stimulation with microbial products (Reis e Sousa et al., 1999) and that such DC are then able to transfer development of protective Th-1 cell responses to mice in such infections as Leishmania and tuberculosis (Ahuja et al 1999; Tascon et al., 2000). Also IL-12 production by DC has been shown in mice early after injection of microbial antigens (Reis e Sousa et al., 1997). In contrast IL-10 is an immunosuppressive cytokine which DC also produce and which can down regulate IL-12 production and so reduce Th1 development (Moore et al., 2001). Addition of IL-10 to DC can reduce their expression of IL-12 and also their ability to induce IFN-γ producing T cells *in vivo* (De Smedt et al., 1997). Conversely, endogenous inhibition of IL-10 expression in DC can boost Th1 immunity to *Chalmydia* infection (Igietseme et al 2000). Both IL-12 and IL-10 are produced in the skin of mice infected with normal or irradiated cercariae of *S. mansoni* (Hogg et al. 2003) and reciprocal regulatory effects of these two cytokines were recently demonstrated using irradiated infections (Hogg et al., 2003b). IL-12-/- mice vaccinated with GI failed to develop IFN-γ in the SLN but notably showed a marked increase in IL-10 production in the skin and SLN. This resulted in reduced skin inflammation. Conversely in GI vaccinated IL-10-/- there was a marked increase in IL-1β and IL-12p40 in the skin and in IL-12p40 and IFN-γ in the SLN. Such mice showed

a marked increase in skin inflammation. This shows that IL-10 production in the skin during GI has an anti-inflammatory role preventing excessive dermal inflammation.

Earlier, Ramaswamy et al., (2000) had reported that *S. mansoni* schistosomula are able to produce significant amounts of prostaglandin E2 (PGE2) following incubation with linoleic acid, a free fatty acid found on the surface of the skin and that they were also able to induce PGE2 production by mouse keratinocytes. This PGE2 led to IL-10 production by keratinocytes as PGE2 is a potent inducer of IL-10 production which in turn leads to reduced IL-12 production (van der Pouw Kraan et al, 1995; Harizi et al., 2002). This PGE2/IL-10-inducing effect was associated with a fraction <30 kDa molecular size in the parasite secretions, and addition of this fraction or of parasite-stimulated keratinocyte culture supernatant to Con A-stimulated spleen cells caused the suppression of cell proliferation. It was proposed that this PGE2 -dependent, IL-10-mediated effect would inhibit skin inflammatory responses which would help parasite survival. With regard to the present observations it is interesting that IL-12 production can be inhibited in DC by agents that increase cyclic AMP, which include PGE2 (Kalinski et al., 1997). Kalinski et al., (2001), showed that PGE2 could enhance IL-12p40 mRNA expression and protein secretion in human TNF-α-stimulated immature DCs. However, this effect was not accompanied by the induction of IL-12p35 expression and thus not by the secretion of the bioactive IL-12p70 heterodimer. When the DCs were stimulated with LPS, as in the present studies, PGE2 inhibited the production of both IL-12p70 and IL-12p40. PGE2 was also reported to inhibit TNF-α mRNA, protein level and release from bm-derived DCs stimulated with LPS (Vassiliou et al., 2003). So it is possible that the observed reduction in LPS-induced IL-12p70 in the DC/schistosomula co-cultures is mediated at least in part by PGE2 released by the co-cultured schistosomula.

Angeli et al. (2001) found that LC, although activated by infection, did not migrate from the skin and that this was due to PGD2 which is produced by the parasite and may be produced in the skin by the release by the penetrating larvae of large amounts of PGD2 synthase. They did not detect DC accumulation in the SLN up to day 10 post infection and it was suggested that the reduced emigration of LC and accumulation of DCs in the SLN may play a key role in control of cutaneous immune responses and again facilitate parasite survival.

Ramaswamy et al. (2000) also reported that γ-irradiation attenuation of parasites significantly abrogated their ability to induce PGE2 or IL-10 from skin cells and it was suggested that this could account for the enhanced skin inflammation and protection seen with GI. Hogg et al., (2003b) also described lower IL-10 in the skin of mice given GI compared with NI at day 4 post infection but since the levels of IL-10 in the skin in GI were substantial they did not consider that this difference accounted for the difference in inflammation. The idea that attenuated parasites may be less effective in producing immunosuppressive molecules like PGE2 and PGD2 and so allow more effective immune responses to develop could also apply to the RoNI model and it would be interesting to see if the RoNI treatment prevented such PGE2 and PGD2 production.

In the present studies, the inhibitory effect on cytokine and in particular IL-12p70 was much less marked when schistosomula were cultured for 24hr rather than when they were used at 3hr. So what might the 3hr schistosomulum produce that is not present after 24hr culture? The cercarial stage has pre- and post-acetabular glands which contain a number of different products which may be released during penetration. One of these is a serine proteinase with elastase activity (Mckerrow et al., 1985) and this is reported to be the only protease present in the acetabular gland secretions (Salter et al., 2000). However, this protease is rapidly released during mechanical transformation and Dalton et al. (1997) found that 3 hr cultured schistosomula no longer contained any of this enzyme. Dalton et al. (1997) did however, describe the presence of the cysteine protease, cathepsin L in the postacetabular glands of cercariae and this could be detected at a low level in extracts of 3hr schistosomulum. However, it is not known if this enzyme is released from the glands or if the protease found in the extracts of 3hr schistosomula is present in remnants of the glands or in the body of the larvae where it is also found in the adult.

Faveeuw et al, (2003) have recently shown that the post-acetabular glands of freshly transformed schistosomula contain glycoproteins with core α3-fucose and core β2-xylose specificities which were also found in abundance in egg antigens. Egg derived glycoconjugates containing α3-fucose e.g the Lewis x trisaccharide (Le^X) have been shown to promote Th2 responses when injected *in vivo* (Okano et al, 2001) and are

also able to promote Th2 responses when mixed with DC which are then injected into mice (Faveeuw et al, 2003). Several species of organism interact with DCs via CHO structures on the organism and C-type lectins on the DC. Amongst these is the DC specific lectin DC-specific ICAM (intercellular adhesion molecule)-3-grabbing non-integrin (DC-SIGN) and this has recently been shown to bind to Le^X specificities and to Le^X -rich *S. mansoni* antigen (Appelmelk et al., 2003). Unlike the Toll-like receptors (TLR) which recognize specific pathogen components such as LPS and then induce intracellular signalling resulting in upregulation of cytokine production and costimulatory molecules (Underhill et al., 2002), the C-type lectins seem to be involved in internalization of the organisms for antigen processing rather than signal transduction (Figdor et al., 2002) but engagement of DC-SIGN by zymosan can lead to the production of IL-10 (Cambi et al., 2003). Recently, Thomas et al. (2003) have reported that Le^X presented on lacto-N-fucopentaose III can signal through TLR4 which has previously only been associated with Th1-driving molecules. Interestingly the stimulated DCs failed to upregulate surface marker expression as reported by MacDonald et al. (2001) and similarly drove a Th2 response *in vitro*. It is suggested that opposing effects on TLR4 engagement by Th1- promoting (e.g. LPS) or Th2 - promoting (e.g. Le^X) may be controlled by the intensity of the TLR4 engagement with accessory molecules playing a role. Van der Kleij et al. (2002) showed that lipid derived from schistosomal eggs or adult worms e.g., schistosome-specific di-acylated phosphatidylserine activated TLR2 also directing DCs into a Th2 promoting DC phenotype, while the presence of mono-acylated lyso-phospatidylserine (lyso-PS) induced maturation into a DC phenotype that promotes development of T regulatory cells that inhibit proliferation of bystander T cells through IL-10. These interesting pro–Th2 effects of Le^X - containing schistosome molecules have been focussed on egg products which is known to induce strong Th2 responses, but the presence of this structure in the larval stages might also be involved in immuno-regulatory processes associated with this stage of the life cycle including perhaps the observed effects on DC responses reported here. However, it is not yet known if such glycosylated molecules are even secreted from the schistosomula glands *in vivo* or whether they are transiently expressed. Apart from the possible involvement of the remnants of molecules in the penetration, transformation is associated with rapid changes to the membrane with loss of the carbohydrate rich glycocalyx but with the appearance of new antigens (Bickle et al, 1986). Persistence of components of the glycocalyx or

transiently expressed surface associated molecules could also be responsible for the effects of 3hr schistosomula.

Regarding how schistosomula products might induce differential effects on different cytokine expression following LPS activation, it is known that the DC surface has a complex array of surface receptors including the C-type lectin receptors, the Toll-like receptors (which include 10 different family members and also non-Toll-like pattern recognition receptors (Kelsall et al., 2002). Other infectious organisms and their products are also known to modulate DC functions and in some situations differential effects on DC cytokine production has been shown. Urban et al. (1999) reported that prior incubation of human DC with *Plasmodium falciparum* - infected red blood cells inhibited subsequent LPS -induced upregulation of surface activation markers including HLA-DR, CD80, CD86 and CD40. Cholera toxin, a potent mucosal vaccine adjuvant inhibited the production of IL-12p70 and TNF-α but not IL-10, IL-6, TGF-β or prostaglandin E2 by LPS-activated blood monocyte-derived DCs (Gagliardi et al., 2000) and these were able to prime naïve T cells *in vitro*, skewing polarization to a Th2 response. However, unlike in the present studies, the cholera toxin-treated DCs up-regulated the expression of HLA-DR molecules, B7.1 and B7.2 co-stimulatory molecules. More similar to the studies in this Chapter, Van Overtvelt et al. (1999) showed that infection of human monocyte-derived DCs with *T. cruzi* significantly reduced the LPS-induced secretion of IL-12, TNF-α and IL-6, as well as the up-regulation of HLA-DR and CD40 molecules. The same effects were induced by *T. cruzi*-conditioned medium, indicating that these inhibitory effects were mediated by released soluble factors and a small family of type 1 Glycoinositolphospholipids (GIPLs) found on the cell-surface of the epimastigote and metacyclic forms of *T.cruzi* have been shown to have similar effects (Brodskyn et al. 2002). GIPLs had differential effects on LPS-induced cytokine production. There was a significant decrease in secretion of TNF-α, IL-10 and IL-12 but not IL-8 from both LPS-stimulated human macrophages and DCs. So in contrast to the present studies, both IL-12 and IL-10 were affected. However, Weigt et al. (2003) reported that mycoplasma-derived lipopeptide 2 induced differential IL-10/IL-12 responses in DCs upregulating IL-10 only and resulting in unpolarized immune responses. Recently, Semnani et al. (2003) reported that human DCs exposed to live *Brugia malayi* microfilariae (MF) up-regulate both the

cell surface and gene expression of CD54 (ICAM-1), and result in a 3-fold increase in DC death compared with MF-unexposed DCs, primarily due to apoptosis. They demonstrated, using microarray and real-time RT-PCR, that live MF up-regulate the expression of mRNA of proinflammatory molecules such as IL-8, RANTES, IL-1β and TNF-α in DCs, the presence of which is also detected at the protein level, while inhibiting the production of IL-12 (p40 and p70) and IL-10. Soluble excretory-secretory products from live MF diminished IL-12 and IL-10 production and induced DC death, although to a lesser degree.

Although co-cultured schistosomula failed to activate DC as judged by surface marker and cytokine expression, the studies in Chapter 4 showed that DCs had a marked effect on the level of schistosomula mediated IFN-γ produced in recall experiments using RoNI sensitized SLNs. This may be explained by the fact that DC use different receptors for antigen uptake e.g. the C-type lectin receptors like CD205 than for cellular activation e.g. the Toll-like and non-Toll-like pattern recognition receptors (Kelsall et al., 2002). So in the recall experiments the DCs may simply enhance antigen presentation to the population of SLN cells amongst which Th1 cells predominate.

In conclusion, the experimental studies in this Chapter were initiated to see if the Th1 biased response that develops following *S. mansoni* infection of mice and which is markedly enhanced following *in vivo* termination of the infection with the drug Ro11-3128 might be influenced by direct schistosomula/DC interactions leading to pro-Th1 activation of the DCs and whether Ro11-3128 treatment of the larvae would enhance any such activation. The results showed that incubation with a large range of concentrations of larvae failed to induce upregulation of MHCII or the co-stimulatory molecule, CD86 and CD40 in bm-DCs and this was not affected either by irradiation of the larvae or by Ro11-3128 treatment. LPS induced the expected upregulation of these molecules but co-cultures with the schistosomula neither increased or decreased this expression. Similarly, schistosome/bm-DCs co-cultures failed to induce cytokine production but rather reduced LPS induced expression in a dose dependent way and with differential effects on different cytokines, IL-12 being most affected and IL-10 not affected. These effects could not be attributed to toxic effects of the larvae on the cells but further work to confirm the specificity of the response is needed. If these

direct effects are confirmed they may represent another way in which schistosomula products modulate skin inflammation.

The failure to demonstrate an effect of Ro11-3128 treatment of schistosomula on DC activation suggests that other features of the Ro11-3128 terminated infections promote the high Th1 responses. The tissue damage caused by the death of larvae in the skin may induce production of pro-inflammatory factors from keratinocytes and other cells recruited to the skin (as in the case of the GI model, Riengrojpitak et al., 1998), and activation of Langerhans cells, the skin DCs, may occur secondarily to this. The inflammatory response induced by the drug-terminated infections is persistent and in fact does not peak in the skin until day 7 post infection as judged by IL-1β mRNA production (Zhang et al. – manuscript in preparation). Furthermore, antigen-specific IFN-γ continues to rise in the SLN until at least day 21 suggesting persistent antigen stimulation probably by persistence of drug-treated schistosomula in the nodes. So it is suggested that these persistent inflammatory responses in the skin and SLN are responsible for the generation of a strong protective Th1 response rather than simply direct effects of Ro11-3128 treated schistosomula on DC activation.

CHAPTER 6

GENERAL DISCUSSION

To design an anti-schistosome vaccine that will effectively and significantly reduce the incidence of severe disease, it is vital to understand the mechanisms of immunity induced in schistosomiasis. In experimental animals the previously most effective and extensively studied immunization regimens were based on γ-irradiated cercarial infections (GI). In order to stimulate optimal levels of immunity (60-70%) the immunizing irradiated larvae need to survive through to the lung stage. Based on earlier observations, work in this thesis showed that even higher levels of protection (>90%) could be induced in C57BL/6 mice by percutaneously applied *Schistosoma mansoni* infections which were treated with 200 mg/kg of the drug Ro11-3128, on day 2 post-infection (RoNI) when the larvae were in the skin. However, combining both types of attenuations (irradiation and drug-treatment) to the schistosomula (RoGI) induced poor levels of resistance (~ 30%).

All of these attenuated infections as well as normal unattenuated infections (NI) resulted in a Th1 biased cytokine recall by skin draining lymph node (SLN) cells (IFN-γ > IL-4). At day 7 post-infection the IFN-γ response was highest in the RoGI and GI, whereas the RoNI response was lower and more comparable to that of the NI in one experiment and higher than NI in another. However, the drug-treatments (RoNI and RoGI) induced enhanced and more prolonged antigen-specific IFN-γ cytokine responses in the SLN compared with the NI and GI at day 21 post-infection. This can be explained by the fact that the vast majority of the NI and GI larvae would have left the skin and migrated to the lungs before day 21 (Mangold and Dean, 1983) whereas the drug-treated schistosomula either by their death or persistence in the skin/SLN (Mountford et al., 1988; Mountford et al., 1989) provides stronger local stimulation. The pattern of higher IFN-γ production by SLN cells in the RoGI than in RoNI at day 7 and the reversal at day 21 is consistent with the idea that the larvae in RoGI die more rapidly after infection in the skin rather than in SLN (Mountford et al., 1988; Mountford et al., 1989) compared to RoNI leading to higher initial antigen production and cytokine responses but less persistent responses. The major difference between RoNI and RoGI was the higher IFN-γ responses in the spleen in RoNI at day 21. More prolonged stimulation of the SLN by RoNI could explain this stronger systemic spread of antigen specific

effector/memory Th1 cells. It would be interesting to extend the time of observation of the immunological and immuno-histological responses to the persistent schistosomula in the SLNs beyond the 21 days followed in the present studies.

There is strong evidence that the RoNI unlike the GI does not rely on the arming of the lungs by death of attenuated larvae, since extensive histology of the lung failed to find either parasites or reactions at day 14 or 21 post-RoNI treatment (in this model using 200mg/kg) in contrast to a parallel study using GI (Quentin Bickle-personal communication). Also extensive cytokine and chemokine mRNA studies showed no evidence of inflammatory responses in the lungs at any time following RoNI, as judged by production or presence of antigen-specific or non-specific IFN-γ producing cells in the lung tissue or in broncho-alveolar lavage during the time schistosomula are expected to reach and die in the lungs i.e., up to 21 days post infection (Yaobi Zhang-personal communication). Furthermore, double treatment with Ro11-3128 (on days +2 and +4) which would be expected to cause even more restriction on the survival of the RoNI, and again histology failed to demonstrate any parasites or parasite reactions in the lung at day 14 post-infection, in contrast to GI (Quentin Bickle-personal communication). However, this regimen induced comparable levels of protection to the single treatment. Further work could focus on comparison of the survival/migration in skin/SLN following the RoNI single and double treatments and the RoGI protocols, in addition to measuring and comparing IFN-γ responses in the SLN and spleen. Monitoring cytokines associated with induction of Th1 responses in the skin/SLN would be interesting e.g., IL-12, IL-23, IL-27.

Looking at the serum antibody responses on day 21 post vaccination from mice given the four treatments (NI, GI, RoGI and RoNI) the levels were comparable and significantly raised among the 3 attenuated infections (GI, RoNI and RoGI), whereas it was raised but to a lesser extent with NI. So this measure did not distinguish responses between the protective and non-protective regimes.

Antigen specific IL-10 production also occurred and tended to correlate with IFN-γ levels. This suggested that IL-10 rises along with IFN-γ until it is able to terminate the IFN-γ response (Wynn et al., 1994). The antigen-specific IL-10 production by SLN cells from RoGI was higher than in the RoNI at day 7 post-infection, but by day 21

post-vaccination when the levels of IL-10 had dropped there was no significant difference between RoNI and RoGI IL-10 levels. This higher IL-10 at day 7 did not cause lower IFN-γ responses but rather correlated with higher levels. It is not known whether this higher level of IL-10 during the early part of RoGI results in more rapid attenuation in IFN-γ response or could inhibit later macrophage mediated inflammatory responses to challenge larvae, as was shown in P strain mice which develop poor protection when given irradiated cercariae. Thus specific antigen stimulation of spleen cells from P strain mice produced levels of IFN-γ comparable to the highly protected C57BL/6 mice, but made 3x more IL-10 and >2x IL-4 than the C57BL/6 mice (Oswald et al., 1998). This observation together with the demonstration that IL-10 KO mice develop higher levels of protection than wild-type controls (Hoffmann et al., 1999) led to the suggestion that effective schistosome vaccines focussed on induction of Th1 protective responses should not induce high IL-10 levels. The present results are consistent with this notion as the protective RoNI regimen produced comparable IL-10 to the GI in the early after vaccination in C57BL/6 mice but this did not prevent the subsequent development of high IFN-γ levels. However, the RoNI model shows that it is possible to get very high levels of protection (comparable to those in IL-10 KO mice) even if some IL-10 is produced early during the vaccination procedure.

The above *in vitro* studies suggested that IFN-γ was important in the RoNI-induced immunity, and to confirm this *in vivo* studies were carried out in gene knock-out (KO) and anti-cytokine treated mice. Vignali et al., (1989a) had previously shown that immunity in GI and RoNI can be ablated or reduced following treatment during challenge infection with anti-CD4 antibody but not with anti-CD8 antibody. So this suggested that the elimination of challenge parasites was due to the CD4-T cell component of CMI responses. In this study the immunological specificity of the RoNI protocol was shown by the complete lack of protection in B6.RAG-1-/- mice which are deficient in T and B cells. Vaccination of IFN-γ -/- mice showed a 53-54% reduction in protection, but depletion of IFN-γ with neutralizing monoclonal antibody during the challenge phase led to a loss of protection. μ-MT mice showed almost comparable protection to wild-type controls (82% compared with 94% in controls) showing that B cells and antibody are not significantly involved in this protection. It is concluded that the protection following RoNI is mediated almost entirely by IFN-γ mediated mechanisms and antibody is not involved. It is also suggested that the high efficacy of

the RoNI is related to the induction of very strong and protracted Th1 responses in the skin which, unlike the RoGI, disseminate systemically giving rise to memory in other lymphoid tissues (as seen by elevated but persistent IFN-γ responses in the spleen). but which does not seem to require parasite induced inflammation in the lungs as is needed for high levels of immunity in the GI. Although the μMT mice, which make no antibody, were fully protected the lack of a protective role for antibodies in the single RoNI could be confirmed by passive transfer experiments and it would be of particular interest to investigate the role of antibody in mice given repeated RoNI as, in the case of repeated GI, antibody comes to play the key protective role (Kelly and Colley, 1988; Mangold and Dean, 1986).

Parallel approaches to investigate why RoNI is so effective in inducing immunity were concerned with the direct effects the drug has on antigen presentation to the host by inducing membraneous blebs at the larval surface. *In vitro* studies showed that Ro11-3128 in its native form, at doses of 1.25-2.5μg/ml, caused damage to schistosomula resulting in the production of membraneous blebs at their surface. The antigenic composition of these blebs has been shown to be different from that of the surface of the penetrating larvae e.g., in expressing the membrane glycoprotein Sm16, a vaccine candidate antigen (Bickle et al., 1986). Other studies by Smith et al., (1994) showed that the particulate matter released from the drug-treated parasites was responsible for increased stimulatory activity as measured by production of IL-3/GM-CSF from antigen sensitized T cells. In the present study it was shown that drug-treated 20 krad γ-irradiated schistosomula (the poorly protective RoGI) also produced blebs in *in vitro* cultures, indicating that production of membraneous blebs *per se* did not account for the superior immunity induced by unirradiated drug-treated infections. No differences were observed between RoGI and RoNI parasites regarding survival or percentage of parasites with drug-induced morphological changes (production of blebs). However, the antigenic composition of the membraneous blebs and soluble antigens produced/released from RoGI parasites was never tested. It would, therefore, be interesting to know if they are antigenically different from RoNI parasites by using Western blotting techniques or metabolic labelling and co-precipitation. It would also be interesting to compare skin/SLN cellular responses following RoNI and infections terminated by Ro15-5458 another drug which is effective against the skin stage schistosomula but which induce low levels of immunity and which does not induce the

production of membraneous blebs at the surface (Bickle et al., 1990). The demonstration by Smith et al (1994) of enhanced antigen presentation by Ro11-3128-treated schistosomula was further investigated by culturing parasites and cells from the inguinal and axillary lymph nodes of either immunized or non-immunized animals and cytokine production measured. No IFN-γ production could be detected with RoNI-sensitized SLN cells and schistosomula alone, but addition of *in vitro* bone-marrow-derived dendritic cells (bm-DCs) induced IFN-γ production. In this situation there was evidence in some experiments that Ro11-3128 increased the recall response indicating that the altered antigen production by the parasites by the direct effects of the drug may also be involved in inducing the high levels of response seen in the skin during drug-terminated infections. However, the soluble and membraneous material contained in supernatants of drug-treated schistosomula did not induce IFN-γ production by RoNI-sensitized SLN cells contrary to what was shown by Smith et al., (1994). To directly assess if altered antigen presentation itself was solely responsible for inducing high levels of immunity, mice were immunized with 500 F/T drug-treated parasites and any soluble released material intradermally. This did not induce marked IFN-γ production by the spleen cells (at day 21) compared with the RoNI model and did not significantly reduce worm burdens. This work supports the notion that optimal protection due to RoNI requires protracted survival of the drug-treated schistosomula rather than any specific effect of the drug on the parasite surface. From the viewpoint of vaccine design this emphasizes the value of protracted stimulation of the SLN.

The possibility that Ro11-3128 might have a direct immunomodulatory effect on host cells was investigated using spleen cells cultured with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) in the presence of a range of Ro11-3128 concentrations. Production of IFN-γ in this system involves interactions of activated macrophages, natural killer cells and T cells allowing for assessment of the drug on induction of immune responsiveness. No enhancement of IFN-γ production was seen at any concentration but at high drug concentrations (5-10μg/ml) inhibition was seen. So there was no evidence of an adjuvant effect of Ro11-3128 *per se* although it might be of value to confirm this by looking at drug effects on *in vivo* immune responses to a defined antigen or to a live antigen such as BCG.

In Chapter 5 studies were carried out to investigate if the Th1-skewed response induced by exposure to larval schistosomes *in vivo* was due to direct effects of larval parasites

on dendritic cells and if this was influenced by drug treatment. Addition of varying numbers of living schistosomula (50-10,000/ml) to bm-DCs did not significantly affect (up- or down- regulate) MHCII and CD86 expression on the surface of DCs. When bm-DCs were partially activated by addition to the cultures of a range of concentrations of TNF-α or LPS the parasites were still not able to enhance expression of MHCII, CD86 and CD40. Addition of Ro11-3128 to the cultures did not affect the response. Schistosomula also failed to induce significant levels of cytokine production (IL-12p70, TNF-α, IL-10 or IL-6). These effects were also independent of the presence of Ro11-3128 in the cultures. However, in the presence of LPS co-culture with schistosomula did, in fact, reduce the levels of cytokine production by bm-DCs in a dose dependent manner. A marked reduction occurred with high numbers of larvae (2,500-10,000 P/ml), while this inhibition was less with lower numbers 625-1,250/ml. There were differential effects on the different cytokines, IL-12p70 being most affected (mean reduction of 80%), followed by IL-6 and TNF-α (mean reduction of 34% and 32%, respectively). IL-10 was relatively unaffected. The lack of effect of schistosomula on CD86 and MHC class II expression and the negative effects on cytokine expression were unaffected by the addition of Ro11-3128 to the cultures.

A possible explanation for this was that either the schistosomula products failed to signal DC activation and therefore inhibit different cytokine pathways, or that schistosomula products (enzymes, etc.,) selectively degrade secreted cytokines (IL-12p70 >> IL-10) from bm-DCs, or that they damage/kill DCs resulting in this differential effect on cytokine production. However, regarding the last possibility the same differential effects on cytokines was obtained when parasites and bm-DCs were cultured together for 24h prior to stimulation with LPS. Furthermore, bm-DCs cultured with freshly transformed schistosomula were 70-90% viable as measured by Trypan blue staining and AnnexinV and Propidium Iodide staining. The other possibilities could be investigated by (i) directly testing whether cytokines are degraded in the presence of schistosomula, and (ii) looking at the mRNA expression of the different cytokine genes in relation to housekeeping genes. The possible role of PGE2 released by the parasites or induced by keratinocytes in mediating these effects could be studied by addition of indomethacin to the cultures. It would be interesting to study whether the schistosomula-treated bm-DCs showed inhibition of other functions such as the ability

to process and present antigens, using ovalbumin and ovalbumin specific transgenic T cells (Hsieh et al., 1993).

The inhibitory effect on cytokine production induced by 3hr schistosomula was much less pronounced with schistosomula pre-cultured for 24hr in medium alone before use. Factors which might explain this are the release of oligosaccharides which interact with receptors on the DC surface. For example, the Lewis x (Le^x) oligosaccharide has been reported to interact with DC-SIGN (Appelmelk et al., 2003) and Lewis x has been shown to be present in the cercarial glycocalyx (Harn et al., 1987; Ko et al., 1990), remnants of which would survive at the surface of the newly transformed schistosomula but subsequently be lost as the new heptalaminate membrane fully forms. In addition, Faveeuw et al., (2003) showed that core α3-fucose and core β2-xylose determinants (which contain Lewis x) are expressed in the excretory-secretory systems of schistosomula and certain of these would be released during culture.

This demonstration of inhibitory effects of living schistosomula on DCs is consistent with a recent report by Trottein et al., (2004) who compared the transcription of genes induced in immature mouse DCs (called D1, a DC line) by *S. mansoni* eggs and skin stage schistosomula, and found that schistosomula-treated DCs did not secrete TNFα, IL-2, IL-1α, IL-6, IL-12p40 nor IL-12p70. The authors also mentioned (data not shown) that the general effects described exerted by the live schistosomula on DC were not mimicked by their corresponding antigen extracts and suggested that physical contact, rather than soluble factors, are involved in these phenomena. By looking at mRNA expression they found other inhibitory effects on DCs. The schistosomula, unlike the eggs, did not up-regulate H-2M, which plays a crucial role in the peptide loading of MHCII molecules, the costimulatory molecules CD40 and ICAM-1 or chemokines and the Cathepsins D and L, which are believed to remove invariant chain from its complex with MHCII molecules, were down-regulated by schistosomula suggesting a reduction in the antigen processing capacity exerted by the larval stage on DC. So this work together with the studies in this thesis show inhibition of DC functions suggesting additional ways to those already described (Angeli et al., 2001; Ramaswamy et al., 2000; Trottein et al., 2004) in which schistosomula may inhibit inflammatory processes in the skin. Although this can be considered an advantage to survival of the parasite it fails to give any insight into how the Th1 response which ultimately does develop is induced.

Finally, what has been learnt from the RoNI model which may have relevance for vaccine development? There was no evidence that specific drug-induced antigen presentation or adjuvant effects were involved. Rather the data suggests that the drug-treated infections promote strong Th1 responses in the SLNs. However, high antigen specific Th1 responses in the SLN are not sufficient to ensure high levels of immunity as the poorly protective RoGI induced comparable protracted IFN-γ responses to the highly protective RoNI in the SLN at both 7 and 21 days post-infection which were both significantly higher than that induced by the GI infection by day 21 p.i.. The 20krad GI is characterised by the irradiated larvae migrating to the lungs and there is strong evidence that this phase is essential to recruit/boost cellular responses primed in the skin (e.g., Coulson and Wilson, 1997). However, the RoNI does not appear to transit to the lungs and induce inflammation there but rather the RoNI is distinguished from the RoGI by evidence of more prolonged stimulation of the SLN response leading to higher antigen specific systemic IFN-γ responses in the spleen. It is proposed that the protracted survival of the RoNI parasites results in local stimulation of a strong local response which can lead to systemic spread of memory cells to the lung lymph nodes/lungs where they can respond rapidly to larvae of a challenge infection as seen by a rapid rise in IFN-γ and chemokine levels in the lungs post-challenge (Dr Yoabi Zhang- personal communication), i.e., a systemic Th1 mediated protection, and so provides a paradigm for development of vaccine strategies that mimic this. In this regard several studies demonstrated the efficacy of prime-boost vaccination strategies in generating cellular immunity to a variety of pathogens (Woodland, 2004). This strategy is based on T cell priming with DNA vaccines or recombinant virus followed by boosting with a heterologous recombinant virus expressing the same antigen gene. Examples of such studies include, *M. tuberculosis* (Tanghe et al., 2001), HIV (Amara et al., 2001), malaria (Bruna-Romero et al, 2001;Gilbert et al., 2002), herpes simplex virus (Meseda et al., 2002), leishmania (Gonzalo et al., 2002), Ebola virus (Sullivan et al., 2000), *Listeria monocytogenes* (Fensterle et al., 1999), hepatitis C virus (Matsui et al., 2003), hepatitis B virus (Pancholi et al., 2001). The studies in the RoNI indicate that such prime-boost vaccination could be administered in the same site e.g., the skin, provided protracted responses were induced. On the other hand, to mimic the GI it would be necessary to prime in the skin and then boost in the lungs. Such an approach may well be possible long term as successful vaccination with recombinant viruses via

the intranasal route has been reported (Goonetilleke et al., 2003) for *Mycobacterium tuberculosis* infections in mice. It should be emphasized however, that following repeated vaccination with GI, protective antibody responses are induced (Mangold and Dean, 1986; Wynn et al., 1996), and the need for recruitment of sensitized cells to the lungs to arm the lungs is no longer needed as depletion of CD4-T cells is without effect (Kelly and Colley, 1988). Nevertheless, the mechanisms of immunity which might be effective in human is unknown and so development of vaccine strategies that stimulate a range of immune responses should be investigated. From the perspective of the Th1 axis, the main challenge now is to identify the likely vaccine candidate antigens to express in vectors for use in the prime-boost strategies.

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APPENDIX

A.1 E/LAC Medium:

The main constituents of Earle's medium were Earle's Balanced Salt and Lactalbumin Enzymatic Hydrolysate, hence the abbreviation **E/LAC**. E/LAC is made up of: 8.64g Earle's Balanced salts [EBSS] (Sigma, UK), 0.85g sodium bicarbonate [$NaHCO_3$] (Sigma, UK), 5.00g Lactalbumin Enzymatic Hydrolysate (Sigma, UK) / Litre MilliQ water (pH 7.4). This medium was then filtered sterilized and stored at 4°C.

A.2 M169 Medium:

Table A.2 . *Schistosome culture medium M169.*

Component	Stock Conc.	Add stock	Working conc.
BME (Basal Medium[Eagle]) ~		1 Litre	
Lactalbumin Hydrolysate *		1 g	1 g/Litre
Glucose *		1 g	11.1 mM
Hypoxanthine *	10^{-3} M ξ	0.5 ml	5 x 10^{-7} M
Serotonin *	10^{-3} M ξ	1 ml	10^{-6} M
Insulin, crystalline *	8 mg/ml ξ	1 ml	8 µg/ml
Hydrocortisone *	10^{-3} M ξ	1 ml	10^{-6} M
Triiodothyronine *	2 x 10^{-4} M ξ	1 ml	2 x 10^{-7} M
MEM vitamins ~	100x ξ	5 ml	0.5x
Schneider's medium *	1x	50 ml	5%
HEPES ~		2.4 g	10 mM
NaOH	5 N		pH 7.4
$NaHCO_3$		2.2 g	26 mM
MilliQ water		1 Litre	

Adapted from Basch P. (1981).

* = Products obtained from Sigma, UK.

~ = Products obtained from Gibco, UK.

ξ = stocks frozen at -20°C. Working concentration is based upon a theoretical total volume of 1 litre.

M169 Medium was filter sterilized and stored at 4°C.

A.3 FACS-EDTA buffer:

1 % Horse serum (Sigma, UK), 1x PBS (Sigma, UK), 0.1% sodium azide [NaN_3], 0.05 M EDTA (Ethylenediaminetetracetic Acid).

A.4 Gey's Solution:

Stock	Components	Weight (g)	Made up to
STOCK A	NH_4Cl	35.00 g	
	KCl	1.85 g	
	$Na_2HPO_4.2H_2O$	7.50 g	
	KH_2PO_4	0.12 g	
	D-glucose	5.00 g	
	Phenol Red (Optional)	0.05 g	
	MilliQ water (Triple Red Lab. Technology, UK)	1000 ml	1000 ml

Stock	Components	Weight (g)	Made up to
STOCK B	$MgCl_2.6H_2O$ (Magnesium Chloride Hexahydrate)	0.42 g	
	$MgSO_4.7H_2O$ (Magnesium Sulphate Heptahydrate)	0.14 g	
	$CaCl_2$	0.34 g	
	MilliQ water (Triple Red Lab. Technology, UK)	100 ml	100ml

Stock	Components	Weight (g)	Made up to
STOCK C	$NaHCO_3$	2.25 g	
	MilliQ water (Triple Red Lab. Technology, UK)	100 ml	100ml

1x Gey's Solution was made of 20 parts STOCK A + 5 parts STOCK B + 5 parts STOCK C + 70 parts double distilled water. It was then filter sterilized and stored at 4°C, until use.

A.5 <u>10x PBS Buffer (Phosphate buffered solution) :</u>

This is used for ELISA's and any non-sterile technique.

80.0g NaCl, 2.0g KCl, 11.6g Na_2HPO_4, 2.0g KH_2PO_4, and made up to 1 litre with distilled water (pH 7.2).

A.6 <u>Reagents provided in Annexin V-FITC staining Kit I</u> :

a- <u>FITC-conjugated Annexin V</u>:

Buffered in 50mM Tris, pH 8.0, 80 mM NaCl, 1mM EDTA, 0.09 % (w/v) sodium azide. Stored at 4°C. Used at 5µl per test ($1x10^5$ cells).

b- <u>Propidium Iodide Staining Solution (PI)</u>:

50µg/ml stock solution of PI in 1x PBS buffer, pH 7.4. Stored at 4°C.

c- <u>10x Binding Buffer</u>:

0.1M HEPES/NaOH, pH 7.4; 1.4M NaCl, 25mM $CaCl_2$. Sterilized with 0.2µm filter. Diluted to 1x prior to use. Stored at 4°C.